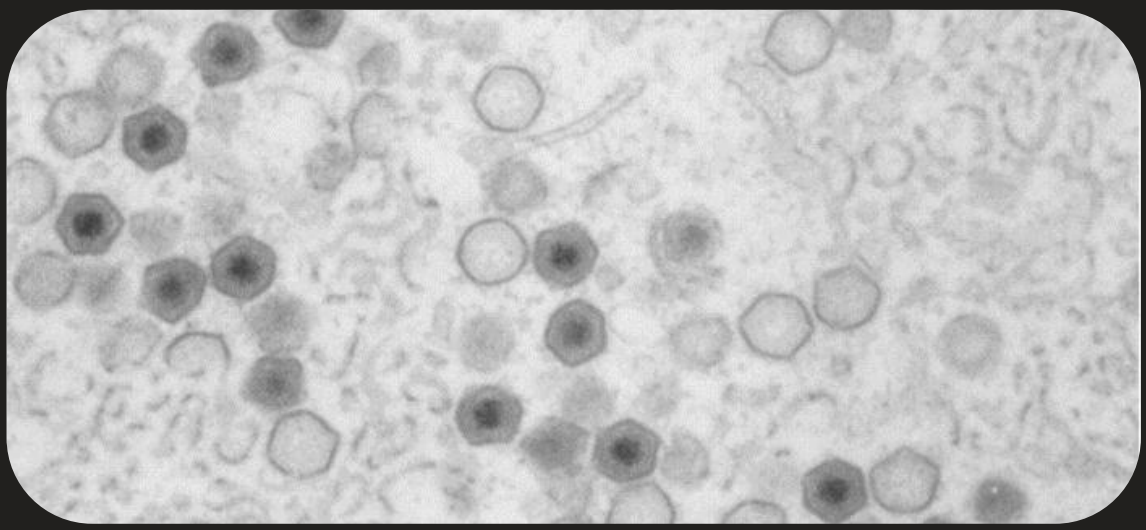
Strategies of the African Swine Fever Virus to manipulate innate immunity

Sónia Ventura



Dissertation presented to obtain the Ph.D degree in Biology

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras,
June, 2012

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Knowledge Creation

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Backcover image: ASFV assembly

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Abbreviations

APCs	Antigen Presenting Cells
ASF	African Swine Fever
ASFV	African Swine Fever Virus
ATF2	AcTivating Factor 2
BVDV	Bovine Viral Diarrhoea Virus
CARD	CAspase Recruitment Domain
CBP	CREB-binding protein
CCR	CC Chemokine Receptors
CD2v	CD2 like protein
CLR	C-type Lectin Receptors
CREB	cAMP-Response Element-Binding protein
CTL	Cytotoxic T Lymphocyte
DAI	DNA-dependent Activator of IFN-regulatory factors
DBD	DNA Binding Domain
DC	Dendritic Cell
dsRNA	double stranded RNA
EBV	Epstein-Barr Virus
eIF	eukaryotic Initiation Factor
ELISA	Enzyme-Linked Immunoabsorbent Shift Assay
EMCV	EncephaloMyoCarditis Virus
ER	Endoplasmic Reticulum
FADD	Fas-Associated protein with Death Domain
FBS	Foetal Bovine Serum
GAS	Gamma Activated Site
GFP	Green Fluorescence Protein
HA	Haemaglutinin
HCMV	Human CytoMegaloVirus
HCV	Hepatitis C Virus
HDAC	Histone DeACetylase
HHV8	Human HerpesVirus 8
HIV	Human Immunodeficiency Virus
HMGI(Y)	High-Mobility Group (HMG) chromatin associated protein
HSV	Herpes Simplex Virus
HVS	HerpesVirus Saimiri
IFN	Interferon

IFNAR	Interferon Alpha Receptor
IFNGR	Interferon Gamma Receptor
Ig	Immunoglobulin
IKKε	Inhibitory protein κB-ε
IL	InterLeukin
iNOS	inducible Nitric Oxide Synthase
IRAK	IL-1 Receptor-Associated Kinase
IRF	Interferon Regulatory Factor
ISG	Interferon Stimulated Gene
ISGF3	IFN Stimulated Gene Factor 3
ISRE	IFN-Stimulated Response Element
Jak	Janus activated kinase
KSHV	Kaposi's Sarcoma associated Herpesvirus
LPS	LipoPolySaccharide
LRRs	Leucine Rich Repeats
MAVS	Mitochondrial Antiviral Signalling
MDA-5	Melanoma Differentiation-Associated gene 5
mDCs	Myeloid Dendritic Cells
MGF	Multi Gene Family
MHC	Major Histocompatibility Complex
MHV68	Murine gamma-Herpesvirus 68
M-MLV	Murine Molony Leukaemia Virus
MyD88	Myeloid Differentiation primary response gene 88
ND10	Nuclear Domains 10
NFAT	Nuclear Factor of Activated T cells
NF-κB	Nuclear Factor κB
NK	Natural Killer
NLR	NOD-Like Receptors
NO	Nitric Oxide
NOD	Nucleotide binding and Oligomerization Domain
OAS	2´,5´-Oligoadenylate Synthetase
ORF	Open Reading Frame
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate Buffered Saline
pDCs	Plasmocytoid Dendritic Cells
PI3K	Phosphatidylinositol 3-Kinase
PIAS	Protein Inhibitor of Activated STAT

PKC	Protein Kinase C
PKR	dsRNA-dependent Protein Kinase R
PML	Promyelocytic Leukaemia Protein
Poly (I:C)	Polyinosine-Polycytidylic Acid
PRD	Positive Regulatory Domain
PRR	Pattern Recognition Receptor
RBC	Red Blood Cell
RIG-I	Retinoic Acid Inducible Gene I
RLR	Rig-I Like Receptor
RNAi	RNA Interference
RSV	Respiratory Syncytial Virus
SeV	Sendai Virus
SOCS	Suppressors of Cytokine Signalling
ssRNA	single stranded RNA
STING	STimulator of Interferon Genes
SV40	Simian Virus 40
TBK-1	Tank-Binding Kinase-1
TBP	Tata-Binding Protein
TCR	T-Cell Receptor
Th	Helper T Lymphocyte
TIR	Intracellular Toll-IL-1 Receptor
TIRAP	TIR Domain-Containing Adaptor Protein
TLR	Toll Like Receptor
TNFα	Tumour Necrosis Factor A
TRAF	TNF Receptor Associated Factor
Treg	Regulatory T Lymphocyte
TRIF	TIR Domain Containing Adaptor Inducing IFN
VRE	Virus-Responsive Element
VSV	Vesicular Stomatitis Virus
VV	Vaccinia Virus

Resumo

Esta tese teve como objectivo determinar os mecanismos e consequências de dois genes virais de evasão à resposta do interferão (IFN), expressos pelo economicamente importante, e frequentemente fatal, Vírus da Peste Suína Africana (VPSA). De modo a sobreviverem, os vírus de ADN, tal como o VPSA, têm frequentemente múltiplas estratégias/genes que modulam positiva ou negativamente a biologia celular do hospedeiro, bem como a resposta imunitária. Os dois genes aqui apresentados funcionam em benefício do vírus, inibindo um das principais componentes da resposta imune inata, a resposta do IFN.

O gene I329L foi recentemente reportado como sendo capaz de inibir as respostas celulares, controladas pelo TLR3, que levam à indução e secreção de IFN-β, bem como à activação do NF-κB. Aqui, é demonstrado que o I329L não só inibe a indução e secreção de IFN-β pelo TLR3, mas também inibe a activação do NF-κB após estimulação pelo TLR4. Demonstrou-se ainda, bioquimicamente, que a proteína I329L interage com a proteína adaptadora TRIF, o que é consistente com a inibição observada de ambas as vias do TLR3 e TLR4. De forma a caracterizar a modulação da resposta do IFN tipo I pelo I329L, bem como determinar o papel de cada domínio do I329L nesta inibição, foram construídos plasmídeos que expressam mutantes truncados, com apenas o domínio extracelular ou intracelular. Estes mutantes foram testados por ensaios de luciferase. O domínio extracelular apenas inibe a activação do IFN-β e NF-κB induzida por estímulo com Poly (I:C), enquanto o domínio intracelular é capaz de inibir a mesma via quando esta é induzida por expressão ectópica de TRIF. Além disso, demonstrou-se que a proteína I329L é proteoliticamente processada durante a infecção viral e após

estimulação da via do TLR3. Com base nos resultados, é proposto um modelo em que o domínio extracelular inibe a activação da resposta pelo TLR3 através da formação de um heterodímero não-funcional I329L-TLR3, e o domínio intracelular interfere com a transmissão do sinal através do TRIF.

Demonstrou-se que duas variantes distintas do gene não-conservado MGF360-18R do VPSA, uma da estirpe patogénica Benin97/1 e outra da estirpe adaptada à cultura celular Ba71V, inibem a indução do IFN-β, e a resposta do hospedeiro à expressão de IFN tipo I e tipo II. Ambas as variantes da proteína MGF360-18R afectam a proteína MAVS, uma proteína adaptadora da via citosólica RLR que é essencial para a indução do IFN-β. Por outro lado, apenas a variante 'patogénica' afecta o factor de transcrição IRF-3, o que confere uma vantagem adicional ao vírus, em resultado de uma supressão mais eficiente da resposta do IFN, *in vivo*. Adicionalmente, demonstrou-se que ambas as variantes da proteína MGF360-18R impedem a resposta da célula à expressão de IFN tipo I e tipo II (via da Jak-STAT), induzindo a degradação do factor de transcrição STAT1 pelo proteasoma 26S.

Summary

The objective of this thesis was to determine the mechanisms and consequences of two non-homologous host evasion genes of the economically important, frequently fatal African Swine Fever Virus (ASFV). In order to survive, large DNA viruses, such as ASFV, typically have multiple genes/strategies for positive and negative modulation of host cell biology and immune responses. The two genes presented here inhibit a major component of innate immunity, the Interferon (IFN) response, and so function to the benefit of the virus.

The conserved I329L gene was recently reported to impair the cellular responses controlled by TLR3 that lead to both IFN-β secretion and NF-κB activation. Here, this observation is extended by demonstrating that I329L not only inhibits both induction and secretion of IFN-β, but also inhibits TLR4 stimulated activation. The I329L protein was also biochemically demonstrated to target the adaptor protein TRIF, consistent with the observed inhibition of both TLR3 and TLR4 pathways. To further characterize the modulation of the type I IFN response by I329L, as well as to assess the role of each domain, truncation mutants expressing either the ectodomain or the intracellular domain were designed and tested by luciferase reporter assays. The extracellular domain inhibited activation of IFN-β and NF-κB *via* Poly (I:C) in a dose dependent manner, whereas the intracellular inhibited activation of IFN-β stimulated by ectopic TRIF. In addition, the I329L protein was shown to be proteolytically processed during virus infection or after stimulation of the TLR3 pathway. On the basis of these results, a testable working model is proposed, with the I329L extracellular domain inhibiting activation of the TLR3 response through the formation of a non-signalling I329L-TLR3 heterodimer, and the I329L intracellular domain interfering with signal transmission through TRIF.

Two distinct variants of the non-conserved MGF360-18R gene, one from the pathogenic Benin97/1 virus and the other from the Ba71V tissue culture adapted virus, were shown to inhibit both the induction of IFN-β and the host cell response to type I and type II IFN. Both variants of the ASFV MGF360-18R protein target MAVS, a key adaptor protein of the RLR pathway for induction of IFN-β, while only the 'pathogenic' variant targets IRF-3, which may give the virus an extra advantage as a result of a more efficient suppression of the IFN response *in vivo*. Additionally, both variants of the ASFV MGF360-18R protein were shown to impair the host cell response to both IFN-α and IFN-γ (Jak-STAT pathway), inducing STAT1 degradation by the 26S proteasome.

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Chapter 1

Introduction

1. Introduction

The immune system of multicellular organisms evolved as a direct consequence of the selective pressure imposed by infectious microorganisms. The most ancient defence mechanisms, also known as the innate immune system, evolved to initiate an immediate and robust response against the invading microbes, and depend on relatively few germ-line encoded receptors. Emergence of the vertebrates was accompanied by the additional evolution of the adaptive immune system, which introduced a fundamental evolutionary advancement: more precise mechanisms of immune recognition and long-term immunological memory, based on an essentially infinite repertoire of receptors generated by gene rearrangements (reviewed by Hirano *et al*., 2011). [1]

In the mammalian immune system, these two very different, innate and adaptive immune systems are interconnected, enabling efficient detection and protection against a wide variety of rapidly-evolving pathogens that can cause disease. The adaptive immune response requires signals that provide information about the origin of the antigen and the type of response to be induced. These signals are thought to be provided by the innate immune system. Understanding the molecular mechanisms responsible for the generation of these signals would be crucial for the development of new approaches to vaccine formulation and immunotherapy (reviewed by Schenten & Medzhitov, 2011). [2]

1.1. Immune response to viruses

Viruses are particularly well adapted pathogens, capable of parasitizing all cellular life forms, exploiting the host's cellular machinery for their survival and replication. Over millions of years of evolution, the organisms that are hosts to viruses have evolved anti-viral defences, but viruses have also responded through the evolution of multiple strategies to modulate, or inhibit host defences, so that the virus is able to complete the infectious process and then infect new hosts. The interactions between virus-infected cells and the host defence mechanisms determine the harmful pathological consequences that can occur during viral infection. These consequences reflect, not only the strategies that a given virus uses during infection, but also how the host resists infection. A highly virulent virus is not necessarily the most successful, as its very pathogenicity could lead to death of the host before the virus has spread. The concept that viruses benefit by mutating to less virulent forms, led to the assumption that virulence can be the result of incomplete adaptation of the virus to the host. A virus–host association that has existed for a long period is likely to have evolved a relationship in which the host suffers little or no harm. However, when a virus extends its host range into a new species, for example, recently emerged haemorrhagic viruses, typically will be much more virulent in the new host than in the old [3,4].

The effect of viral diseases on the well-being of societies is a powerful motivation to comprehend the nature of viruses, how they replicate and how they cause disease. The understanding of viral mechanisms of infection, as well as its evasion strategies, may provide a rational basis for the development of more effective means for prevention, diagnosis and treatment of virus diseases through the production of vaccines,

diagnostic reagents and techniques, and antiviral drugs. Virus host evasion mechanisms have also an enormous potential as a source of strategies for immunomodulation [3].

The host defence mechanism against viral infection consists of a complex relationship between components of the innate and the adaptive immune system. A key point in trying to understand this balance is the biology of the virus and its life style; as an obvious example, highly acute viruses, such as the recently virulent avian influenza, present a totally different challenge to the immune system than persistent viruses such as HIV, and this will not only dictate the most appropriate immune effector response necessary for effective protection, but also provide the rational framework for the development of novel therapeutic approaches.

The innate immune response represents a rapid first line of defence, and it is sometimes sufficient by itself to clear a viral infection. In these circumstances, innate immune mediators play a very important role in keeping the virus load low. Equally important, components of the innate response shape the adaptive immune response and direct the subsequent effector phase.

When virus replication outpaces innate host defence, second line defences (adaptive immune response) are necessary. The adaptive immune response towards viruses is mediated by T and B cells expressing antigen-specific receptors and responsible for cellular and humoral immunity, respectively. Activation and differentiation of T and B cells is instigated and controlled by cell interactions between T cells and antigen presenting cells and T cells and B cells, leading to the generation of the proper type of adaptive immune response. The optimal activation of the innate immune system is also important for shaping the inflammatory response, which is essential for clearance of

virus infection, and may result in associated immunopathology. Appropriate regulation of all these processes is necessary in order to have a successful immune response against an infection. Dissection of the critical cellular pathways that control these processes will eventually unveil opportunities for manipulating the host immune response to control viral infection and control pathogenesis (reviewed by Christensen & Thomsen, 2009) [5].

Both innate and adaptive immune responses and their role in protecting against viral infections will be briefly described below.

1.1.1. Innate immunity

Upon microbial infection, host survival critically depends on the establishment of a rapid and appropriate innate response. Characteristically, innate immune responses start within minutes or hours of infection, while activation of an effective antibody and activated cytotoxic lymphocyte response to the infectious agent takes several days. Initial control of viral spread is thus the responsibility of innate immunity. In addition, innate immunity, largely through the spectrum of secreted chemokines and cytokines, regulates the direction of the adaptive immune response. However, given that continued activation of the innate response may cause damage to the host, it must be itself tightly regulated and transient [6,7].

Cells of both hematopoietic and nonhematopoietic origin undertake the responsibility of orchestrating the innate immune response. Cells of hematopoietic origin include monocytes, macrophages, natural killer (NK) cells, mast cells, neutrophils and eosinophils. Other cellular elements involved in both innate and adaptive responses are NK T cells and dendritic cells. In addition to hematopoietic cells, the skin and

the epithelial cells lining the respiratory, gastrointestinal, and genitourinary tracts bear receptors that recognize conserved molecular structures expressed by a large variety of microbes, and so may also provide a component of innate immunity [6,8].

Cellular and humoral elements (complement system) of the innate immunity work together to constrain the virus spread and eliminate virus-infected cells, building an effective defence system against pathogenic microorganisms. The secretion of high levels of cytokines directs not only the activation and differentiation of the adaptive immune response but also the subsequent recruitment of antigen-primed effector T cells to the sites of viral replication (reviewed by Christensen & Thomsen, 2009) [5].

When exposed to a pathogen, epithelial cells and tissue resident macrophages are potent producers of cytokines. These are polypeptides that act as immunomodulating agents. They coordinate important aspects of the immune response, including inflammation, cellular recruitment, activation, proliferation and differentiation, being critical to the development and functioning of both the innate and adaptive immune responses. According to their function, cytokines can be divided into proinflammatory cytokines, anti-inflammatory cytokines and chemokines (chemoattraction mediators). Finally, cytokine expression is not restricted to cells of the immune system, e.g. epithelial cells can secrete cytokines such as IFN [6,9].

The initial host response to viral infection includes the production of potent cytokines by infected cells. These cytokines bind to receptors on sentinel DCs, macrophages and neighbour uninfected cells, leading to a cascade response, with simultaneous or sequential expression of more cytokines, mainly by the activated cells of the innate immune system. The first cytokines to be produced are the type I interferons

IFN-α and IFN-β, followed by TNF-α, IL-6, IL-12, IL-18 and type II interferon, IFN-γ. In addition to inducing a local antiviral response, cytokines also have a more general effect by inducing acute-phase proteins that are important and necessary for tissue damage repair and to clear infection (reviewed by Christensen & Thomsen, 2009) [5].

TNF-α, a proinflammatory cytokine, is able to regulate the expression of adhesion molecules on the endothelium of nearby capillaries. This action induces changes which attract and facilitate the extravasation of leukocytes to the site of infection. When binding to receptors on infected cells, TNF-α is also able to induce an antiviral response that eventually leads to apoptosis (reviewed by Rahman & McFadden, 2006) [10].

Type I IFNs are key contributors for both the innate and adaptive immune responses to viral infection. When an infected cell produces and releases type I IFNs, they will bind to the type I IFN receptors (IFNAR) of neighbouring cells. The consequent transcription of over 300 antiviral genes results in the inhibition of several steps of the viral life cycle. Furthermore, IFN-α/β are able to amplify the IFN original signal, inducing an augmented antiviral state that result in secretion of high levels of cytokines and chemokines. Cells of the innate immune system are recruited to virus-infected tissues, where they are activated and in turn facilitate the induction of the adaptive immune response (reviewed by Le Bon & Tough, 2008) [11]

Chemokines coordinate the localization and collaboration between the cells of the immune system. Functionally, they can be divided into inflammatory (inducible), homeostatic (constitutively expressed) and dual-functional chemokines. Although most chemokines are secreted molecules, they are most likely mainly sensed by leucocytes while

bound to the extracellular matrix or to cell surfaces through proteoglycans. Chemokines need to interact with specific cell surface receptors in order to exert their biological function. The two major families of chemokine receptors are the CXC chemokine receptors and the CC chemokine receptors (CCR), which bind CXC and CC chemokines, respectively (reviewed by Christensen & Thomsen, 2009) [5].

The intracellular signal transduction pathways responsible for expression of the multiple cytokines and chemokines released during viral infection, are activated as a consequence of pathogen recognition receptor (PRR) signalling in cells such as epithelial cells, macrophages and DCs. Germline-encoded PRRs are able to detect and distinguish between self and invariant microbial molecular structures (Pathogen Associated Molecular Patterns – PAMPs) shared by all pathogens of a given class. These molecular signatures are usually indispensable for the pathogen life cycle and are different from the molecular structures found in the host. In turn, the PRRs are similarly invariant. In response to PAMP recognition, PRRs execute the first line of host defensive responses and later participate in the control and direction of the second line of host defence, the adaptive immunity. The selective specificity of PRRs avoids activation of the immune system by self molecules. However, viruses usually replicate using host strategies and consequently generate molecular structures that resemble the molecular patterns found in the host. This poses a particular problem for innate recognition of viral infections (reviewed by Diebold, 2010) [12].

PAMPs, according to their origin and nature, activate distinct classes of PRRs, which include Toll-like receptors (TLRs) retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs), nucleotide binding and

oligomerization domain (NOD)-like receptors (NLRs), C-type lectin receptors (CLRs) and DNA receptors (cytosolic DNA sensors). This variety of PRRs ensures the existence of multiple sensor systems that can detect and respond to almost any infection of the host. During viral infections, nucleic acid- and glycoprotein-PAMPs interact with particular classes of PRRs, which include certain Toll-like receptors, retinoic acid inducible gene-I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), and the cytosolic DNA receptors. Interplay between TLRs and RLRs in different cell types during viral infection plays an important role in antiviral responses, as well in controlling adaptive immunity. While the cytoplasmic PRRs are responsible for limiting virus spread locally and for generating an inflammatory environment, the nucleic acid-sensing TLRs are crucial for orchestrating the adaptive anti-viral immune response that eventually leads to the elimination of the virus and virus-infected cells. To circumvent the fact that viral nucleic acids are structurally similar to eukaryotic nucleic acids, recognition occurs in a specialised endosomal compartment and not at the cell surface, in contrast to the TLR sensing bacterial, fungal and protozoan ligands (reviewed by Christensen & Thomsen, 2009, Diebold, 2010 and Kaway & Akira, 2011) [5,12,13].

PAMPs	TLR usage	PRRs involved in recognition
DNA	TLR9	AIM2, DAI, IFI16
RNA	TLR3, TLR7, TLR8	RIG-I, MDA5, NALP3
Structural protein	TLR2, TLR4	

Table 1.1 – Viral PAMP detection by TLRs and other PRRs.
(adapted from Kaway & Akira, 2011)

Sensing of the invading viral pathogen through the appropriate PRR(s) triggers multiple and distinct signalling pathways, activating transcription factors such as interferon regulatory factor (IRF) 3 and 7 as well as Nuclear Factor-kappaB (NF-κB). Activation of these pathways leads to the secretion of proinflammatory cytokines and chemokines that are involved in both innate and adaptive immunity. Some of the most critical mediators in the innate host response to viral infection are the type I IFNs (e.g. IFN-α and IFN-β). Type I IFNs induce the expression of hundreds of IFN-stimulated genes that may have direct antiviral activity and/or modulate innate and adaptive immunity by activating immature DCs, enhancing NK-cell function and promoting survival and effector functions of T and B cells (reviewed by Christensen & Thomsen, 2009) [5].

1.1.2. Adaptive immunity

The adaptive immunity consists of both cellular and humoral immune responses. Cell-mediated immunity involves the activation of cells from both the innate (macrophages, NK cells) and adaptive immune responses (antigen-specific cytotoxic T-lymphocytes), responsible for the release of several cytokines in response to an antigen. The humoral immune response is mediated by the secretion of clonally distributed antigen-specific recognition receptors (antibodies) produced by cells of the B lymphocyte lineage. Together, T and B cells are able to identify and eliminate pathogens through the expression of antibodies. In addition, they are able to generate long-lived immunological memory, which will allow the organism to respond more rapidly to a secondary infection by the same pathogen. This competence of the “memory” components of the adaptive immune system is the fundamental basis of vaccines (reviewed by Bonilla & Oettgen, 2010) [14].

For T cells, two main functionally distinct sublineages exist, one expressing an αβ T cell receptor (TCR) and the other expressing a γδ TCR. αβ TCRs specifically recognize antigens bound to MHC molecules, and participate in the activation of T cells in response to the presentation of antigen. The γδ T cells, however, are thought to participate at the levels of both innate and adaptive immunity, and some, at least, recognize antigens directly, without the requirement for antigen presentation (reviewed by Born *et al*., 2011 and Chen, 2011) [15,16]

Immature αβ T cells leave the thymus as either CD4+ or CD8+ T cells. During viral infection, CD4+ T cells can differentiate into a variety of effector subsets, while CD8+ T cells differentiate into cytotoxic T lymphocyte (CTL), killing virus-infected cells, and also capable of releasing a range of effector cytokines. A subset of CD4+ T cells, known as regulatory T cells (Treg), regulates immune responses by suppressing them. T cells can only recognize peptides that have been degraded and bound to MHC class I or II. The MHC class I molecules (displaying endogenous peptides) are expressed on most somatic cells and interact with CD8+ T cells, whilst MHC class II (displaying exogenous, phagocytosed peptides) have a more limited expression, being restricted to professional APCs (such as DCs or B cells), and interact with CD4+ T cells (reviewed by Bonilla & Oettgen, 2010) [14].

Upon interaction with cognate antigen presented by professional APCs, such as DCs, CD4+ T cells can differentiate into classical Th1 cells and Th2 cells, the more recently defined Th17 cells, follicular helper T (Tfh) cells, and induced regulatory T (iTreg) cells. Differentiated Th cell subsets secrete different cytokines that engage other leucocytes including macrophages, mast cells, eosinophils, neutrophils, natural killer (NK) cells, and B cells. Moreover, they can

express different profiles of cell-surface molecules that determine their effector cell capacity. The effector T cells had been thought to be terminally differentiated lineages, but it now appears that there is considerable plasticity allowing for conversion to other phenotypes (reviewed by Zhou *et al*., 2009) [17].

When immature CD8+ T cells interact with MHC class I – peptide complexes presented by professional APCs, they differentiate into CTLs that actively destroy any infected cells presenting the recognized foreign peptides. Activated CTLs up regulate perforin expression, which is stored in cytotoxic granules and released upon the recognition of an infected cell. Perforin is a pore-forming protein that leads to osmotic lysis of the target cells and subsequently enables granzymes to enter the target cells and initiate apoptotic cell death. In addition, the high levels of cytokines secreted by CD8+ T cells, induce an antiviral state in neighbouring cells, and apoptosis of the infected cell (reviewed by Smith-Garvin *et al*., 2009) [18]. Unfortunately, in cases of large-scale killing of virus-infected cells, the CTL activity may result in some degree of damage to the host organism.

Naive B cells express clonally distributed Immunoglobulin antigen receptors on their surfaces that can be activated in a T-cell dependent or independent manner; however, two signals are always required to initiate activation. During T cell-dependent activation, an APC (macrophage or DC) presents a processed antigen to a Th cell, priming it. When a B cell processes and presents the same antigen to the primed Th cell, the T cell releases cytokines that activate the B cell. B cell activation can also occur through recognition of native antigen, or if a macrophage presents several copies of the same antigen in a way that causes cross-linking of antibodies on the surface of B cells. As a result of the subsequent initiation of specific signal transduction

pathways, naive B cells undergo clonal proliferation and terminal differentiation into short-lived antibody producing plasma cells or long lived memory B cells. Antibodies of all Ig classes can be produced in response to viral infections, and can significantly influence the outcome of the infection (reviewed by Bonilla & Oettgen, 2010) [14].

The function of antibodies in response to viral infections can be diverse. The major mechanism is antibody-mediated viral neutralization, occurring when antibodies bind the virus molecule that interacts with its cell-surface receptor, preventing virus attachment. Antibodies can also aggregate many infectious particles, resulting in their phagocytosis, therefore reducing the number of viruses that can effectively infect cells. Additionally, antibodies can act in concert with the complement system, IFN and other cytokines in order to clear viruses from persistently infected cells.

Following virus elimination, the pool of specific T and B cells substantially contracts, leaving a small population of antigen-primed memory cells, from which two major subsets of memory T cells are evidenced. A first line of specific defence is provided by effector memory T cells, in case of reinfection with the same or an antigenically related pathogen. Additionally, an expanded population of so-called central memory cells persists in the secondary lymphoid organs; these cells serve as a pool from which secondary waves of effector T cells may rapidly be derived, should the pathogen challenge overwhelm the forward defences (reviewed by Bonilla & Oettgen, 2010) [14].

In conclusion, evolution has shaped the immune system according to its challenges resulting in different strategies to control different infections. These strategies are selected according to the replication site of the organism (intracellular or extracellular), and other critical

factors such as the route of entry and form of antigen presentation pathway used by the pathogen, which in turn determine selection of the most appropriate component of the immune response (e.g. antibody or T cell). Whatever the balance of the acquired immune system that is selected, it must be precisely regulated. It is necessary to have a fast and potent innate response as a first line of defence and as a signal to activate the specific and adequate adaptive immune response, in which both T cells and B cells act in concert to clear virus infection. It is equally important to terminate the response (see Fig.1.1).

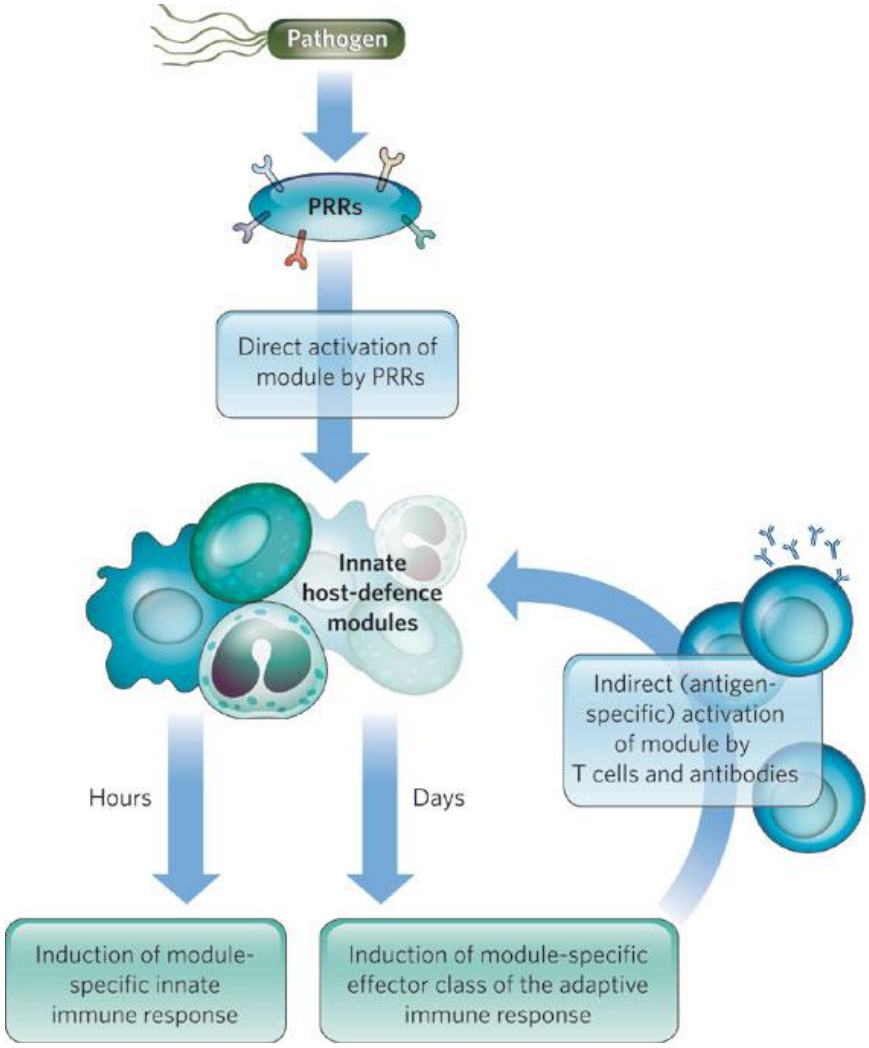

Figure 1.1 – Activation of host-defence mechanisms.

Host-defence mechanisms can be induced directly, by engagement of PRRs, or indirectly, by T cells and/or antibodies. Each module is characterized by distinct antimicrobial defence mechanisms and can instruct the adaptive immune system to mount a response involving a module-specific effector class. After an adaptive immune response has been initiated, it results in antigen-specific activation of the same innate immune module that instructed the adaptive immune response (adapted from Medhzitov, 2007).

1.2. The Interferon System

In 1957, Isaacs and Lindenmann described the occurrence of a factor secreted by infected cells, able to inhibit viral replication in cells infected with homologous or heterologous viruses. They baptized this factor interferon (IFN) and conclusively demonstrated that IFN was a cellular product, acting to protect cells from viral infections [19,20,21].

The IFNs are now also recognized as central regulatory mediators of the immune response. The functions of IFNs are represented by three major biological activities: antiviral activity, antitumor activity and immunoregulatory activity. Fifty years have passed since the discovery of the interferon system, and much has been learnt about induction of IFN, IFN receptor signalling and IFN-dependent antiviral immunity. IFNs produced by infected cells are released and stimulate an antiviral state in neighbour cells, inducing the expression of proteins that interfere with viral processes, whereby viral replication is blocked or impaired. IFNs also have a major role in activation of the adaptive antiviral immune response. Immature plasmacytoid DCs (pDCs) are natural IFN-producing cells, and one of the key cells in the IFN-α response to immune stimuli. pDCs differentiate into mature antigen-presenting DCs, which have a crucial role in T and B cell activation (reviewed by Fitzgerald-Bocarsly & Feng, 2007) [22].

Interferon was the earliest described member of the class of protein molecules now known as cytokines: a soluble product released from stimulated cells that serves to communicate between cells of the immune system [22]. IFNs are key cytokines in the establishment of a multifaceted antiviral response. Three distinct types of IFNs are now recognized (type I, II, and III), based on their structural features, receptor usage and biological activities.

Type I IFNs (IFN-α/β/ω/ε/κ in humans) possess strong antiviral activity, and are able to induce a potent antiviral state in a wide variety of cells. However only IFN-α and IFN-β are induced directly in response to virus infection. In humans, there are 30 genes coding for type I IFN, including 13 IFN-α genes, one IFN-β gene, one IFN-ω gene, one IFN-ε gene, one IFN-κ gene and 13 additional pseudogenes of the IFN-α and -ω families. The functional activities of this complex gene family are yet to be explored. These molecules signal through a ubiquitously expressed receptor composed of two chains: IFN-αR1 and IFN-αR2 (reviewed by Chelbi-Alix & Wietzerbin, 2007 and Hardy *et al*., 2004) [23,24].

There is only one type II IFN, known as IFN-γ, and which is secreted mostly by activated Th1 cells and NK cells and stimulates cell-mediated immune responses that are critical for the development of host protection against pathogenic intracellular microorganisms, such as the activation of macrophages for microbicidal activity. It also plays a central role in the development of antitumor immune responses, and it can amplify the induction of antiviral activity by IFN-α or -β, although antiviral activity is not the primary biological function of IFN-γ. Type I and type II IFNs often work together to activate a variety of innate and adaptive immune responses that result in the induction of effective antitumor immunity and the elimination of viral infections. It signals via a ubiquitously expressed receptor composed of the IFN-γR1 and IFN-γR2 subunits (reviewed by Young & Bream, 2007) [25].

The most recent addition to the IFN family, the type III IFNs, demonstrate structural features of the IL-10-related cytokines, but also elicit a similar antiviral response as IFN-α/β in a variety of target cells. In humans, the type III IFN family is composed by 3 distinct but closely related IFN-λ proteins: IFN-λ1, -λ2, and -λ3. The IFN-λ proteins bind

and signal through a receptor complex composed of the unique IFN-λR1 chain. Like the type I IFNs, their tissue distribution, specificity and regulatory mechanisms are not well understood (reviewed by Donnelly & Kotenko, 2010) [26].

Different human IFN-α's and IFN-β, IFN-γ, and IFN-λ's can establish an anti-viral state in vitro; this is the essential signature of IFNs. However, the existence of three types of IFN, using three different receptors, raises the possibility of different roles in host defence against viruses and other pathogens.

1.2.1. Induction of IFN expression

Upon viral infection, cells are able to recognize the invading microorganism and, through multiple distinct routes that culminate in the induction of IFN, rapidly initiate antiviral mechanisms. The importance of any individual route of IFN induction depends upon the specific virus, the nature of the cell being infected or the stage of infection. Cells express PRRs that recognize viral PAMPs, differentiating them from self. Whenever a cell senses a virus infection, signal transduction pathways are activated, inducing the expression of type I IFN, a major component of the innate immune system. Type II IFN, in turn, is produced by activated lymphocytes and further amplifies the IFN response to infection (reviewed by Zhang *et al*., 2008) [27].

Both type I and type II IFNs are important in the coordination of the innate and adaptive immune responses to a viral infection, making IFNs powerful and eventually hazardous molecules, if not tightly regulated. For this reason, mechanisms have evolved to control the expression of IFN at both transcriptional and post-transcriptional

levels. The pathways leading to expression of either type of IFN are quite distinct, and so will be described separately.

1.2.1.1. Induction of Type I IFN

Virus infection of a cell induces the development of an antiviral state within the infected cell and due to the concomitant secretion of IFN, establishes an anti-viral state in nearby cells. The first potent IFN inducer to be identified was double-stranded RNA (dsRNA), a molecular pattern associated with viral infection, because it is produced by most viruses at some point of their replication [28]. It was postulated that dsRNA can mimic IFN induction by viruses. This finding facilitated investigations of the mechanism of IFN induction without the complexity of an associated viral infection [23]. Currently, the best studied model is the production of IFN-β in fibroblast cells in response to either RNA viruses such as Sendai virus (SeV) or the synthetic chemical that mimics dsRNA, the polyinosine-polycytidylic acid (Poly (I:C)).

Type I IFN expression can be induced by several different mechanisms. However, the downstream kinases and transcription factors are common to all. Virus- or dsRNA-induced expression of type I IFN is controlled by sequences present in the 5' flanking region of the IFN-α/β genes.

Two families of transcriptional factors play a major role in the transcriptional activation of type I IFN genes: the family of NF-κB and the family of interferon regulatory factors (IRF).

In unstimulated cells, NF-κB proteins (p65 and p50) exist as homo- or heterodimeric proteins which are retained in the cytoplasm by association with inhibitory κB (IκB) proteins. Upon virus infection,

stimuli derived from the antiviral response activate the IκB kinase (IKK). This kinase is responsible for the phosphorylation of serine residues within the N-terminal destruction box of IκB proteins (e.g. S32 and S36 of IκBα). Phosphorylated IκB is subsequently ubiquitinated and degraded by the proteasome, thus unmasking the nuclear localization signal of the NF-κB proteins, which translocate into the nucleus and bind to type I IFN promoter [29].

The family of IRF transcription factors mediate virus-, bacteria- and IFN-induced signalling pathways and as such play a critical role in antiviral defence, immune response, cell growth regulation and apoptosis. To date, IRF-1, IRF-3, IRF-5, IRF-7 and IRF-9 have been described as major regulators of type I IFN transcription, in concert with the transcription factor NF-κB [30].

The availability of genetically modified mice, which have distinct IRF deleted, has revealed the function of the members of the IRF family. Table 1.2 summarises what is known about IRF involved in the transcription of type I IFN genes:

IRF	Defects
IRF-1	Apoptosis, iNOS, IL-12
IRF-3	Down modulation of type I IFN induction Increased susceptibility to infection
IRF-5	Induction of inflammatory cytokines (IL-6, TNF-α and IL-12)
IRF-7	Block in the type I IFN induction
IRF-9	Type I and II IFN signalling, induction of IRF7, IFN-α and ISG

Table 1.2 – Phenotypic changes in IRF null mice.
(adapted from Paun & Pitha, 2007)

IRF-1 was identified by its ability to bind to the positive regulatory domain 1 (PRDI) in the virus-responsive element (VRE) of the IFN-β gene, where it was assumed to function as an activator of transcription [31]. However, although IRF-1 is present in the IFN-α and IFN-β enhanceosomes, binding to the respective promoter regions, it does not have a critical role in the virus stimulation of type I IFN genes. Instead, IRF-1 was shown to be involved in the antiviral defence mediated by IFN-γ and to play a critical role in the inducible expression of MHC class I and apoptosis [32,33]. More recently, it was demonstrated that IRF-1 is not required for IFN expression, but it is needed for expression of interferon-stimulated genes (ISGs) [34]. Thus, IRF-1 may uniquely control IFN-independent signalling events that lead to ISG expression and antiviral immunity.

IRF-3 and IRF-7 were identified by their ability to activate the promoters of IFN-α and -β genes. The identification of these two IRFs and their role in the transcriptional activation of Type IFN genes had a major impact on the understanding of the molecular mechanism of the pathogen-induced innate antiviral response [35,36]. Although pathogen recognition may be mediated by distinct cellular receptors and signalling pathways, they all lead to the activation of IRF-3 or IRF-7 which are critical for the transcriptional activation of Type I IFN genes [37,38]. The IFN-β enhanceosome not only contains IRF-3 but also IRF-7 [39]. In addition, several authors have gathered evidence suggesting that relative levels of IRF-3 and IRF-7 in cells determine the levels of expression of individual IFN-α subtypes (reviewed by Paun & Pitha, 2007) [40].

IRF-3 is ubiquitously expressed in a variety of cells, but remains in the cytoplasm as an inactive monomer until detection of a viral PAMP, such as dsRNA, a common signature of virus-infected cells [35]. Both

TLR-3 and RIG-I/MDA-5 signalling pathways lead to the phosphorylation of IRF-3 at the carboxyl-terminal region (serines 385 and 386) and at the serine/threonine cluster (between region 396 and 405), by the IKK-related kinases, TANK-binding kinase (TBK)-1 and IKKε. Serine 386, at C-terminal region, is critical for activation, as it is predicted to lead to a conformational change that allows IRF-3 to homo- or heterodimerize with IRF-7 [38,41,42]. Following translocation to the nucleus, IRF-3 associates with the co-activator CREB binding protein (CBP)/p300 and stimulates transcription of IFN-β [43], as well as some ISGs, such as CCL5/RANTES and ISG54 [44].

Several observations underline the importance of IRF-3 in the induction of the antiviral response. First, being ubiquitously expressed, IRF-3 is capable of stimulating the antiviral response and synthesis of IFN-β in all varieties of infected cells. Second, several viruses target IRF-3, thus preventing the induction of Type I IFN. IRF-3 is required for type I IFN induction triggered by TLR3/TLR4, cytosolic RNA sensing or cytosolic DNA sensing pathways in many cell types, including cDCs, but it is not required for type I IFN induction in pDCs [40].

Like IRF-3, the IRF-7 transcription factor is also expressed as an inactive monomer in the cytoplasm of cells, and after virus induction it is phosphorylated on C-terminal serine residues (serines 477 and 479) by the same kinases, TBK-1 and IKKε. The activated IRF-7 forms either homodimers with itself or heterodimers with IRF-3, and then translocates into the nucleus. It has been described as being critical for the induction of IFN-α and IFN-β gene expression, functioning even in the absence of IRF-3 [45]. IRF-7 is also able to form complexes with myeloid differentiation primary response gene 88 (MyD88), IL-1 receptor-associated kinase (IRAK)-4, IRAK-1 and TNF receptor associated factor (TRAF)-6 upon TLR7/TLR9 stimulation, resulting in

IFN-α production by an IKK-α (and not TBK1) dependent pathway [46,47,48].

Constitutive expression of IRF-7 is restricted to some lymphoid cells, particularly pDCs that express high amounts of IFN-α in response to TLR7/8 and TLR9 activation [40]. When induced to differentiate, monocytes express IRF-7, which was shown to be a key regulator of the differentiation of monocytes to macrophages [49]. Expression of IRF-7 can be induced in most cells types, not only by Type I IFN but also by TNF-α [50]. Finally, IRF-7 has a short half-life, a characteristic that may play a role in the regulation of the transient expression of IFN-α genes [51].

Constitutive expression of IRF-5 is restricted to few cell types, such as monocytes and DCs that express high levels of IFN-α upon viral infection. IRF-5 is mainly expressed in the cytoplasm of non-infected cells and, upon viral infection, is phosphorylated and activated by distinct kinases. The activated IRF-5 forms either homodimers with itself or heterodimers with IRF-3 and then translocates into the nucleus. Both RNA and DNA viruses can activate IRF-5 nevertheless this activation is virus-specific [52]. Like IRF-7, MyD88-mediated activation of IRF-5 involves the formation of a complex composed by MyD88, IRAK4, IRAK1 and TRAF6. Most likely, this complex preferentially assembles with IRF-7 [46,53].

IRF-5 seems to have a role in DNA-damage-induced apoptosis and the innate immune antiviral response [54]. Activated IRF-5 contributes to the induction of IFN genes. However, in the presence of high levels of IRF-4 or MyD88-activated IRF-7, the role of IRF-5 is limited to the transcriptional regulation of early inflammatory chemokines and cytokines [55]. Since most of these chemokines have lymphocyte-

chemotactic activity, it was suggested that IRF-5 may have an important role in lymphocyte trafficking [56,57]. Recently, a new role was described, in which IRF-5 expression in macrophages is responsible for initiating a potent Th1-Th17 response [58].

The transcription of the IFN-β gene requires an enhancer element located upstream of the core promoter that is recognized by three distinct sets of transcription factors (NF-κB, IRFs and ATF-2/cJun) and by the high-mobility group (HMG) chromatin-associated protein HMGI(Y) [59]. This enhancer element is composed by four positive regulatory regions (PRDI-IV): PRDI and PRDIII sites are for binding of IRF-7 and IRF-3, the PRDII site is for binding of NF-κB, and PRDIV site is for binding of ATF-2/cJun heterodimers. Virus infection leads to coordinated activation of all three types of transcription factors, which assemble on the IFN enhancer region to form a large, multi-subunit complex known as the IFN-β enhanceosome [32]. In a first phase, after being delivered to a single IFN allele, NF-κB plays a crucial role in the recruitment of the remaining factors to the enhanceosome, leading to IFN-β transcriptional activation which, in turn, activates transcription of IRF-7. At a second phase, the increasing levels of IRF-7 trigger enhanceosome assembly on multiple IFN-β alleles, thus amplifying the production of IFN-β [60]. In order to have an optimal induction of the IFN-β promoter, cooperation between all transcription factors is required. Since virus infection is the only known signal that can activate all of the IFN-β transcriptional activators simultaneously, enhanceosome assembly will not occur in response to other signals that can separately activate each of the transcription factors [32,61]. However, under conditions in which NF-κB or ATF-2/c-Jun are not activated, expression of IFN-β has been reported. In opposition, IRF-3 and IRF-7 are indispensable for induction [62].

The promoter region of IFN-α genes contains only binding sites for IRFs, lacking binding sites for NF-κB. Although the identity of the IRF members that stimulate IFN-α transcription is uncertain, there is some evidence that IRF-7 is required for induction. In pDCs, which constitutively express IRF-7 and induce the expression of massive amounts of type I IFN, the induction of IFN-α is not dependent on primary induction of IFN-β and its feedback loop [62].

The activation of the different mechanisms leading to IFN expression requires, as a first step, recognition of the viral infection by the host cell. The discovery of pattern associated molecular patterns (PAMPs) and their recognition by cellular pattern recognition receptors (PRRs) has revolutionized our understanding of innate immunity, and explains how and why a virtually unlimited number of pathogens can be recognized by a small number of innate immune receptors, triggering anti-microbial responses.

PRRs recognize common patterns of a large number of microbial molecules, and must be able to differentiate microbial molecules from host molecules that often have substantial structural similarity. An inappropriate decision in this self-nonself discrimination can be fatal to the host. This is particularly important for recognition of viral components that are similar to self components. At first sight, and paradoxically, host receptors specialized in virus detection have evolved to recognise a feature that is common to all living organisms: the nucleic acids. In order to be able to discriminate self nucleic acids from non-self, viral sensors may be compartmentalized in locations where the host cell component is not found, for example, viral DNA is sensed in the cytoplasm and viral RNA is detected in the endosomes of infected cells.

There are at least two major complementary receptor systems (see Fig. 1.2 and 1.3) that detect most viral products: one class of receptors detects viral nucleic acids in endosomes of specialized cell types, whilst the second class of receptors are expressed ubiquitously and localized in the cytosol, where they are able to detect viral nucleic acids produced upon infection [63]. In addition to viral nucleic acids, several viral proteins have been shown to induce IFN, although this is not a general feature of viruses. For example, the fusion (F) protein of respiratory syncitial virus (RSV) and the glycoprotein G of vesicular stomatitis virus (VSV) can activate the synthesis of IFN type I through a TLR4-dependent pathway [64].

The distinct classes of antiviral PRRs and the strategies employed by the host for the successful detection of a viral infection will be briefly described.

1.2.1.1.1. The Toll like receptor pathway

Toll-like receptors (TLRs) are a family of PRRs that play central roles in innate immune defence against infection by binding to microbial molecules.

All TLR family proteins consist of an extracellular ligand binding domain, a single transmembrane TM domain and an intracellular signalling domain. The extracellular domains of TLRs possess N-terminal leucine-rich repeats (LRR) motifs, known to be important for ligand binding and associated signalling. Each TLR LRR structure is distinct, having specific adaptations that allow for improved interaction with either its respective ligand or a coreceptor-ligand complex. Given that the overall shape of the TLR-ligand complexes is strikingly similar, ligand-mediated activation of the receptors can lead to the formation of homo- or heterodimers. Dimerization of the extracellular domains

requires involvement of the TM domains and directs juxtaposition and activation of the intracellular domains. The intracellular signalling domains contain an intracellular Toll/IL-1R (TIR) motif, important in protein-protein interactions. This motif is also present in the signalling adaptors that are recruited to the ligand-activated TLR TIR domains, forming the first step in the signalling cascade leading to the expression of multiple genes involved in innate and adaptive immunity, including type I IFN (reviewed by Kang & Lee, 2011) [65].

TLRs are primarily expressed in sentinel APCs of the immune system such as macrophages and DCs, but can also be present in epithelial cells. The cellular expression of the different TLRs is heterogeneous: For example, TLR3 is expressed by mDCs and NK cells, whereas TLR7 and TLR9 are expressed by macrophages and both mDCs and pDCs, the latter being known to produce high levels of type I IFN in response to viral infection (reviewed by Moresco *et al*., 2011) [66].

The cellular localization of TLRs has important consequences for ligand accessibility and can also affect downstream signalling pathways. TLR1, TLR2, TLR4, TLR5 and TLR6, involved in the recognition of microbial membrane components, are located on the cell surface, while TLRs recognizing microbial nucleic acids are mainly located within endolysosomal compartments (TLR3, TLR7, TLR8, and TLR9). TLR4, normally present at the surface can also enter the endocytic pathway following ligand-mediated activation. In each case, the TIR motif resides in the cell cytoplasm while the LRR domain is positioned to detect either extracellular PAMPs (outside of the cell) or PAMPs acquired during sampling (within the endosomes). Compartmentalization of TLRs is used by the host cell as a strategy to limit access to self molecules. The endosomal localization of nucleic acid-sensing TLRs (TLR3 for dsRNA, TLR7/8 for ssRNA and TLR9 for

CpG DNA motifs) prevents the recognition of self nucleic acids and activation of signalling pathways in the absence of infection. In addition to its intracellular expression, TLR3 was also detected on the surface of a few cell types, including fibroblasts, but until now no studies were published on the comparison of the physiological significance of intracellular versus cell surface TLR3 (reviewed by Barton & Kagan, 2011) [67]. Trafficking of endosomal receptors to endolysosomal compartments by UNC93B1 and proteolytic regulation of some TLRs (TLR7 and TLR9) are other strategies used to further control receptor activation [68,69,70].

In addition to recognizing distinct ligands, individual TLRs trigger different signal transduction pathways. This specificity is achieved by the engagement of different adaptors to different receptors, through interaction between the corresponding TIR domains. The particular signalling adaptor used determines which signalling pathway will be activated: TIR-containing adaptor MyD88 induces a pro-inflammatory response dependent on the activation of NF-κB and mitogen-activated protein (MAP) kinase, whereas TIR domain-containing adaptor protein inducing IFN-β (TRIF) is responsible for activation of IRF-3, IRF-7 and NF-κB, culminating in the induction of type I IFN and inflammatory cytokines (reviewed by Moresco *et al*., 2011) [66].

Most TLRs recruit MyD88, although some require the sorting adaptor TRIF-related adaptor molecule (TRAM) to facilitate binding between the receptor and the signalling adaptor MyD88 in order to initiate the signalling transduction pathway [71]. Activated TLR7, TLR8 and TLR9 also signal through MyD88, not only to induce expression of pro-inflammatory cytokines and chemokines, but also to initiate the expression of IFN-α, through IRF-7 (but not IRF-3). Upon TLR7/9 stimulation, IRF-7 forms complexes with MyD88 and TRAF6. After

being phosphorylated in an IRAK1-dependent manner, IRF-7 translocates to the nucleus and binds the promoter of the IFN-α gene [46]. Combining the observations that pDCs have an efficient mechanism for retaining CpG DNA in the endosomes, and also have a high constitutive expression of IRF-7, it is not surprising that these cells express huge amounts of IFN-α.

The signalling of TLR3 is induced through TRIF, and not MyD88. Activated TRIF associates with TRAF3 and TRAF6 and subsequently, with the noncanonical kinases TBK-1 and IKKε (Fig.1.2), phosphorylates IRF-3 and IRF-7, leading to its dimerization and nuclear translocation to bind the promoter of type I IFN. TRIF also mediates the activation of NF-κB and activating protein 1 (AP-1) through the complex of kinases IKK-α/β/γ. These two transcription factors translocate into the nucleus, together with IRF-3 and IRF-7, and bind to the PRDI-IV positive regulatory elements of the IFN-β enhancer region [72]. TLR3-mediated signalling also leads to phosphorylation of specific tyrosines and the recruitment of phosphatiylinositol 3-kinase (PI3K), essential for full activation of IRF-3 [73,74].

Virus infected cells mainly depend on TLR3, TLR7/TLR8 and TLR9 to induce the expression of type I IFN, following detection of viral nucleic acids. However, TLR4 is also capable of inducing type I IFN by the recognition of non-nucleic acid ligands, such as lipopolysaccharide (LPS). Upon LPS-binding, TLR4 initiates signalling transduction pathways through both MyD88 and TRIF adaptors. Signalling through MyD88 requires TRAM [71] and culminates in the activation of NF-κB, inducing an early pro-inflammatory response. TLR4 is then internalized into the endosome [75], where it requires the TIR domain-containing adaptor protein (TIRAP) to bind TRIF [76]. Through this pathway,

TLR4 induces both a late pro-inflammatory response (NF-κB dependent) and type I IFN expression.

Recently, a TLR2-dependent antiviral signalling pathway leading to the production of type I IFN was reported in inflammatory monocytes. Like TLR4, TLR2 recognizes certain viral proteins, and when it does so, it is internalized into endosomal compartments. However, in contrast with TLR4, all TLR2 signalling is MyD88 dependent. Thus, inflammatory monocytes are able to use TLR2 to activate unique MyD88-dependent pathways culminating in the activation of IRF3, IRF7 and NF-κB [77].

TLR signalling and subsequent functions must be under tight negative regulation because excessive activation over time contributes to the pathogenesis of autoimmune, chronic inflammatory and infectious diseases. This regulation is achieved by several kinds of mechanisms, from which degradation, deubiquitination, and competition are most frequently observed. The establishment of these regulatory mechanisms usually use a mode of negative feedback. Termination of TLR signalling occurs in response to its overactivation, and can be accomplished either by inducing expression of negative regulators or by processing constitutively expressed factors (reviewed by Wang et al., 2009) [78].

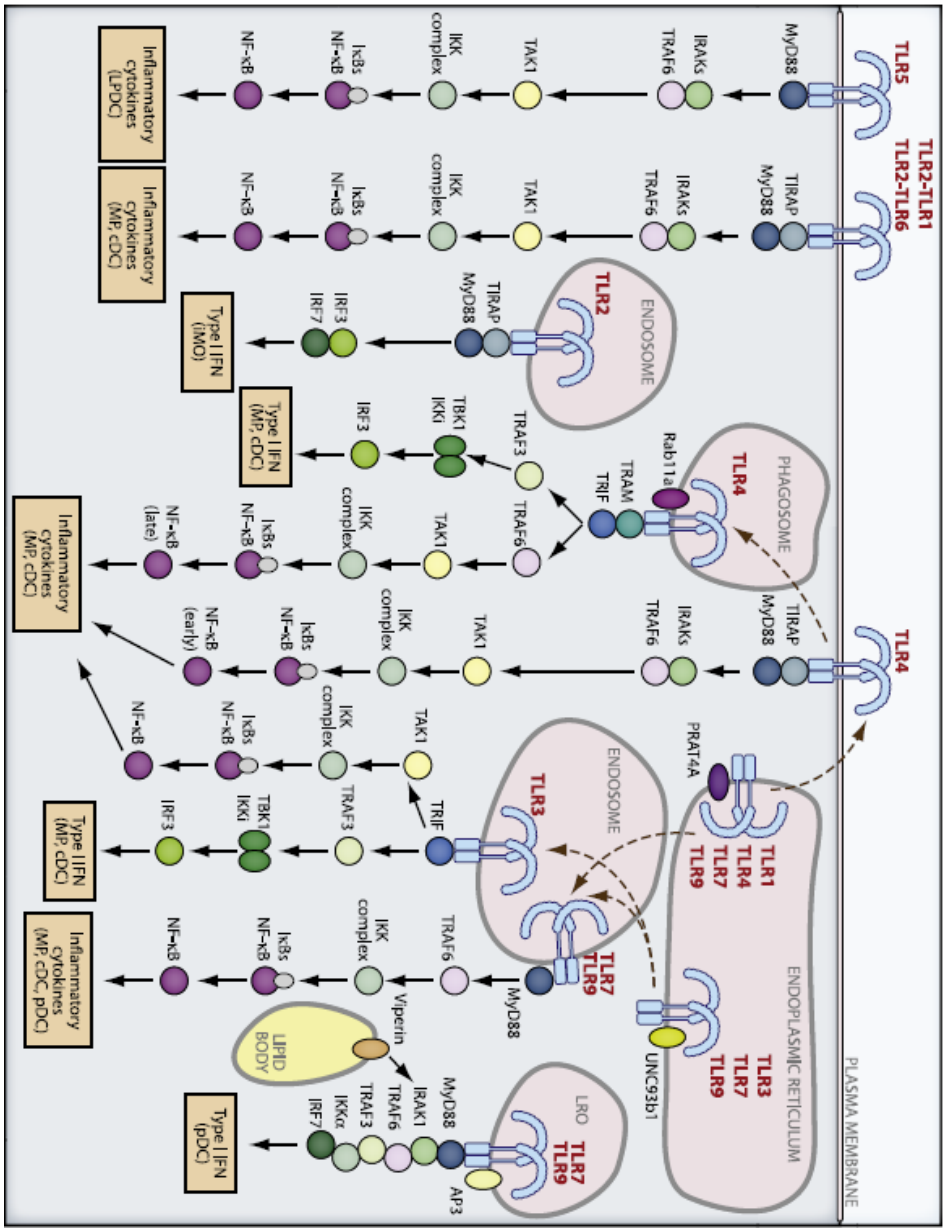

Figure 1.2 – TLR trafficking and signalling. (from Kaway & Akira, 2011)

1.2.1.1.2. The cytosolic recognition pathway

All viruses, even those replicating within the nucleus (herpes viruses), include a cytoplasmic phase in their replication strategy; for example, viral genome amplification and/or mRNA metabolism and viral protein expression. Within the cytosol, there are specific PRRs that recognize viral nucleic acids, such as RIG-I-like receptors (RLRs), nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs), and DNA receptors (cytosolic DNA sensors). Like TLRs, cytoplasmic sensors activate signalling transduction pathways leading to the production of type I IFN and pro-inflammatory cytokines (reviewed by Wilkins & Gale, 2010) [79,80]

The RLR family is composed by three RNA helicases, RIG-I, MDA-5 and Laboratory of Genetics and Physiology (LGP)-2. RIG-I and MDA5 are ubiquitously expressed in most cell types and are able to recognize viral RNA in the cytoplasm, leading to induction of IFN. On the other hand, LGP2 acts as a negative regulator of IFN gene expression, most probably by masking viral dsRNA from recognition by RIG-I and MDA5 [81,82].

RIG-I and MDA5 contain a C-terminal DExD/H box RNA helicase domain and two N-terminal caspase activation and recruitment domains (CARDs). Interaction of the DExD/H box with viral RNA induces its ATP catalytic activity and leads to conformational changes that allow the interaction between the CARD domains of the helicases and a CARD domain containing adaptor, initiating a downstream signalling cascade [82]. RIG-I and LGP2 have a C-terminal repressor domain, which maintains the molecule in an inactive conformation until binding to a nonself ligand.

Although structurally similar, RIG-I and MDA5 are not redundant and are responsible for IFN induction by different sets of viruses. This virus specificity may be the result of the distinct recognition of particular RNA structures or nucleotide composition by each sensor. For example, RIG-I specifically binds to a free 5´-triphosphate RNA structure. This feature probably allows for discrimination between self and non-self RNA, since 5´-ends of most endogenous RNAs are either capped or post-translationally modified to remove the 5'-triphosphate [73,82,83,84].

The adaptor for RIG-I and MDA-5 was identified and, although named differently by different groups, the recommended name is now mitochondrial antiviral signalling protein (MAVS) [37,84,85]. The MAVS protein is found in the outer mitochondrial membrane, a location essential for its function [38,85,86]. The interaction between the CARD domains of RIG-I and MDA5 with the CARD domain of mitochondrial MAVS leads to the activation of two IκB kinase-related kinases, TBK-1 and IKKε, responsible for the phosphorylation of IRF-3 and IRF-7. In addition, MAVS also activates NF-κB by a TRAF6 dependent pathway. The activated transcription factors translocate into the nucleus and initiate transcription of IFN genes (see Fig.1.3) [37,86,87]. MAVS is also present on peroxisomes in several cell types. Peroxisomal MAVS leads to the activation of IRF-1 and IRF-3, which trigger the rapid and direct expression of ISGs. This differential placement of MAVS allows for the diversification of the signalling pathways that are activated after RLR-ligand binding. Peroxisomal MAVS is essential for rapid ISG expression independent of type I IFN, thus initiating an immediate, although transient, antiviral response. Mitochondrial MAVS induces ISGs with delayed kinetics and primarily dependent on type I IFN secretion, promoting a more sustained response later during infection. [88,89]

Recognition of viral DNA in the cytoplasm is carried out by specific DNA sensors. The first cytoplasmic DNA receptor to be identified was the DNA dependent activator of IFN-regulatory factors (DAI). This protein binds B-form DNA (particularly poly(dA:dT)), triggering activation of NF-κB, IRF-3 and possibly IRF-7, thus being responsible for DNA-dependent type I IFN induction in some cell types. However, cells that do not express DAI are still able to respond to viral DNA in the cytoplasm, suggesting that other DNA receptors must exist [90,91]. Recently, two additional proteins involved in the detection of cytosolic DNA and subsequent IFN induction have been identified: RNA polymerase III and interferon-inducible protein 16 (IFI16) (see Fig. 1.3) [92]. RNA polymerase III acts indirectly by transcribing AT-rich DNA into uncapped 5′ triphosphate–bearing RNA, which serves as an agonist for RIG-I [93,94]. IFI16, a member of the pyrin and HIN200 domain (PYHIN)–containing protein family, is a sensor for intracellular non–AT-rich dsDNA [73]. These studies have shown that detection of cytosolic DNA probably requires multiple and possibly redundant sensors that converge on the signalling molecule STING and the kinase TBK-1 and lead to activation of the transcription factor NF-κB and TBK1-mediated phosphorylation of the transcription factor IRF3.

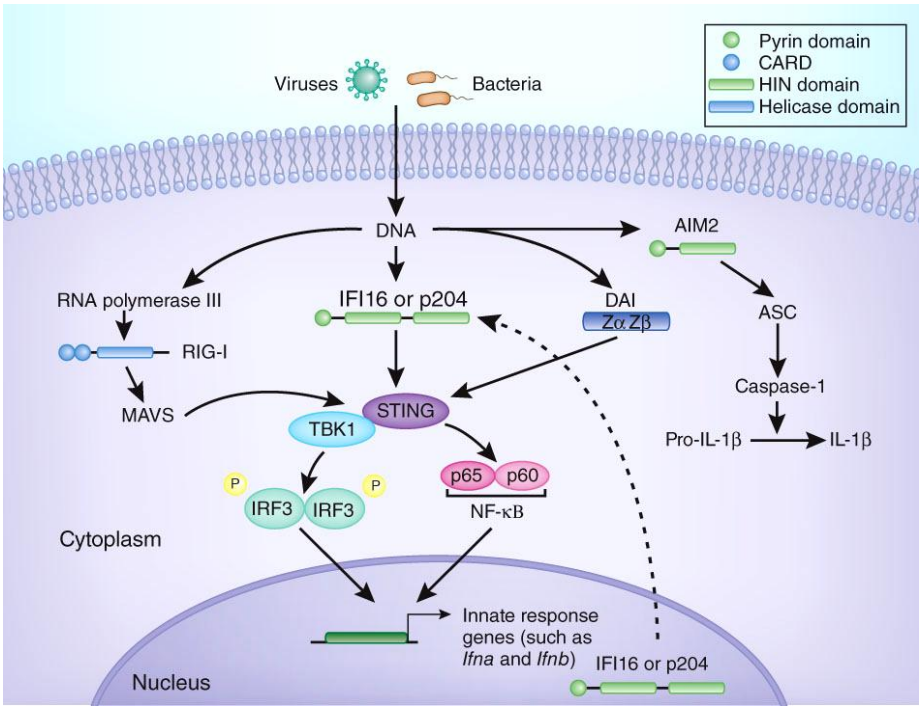

Figure 1.3 – Cytosolic recognition pathways. (from Goubau *et al.*, 2010)

1.2.1.2. Induction of type II IFN

When pathogens invade the host, an early pro-inflammatory response is initiated. Both macrophages and NK cells secrete type I IFNs. Later, differentiated T cells, both CD4+ and CD8+, secrete type II IFNs [7,95]. The majority of IFN-γ produced in response to infection is thus not directly induced by invading pathogens but is instead part of a secondary response. The main cytokine, among others, in the induction of IFN-γ is IL-12, which is also involved in the development of a Th1 response, and can also act synergistically with other cytokines such as IL-2, TNF-α, and in particular IL-18 [7]. NK cells and CTLs are considered to be the main source of IFN-γ. However, other cell types, such as macrophages and DCs, have also been reported to produce type II IFN under specific conditions [7,96].

1.2.1.3. Induction of type III IFN

In 2003, a novel class of IFNs has been identified, and named type III IFNs or IFN-λ. Type III IFNs have functional similarities with type I IFNs, but unlike type I IFNs, which exert antiviral activity on all cell types, type III IFNs target primarily epithelial cells, and consequently play an important role in innate antiviral defences at epithelial surfaces, which constitute a major portal of entry for viral infections [97].

In addition to having similar functions, type I and type III IFNs also have similar expression patterns. In fact, it was determined that type III IFN genes are expressed in response to most classes of viruses and to a variety of TLR agonists, the same stimuli responsible for expression of type I IFN genes. Computer analysis of promoter sequences of type III IFN genes predicted the existence of potential binding sites for several transcription factors, some already known to be involved in the regulation of type I IFN genes transcription, e.g., AP-1, NF-κB, and various IRFs [26]. Accordingly, it was recently demonstrated that both classes of IFNs are induced by transcriptional mechanisms involving IRFs and NF-κB. However, while IFN-β induction requires the coordinated action of a multifactor enhanceosome, and IFN-α expression is activated by multiple IRF-binding cis-promoter elements, the type III IFNs are induced through independent actions of IRFs and NF-κB. Hence, it was proposed that IFN-λ expression is more flexible than IFN-α/β expression, which could allow expression of type III IFNs in response to a wider range of stimuli compared with type I IFNs (reviewed by Iversen & Paludan, 2010) [98].

1.2.2. Signalling responses to IFN

Although type I IFNs (IFN-α/β), type II IFN (IFN-γ) and type III IFNs (IFN-λ) bind to distinct receptors, they can all activate a common

intracellular signalling pathway, regulating many of the same biological activities, including a range of antiviral immune responses [26]. The JAK-STAT pathway was the first signalling pathway shown to be activated by IFNs (Fig.1.4), and extensive studies over the years have firmly established its functional relevance in the interferon system.

Type I IFNs are secreted factors that are recognized by a cell surface transmembrane receptor – the type I IFN receptor. This protein is a heterodimer composed of two subunits, IFN-α receptor 1 (IFNAR1) and IFNAR2, which cytoplasmic domains are associated with the inactive Janus tyrosine kinases, Tyk2 and Jak1, respectively. Prior to stimulation, IFNRA2 is also bound to STAT2 that is, in turn, weakly associated with STAT1. Upon IFN binding to the receptor and subsequent stimulation, the two subunits of the receptor associate and facilitate the activation of Tyk2 and Jak1. The phosphorylation of the tyrosine at position 466 (Tyr^{466}) on IFNAR1 by Tyk2, creates a docking site for the SH2 domain of STAT2, and its subsequent phosphorylation by Tyk2 at Tyr^{690}, while Jak1 phosphorylates STAT1 on Tyr^{701} [64,99,100]. The activated STATs dissociate from the receptor forming a stable heterodimer and associate with IRF-9, forming the ISGF3 tertiary complex that translocates into the nucleus. In this complex, IRF-9 is the major DNA binding component and, in the nucleus, binds to IFN-stimulated response elements (ISRE) present in the promoter region of IFN-stimulated genes (ISGs), inducing their transcription. IRF-9 can also form a DNA binding complex with STAT1 homodimers and with STAT2 alone, and these complexes can bind to DNA with the same specificity as ISGF3 (reviewed by Paun & Pitha, 2007) [40].

Besides tyrosine phosphorylation, additional posttranslational modifications are involved in the type I IFN-induced activation of the Jak-STAT pathway. It was recently proposed that a CREB-binding

protein (CBP)-mediated acetylation cascade, together with serine phosphorylation, also plays a critical role in type I IFN intracellular signalling. The cytoplasmic CBP protein is a mediator for the acetylation of cytokine receptors and their downstream signalling molecules, e.g. IFNRA2, IRF9, STAT1 and STAT2. Acetylation plays a major role in the complete formation and activation of the ISGF3 complex, thus mediating cytokine receptor signal transduction (reviewed by Tang *et al*., 2007) [101].

The type II IFN receptor is also a heterodimer composed of two subunits, the IFN-γ receptor 1 (IFNGR1), which associates with Jak1, and the IFNGR2, which constitutively associates with Jak2. Dimerization of the receptor, upon binding of IFN-γ, leads to association of Jak1 and Jak2 and subsequent activation of Jak2 which, in turn, phosphorylates Jak1. After being phosphorylated by activated Jak1 and Jak2, the C-terminus of IFNGR1 creates a pair of binding sites for STAT1, allowing for its phosphorylation at Tyr^{701}. The phosphorylated STAT1 homodimer dissociates from the receptor and translocates into the nucleus, where it binds to unique elements of IFN-γ stimulated genes, the gamma-activation sequence (GAS), and induces transcription. Of note is the fact that type I IFN stimulation is also able to form STAT1-homodimers, leading to the induction of ISGs containing GAS elements in their promoter region (reviewed by Goodbourn *et al*., 2000) [99].

IFN-λs exert their biological activities by signalling through a heterodimeric receptor complex composed of IFN-λ receptor 1 (IFN-λR1) chain and the shared IL-10R2 chain, which is also a part of the receptor complexes for IL-10, IL-22, and IL-26. Type III IFN receptor is different from other IFN receptors, but activation through either IFN-λ or IFN-α receptor complexes results in initiation of the same Jak-STAT

signal transduction cascade. Although IFN-λR1 is constitutively expressed by a broad range of cell lines and tissues, there are many cell types that do not express IFN-λ receptors, and, as a consequence, cannot respond to this cytokine (reviewed by Donnely *et al*., 2010) [26].

Type I and type II IFN also induce phosphorylation of STAT1 on Ser^{727}, and although this phosphorylation is not required for either nuclear translocation or DNA binding, it is essential for full transcriptional activity of STAT1. This reaction is catalysed by protein kinase C isoform PKC-δ, which is activated by the PI3K pathway, and directly interacts with STAT1 [102,103].

An effective antiviral response requires a rapid and efficient induction of ISGs by the Jak-STAT pathway, in response to IFNs released by infected or activated cells. However, this response must be tightly regulated and terminated once the viral threat is over, in order to avoid damage to the host, and several negative regulators of the Jak-STAT signalling have been already described.

Phosphatases are important regulators of kinase based signalling cascades, inducing dephosphorylation of specific amino acid residues and, consequently negatively regulating activation of effector proteins. In particular, SH2-containing phosphatase (SHP)-1, SHP-2 and protein tyrosine phosphatase 1B (PTP1B) dephosphorylate tyrosine residues on the IFN receptors or on Janus kinases, preventing tyrosine phosphorylation of STATs and their subsequent activation [104,105].

At cytoplasmic level, the Jak/STAT signalling pathway can be inhibited by a negative feedback inhibitor – the suppressor of cytokine signalling (SOCS). Following cytokine signalling, SOCS are activated and recruited to ligand-receptor complexes, causing inhibition or triggering

protein turnover of the IFN receptor through ubiquitin-proteasome-mediated degradation, preventing STAT activation [104,106,107]. PIAS – proteins that inhibit activated STAT, are a family of proteins able to inhibit active STATs at the nuclear level. PIAS1 interacts with tyrosine phosphorylated STAT1, blocking its DNA binding ability, while PIASy acts as a transcriptional co-repressor of STAT1 [104,108]. PIAS proteins can also act as platforms to facilitate both removal and recruitment of other regulatory proteins, such as SUMO proteins [109].

STAT1 activity can be regulated by another cytoplasmic inhibitor, the small ubiquitin-like modifier (SUMO) proteins. SUMO posttranslational modification (SUMOylation) is a dynamic and reversible process that can both decrease STAT1 tyrosine phosphorylation and indirectly facilitate STAT1 dephosphorylation. Phosphorylation of STAT1 at Tyr701 and SUMOylation at the adjacent Lys703 are mutually exclusive, such that Tyr701-phosphorylated STAT1 remains unSUMOylated [110,111]. This results in the formation of semiphosphorylated STAT1 dimers that function as competitive polymerization inhibitors, increasing the solubility of fully active STAT1 molecules, thus inducing its dephosphorylation [110]. Together, these two mechanisms are able to diminish the amount of transcriptionally activate STAT1 in the cell nucleus, protecting cells against hyper responsiveness to IFN-γ [112].

STAT1β is a truncated form of STAT1, lacking the transactivation domain (TAD). This is a naturally occurring form of the protein, formed by differential splicing, that can replace STAT1 in homo- or heterodimers formed following receptor activation. However, STAT1β is non-functional, thus unable to induce IFN-γ dependent transcription. Nevertheless, in response to IFN-α/β, it retains the ability to be incorporated in the ISGF3 complex, and so participate in transcription,

probably due to the presence of a functional STAT2. Therefore, this protein can function as a dominant negative regulator, either by binding to DNA as a non-functional factor, or by interacting and sequestering functional STAT1 [99,104].

Post-translational modification of STAT proteins via ubiquitination is another important means to regulate STAT signalling. Conjugation of ubiquitin to proteins results in their degradation by the 26S proteasome-dependent pathway. This is a common mechanism for the regulation of several cellular processes. Ubiquitination of target proteins requires three enzymes, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3). E3 ligase confers specificity to the reaction, interacting with both E2 and the target protein to facilitate transfer of ubiquitin to the substrate. Regulation of IFN-activated STAT1 levels by the ubiquitin-proteasome pathway was demonstrated in 1996, by Kim and Maniatis [113] and, some years later, PDLIM2 protein was identified as a STAT ubiquitin E3 ligase [114]. It was later reported that phosphorylation of PDLIM2 Ser-137 is required for Ub-P-STAT1 formation and degradation by the 26 S proteasome system [115]. Ubiquitination is the only regulatory mechanism that results in reduced levels of STAT1 in the cell, helping to restrain the inflammatory response.

The Jak-STAT pathway only accounts for some of the biological functions exerted by IFNs in the organism. The PI3K pathway was already mentioned as necessary for the full activation of STAT1. In addition, this pathway can also induce downstream survival or death pathways, in response to IFN [103,116]. Other pathways are also involved in IFN-mediated signalling, such as CRKL, PI3K, and p38 kinase pathways [103,116].

The CRKL proteins were first identified as cellular homologues of viral CRK. These are adaptor proteins that facilitate the formation of various signalling complexes in response to various stimuli, including IFN. In response to type I and type II IFN, Tyk2 phosphorylates CRKL, which binds to STAT5 and forms a complex that translocates into the nucleus, binding to GAS elements present in certain ISGs. In addition, activated CRLK can also activate RAP1, generating growth-inhibitory responses [102,103].

The p38 protein is a member of the mitogen-activated protein kinase (MAPK) family. Members of this family are typically involved in the regulation of gene transcription, apoptosis, and cell cycle. The p38 protein, in particular, was shown to be necessary for the transcriptional regulation of ISGs, in response to type I IFN. This serine-threonine kinase does not affect the activation of the Jak-STAT pathway, but contributes to the growth-inhibitory effects of interferon [103].

In order to exert its diverse biological functions, the produced IFN must activate more than one signalling pathway. A combination of more than one signalling cascade is often required to generate a given biological response. For example, both STAT- and p38-signalling pathways are required to elicit the antiviral effects of IFNs [103].

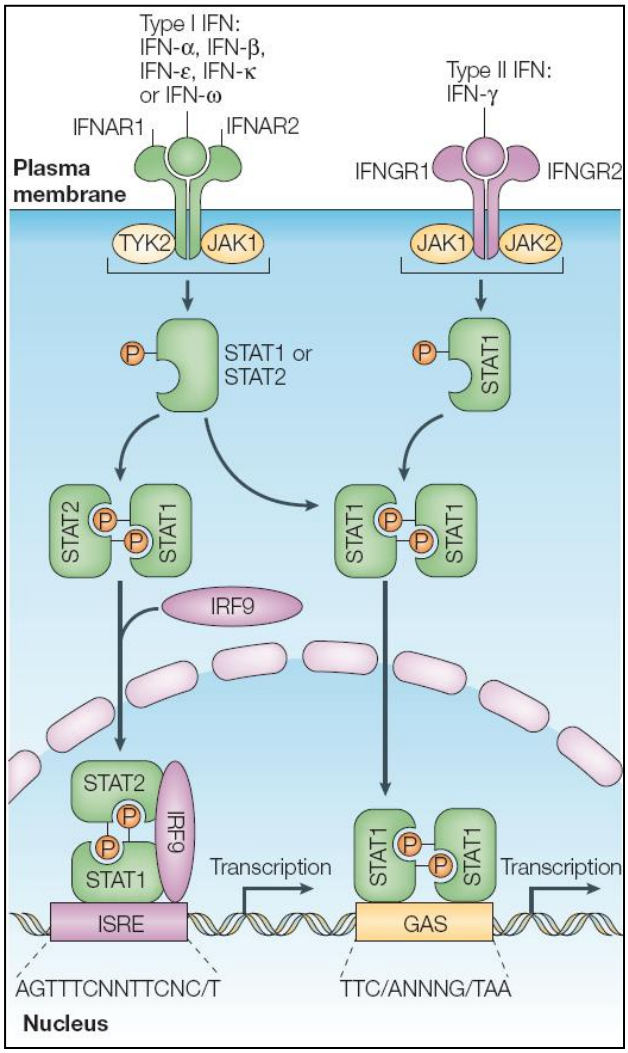

Figure 1.4 – **Activation of classical JAK-STAT pathway by type I and type II IFNs**. (from Platanias *et al.*, 2005).

1.2.3. IFN-induced antiviral state

The induction of an anti-viral state is achieved through the IFN-induced expression of specific sets of genes, the interferon stimulated genes (ISGs), which will limit virus replication and its subsequent spread to neighbouring cells. The best characterized IFN inducible components

are the enzymes dsRNA-dependent protein kinase (PKR), 2',5'-oligoadenylate synthetase (2'5'OAS), and Mx proteins.

Upon binding to dsRNA, the serine/threonine kinase PKR is activated and catalyses phosphorylation of different substrates, such as the α-subunit of the eukaryotic translational initiation factor eIF2 (eIF2α). PKR-induced phosphorylation of eIF2α prevents its recycling, thus resulting in the inhibition of initiation of translation. In addition, phosphorylated eIF2α mediates autophagy, enabling controlled degradation of cell contents. PKR also acts on additional substrates, regulating other antiviral mechanisms such as control of cell proliferation and apoptosis [64,100,117].

The 2'5'OAS protein is also activated by its binding to dsRNA. This protein is then able to oligomerize ATP through a 2'5' phosphodiester linkage, synthesizing 2',5'-oligoadenylates. These molecules bind to endoribonuclease L (RNaseL) with high affinity. The subsequent activation of RNaseL leads to cleavage of ssRNA (including mRNA) and in the inhibition of protein synthesis. 2'5'OAS was also suggested to be involved in IFN-induced apoptosis [64,117].

Mx proteins are IFN-inducible GTPases that belong to the dynamin family of large GTPases. These proteins have been shown to induce an antiviral response against RNA viruses. Mx proteins, in particular, inhibit viral replication at very early stages of the infection, through the recognition of nucleocapsid-like structures. In contrast to PKR and 2'5'OAS, Mx proteins seem to exhibit a certain degree of specificity to certain viruses [100,118].

Other proteins, such as ISG15, ISG54 and ISG56, ISG20, promyelocytic leukaemia (PML) and tripartite motif (TRIM) proteins also play significant roles in the IFN-induced antiviral response. ISG15

is expressed following IFN stimulation and binds covalently to different proteins, protecting them from degradation. Several of these proteins are involved in the induction of IFN and regulation of IFN impact on the antiviral response, e.g. JAK1, STAT1, PKR, RIG-I and IRF-3. Therefore, one of ISG15 major roles appears to be the up-regulation of the IFN response [64].

ISG54 and ISG56 are induced by viral stress. These proteins inhibit translation by interaction with different subunits of the elongation initiator factor eIF3 complex [119]. The ISG20 protein specifically degrades ssRNA through its IFN induced 3'-5' exonuclease activity. When overexpressed, ISG20 inhibits replication of VSV, influenza A virus (FluAV), EMCV, and HIV-1 [117]. The PML gene encodes multiple isoforms, as a result of alternative mRNA splicing, which shuttle between the cytoplasm and the nucleus. PML nuclear isoforms are localized in distinct subnuclear structures known as PML-nuclear bodies (PML-NBs), PML oncogenic domains (POD), nuclear domain 10 (ND10), or Kremer (Kr) bodies. The major function of PML-NBs is to suppress viral transcription, and this activity is enhanced in response to the production of type I or type II IFNs [120].

The TRIM proteins control normal cellular functions. These proteins are constitutively expressed but are up regulated in response to type I IFN in certain cell types. This suggests their involvement in antiviral responses. TRIMα5 was reported to be involved in blocking the ubiquitination of HIV-1 capsid protein [117].

Interferons also induce the activation of downstream signalling pathways that direct the expression of genes involved in the establishment of a pro-apoptotic state or cell-cycle arrest in target cells [62].

1.2.4. Immunomodulation by IFN

The most studied property of the interferon system is its ability to establish an antiviral state. In addition, this system has been shown to be linked to several effector responses of both the innate and the adaptive immune system, with the ultimate goal of eliminating virus infected cells. Accordingly, type I IFNs also regulate the activities of natural killer (NK) cells and cytotoxic T cells (CTLs).

Most somatic cells are capable of producing type I IFN when infected by an intracellular pathogen. However, specialized antigen-presenting pDCs can produce extremely high levels of IFN-α/β upon stimulation of PRRs with virus-derived nucleic acids, without suffering viral infection [121]. This is suggestive of a key role for type I IFN in priming adaptive responses.

The activity exerted by type I IFNs is dependent on the cellular context. Recognition of viral nucleic acids and associated activation of IFN, can initiate cell-integrated apoptosis, as well as the expression of ligands for activation of NK cells and CTLs. Type I IFNs can also act on neighbouring uninfected cells, to induce an alert state. In the absence of other signals, the expression of IFN-inducible genes is activated, with the result that the cells are more sensitive to detection and elimination of the virus [63].

Recently it was demonstrated that IFN-α/β can directly or indirectly influence the fate of both CD4+ and CD8+ T cells during the initial phases of antigen recognition, by enhancing their IL-15-induced proliferation. Secretion of IL-15 by APCs also plays a critical role in the proliferation and maintenance of NK cells. Type I IFNs can also cooperate with T cell receptor and co-stimulatory signals to drive clonal expansion and differentiation of naive CD8+ T cells into effector cells,

and help to sustain the survival of the proliferating cells. Some studies suggested that there are two types of CD8+ T cells, the ones that require type I IFN for expansion and the ones that do not. However, only type I IFN-dependent CTLs provide protective immunity to viral infection [63].

Type I IFNs access and regulate antigen-presenting pDCs, enhancing their ability for cross-presentation of the antigen through the up-regulation of the MHC class I pathway. Together with the IFN-induced up-regulation of other chemokines and co-stimulatory molecules, type I IFNs influence the efficient homing of DCs into secondary lymphoid organs and, consequently, CD4+ and CD8+ T cell responses, including the induction of CTLs [122].

IFN-α/β can also control CD4+ T helper (Th) cell functions, by regulating the development and stability of long-lived memory cells. Type I IFN supports Th1 differentiation, activation of Stat4 signalling and IFN-γ production. At the same time, IFN-α/β acts to restrict the development of alternative populations and their associated effector functions, such as Th2 and Th17 [123].

Regarding the humoral acquired immunity, type I IFNs were shown to promote the generation of antibodies, their switching to opsonizing IgG classes and the establishment of long-lasting B cell memory. These effects are achieved by the action of IFN-α/β on both DCs and B lymphocytes [11]

Type I IFNs can also enhance NK cell-mediated cytotoxicity through their collaboration with NK receptors and consequent elimination of infected cells [63].

All type I IFN members share the capacity of augmenting the expression of MHC class I proteins, therefore promoting CD8+ T cell responses, yet only IFN-γ is capable of inducing the expression of MHC class II proteins, hence promoting CD4+ T cell responses [99,100].

Type II IFN affects the processing of key cellular components for the adaptive immune responses. These include the immunoproteasomes that generate antigenic peptides and also the cellular components that target the peptides for interaction with MHC class I molecules. IFN-γ also acts directly on B cells, increasing the expression of MHC class II and thus increasing antigen presentation, and also in programming immunoglobulin class switching to particular subclasses [124].

Finally, IFN-γ is able to regulate the balance between Th1 and Th2 cells, and the activation of macrophages, as the latter use a variety of IFN-γ induced mechanisms to kill infected cells, such as production of reactive oxygen intermediates [99,100].

1.3. Viral Mechanisms of Immune Evasion

The continuous interaction between viruses and their respective hosts during the course of evolution, has shaped and determined the survival strategies used by viruses and their hosts. As intracellular pathogens, viruses must enter the cell and take control of its machinery with the purpose of replicating and disseminating themselves. In turn, the vertebrate host has evolved an elaborate system of innate and adaptive antiviral immune mechanisms, in order to recognize and destroy pathogen-infected cells. This created a selective pressure on viruses, ultimately leading to the evolution of multiple strategies for virus host evasion. The strategies employed by viruses to evade and

elude the host defence mechanisms can be divided into three main categories: (1) to avoid recognition by the humoral immune response, (2) to interfere with the functioning of the cellular immune response, and (3) to interfere with immune effector functions. The list of such viral mechanisms to manipulate the host immune response is extensive. Some will now be briefly described.

1.3.1. Impairment of the humoral immune response

Effective evasion from the anti-viral B-cell immunity is particularly important for viruses that are sensitive to neutralizing antibodies. A typical example is the influenza virus. The human immune system can rapidly and successfully develop protective immunity against influenza virus type A infections, mostly by directing neutralizing antibodies against the major surface protein of the influenza virus, haemagglutinin. In order to escape this recognition strategy, the virus evolved two forms of antigenic variation, the antigenic drift and the antigenic shift [125]. Both lead to the insertion of changes in critical residues of the haemagglutinin protein on the viral surface. As a result, antibodies produced in response to the viral infection will be unable to bind the epitope, allowing the virus to escape from antibody recognition and neutralization.

1.3.2. Impairment of the cellular immune response

Cell-mediated immune responses play a major role in the elimination of virus infections. By targeting MHC class I and II biogenesis and transportation pathways, viruses are able to interfere with the activation of $CD8_+$ and $CD4_+$ T cells by blocking antigen presentation. Several strategies have been developed by viruses to achieve this purpose, affecting nearly all steps of generation, processing and

presentation of viral peptides or even the processing of MHC molecules.

For example, the Epstein-Barr virus (EBV) codes for a protein called nuclear antigen (EBNA)-1 that efficiently blocks antigen processing by interfering with proteasomal degradation of viral proteins. The Gly-Ala co-repeat (GAr) in EBNA-1 is a cis-acting inhibitor of ubiquitin-proteasome proteolysis, thereby inhibiting the generation of viral epitopes and escaping CTL detection [126].

After the degradation of viral proteins by proteasomes in the cytosol, the resulting peptides pass to the membranes of the endoplasmic reticulum (ER) through translocation by the transporter associated with antigen presentation (TAP), for assembly into MHC class I complexes. Throughout its life cycle, the human cytomegalovirus (HCMV) produces a viral inhibitor of TAP (US6) that stops peptide transport to the MHC class I, limiting the presentation of abundantly expressed structural viral antigens, such as glycoprotein B [127].

In addition to mechanisms that interfere with viral peptide presentation to T cells, viruses have also evolved strategies to prevent MHC class I expression on the cell surface, to promote intracellular retention of the MHC complexes, or even to lead to the degradation of MHC class I molecules [126]. The U21 protein, expressed by the human herpesvirus 7 (HHV-7) is able to bind to and reroute properly folded class I molecules from the ER to a lysosomal compartment [128]. To target MHC class I molecules for proteolytic degradation, HCMV express proteins US3 and US11, responsible for the ubiquitination of the MHC class I molecules, which are then transported from the ER back into the cytosol, where they are degraded by the proteasome [129,130].

Downregulation of MHC class I complexes on the cell membrane by viral proteins results in recognition and killing of the infected cells by NK cells. Therefore, viruses have also developed strategies to circumvent NK cell-mediated killing, such as the expression of MHC homologues by HCMV [126].

Using a different strategy, HCMV and murine cytomegalovirus (MCMV) interfere with CD4+ T-cell recognition of infected cells by downregulating MHC class II expression on endothelial and epithelial cells [131]. This affects the activation of CD8+ T cells and B cell development, disturbing the immune control of viral disease.

An MHC complex is recognized by T cells expressing complementary T cell receptors (TCR) loaded with a peptide. Simultaneous interaction of the MHC molecule with CD4 or CD8 molecules expressed on helper and cytotoxic T cell precursors serves as a co-stimulus, respectively. The human herpesvirus (HHV)-6A is able to downregulate the CD3/TCR complexes at a transcriptional level [132]. The downmodulation of the levels of surface CD4 on infected cells is a strategy used by the human immunodeficiency virus (HIV). The HIV encoded proteins Vpu, Env and Nef, use different mechanisms to downmodulate CD4 at different points during infection [133].

Viral modulation at the level of the MHC class I and II biogenesis and transportation pathways affects antigen presentation, thus preventing a sustained immune response (see Table 1.3).

If viral antigens are still successfully presented, viruses escape the immune response by interfering with B and T cell effector functions. T cell responses can be inhibited at the level of cytokine induction (see Table 1.4), or CTL mediated apoptosis (see Table 1.6).

1.3.3. Viral interference with immune effector functions

In order to replicate and spread in a host population, the virus depends on highly specific interactions of viral proteins with infected cells, which result in the subversion of multiple cellular signal transduction pathways. These strategies are not restricted to modulation of host immunity, for example, viral proteins can manipulate cell cycle progression of the infected host cell, in order to replicate [134].

The attenuation of the various effector mechanisms of the host's immune response is equally important for successful propagation of a virus. The first barrier that viruses need to overcome is the innate immune response. Several viral strategies are already known, including the interference with cytokine synthesis and function (Table 1.3), particularly the inhibition of IFN (to be discussed in detail in the next section), interference with the complement system (Table 1.4), and inhibition of apoptosis (Table 1.5). In addition, viruses also encode functional Fc receptors, thereby interfering with virus neutralization or antibody-mediated cytotoxicity. These receptors can also hide antigenic structures by coating the virus or infected cell with immunoglobulins [126].

Viruses have also evolved strategies to subvert phagocytic activity. Some viruses evade nitric oxide and reactive oxygen radicals generated by macrophages. Others, such as herpesviruses and poxviruses, express surface proteins that mimic CD200, a host regulator that delivers inhibitory signals to macrophages [135].

Virus protein/gene	Mechanism	Reference
HSV-1 – ICP47	Blocks TAP function	[136]
HVS – ORF14	Binds MHC class II	[137]
AdV – E1A	Inhibits MHC class II gene transcription	[138]
KSHV – K3 and K5	Reduces MHC class I surface expression	[139]
HPV – E5	Downregulates MHC class I and II surface expression	[140,141]
HCMV – UL37	Viral MHC-like protein	[142]

Herpes simplex virus-1 (HSV-1); Human papilomavirus (HPV); Herpesvirus saimiri (HVS); Human cytomegalovirus (HCMV); Adenovirus (AdV); Kaposis' associated herpesvirus (KSHV)

Table 1.3 – Modulation of antigen presentation pathway.

Virus protein/gene	Mechanism	Reference
VV – B15R	Binds and inhibits IL-1β	[143]
HCMV – US28	Chemokine receptor viral homologue	[144]
EBV – LMP-1	Binds TRAFs; activates TNFR	[145]
HVS – ORF13	Viral IL-17 homologue; T-cell mitogen	[146]
KSHV – ORF74	Chemokine receptor viral homologue	[147]
HIV – Tat	Chemokine-like activity	[148]
MCV – MC54	Inhibits IL-18 induced IFN-γ production	[149]
MHV68 – M3	Viral CKBP; inhibits chemokine system	[150]
VacV-A41L	Viral CKBP; anti-inflammatory properties	[151]

Vaccinia virus (VacV); Epstein-Bar virus (EBV); Kaposis' associated herpes virus (KSHV); Human immunodeficiency virus (HIV); Herpesvirus saimiri (HVS); Molluscum contagiosum virus (MCV)

Table 1.4 – Modulation of cytokines and cytokine responses.

Virus protein/gene	Mechanism	Reference
HSV – gE and gI	Forms IgG Fc-like receptors	[152]
CPXV – IMP	Sequesters C3 and C4; blocks complement cascade	[153]
VACV – VCP	Sequesters C3 and C4; blocks complement cascade	[154]
HVS – ORF15	Viral CD59 homologue; blocks terminal complement cascade	[155]

Herpes Simplex virus (HSV); Cowpox virus (CPXV); Vaccinia virus (VACV); Herpesvirus saimiri (HVS)

Table 1.5 – **Modulation of complement responses.**

Virus protein/gene	Mechanism	Reference
HPV – E6	Targets p53 for proteolytic degradation	[156]
EBV – LMP-1	Up-regulates expression of cellular bcl-2	[157]
HCMV – UL37	Blocks apoptosis by death receptors	[158]
KSHV – K13	Prevents caspase activation by death receptors	[159]
MHV – M11	Bcl-2 anti-apoptotic gene homologue	[160]
ASFV – A224L	Viral IAP homologue; inhibits caspase activation	[161]

Human cytomegalovirus (HCMV); Murine herpesvirus (MHV); Kaposis' associated herpes virus (KSHV); African swine fever virus (ASFV); Human papilomavirus (HPV)

Table 1.6 – **Modulation of apoptosis.**

Most large DNA viruses code for host evasion proteins that have sequence homology to cellular genes. However, non-homologous virus host evasion genes have recently been identified and described [162]. Such genes represent a challenge, since their function can only be determined by functional assays.

In conclusion, viruses evolved a plethora of mechanisms that favours their replication and survival. The knowledge of these evasion strategies will help in the understanding of immune and inflammatory responses and in the design of novel and effective antiviral treatments and vaccines [163].

1.4. Viral Evasion of Interferon responses

The downregulation of the interferon system, a powerful and first line of defence against virus infections is, unsurprisingly, a priority for most viruses. The viral strategies are numerous and include the inhibition of IFN production, the inhibition of IFN-mediated signalling pathways, and blocking the action of IFN-induced enzymes with antiviral activity.

Given that the induction of IFN is generated in a cascade-like manner, viruses have evolved several molecular mechanisms that act in concert over different steps in the pathway to subvert the IFN response. Many viral antagonists are multifunctional proteins that interact with multiple host components, thereby increasing the efficiency of their host evasion and also allowing the virus to manipulate different biological processes in infected cells. The size of viral genomes contrasts with the number of mammalian genes dedicated to host defence mechanisms, providing an additional selective pressure for the evolution of such viral multifunctional proteins [81].

During the past few years, much has been learned about the molecular mechanisms used by viruses to manipulate and escape the host interferon response. The exact strategy exploited by a virus will presumably depend on the biology of the infection and will be a major factor that will influence the pathogenesis of that virus infection [64].

1.4.1. Inhibition of IFN production

Regarding the inhibition of interferon production, several viral strategies have been identified and characterized (see Table 1.7). It has been observed that almost 50% of the viruses for which IFN antagonists have been identified, interfere with multiple steps of the

IFN response. This clearly depicts the necessity for viruses to successfully circumvent the IFN response [62].

In order to avoid recognition by pattern-recognition receptors (PRRs), viruses usually manipulate production of PAMPs to achieve a minimum level of expression, thus minimizing IFN production in response to the viral infection. This can be achieved, for example, by minimizing the production of dsRNA through the regulation of virus transcription and replication or, as used by paramyxoviruses and picornaviruses, by capping viral RNA, rendering it indistinguishable from cellular mRNA [64].

The majority of IFN antagonists exert their action by one of four different strategies: (I) general inhibition of cellular gene expression, (II) sequestration of molecules in the IFN circuit, (III) proteolytic cleavage of innate immune components, or (IV) targeting these components for proteasomal degradation [62].

By inhibiting the host cell gene expression and/or protein synthesis, viruses affect several cellular functions, including the IFN response. Several mechanisms used by viruses are already known, such as the matrix (M) protein of vesicular stomatitis virus (VSV), responsible for the inhibition of basal transcription, impairment of nuclear-cytoplasmic transport of RNAs and proteins and inactivation of translation factors. The NS1 protein of influenza A virus (FluAV) inhibits processing and export of cellular mRNAs [164].

The sequestration of the ligands that bind to host cell PRRs is another efficient viral mechanism to inhibit the induction of IFN expression. Very well known examples, among others, are the NS1 protein of influenza A virus, and the VP35 protein of Ebola virus. These bind

dsRNA and can inhibit not only the induction of IFN but also dsRNA inducible proteins such as the enzymes PKR and 5'OAS [62].

Some viral antagonists directly inhibit components of the TLR and RLR signalling pathways, blocking IFN production and suppressing host antiviral signal propagation. The 3Cpro cysteine protease of coxsackievirus B3 (CVB3) cleaves two key adaptor molecules of the innate immunity: MAVS and TRIF, thereby blocking TLR3, TLR4 and RLR signalling [165]. The hepatitis A virus (HAV) uses a 3Cpro homologue to achieve the same result [166]. The Vaccinia virus protein A46R not only inhibits TLR3 signalling through TRIF-mediated IRF-3 activation, but is also capable of binding to other TIR adaptors such as MyD88 and TIRAP, interfering with the activation of NF-κB and MAP kinase [167].

By inhibiting the post-translational attachment of ubiquitin or ubiquitin-like modifiers (ULMs) to host cell proteins, viruses are able to deregulate many cellular processes, including the generation of innate and adaptive immune responses to pathogens [168]. For example, the Npro protein of both bovine viral diarrhoea virus (BVDV) and classical swine fever virus (CSFV) induce proteasome-dependent degradation of IRF3 [64]. HIV-1 proteins Vpr and Vif also mediate IRF3 ubiquitination, leading to proteasomal degradation [169]. Influenza A virus NS1 binds and inhibits TRIM25, an E3 ligase required for ubiquitin-dependent interaction between RIG-I and its adaptor MAVS. NS1 is thus able to prevent activation of IRF3-dependent IFN secretion [170].

1.4.2. Inhibition of IFN-mediated signalling

Interferon-mediated signalling not only induces the expression of host cellular anti-viral proteins but also stimulates antigen presentation through increased MHC expression. Considering such crucial roles in anti-viral immunity, it is not surprising that viruses have evolved strategies to inhibit the signal transduction pathways triggered upon binding of IFN to its specific receptor.

Type I and type II IFN signal through distinct receptors, activating downstream components that can be either unique or common to both signalling pathways. Thus, viruses can block the impact of IFN at several levels, inhibiting only one of these two pathways or both (see Table1.8). Poxviruses encode soluble versions of cellular cytokine and cytokine receptors, which can interfere with the normal function of the target cytokines or receptors. For example, B8R protein of VACV binds to soluble IFN-γ and prevents its binding to the cellular receptor. In this way, this virus simultaneously inhibits the antiviral effects due to signalling through the type II IFN receptor, and also the immunoregulatory functions of IFN-γ [171].

Modulation of STAT activity is a very common viral strategy. For example, the Dengue virus NS5 protein mediates ubiquitination and proteasome-dependent degradation of STAT2 [172]. Members of paramyxoviruses encode two different but genetically related proteins, C and V, which interfere with STAT function. According to the strain of the virus, these IFN antagonists act by binding to STAT proteins inducing their degradation, or by inhibiting the Jak kinases [164].

Inhibition of signalling through the STAT proteins can also be indirect. The VP24 protein of Ebola virus (EBOV) interacts with the NPI-1 subfamily of karyopherin-α proteins (responsible for transporting

dimerised phospho-STAT1 to the nucleus), inhibiting nuclear accumulation of STAT [173]. Other indirect mechanism to block IFN signalling includes the rapid induction of SOCS-3 expression, a cellular inhibitor of the Jak-Stat pathway. This is a strategy explored by multiple viruses, such as the FluAV, RSV, HCV, and HSV [62].

1.4.3. Inhibition of IFN-induced effector proteins

Another efficient way to interfere with the interferon response is by directly targeting the proteins that mediate the antiviral state.

Viruses employ different strategies to either inhibit or prevent the activation of the IFN-inducible antiviral effector protein PKR, one of the major host responses to viral infection. Active PKR dimers phosphorylate eIF-2α, preventing the formation of the ternary translational complex, thereby repressing translation of RNAs. Since viruses require the machinery of the host cell for the translation of viral proteins, inhibition of PKR is mandatory [174]. Some viruses express RNA-binding proteins that sequester viral dsRNA, thus preventing the activation of PKR. The Hepatitis C virus (HCV) protein NS5a is able to interact with and inhibit PKR. In addition, the HCV E2 glycoprotein competes with eIF-2α for binding to PKR, thus preventing the inactivation of translation by PKR. Another strategy used by viruses is to encode small RNAs which compete with dsRNA for binding to PKR, hence inhibiting its activation [118]. Direct binding and inhibition of PKR is a strategy explored by the KSHV protein vIRF-2, which prevents PKR activation by inhibiting the autophosphorylation of the protein [174]. The cellular PKR inhibitor p58(IPK) is activated during FluAV, TMV, and TEV infection and contributes to negative regulation of PKR by direct protein–protein interaction [62].

The ISG15, ubiquitin-like protein, has also been shown to be targeted by viruses. The N-terminal domain of the L protein of Crimean Congo hemorrhagic virus (CCHV) has de-ISGylating and de-ubiquitinating activity, while influenza B virus NS1 protein inhibits ISG15 by direct binding [64]. Adenovirus, HSV-1, EBV, and HCMV, are able to disrupt PML nuclear bodies (ND10) by proteasome-dependent degradation, although the relevance of this is debated [118].

1.4.4. Applications of viral inhibitors of IFN responses

It is clear that viruses have evolved multiple mechanisms to evade the interferon response. Viral genes that circumvent the interferon response can have direct applications in the rational development of novel strategies to control viral infections, for example, attenuated virus vaccines with virus host evasion genes deleted. Typically these host evasion genes are non-structural, and non-essential for virus growth *in vitro*. Therefore viruses with targeted deletions of genes that code for IFN antagonists are promising candidates for live attenuated vaccines. This approach has already been successful for viruses such as influenza virus, and bovine respiratory syncitial virus (reviewed by Haller *et al.*, 2005) [81]. A major complication that may arise is that IFN antagonists are often multifunctional, and so a gene deletion may "over-attenuate" the virus; for example, an IFN-sensitive virus may be difficult to grow in culture due to the IFN response of tissue-culture cells [64].

Viral antagonists may also be targets for novel antiviral drugs and tools for immunomodulation. The fact that different viruses target the same cellular proteins raises the possibility of designing an antiviral drug with a wide spectrum of activity.

Our present knowledge of the interplay between viruses and the IFN system is still limited to some extent because the IFN system is still far from being understood. Further studies and understanding on how viruses block this response provide a better control of virus infections through novel vaccines and drugs, and also reveal as yet unknown functions of the IFN system.

Virus (protein)	Mechanism
ECMV (leader protein)	Prevents IRF3 dimerization
BUNV (NSs)	Inhibit RNA polymerase II
Reovirus (σ3/A)	Sequesters dsRNA
HCV	Inhibits TLR signalling and MAVS
Influenza A virus (NS1)	Inhibits MDA5 and RIG-I
HPV16 (E6); HSV (ICP0); BRSV	Inhibit or degrade IRF-3
HPV (E7); AdV (E3)	Interfere with IKK complex
KSHV (IRF orthologues)	Interferes with IFN-β promoter activation
ASFV (A238L)	Competitive non-functional IκB homologue.

Encephalomyocarditis virus (ECMV); Bunya virus (BUNV); Hepatitis C virus (HCV); Bovine respiratory syncitial virus (BRSV); Herpes simplex virus (HSV); Human papilomavirus (HPV); Adenovirus (AdV); Kaposis associated herpesvirus (KSHV); African swine fever virus (ASFV)

Table 1.7 – Inhibition of interferon production.
(adapted from Versteeg & García-Sastre *et al.*, 2010)

Virus (protein)	Mechanism
HCMV	Targets Jak1 for proteasomal degradation
SeV; BRSV	Induces degradation of STATs
HCV; SeV; HCMV	Sequesters STATs or alters their phosphorylation
HCV (Core); HSV (UL13, UL41)	Induces SOCS-3

Herpes simplex virus (HSV); Human cytomegalovirus (HCMV); Sendai virus (SeV); Bovine respiratory syncitial virus (BRSV); Hepatitis C virus (HCV)

Table 1.8 – Inhibition of interferon signalling.
(adapted from Versteeg & García-Sastre *et al.*, 2010)

1.5. African Swine Fever Virus

African swine fever virus (ASFV) was first described by Montgomery, in Kenya, in 1921, when the virus spread from infected warthogs (*Phacochoerus aethiopicus*) to the domestic pigs (*Sus scrofa*) introduced by European colonists, causing a disease with high mortality [175]. In pig populations, this virus is highly contagious and can be easily transmitted to healthy swines from diseased or carrier pigs, by biological vectors (e.g. ticks) or by the ingestion of contaminated pig products. Unlike domestic swine, wild natural hosts (the warthog, the bushpig (*Potamochoerus porcus*) and the giant forest hogs (*Hylochoerus* spp.)) infected with ASFV are generally asymptomatic with low viraemia titers, reflecting the long term host-pathogen co-evolution. These species are thought to be natural reservoirs of the disease in Africa. This virus also infects different species of soft ticks (*Ornithodorus mobata* and *O. erraticus*), where it can persist for long periods of time [176,177]. The asymptomatic wild suids and the transmission among ticks allows a sylvatic cycle that can be maintained indefinitively in Africa [178].

African swine fever is an exceptionally serious disease of domestic pigs, with severe sanitary and socio-economical consequences. The lack of a vaccine or an effective treatment makes it an expensive disease to eradicate. Control is based on laboratory diagnosis and the enforcement of strict sanitary measures that involve elimination of all infected and susceptible animals. All these factors translate into a significant impact on the national and international trade of pigs and pig products, and therefore the World Animal Health Organisation (previously called l'Office International des Épizooties (OIE)) listed ASF as a notifiable disease [179].

Five different epidemiological scenarios regarding ASF spreading and endemicity have been identified and described. The most ancient scenario occurs is eastern and southern African countries, where the disease is maintained by a sylvatic cycle where wild suids and soft ticks (*O. mobata*) act as ASFV reservoirs. Domestic pigs contract the disease mainly by the ingestion of tissues from acute-infected warthogs or bites from infected ticks. However, in other situations, transmission may occur through direct contact between domestic pigs or indirect contact between pigs and pork products, without apparent involvement of soft ticks. Understanding the different epidemiological scenarios and the characteristics of the disease is critical for developing successful contingency and eradication plans in affected areas [180].

Since its first appearance, in 1921, the disease remained confined to Africa until it was introduced in Portugal in 1957, causing a hyperacute disease with 100% mortality. Until the 1990s, several ASFV outbreaks where reported in European countries. Except for Sardinia (Italy), where the disease is endemic, all these countries managed to eradicate ASF. During the late 1970s, ASFV outbreaks were reported in some Caribbean islands, including Cuba and the Dominican Republic. The disease was successfully eradicated by depopulation [178].

At the beginning of 2007, the Caucasus region (Georgia) was affected and ASF has since spread to the neighbouring countries of Armenia, Azerbaijan and Russia. The genetic characterization of all ASFV isolates found in the Caucasus region and Russian Federation suggests that only one virus arrived in the area in 2007 and subsequently spread. This initial ASF virus is closely related to isolates

typically found in Mozambique and Madagascar [180,181]. Since the introduction of ASFV in this region, more than 270 outbreaks have been reported to the OIE. The situation is currently out of control, with devastating consequences for pig industry and Russian economy. There is a considerable risk of introduction of the disease in European countries, since some of the outbreaks occurred very near the EU border. Another threat to Europe comes from African countries. Major outbreaks of ASFV in Africa are regularly reported to the OIE, and there is an increasing commercial trade between countries.

1.5.1. Virus structure and genome organization

African swine fever is a complex large icosahedral and enveloped double-stranded DNA virus. Unique characteristics of its structure and genomic organization led to its inclusion as the only member of the family *Asfarviridae*. It is the only known DNA virus to be an arbovirus, infecting soft ticks of the *Ornithodoros* genus.

Virions have a complex multi-layered structure, composed by a 30nm nucleoid (forming the nucleoprotein system, composed by the viral genome and different enzymes required for replication), surrounded by an 80 nm core shell, a first lipid layer (inner envelope) and a 170-190 nm icosahedral capsid. The extracellular virions acquire an external membrane during the budding through the cellular plasma membrane [182].

The ASFV genome varies in length between 170 and 190 kb, 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 [183].

This virus contains a number of open reading frames, ranging from 160 to 175, depending on the isolate. Of these, 110 are present as a single copy on 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 [178]. 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 [178].

1.5.2. Pathogenesis and host immune response

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 [184].

Macrophages and the monocyte lineage are the cells primarily infected by ASFV, and there is some evidence that endothelial cells can also be infected later in the infection [185]. 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 also contribute to the pathogenesis [178,185]. The extent of lymphocyte apoptosis correlates with the level of ASFV replication and the virulence of the virus isolate [186]. In the bushpig, there are lower levels of apoptosis and absence of clinical signs together with a containment of virus replication [187]. 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 [186]. In agreement with this hypothesis is the fact that increased levels of TNF-α, lL-1α, lL-1β and lL-6 were observed in sera from experimentally infected pigs, coinciding with the onset of clinical symptoms [188] and also an increased number of macrophages expressing these cytokines in areas of lymphocyte apoptosis [189]. On the other hand, another study revealed that the transcriptional levels of TNFα and lL-6 were increased in macrophages infected with the low virulence NH/P68 isolate compared to the highly virulent L60 isolate [190].

During chronic infections, a characteristic feature of ASF is the development of a clear hypergammaglobulinaemia [191]. In fact, hypergammaglobulinaemia was found to be associated with the development of clinical signs after infection with the NH/P68 isolate [184].

Activation of the B cells as a necessary prelude to hypergammaglobulinaemia may result from a direct mitogenic property of the virus and also through factors secreted by infected macrophages [192]. Indeed, ASFV infected macrophages produce a protein, p36, which induces an increase in serum levels of two major

B-cell stimulatory cytokines: IL-4 and IL-10 when inoculated in mice [193]. Activation of the B cells is followed by extensive apoptosis of the same cells, and given that apoptosis of T cells precede that of B cells [186] it was suggested that B lymphocytes enter apoptosis because they are activated in the absence of a rescue signal (*e.g.* CD40L) provided by T cells [192].

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 [184]. 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 virulent isolate [194,195]. In a later study, 85% of the animals receiving anti-ASFV antibodies survived infection with the E75 virulent isolate [196]. 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 [197,198]. However, in later studies it was shown that the immunization against p54 and p30 only conferred protection to 50% of tested animals[199], and the only detected effects were a delay in onset of clinical disease and reduction of viraemia [200].

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 [184]. In addition to NK cells as mediators of protection, the generation of ASFV specific cytotoxic lymphocytes was demonstrated in the NH/P68 model [201,202]. However, the immunization with a recombinant protein expected to stimulate ASFV-specific CTL activity, failed to protect against the infection with the highly virulent L60 isolate [203]. 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 [202].

Finally, both IFN-α and IFN-γ were shown to substantially reduce virus replication in swine monocytes and macrophages [204], and the cooperative action of both was able to cure lytically and persistently infected cells [205]. Although these results were interpreted as evidence for a role of the IFN response in protection, the IFN treatment was done only 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 [205].

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.

1.5.3. Modulation of host defence 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 proteins evolved for host evasion [178].

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 cytokine transcription [178].

One of the first evasion molecules described is the A238L protein with two dual functions: inhibition of NF-κB [206] and NFAT activities [207]. The A238L protein contains ankyrin repeats similar to those present in the IκB inhibitor of the host NF-κB transcription factor in the centre of the protein [183]. In the cytoplasm of normal cells, IκB binds to the NF-κB transcription factor retaining it in an inactive state. Upon stimuli, such as TNF-α or IL-1, the IκB is phosphorylated by IKK, and subsequently degraded by the proteasome, thus liberating NF-κB which translocates and binds to specific DNA sequences in the nucleus. The mechanism suggested for the inhibition of NF-κB mediated transcription of proinflammatory cytokines, chemokines, adhesion molecules and anti-apoptotic genes is through direct binding to NF-κB and thus preventing its binding to DNA [206,208,209]. 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 [207]. In summary, A238L is predicted to act as a potent immunomodulatory protein with diverse inhibitory effects on the transcription of cellular genes regulated by NF-κB and NFAT [178]. In addition, the A238L protein also inhibits COX-2 expression [210], IL-8 induction and TNF-α expression [206,211] , and expression of iNOS

[212]. Several of these functions are inhibited by targeting the p300 coactivator of transcription [211,213].

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) [214]. 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 [178]. The ubiquitin-conjugating enzyme, UBCv, of ASFV has been shown to interact with a host nuclear protein SMCy and is involved in transcriptional regulation [215]. 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 [216].

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 [217]. 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 [218]. Finally, the third protein, EP153R, is a C-type lectin homologue and the first to be described having anti-apoptotic properties, and might also be involved in the control of the activity of cellular p53 [219].

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 [220].

ASFV infection leads to the disruption of the trans-Golgi network with a consequently inhibition of MHC class I surface expression [221], thus providing a possible mechanism for evasion of CTL responses.

The modulation of the interferon response by ASFV has 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. 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 [222]. Notably, in porcine aortic endothelial infected cells, the IFN-α induced MHC class I expression is down-regulated [185].

Recently, the ASFV ORF I329L was described as a host evasion gene capable of inhibiting the induction of type I IFN by activation of the TLR3 pathway. Although the mechanism of action was not elucidated, the data suggest a role in inhibiting the TRIF molecule, a critical adaptor protein of the TLR3 pathway [223]. This non-essential gene

was identified through the combination of bioinformatic analysis and functional assays, an indication of the promising potential of such tools in the identification and characterization of viral host evasion genes.

In order to screen a wide-range of host genes that might be differentially regulated by viruses, the microarray transcriptional profile analysis is a powerful tool. Using this technique, changes in macrophage gene transcription after infection with a highly virulent ASFV isolate, has revealed increased gene expression at 4 hours post infection, followed by a decrease in expression comparable to the mock infected cells at 16 hours post infection. The genes identified in this pattern of expression, included proinflammatory cytokines such as IL-6, TNF-α and IFN-β, chemokines and adhesion molecules. These results suggest that, indeed, ASFV encode proteins that efficiently circumvent the early immune response mounted by the host cell against the virus [224].

1.6. Aim of the project

The work presented in this thesis focuses on identifying and characterizing anti-interferon (IFN) strategies evolved by the African Swine Fever Virus (ASFV), the causal agent of a fatal haemorrhagic disease of domestic pigs, characterized by the absence of clinical symptoms in its natural hosts, the bushpig and the warthog.

The ASFV is a large double-stranded DNA virus and is the only member of the family *Asfarviridae.* Although the virus replicates primarily in macrophages *in vivo* and in macrophages and endothelial cells *in vitro,* surprisingly no individual virus gene inhibiting IFN responses has been described. Large DNA viruses typically have multiple genes/strategies for modulation of host cell biology and immune responses. While many viral genes evolved for host manipulation will have been acquired from the host and function to mimic or block normal cellular functions, the existence of functional evasion molecules without structurally homologous cellular counterparts is now clear, and the possibility of non-homologous ASFV genes evolved for the inhibition of IFN responses was pursued. Two such genes (ORF MGF360-18R and ORF I329L) were selected and their intracellular targets and mechanisms were investigated.

In a previous study performed in this laboratory, several ASFV genes with unassigned functions were tested for their capability to inhibit the expression of IFN-β, including six MGF360 and two MGF530 genes absent in a deletion mutant virus that fails to inhibit type I IFN secretion following macrophage infection. The ASFV ORF MGF360-18R, a member of MGF360, was selected as the focus for this work for its ability to inhibit both IFN-β induction and the impact of type I and type II IFN. A screening through the available genomic sequences of

different isolates of the ASFV revealed that the full length MGF360-18R gene in pathogenic ASFV isolates is partially deleted in the non-pathogenic (OURT88-3) and tissue culture-adapted (Ba71V) strains. These two distinct variants of the non-conserved ORF MGF360-18R were investigated in detail in order to define the mechanisms by which this viral gene evolved for both the manipulation of the IFN response and the modulation of IFN-mediated signalling. The mechanisms of action and potential targets for both MGF360-18R variants are presented.

Recently, the conserved ASFV ORF I329L was described as being able to impair the cellular responses controlled by TLR3 that lead to both IFN-β secretion and NF-κB activation. Bioinformatics analysis of the putative intracellular domain of I329L, at first negative, revealed a short possible region of homology with BOX1 and BOX2 of TLR3-TIR domain. Although both the described homology and the presented results pointed out TRIF as a possible target of this viral host modulation gene, the precise mechanism of inhibition remains to be elucidated. Therefore the aim of this work is to characterize the mechanism by which ORF I329L inhibits the IFN response, defining its target and the I329L domains involved in this inhibition.

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Chapter 2

Mechanism of ASVF ORF I329L-mediated inhibition of type I IFN induction

2.1. Summary

The African swine fever virus (ASFV) is a large double-stranded DNA virus that replicates primarily in macrophages *in vivo* and in macrophages and endothelial cells *in vitro*. It is the only member of the family *Asfarviridae*, and is characterised by its ability to interfere with signalling pathways controlling the transcription of cytokines, including interferons. As the Interferon system plays a major role as an early host defence system against virus infections, ASFV must have evolved a number of counter strategies to antagonise this response.

The work described in this thesis directly demonstrates an I329L-TLR3 interaction and further defines the mechanism of I329L inhibition of the IFN response.

Many viral genes evolved for host manipulation are likely to have been acquired from the host and function to mimic or block normal cellular functions. Recently, the conserved ASFV ORF I329L was reported as being able to impair the cellular responses controlled by TLR3 that lead to both IFN-β secretion and NF-κB activation. Interestingly, a detectable, but very low, homology with BOX1 and BOX2 of TLR3 intracellular TIR domain was observed, raising the possibility that I329L might inhibit activation of IFN-β through interaction with TRIF [1].

Formal biochemical proof of an interaction between the I329L protein and TRIF was obtained. In addition, to further characterize the modulation of the type I IFN response by I329L, truncation mutants lacking either the ectodomain or the intracellular domain (TM/IC and EC/TM, respectively) were designed and tested by luciferase reporter assays for their impact on the TLR3 pathway. Importantly, expression of the intracellular domain alone of I329L inhibited induction of IFN-β and NF-κB activation by ectopically expressed TRIF. This, and the fact

that the extracellular domain of I329L inhibited Poly (I:C) mediated activation of IFN-β, but not activation via ectopically expressed TRIF, demonstrates that I329L inhibits dsRNA activation via its extracellular domain, perhaps through formation of a non-signalling I329L-TLR3 heterodimer, and may also disrupt signal transduction through the impact of its intracellular domain on TRIF.

Finally, evidence indicating proteolytic processing of I329L was obtained by demonstration of such processing as a consequence of viral infection or activation of TLR3 signalling. Consistent with the requirement of this processing for the inhibitory activity of I329L, a mutant I329L lacking a cathepsin L sensitive site continued to inhibit Poly (I:C)-mediated activation, but not activation through ectopically expressed TRIF.

2.2. Introduction

Upon recognition of a viral infection, cells activate different signal transduction pathways that culminate in the expression of a diverse array of cytokines, which act in both an autocrine and paracrine manner to induce the establishment of an antiviral state within the infected and neighbouring cells [2,3]. The interferons (IFNs) are a group of secreted cytokines, and compose a system particularly important for the regulation of the antiviral immune response, inducing the expression of proteins that interfere with viral processes, thus blocking viral replication [4,5].

The first potent IFN inducer to be identified was double-stranded RNA (dsRNA), a molecular pattern associated with viral infection, because it is produced by most viruses at some point in their replication [6]. Distinct pathogen-associated molecular patterns (PAMPs), uniquely

found in microbes (e.g., viral glycoproteins, viral RNA, bacterial endotoxin, bacterial flagella, CpG motifs, etc.) are recognized by specialized pattern recognition receptors (PRRs), such as membrane-bound Toll like receptors (TLRs) or by cytoplasmic receptors (the retinoic acid-inducible gene (RIG)-I, the melanoma differentiation associated antigen 5 (MDA5), and also the DNA-dependent activator of IFN-regulatory factor (DAI)), triggering the release of type I IFN [7,8].

The TLR and the cytosolic pathways both converge on the activation of the downstream kinases and common transcription factors necessary for the induction of type I IFN. Induction of the IFN-β gene requires the activation of two families of transcription factors: the family of Nuclear Factors κB (NF-κB) and the family of Interferon Regulatory Factors (IRFs). Together with a c-jun/ATF-2 heterodimer, IRF-3, IRF-7 and NF-κB form the enhanceosome, a complex that binds to the IFN-β promoter, inducing gene expression [9]. Several studies revealed that binding of IRF-3 and/or IRF-7 is indispensable for induction, but activation of NF-κB and c-jun/ATF-2 may not be essential [3].

The Toll-like receptors are a family of PRRs that play central roles in innate immune defence against infection by binding to microbial molecules. All TLR family proteins consist of an intracellular signalling domain, a single transmembrane TM domain and an extracellular ligand binding domain with N-terminal leucine-rich repeats (LRR) motifs. Each TLR has a distinct LRR structure with specific adaptations that allow for improved interaction with either its respective ligand or a coreceptor-ligand complex. Ligand-mediated activation of the TLR receptors can lead to the formation of homo and sometimes heterodimers. The dimerization of the extracellular domains results in activation of the intracellular domain which contains an intracellular Toll/IL-1R (TIR) motif, important in protein-protein interactions. This

motif is also present in the signalling adaptors that are recruited to the ligand-activated TLR TIR domains, forming the first step in the signalling cascade leading to the expression of multiple genes involved in innate and adaptive immunity, including type I IFN [10]. Recently, a requirement for proteolytic processing of TLRs after ligand binding has been described [11].

Compartmentalization is an important feature of these membrane bound receptors that limits access to self molecules, preventing inappropriate activation of the downstream signalling pathways. TLRs involved in the recognition of microbial membrane components, are located on the cell surface, while those recognizing microbial nucleic acids are mainly located within endolysosomal compartments (e.g., TLR3). TLR4, normally present at the surface can also enter the endocytic pathway following ligand-mediated activation. In each case, the TIR motif resides in the cell cytoplasm while the LRR domain is positioned to detect either extracellular PAMPs (outside of the cell) or PAMPs acquired during sampling (within the endosomes). Trafficking of nucleotide-sensing TLRs to endolysosomal compartments by unc-93 homolog B1 (UNC93B1) protein and proteolytic regulation of some TLRs (TLR7 and TLR9) are other strategies used to further control receptor activation [12,13,14].

Individual TLRs trigger different signal transduction pathways by engaging different adaptors, through interaction between the corresponding TIR domains. The particular signalling adaptor used determines which signalling pathway will be activated. The TIR-containing adaptor MyD88 induces a pro-inflammatory response dependent on the activation of NF-κB and mitogen-activated protein (MAP) kinase, whereas TIR domain-containing adaptor protein inducing IFN-β (TRIF) is responsible for activation of IRF-3, IRF-7 and

NF-κB, culminating in the induction of type I IFN and inflammatory cytokines [15].

The molecular signature of most viruses is double-stranded RNA (dsRNA), produced either as an intermediate of the viral replication cycle (e.g., for dsDNA viruses, such as ASFV) or as part of the viral RNA genome. Viral dsRNA is recognized by the Toll-like receptor 3 (TLR3), inducing a signalling mechanism solely through TRIF, and not MyD88. Activated TRIF associates with TRAF3 and TRAF6 and subsequently with the noncanonical kinases TBK-1 and IKKε (Fig.1.2) which phosphorylate IRF-3 and IRF-7, leading to its dimerization and nuclear translocation to bind the promoter of type I IFN. TRIF also mediates the activation of NF-κB and activating protein 1 (AP-1) through the complex of kinases IKK-α/β/γ. These two transcription factors translocate into the nucleus, together with IRF-3 and IRF-7, and bind to the PRDI-IV positive regulatory elements of the IFN-β enhancer region [16]. TLR3-mediated signalling also leads to phosphorylation of specific tyrosines and the recruitment of phosphatiylinositol 3-kinase (PI3K), essential for full activation of IRF-3 [17,18]. Virus infected cells mainly depend on TLR3, TLR7/TLR8 and TLR9 to induce the expression of type I IFN, following detection of viral nucleic acids. However, TLR4 is also capable of inducing type I IFN by the recognition of non-nucleic acid ligands, such as lipopolysaccharide (LPS). Upon LPS-binding, TLR4 initiates signalling transduction pathways through both MyD88 and TRIF adaptors. Signalling through MyD88 requires TRAM [19] and culminates in the activation of NF-κB, inducing an early pro-inflammatory response. TLR4 is then internalized into the endosome [20], where it requires the TIR domain-containing adaptor protein (TIRAP) to bind TRIF [21]. Through this pathway, TLR4 induces both a late pro-inflammatory response (NF-κB dependent) and type I IFN expression.

Recently, the conserved ASFV ORF I329L was reported as being able to impair the cellular responses controlled by TLR3 that lead to both IFN-β secretion and NF-κB activation. Bioinformatic analysis predicted ORF I329L to be a type I transmembrane protein containing extracellular putative leucine-rich repeats (LRRs) and an intracellular TIR domain homologue. The precise mechanism for the inhibition of TLR3 signalling remains to be elucidated but, based on the results, the authors suggested TRIF as a potential target for I329L inhibitory activity [1]. However, a modelling exercise on this viral protein supported the idea that pI329L may function as a TLR3 decoy, suggesting that the viral protein could hinder TLR3 dimerization, and in doing so, inhibit the downstream signalling pathway [22].

The work presented here is a more profound characterization of the mechanism of action of ASFV ORF I329L. Truncation mutants of I329L lacking either the ectodomain or the intracellular domain (TM/IC and EC/TM, respectively) were designed and tested by luciferase reporter assays for their impact on the TLR3 pathway, revealing a distinct inhibitory role for each of these domains. The TLR3 adaptor protein TRIF was identified as being one of the targets for this viral protein, but an additional role in the direct inhibition of TLR3 dimerization or TLR3-dsRNA binding is also plausible. The proteolytic processing of pI329L is described and its relation to the full inhibitory potential of this viral protein is discussed.

2.3. Materials and methods

2.3.1. Cell culture

Human embryonic kidney (HEK)-293T and Vero cell lines were maintained in Dulbecco´s modified Eagle´s medium (DMEM) (Gibco) supplemented with 100U/ml penicillin G sodium /100µg/ml streptomycin sulfate (Gibco), 2mM L-Glutamine (Gibco) and 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco). The HEK-293-hTLR3 stable cell line, kindly provided by Dr. A. Bowie, was maintained in DMEM supplemented with 100U/ml penicillin G sodium, 100µg/ml streptomycin sulphate, 10µg/ml blasticidin (Invivogen), 2mM L-Glutamine and 10% (v/v) heat-inactivated FBS.

2.3.2. Plasmids

The ASFV I329L full length ORF (Gene ID: 1488904) was amplified by PCR from BA71V isolate genomic DNA and cloned into the pcDNA3 plasmid, in frame with either a carboxyl-terminal or an amino-terminal influenza haemaglutinin (HA) tag. Using QuikChange II Site-Directed Mutagenesis kit (Stratagene), a point mutation leading to a nucleotide substitution (G108A) in the I329L coding region was performed, creating the mutant pcDNA3-I329L^{mut}-HA plasmid, coding for pI329L.G108A.

Truncation mutants of I329L containing the putative ecto- and intracellular domains together with the putative transmembrane domain (EC/TM and TM/IC, respectively) were designed using specific primers to amplify the designated regions by PCR from BA71V isolate genomic DNA. The fragments were then cloned into the pcDNA3 plasmid, in frame with an amino-terminal HA tag.

An alternative expression plasmid for the I329L ORF was constructed replacing the human cytomegalovirus immediate early promoter sequence of the pcDNA3 plasmid with a 282 bp sequence immediately upstream the I329L initiation codon. The I329L full length ORF was cloned into this plasmid, with an amino-terminal HA tag, being expressed under the control of its own promoter (pcDNA3-prom^{I329L}-I329L-HA).

For the construction of a recombinant lentivirus vector (pHR-CMV-HA-I329L-eGFP), the I329L gene was excised from pcDNA3, together with the carboxy-terminal HA tag, and cloned into the vector pHR-CMV-eGFP (kindly provided by Dr. Y. Ikeda), upstream of an internal ribosome entry site (IRES)-driven enhanced green fluorescent protein gene (eGFP). The same procedure was used to clone the TM/IC truncation mutant.

The luciferase reporter plasmids containing the sequences of the IFN-β promoter [pIFΔ(-125/+72)lucter], the NF-κB binding site of the IFN-β promoter [p(PRD2)5tkΔ(-39)lucter], as well as the expression vector for TRIF, RIG-I and MDA-5 were gifts of Dr. S. Goodbourn. The expression plasmid for MyD88 was provided by Dr. A. Bowie. The expression plasmid containing the human TLR3 fused with the yellow fluorescent protein (YFP) was provided by Dr. R. Medhzitov.

The pCMVβ plasmid contains a β-galactosidase gene under the control of human cytomegalovirus immediate early promoter, and serves as an internal control for culture to culture variations in transfection efficiency.

2.3.3. Lentivirus production

Lentivirus was produced by transient co-transfection of HEK-293T cells with the packaging and envelope plasmid together with the empty pHR-CMV-eGFP plasmid or the recombinant I329L and TM/IC plasmids at a weight ratio of 1:1:3, respectively, using FuGENE 6 (Roche) according to the manufacturer's instructions. Supernatants containing the lentivirus were collected at 48h and 72h post-transfection, clarified by filtration, and lentivirus were collected by ultracentrifugation (125,000xg, 3h, 4°C). Virus pellets were resuspended in fresh DMEM, and frozen at −80°C.

2.3.4. Lentivirus transduction of HEK-293T cells

The HEK-I329L, HEK-TM/IC and HEK-eGFP stable cell lines were produced by lentivirus infection of HEK-293T cells with either one of the recombinant plasmids or the empty pHR-CMV-eGFP, respectively, using a multiplicity of infection (MOI) of 10 in DMEM. Confirmation of lentivirus infection was done by detecting eGFP-positive cells by optical microscopy at 48 h post-infection (p.i.), and protein expression was confirmed by Western blot.

2.3.5. Luciferase reporter gene assay

HEK-293T cells ($6x10^4$ cells/well, in a 24 well plate) were co-transfected with 100ng of the indicated luciferase reporter plasmid, 25ng of the β-galactosidase internal control plasmid (pCMVβ) and 300ng of either pcDNA3-I329L-HA or the empty pcDNA3HA, according to the Lipofectamine 2000 (Invitrogen) protocol. Forty-eight hours post-transfection, the cells were either stimulated with 35μg/ml Poly(I:C)

(Amersham Biosciences) for five hours, or left untreated. After the treatment, the cells were lysed.

In an alternative protocol, the cells were co-transfected with 100ng of the indicated luciferase reporter plasmid, 25ng of pCMVβ, the indicated amounts of plasmids expressing the different components of the IFN-β induction pathway and increasing amounts of the pcDNA3-I329L-HA plasmid. The quantity of DNA in each transfection was kept constant by supplementation with empty pcDNA3HA. Forty eight hours post-transfection the cells were lysed. The luciferase activity was measured using the luciferase assay system (Promega) according to the manufacturer`s protocol. The β-galactosidase activity was measured using the Galacton-Plus kit from Tropix (Bedford, MA). The luciferase activity was normalized relatively to the β-galactosidase activity of each sample, as to correct transfection efficiency variations between different cells.

2.3.6. Enzyme-linked Immunoabsorbent Assay (ELISA)

HEK-293T cells ($6x10^4$ cells/well, in a 24 well plate) were transfected with 300ng of the pcDNA3-I329L-HA, the pcDNA3-I329L^{mut} -HA or the non-recombinant pcDNA3HA plasmid, according to the Lipofectamine 2000 (Invitrogen) protocol. Cells were stimulated by co-transfection of 20ng of TRIF expression plasmid. Forty-eight hours post-transfection, the medium of each well was collected and centrifuged for 15 minutes at 1000xg. Quantitative determination of human IFN-β concentration in the supernatants was performed using the Human Interferon β ELISA Kit (PBL Interferon Source), according to the manufacturers' instructions. Absorbance at 450nm was measured using a BioRad ELISA reader (BioRad) and concentration of IFN-β was determined by comparison to a standard curve.

2.3.7. Western blot

HEK-293T cells ($3x10^5$ cells/well, in a 6-well plate) were transfected with 3µg of either pcDNA3-prom^{I329L}-I329L-HA or the non-recombinant pcDNA3HA, and 500ng of TLR3 expression plasmid, according to the Lipofectamine 2000 (Invitrogen) protocol. Forty-eight hours post-transfection, the cells were either stimulated with 100µg/ml Poly(I:C) (Amersham Biosciences), during the indicated amounts of time, or left untreated. In a second alternative protocol, cells were stimulated by co-transfection of 250ng of TRIF expressing plasmid.

In other protocol, Vero cells were infected with ASFV Ba71V strain at a MOI of 3 or mock infected. After incubation for one hour to allow virus adsorption, cells were incubated in culture medium for the indicated times.

Cells were harvested and lysed in lysis buffer (15 mM Tris-HCl, 120mM NaCl, 25mM KCl, 2mM EDTA, 2mM EGTA, 0.1mM DTT and 1%Triton X-100) containing a protease inhibitor cocktail (Sigma). Cell lysates were resolved by sodium dodecyl sulphate-polyacrilamide gel electrophoresis (SDS-PAGE) and proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare). Membranes were blocked with 5% non-fat milk for one hour at room temperature and probed with the following antibodies: rabbit serum anti-I329L (provided by Vivian de Oliveira), rat anti-HA-horseradish peroxidase (HRP) conjugated (high affinity) (Roche), and rat-anti-β-actin-HRP conjugated (Sigma) as loading control. Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody was purchased from Invitrogen. Membranes were developed by enhanced chemiluminescence detection according to the manufacturer's instructions (SuperSignal West Pico Chemiluminescent Substrate,

Thermo Scientific or Luminata Forte Western HRP Substrate, Millipore).

2.3.8. Immunoprecipitation

HEK-eGFP, HEK-I329L and HEK-TM/IC ($5x10^5$ cells/plate, in a 60 mm Ø plate) were transfected with 1μg of TLR3 expression plasmid, according to the Lipofectamine 2000 (Invitrogen) protocol. Forty-eight hours post-transfection, the cells were either stimulated with 100μg/ml Poly (I:C) (Amersham Biosciences) for 15 minutes, or left untreated.

Cells were then harvested and lysed in lysis buffer (15 mM TrisHCl, pH 7.4, 120 mM NaCl, 25 mM KCl, 2mM EDTA, 2mM EGTA, 0.1mM DTT and 1% Triton X-100) containing a protease inhibitor cocktail (Sigma). Immunoprecipitations were performed with Dynabeads protein A (Millipore), using rabbit anti-human TRIF antibody (Cell Signaling) and with Dynabeads protein G (Millipore), using mouse anti-HA (Santa Cruz). Washes were performed using commercially available lysis buffer (Sigma). Elution was done using 2X sample buffer. Immunoprecipitation eluates were resolved on a 12% SDS-PAGE gel and proteins were transferred to a PVDF membrane (GE Healthcare). Membranes were blocked with 5% non-fat milk for one hour at room temperature and probed with rabbit anti-human TRIF (Cell Signaling), mouse anti-human TLR3 (eBioscience), rat anti-HA-HRP conjugated (Roche) and anti-β-actin-HRP conjugated (Sigma) as a loading control for input samples. Horseradish peroxidase-conjugated rabbit anti-mouse and goat anti-rabbit secondary antibodies were purchased from Invitrogen.

2.3.9. Immunofluorescence

HEK-293T cells were seeded on glass coverslips ($1.5x10^5$) and transfected with pcDNA3-prom^{I329L}-I329L-HA or the non-recombinant pcDNA3HA. Forty-eight hours post-transfection, the cells were washed with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde for 20 minutes. The cells were washed with PBS and the coverslips were finally mounted in “Vectashield” (Vector laboratories) and examined under a fluorescence microscope. After washing, the cells were blocked with PBS + 0.05% Tween-20 containing 5% normal goat serum for one hour. Early endosomes were detected with rabbit anti-EEA1 (Sigma), late endosomes were detected with rabbit anti-LAMP1 (Sigma) and TRIF expression was detected with rabbit anti-TRIF (Cell Signaling). To visualize I329L-HA tagged protein, coverslips were incubated with rat-anti HA (high affinity) (Roche). Coverslips were washed and incubated with the secondary antibodies goat anti-rabbit Alexa488-conjugated (Invitrogen) or goat anti-mouse Texas Red-conjugated (Molecular Probes), respectively. Cell nuclei were stained with DAPI. All the incubations were performed at room temperature.

Confocal fluorescent images were obtained by a *DeltaVision Core* wide-field deconvolution inverted-base microscope (Applied Precision/Olympus), with a 100x objective. Images were analyzed by ImageJ 1.43u software. The term colocalization refers to the coincidence of green and red fluorescence, as measured by the confocal microscope.

2.3.10. Statistical Analysis

Data were shown as mean values with standard deviations (SD). Differences between experimental groups were determined by a two-tailed Student *t* test, using Excel software (Microsoft).

2.4. Results

2.4.1. The inhibition of IFN-β induction by I329L is MyD88 independent and not through cytoplasmic sensors

Although the ASFV ORF I329L has been described as an inhibitor of NF-κB activation, acting through a MyD88 independent pathway, the experiments performed did not test the activity of this viral gene in cells specifically activated by ectopic expression of MyD88. In order to definitively exclude this possibility, HEK-293T cells were co-transfected with the MyD88 expression plasmid and increasing quantities of I329L. A luciferase reporter plasmid containing only the NF-κB binding site of the IFN-β promoter, the positive regulatory domain (PRD)-II, was used.

In the positive control, ectopic expression of MyD88 significantly increased the NF-κB promoter-mediated luciferase activity. As presented here, the expression of I329L has no effect on the activation of the NF-κB transcription factor by MyD88 (Fig 2.1). Therefore, the previously described inhibitory activity of I329L must be through a MyD88 independent pathway.

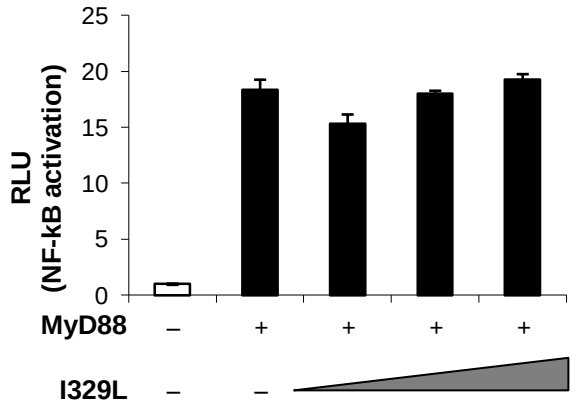


Figure 2.1 - ASFV I329L inhibition of Poly (I:C)-mediated IRF3 and NF-kB activation is MyD88 independent

HEK-293T cells were co-transfected with the NF-kB binding luciferase reporter (PRD-II), the β-galactosidase control plasmid, 40ng of MyD88 expression plasmid (■) and increasing amounts (200-600ng) of pcDNA3-I329L-HA. Luciferase activity was normalized to β-galactosidase activity as a control for transfection efficiency. Data are expressed as means of RLU ± SD of triplicate well from one of three similar experiments.

Both TLR3 and RLR signalling pathways are capable of recognizing dsRNA or its synthetic analogue, Poly (I:C), initiating a cascade of events that lead to the activation of NF-kB, IRF-3 and IRF-7, transcription factors necessary for the induction of IFN-β. Given that I329L has been described as an inhibitor of IFN-β induction in Poly (I:C) stimulated cells, we assessed the effect of this viral gene on the induction of IFN-β by ectopic expression of either RIG-I or MDA-5, the RLR family members involved in viral recognition and induction of type I IFN.

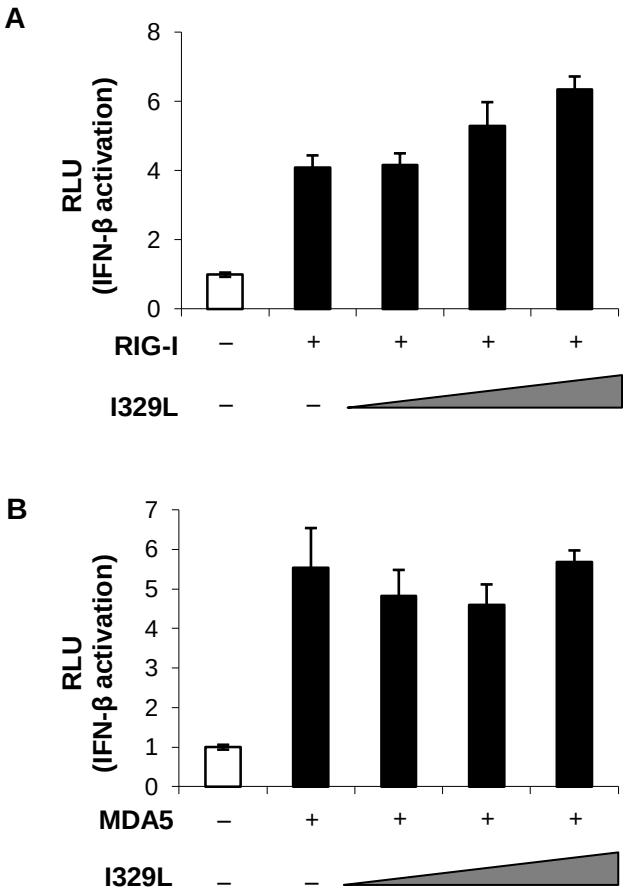


Figure 2.2 – ASFV I329L inhibition of Poly(I:C)-mediated IRF3 and NF-kB activation is independent of the RLR pathway

HEK-293T cells were co-transfected with the IFN-β promoter luciferase reporter, the β-galactosidase control plasmid, 40ng of RIG-I expression plasmid (**A**) or MDA-5 expression plasmid (**B**) and increasing amounts (200-600ng) of pcDNA3-I329L-HA. Luciferase activity was normalized to β-galactosidase activity as a control for transfection efficiency. Data are expressed as means of RLU ± SD of triplicate well from one of three similar experiments.

The results presented here (Fig. 2.2-A and 2.2-B) clearly demonstrate that I329L is not exerting its inhibitory activity over the RLR pathway.

2.4.2. The ASFV ORF I329L colocalizes to the early endosome

The ASFV ORF I329L was previously described to be a surface membrane expression protein, also localizing to the endoplasmic reticulum (ER) and the Golgi complex, inside the cell [1]. However, it was also reported to inhibit both induction of IFN-β and NF-κB activation through the TLR3 pathway, a statement confirmed by the results depicted in Fig. 2.3.

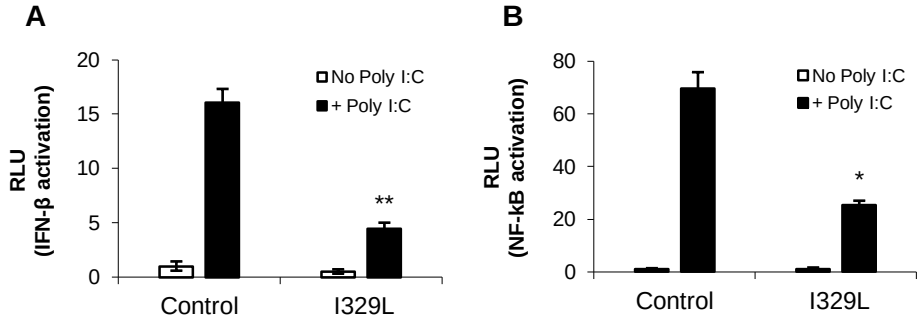


Figure 2.3 – The ASFV ORF I329L inhibits Poly (I:C) stimulated activation of IFN-β transcription in an NF-κB dependent manner.

HEK-293T cells stably expressing TLR3 protein, were co-transfected with the pcDNA3HA (Control) or pcDNA3-I329L-HA plasmid, the β-galactosidase plasmid and either the (**A**) IFN-β promoter (IFNβ) or the (**B**) NF-κB promoter (PRDII) luciferase reporter. For both assays, forty-eight hours post-transfection, the cells were either induced with 35μg/ml Poly (I:C) for five hours (■), or left untreated (□). Luciferase activity was normalized to β-galactosidase activity as a control for transfection efficiency. Data are expressed as means of Relative Luciferase Units (RLU) ± SD of triplicate well from one of three similar experiments. Statistical significance is represented as p≤0.05 (*) or ≤0.01 (**).

The TLR3 recognizes microbial nucleic acids and is mainly located within endolysosomal compartments, although some cell surface expression has been described in fibroblasts. Considering the suggested mechanism of action of I329L, one would expect it to be

associated to such cell compartments. Immunofluorescence assays were performed in HEK-293T cells stably expressing I329L protein with an HA tag.

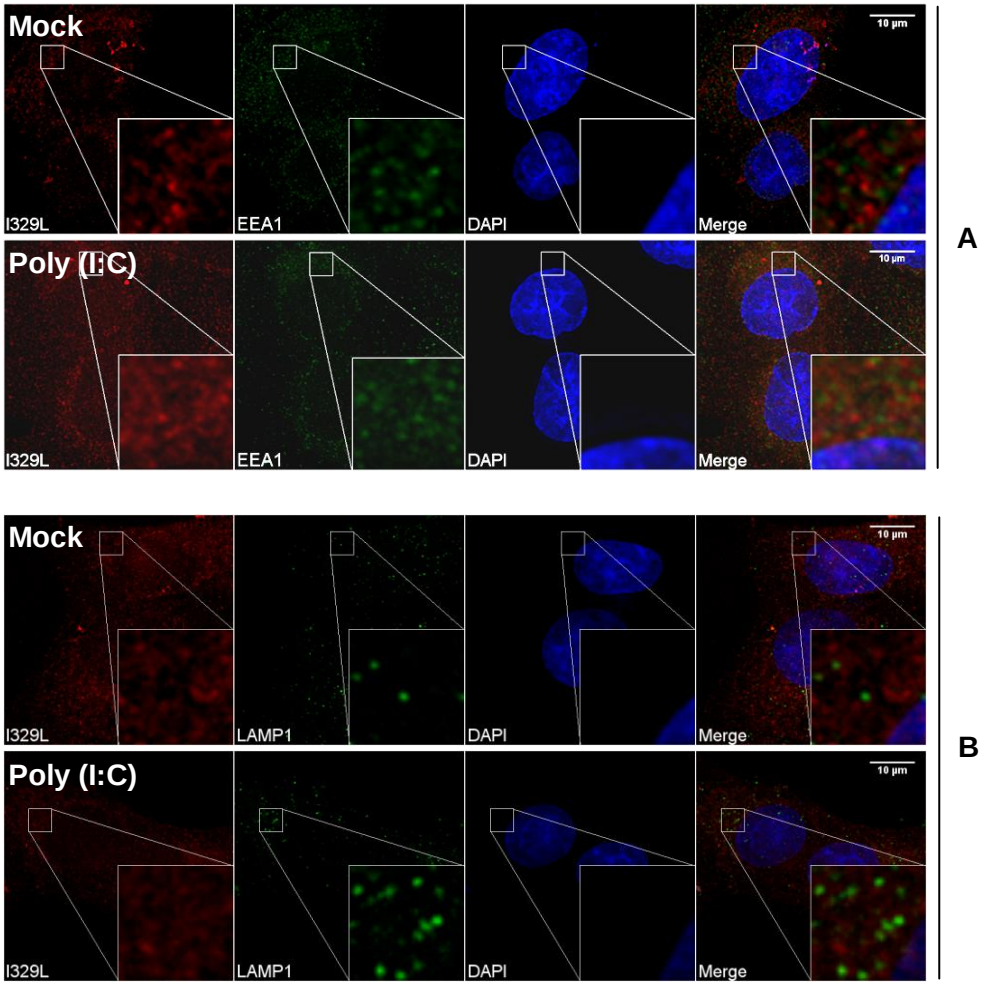


Figure 2.4 – The ASFV ORF I329L colocalizes to the early, but not late endosomes.

HEK-293T cells stably expressing I329L-HA tagged protein were transfected with TLR3 plasmid. Forty-eight hours post-transfection, the cells were either stimulated with 100µg Poly(I:C) for 30min., or left untreated. Early endosomes (**A**) were visualized using rabbit anti-EEA1 antibody and late endosomes (**B**) were visualized using rabbit anti-LAMP1 antibody (red). The I329L protein was visualized using a monoclonal anti-HA antibody (blue). Bar, 10µm.

As presented here, I329L partially colocalizes with early, but not late, endosomes (Fig.2.4). Colocalization with early endosomes requires activation by Poly (I:C), suggesting that I329L is recruited for this subcellular compartment following activation of TLR3 signalling pathway. In non-stimulated cells, I329L is probably localised in the ER and the Golgi complex, as previously reported by de Oliveira *et al.*, 2011.

Whereas the endoplasmic reticulum functions in concert with the Golgi complex to target new proteins to their proper destinations, early endosomes and the Golgi communicate bidirectionally. As such, it is reasonable that a viral membrane protein may be directed to the Golgi complex through the ER, and then sent to the early endosome, where it will inhibit TLR3 signalling.

2.4.3. The ASFV ORF I329L interacts with TRIF

The ASFV I329L protein was proposed to exert its effect in the TLR3 pathway by interfering with the TRIF adaptor. Upon LPS-binding, and an early signalling event through MyD88, TLR4 initiates an endosomal phase, where it is able to induce a late pro-inflammatory response through TRIF. A luciferase assay was performed in which the impact of I329L protein expression in the LPS-mediated activation of NF-κB was assessed.

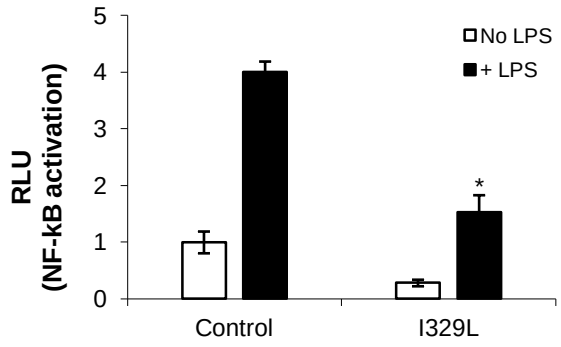


Figure 2.5 – The ASFV ORF I329L inhibits LPS-mediated activation of NF-κB.

HEK-293T cells, were co-transfected with the pcDNA3HA (Control) or pcDNA3-I329L-HA plasmid, the TLR-4 expression plasmid, the β-galactosidase plasmid and the NF-κB promoter (PRDII) luciferase reporter. Forty-eight hours post-transfection, the cells were either induced with 100ng/ml LPS for five hours (■), or left untreated (□). Luciferase activity was normalized to β-galactosidase activity as a control for transfection efficiency. Data are expressed as means of Relative Luciferase Units (RLU) ± SD of triplicate well from one of three similar experiments. Statistical significance is represented as p≤0.05 (*).

The fact that TLR4 is able to initiate an endosomal, TRIF-dependent pathway, and the observation that I329L is able to inhibit LPS-mediated activation of NF-κB (Fig.2.5), are in agreement with the hypothesis that I329L interferes with TRIF.

Immunofluorescence assays were performed to verify if I329L and TRIF colocalize to the same location.

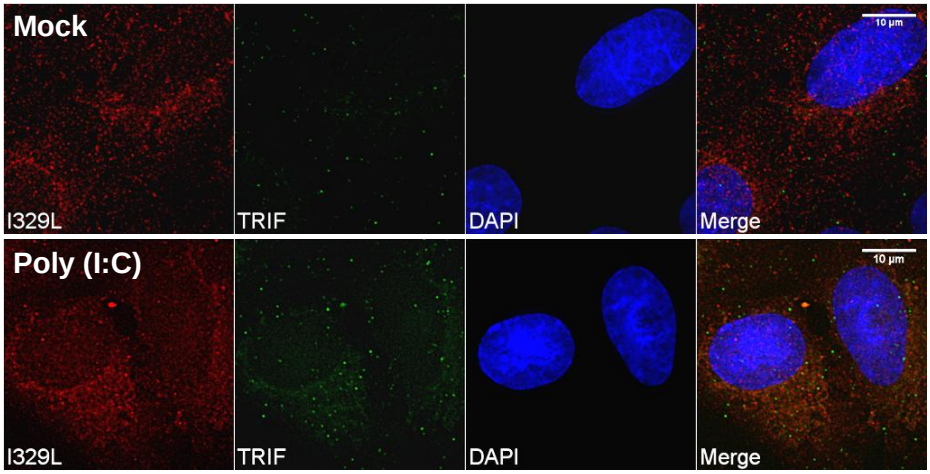


Figure 2.6 – The ASFV ORF I329L colocalizes to TRIF.

HEK-293T cells were co-transfected with pcDNA3-prom^{I329L}-I329L-HA and TLR3 expression plasmid. Forty-eight hours post-transfection, the cells were either stimulated with 100µg Poly(I:C) for 30min., or left untreated. Expression of endogenous TRIF protein was visualized using rabbit anti-TRIF antibody (green). The I329L protein was visualized using a monoclonal anti-HA antibody (red). Bar, 10µm.

As can be seen, I329L and TRIF partially colocalize on Poly (I:C) activated cells, but not on non-stimulated cells. The results presented in both Fig.2.4 and Fig.2.7, suggest that I329L is being recruited to the endosomes of stimulated cells, where it interferes with TRIF, inhibiting cytoplasmic signal transduction.

The colocalization of I329L and TRIF are consistent with an interaction between these two proteins. An immunoprecipitation assay was performed, in order to verify this assumption.

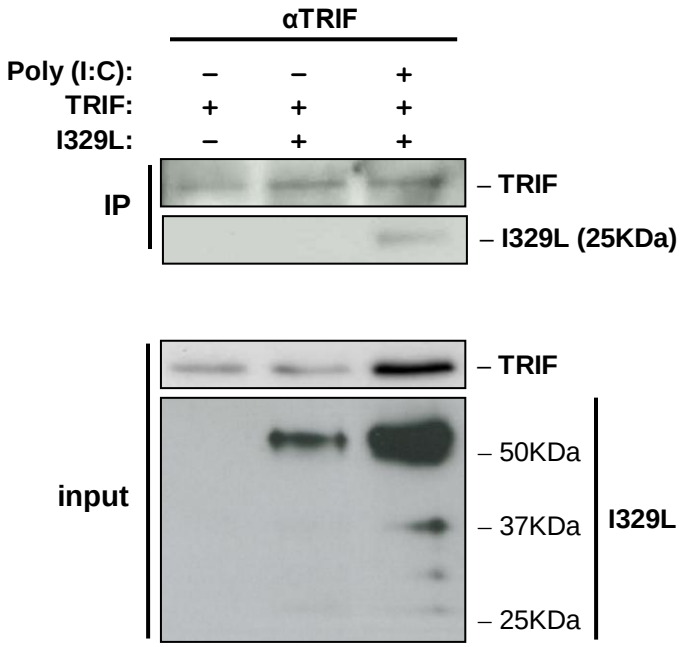


Figure 2.7 – The ASFV ORF I329L co-immunoprecipitates with TRIF.

HEK-293T cells stably expressing the C-terminally HA-tagged I329L protein were transfected with TLR3 expression plasmid. Forty-eight hours post-transfection, the cells were stimulated with 100μg/ml Poly (I:C) for 20 minutes, or left untreated. Cell lysates were immunoprecipitated with rabbit anti-TRIF (αTRIF). The immunoprecipitates (IP) were analyzed by immunoblot with anti-HA or anti-TRIF (upper panel). Expression of TRIF and I329L in the lysates was detected by immunoblot analysis with anti-TRIF and anti-HA (bottom panel).

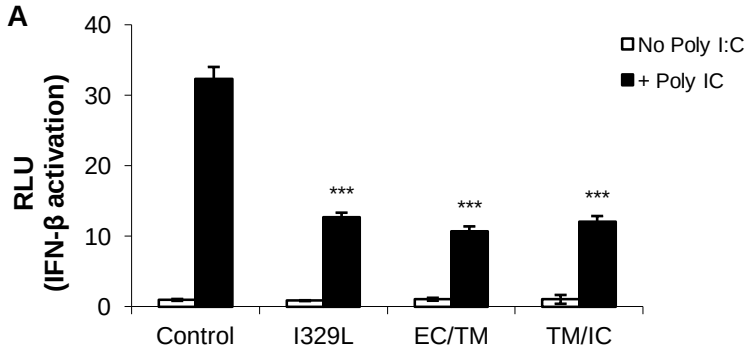
The results presented here (Fig.2.7) confirm that I329L directly interacts with TRIF, interfering with its function as an essential adaptor for the TLR3 signalling pathway. Intriguingly, the HA-tagged fragment immunoprecipitated with TRIF is ~25KDa, suggesting that some cleavage may be occurring prior to interaction of I329L with TRIF.

2.4.4. The ASFV ORF I329L inhibits TLR3 signalling pathway by two distinct mechanisms.

Bioinformatic analysis of the I329L protein sequence, using the TMHMM program, revealed an N-terminal extracellular domain, a transmembrane domain (with a single transmembrane helix) and a C-terminal intracellular domain. Based on these previously published results, and in order to define the domain involved in the inhibition of TLR3 signalling pathway, we cloned I329L truncated mutants containing the transmembrane domain sequence but lacking either the ectodomain (TM/IC) or the intracellular domain (EC/TM).

2.4.4.1. Inhibition of stimulation with Poly (I:C)

Luciferase reporter assays were performed on cells transfected with expression plasmids for the entire I329L and both truncated mutants, in order to assess their impact on the activation of the TLR3 signalling pathway through Poly (I:C).



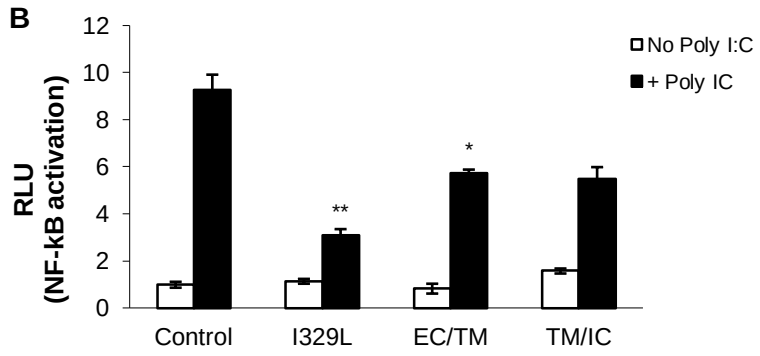


Figure 2.8 – Both domains of the ASFV ORF I329L inhibit Poly (I:C) stimulated activation of IFN-β transcription (A) in an NF-κB dependent manner (B).

HEK293-TLR3 stably expressing cells, were co-transfected with the pcDNA3HA (Control), pcDNA3-I329L-HA, pcDNA3-EC/TM-HA or pcDNA3-TM/IC-HA plasmids, the β-galactosidase plasmid and either the (**A**) IFN-β promoter (IFNβ) or the (**B**) NF-κB promoter (PRDII) luciferase reporter. For both assays, forty-eight hours post-transfection, the cells were either induced with 35μg/ml Poly (I:C) for five hours (■), or left untreated (□). Luciferase activity was normalized to β-galactosidase activity as a control for transfection efficiency. Data are expressed as means of Relative Luciferase Units (RLU) ± SD of triplicate well from one of three similar experiments. Statistical significance is represented as p≤0.05 (*) or ≤0.01 (**).

The results obtained using this reporter indicate that both I329L truncated mutants inhibit IFN-β induction in response to Poly (I:C) (Fig.2.8-A) through an NF-κB dependent pathway (Fig. 2.8-B). The same result was obtained with the expression of the entire I329L molecule.

2.4.4.2. Inhibition of stimulation by ectopically expressed TRIF

The only TLR3 adaptor, TRIF, was suggested as a probable target for this viral protein, so a similar luciferase reporter assay was performed, activating the cells through ectopic expression of TRIF.

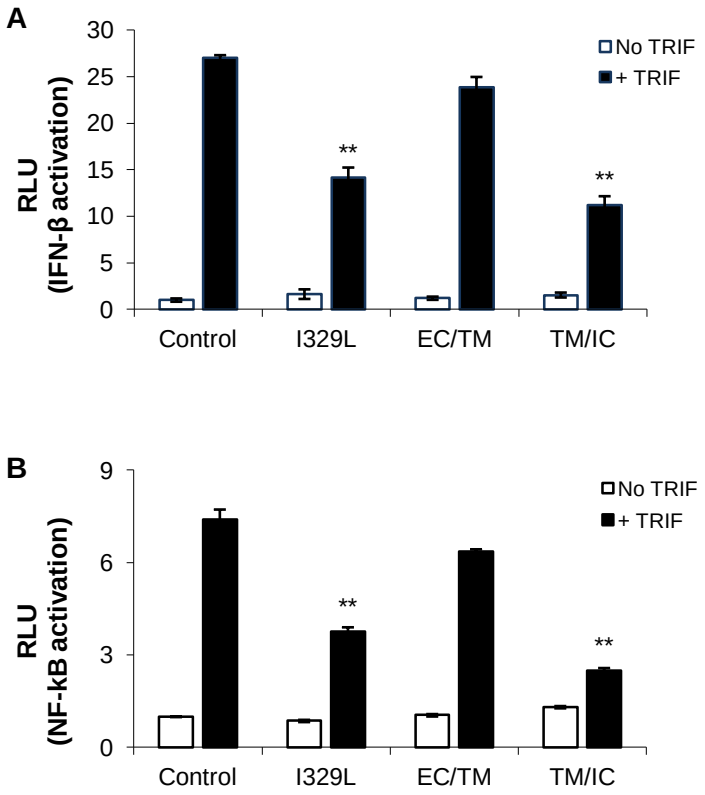


Figure 2.9 – The intracellular, but not the extracellular domain of the ASFV ORF I329L, inhibits ectopic TRIF stimulated activation of IFN-β transcription (A) in an NF-κB dependent manner (B).

HEK-293T cells were co-transfected with the pcDNA3HA (Control), pcDNA3-I329L-HA, pcDNA3-EC/TM-HA or pcDNA3-TM/IC-HA plasmids, the β-galactosidase plasmid and either the (**A**) IFN-β promoter (IFNβ) or the (**B**) NF-κB promoter (PRDII) luciferase reporter. Cells were induced by co-transfection of TRIF expression plasmid. Luciferase activity was normalized to β-galactosidase activity as a control for transfection efficiency. Data are

expressed as means of Relative Luciferase Units (RLU) ± SD of triplicate well from one of three similar experiments. Statistical significance is represented as p≤0.01 (**).

These results clearly reveal an inhibitory role for the I329L intracellular domain in cells induced by TRIF ectopic expression (Fig. 2.9). Strikingly, the extracellular domain has no inhibitory effect on cells induced by TRIF expression, although it is capable of inhibiting Poly (I:C) stimulated induction of IFN-β (see Fig. 2.8).

2.4.4.3. The extracellular domain of I329L inhibits stimulation with Poly (I:C) in a dose dependent manner

A luciferase reporter assay was performed in cells expressing amounts of EC/TM truncated mutant and an IFN-β promoter luciferase reporter, stimulated by Poly (I:C).

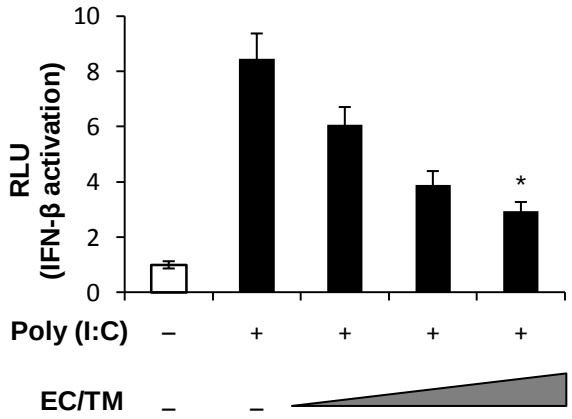


Figure 2.10 - The ectodomain-transmembrane domain fragment of I329L inhibits Poly (I:C)-mediated activation of the IFN-β promoter in a dose dependent manner.

HEK293-TLR3 stably expressing cells were co-transfected with the IFN-β promoter luciferase reporter, the β-galactosidase control plasmid, the non-recombinant pcDNA3HA and increasing amounts (300-900ng) of pcDNA3-

EC/TM-HA plasmid (encoding for the ectodomain-transmembrane domain fragment). Forty-eight hours post-transfection, the cells were stimulated with 35μg/ml Poly (I:C) for five hours (■), or left untreated (□). Luciferase activity was normalized to β-galactosidase activity as a control for transfection efficiency. Data are expressed as means of RLU ± SD of triplicate well from one of three similar experiments. Statistical significance is represented as p≤0.05 (*).

As presented here (Fig.2.10), the inhibition of Poly (I:C)-mediated activation of the IFN-β promoter by the ectodomain-transmembrane domain (EC/TM) fragment of I329L is dose dependent. Taken together with the observation that the EC/TM fragment does not inhibit the TRIF-mediated activation of the IFN-β promoter, these results suggest a role for I329L in inhibiting TLR3 function, either at the level of ligand binding or receptor dimerization.

2.4.5. Proteolytic processing of I329L

Recent evidence suggests that TLR9, TLR7 and TLR3 are proteolytically processed as an essential step in their signalling transmission mechanism [11]. Although I329L is present in transfected cells as a stable molecule of ~50KDa, the availability of a rabbit antibody (prepared by Parkhouse, R.M.E.) against the recombinant I329L protein allowed an examination of the stability of the I329L molecule in virus infected cells.

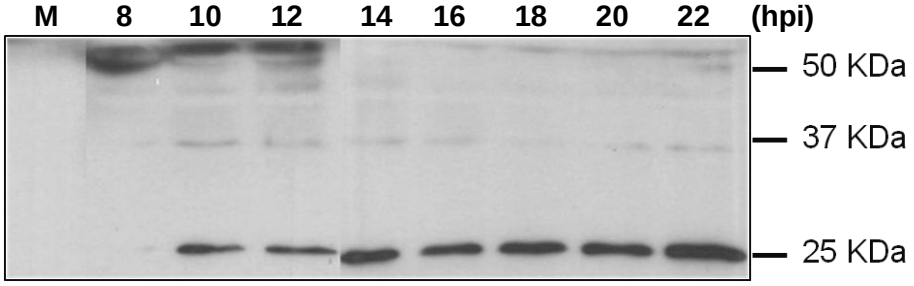


Figure 2.11 – The ASFV I329L protein is proteolytically processed during ASFV infection.

Vero cells were infected with Ba71V ASFV strain at MOI=3 or mock infected (M). Cells were lysed at timepoints 8 to 22 hpi (hours post infection) and immunoblotted with rabbit anti-I329L serum.

As can be seen (Fig.2.11), ten hours after infection of Vero cells with the tissue culture adapted Ba71V strain of ASFV, there is a dramatic conversion of I329L to a molecule of ~25KDa.

This observation raised the question of whether the proteolytic degradation was a consequence of virus infection *per se*, or whether it was triggered by activation of the TLR pathway. Therefore, the stability of I329L was investigated in cells transfected with I329L cloned with a C-terminal HA sequence, and then stimulated with either Poly (I:C) or ectopically expressed TRIF. The cells were then lysed and examined by Western blot.

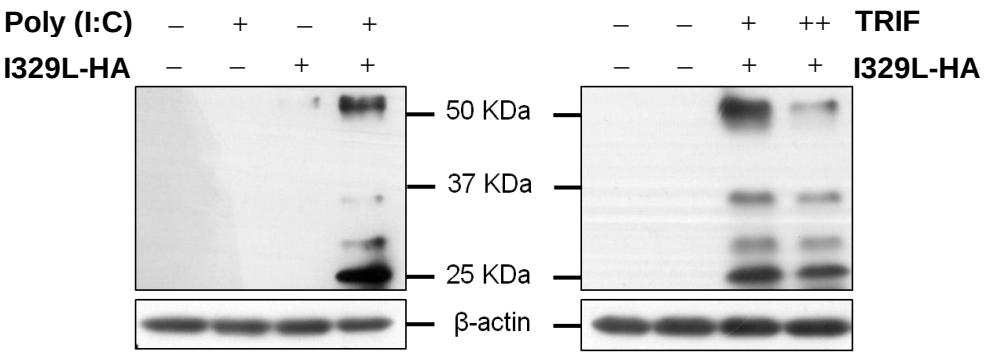


Figure 2.12- The ASFV ORF I329L expression and processing is dependent on activation by either Poly (I:C) or ectopic expression of TRIF and involves proteolytic cleavage.

HEK293T cells stably expressing I329L-HA or non-recombinant eGFP were either induced with 100µg/mL Poly (I:C) for 1 hour or left untreated (**A**). Alternatively, cells were induced by transfection of 500ng (+) or 1µg (++) of TRIF expression plasmid (**B**). The cells were lysed and total cell extracts were immunobloted with anti-HA-HRP conjugated antibody to detect expression of I329L-HA tagged protein. Anti-β-actin antibody was used as loading control.

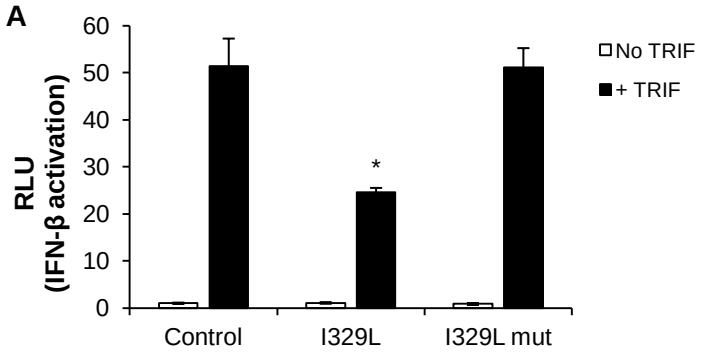
As can be seen, with either stimulus, there was a similar degradation of I329L to a predominant fragment of about 25KDa (Fig.2.12), presumably an N-terminal fragment, as the Western blot was developed with an antibody recognizing a C-terminally expressed HA epitope.

2.4.5.1. I329L has a potential cleavage site for ASFV and cellular proteases, such as cathepsin L.

The observation of proteolytic processing of I329L in virus infected and in Poly (I:C) or ectopic TRIF stimulated, I329L transfected cells, clearly requires proteolytic susceptibility of the molecule. As the processing of TLRs has been shown to be the result of the activity of endosomal

cathepsins S, L and B, the presence of the sequence GGFGKE, recognized by cathepsin L, at aminoacid residues 107-112 in I329L sequence, could be relevant. Additionally, the sequence GGF, at aminoacid residues 107-109, is recognized by the ASFV polyprotein processing protease [23].

Accordingly, this sequence was mutated, at residue 108, to G***A***FGKE (or G***A***F), and the resulting mutant I329L protein was tested for inhibition of induction of IFN-β and NF-κB activation in transfected cells, with the wild type I329L sequence as a control. As can be seen, and in contrast to the control, the mutant I329L had no effect on the activation of either IFN-β or NF-κB luciferase reporters by ectopic expression of TRIF (Fig.2.13), although it resembled the wild type sequence in inhibiting activation stimulated by Poly (I:C) (Fig. 2.14).



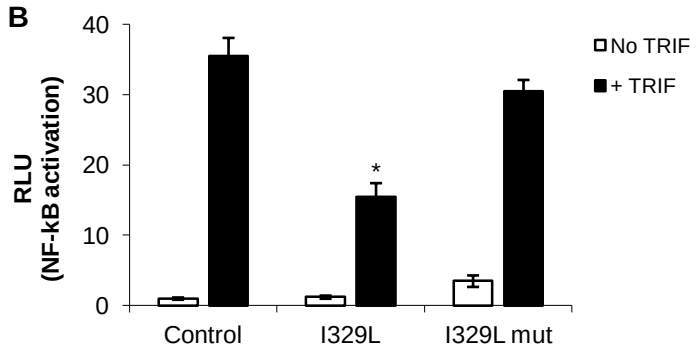


Figure 2.13 - The ASFV mutant I329L gene has no effect on IFN-β induction (A) and NF-kB activation (B) on cells stimulated by ectopic expression of TRIF adaptor.

HEK-293T cells were co-transfected with the non-recombinant pcDNA3HA (Control), pcDNA3-I329L-HA or pcDNA3-I329L.G108A-HA plasmids, the β-galactosidase plasmid and either the (**A**) IFN-β promoter (IFNβ) or the (**B**) NF-κB promoter (PRDII) luciferase reporter. Cells were induced by co-transfection of TRIF expression plasmid. Luciferase activity was normalized to β-galactosidase activity as a control for transfection efficiency. Data are expressed as means of Relative Luciferase Units (RLU) ± SD of triplicate well from one of three similar experiments. Statistical significance is represented as $p \leq 0.05$ (*).

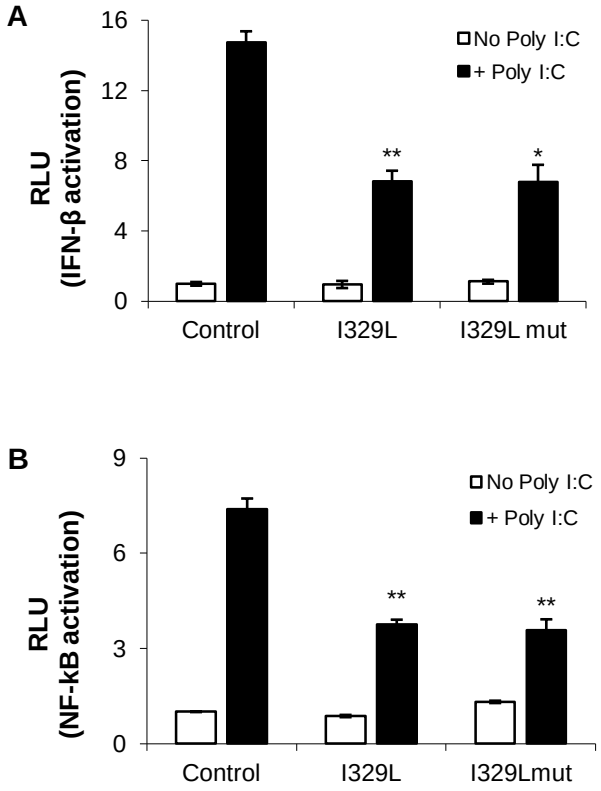


Figure 2.14 – The ASFV mutant I329L gene inhibits Poly(I:C) stimulated induction of IFN-β (A) or NF-κB activation (B).

HEK-293T cells were co-transfected with the non-recombinant pcDNA3HA (Control), pcDNA3-I329L-HA or pcDNA3-I329L.G108A-HA plasmids, the β-galactosidase plasmid and either the (**A**) IFN-β promoter (IFNβ) or the (**B**) NF-κB promoter (PRDII) luciferase reporter. For both assays, forty-eight hours post-transfection, the cells were either induced with 35μg/ml Poly (I:C) for five hours (■), or left untreated (□). Luciferase activity was normalized to β-galactosidase activity as a control for transfection efficiency. Data are expressed as means of Relative Luciferase Units (RLU) ± SD of triplicate well from one of three similar experiments. Statistical significance is represented as p≤0.05 (*) or ≤0.01 (**).

The amount of IFN-β secreted into the supernatants of cells expressing either wild type pI329L, the mutant pI329L.G108A, and stimulated by ectopic expression of TRIF, was determined by ELISA, to examine the impact of these proteins on the expression and secretion of IFN-β. Once again, the mutant failed to inhibit ectopic TRIF stimulation (Fig.2.15).

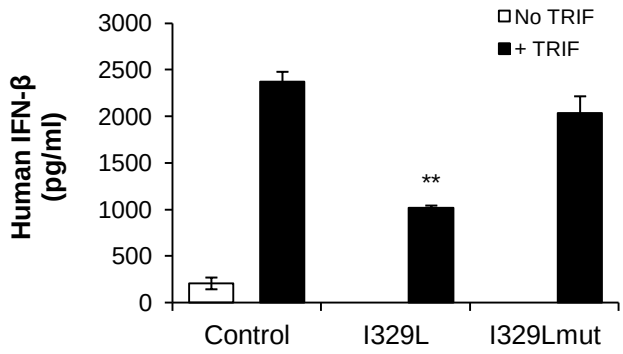


Figure 2.15 – The ASFV mutant I329L has no impact on secretion of IFN-β in cells induced by ectopic expression of TRIF.

HEK-293T cells were co-transfected with the non-recombinant pcDNA3HA (Control), pcDNA3-I329L-HA or pcDNA3-I329L.G108A-HA plasmids. Cells were induced by co-transfection of TRIF expression plasmid. Supernatants were collected and IFN-β concentration (pg/ml) was measured by ELISA. Data are expressed as means ± SD of triplicate well from one of two similar experiments. ** Statistically significant when compared to control vector-expressing cells ($p \leq 0.01$).

It may be concluded, therefore, that the cathepsin L sensitive site in pI329L sequence is required for its inhibition of the TLR3 signalling at the level of TRIF, but not for signalling through Poly (I:C).

2.5. Discussion

African swine fever virus (ASFV) is an economically important cytoplasmically replicating DNA virus of pigs, endemic in many African countries, and which entered Portugal with a disastrous economic impact [24]. There is no vaccine, and although ASFV is known to interfere with signalling pathways controlling the transcription of a large number of immunomodulatory genes such as cytokines [25,26,27], no individual virus gene manipulating the IFN response has been reported. This is all the more surprising as ASFV not only possess an early acute phase in macrophages, but may also persist. It is hard to imagine that a virus with this lifestyle could persist in the face of an efficient IFN response.

Many viral genes evolved to mimic or block host cellular functions are likely to have been acquired from the host. It is also likely that some of the many non-homologous genes of the ASFV will have evolved for host manipulation and will only be detected by functional approaches, as indeed is reported in this thesis.

The search for a TLR agonist in ASFV was prompted by the fact that the virus infects both vertebrate and invertebrate hosts, and only innate immunity, in particular TLR responses, is common to both hosts. Initial identification of an ASFV TLR homologue was negative. Bioinformatic analysis predicted ORF I329L to be a type I transmembrane protein containing extracellular putative leucine-rich repeats (LRRs) and an intracellular TIR domain homologue. Upon more focused bioinformatic analysis, a marginal homology to the TIR domain of TLR3 was reported and, in addition, I329L was recently reported to impair the cellular responses controlled by TLR3 that lead to both IFN-β secretion and NF-κB activation [1]. The precise mechanism for the inhibition of TLR3 signalling was not elucidated and

the adaptor TRIF was tentatively proposed as a potential target for I329L. A modelling exercise on this viral protein supported the idea that I329L might function as a TLR3 decoy, through the formation of TLR3-I329L heterodimers and, in doing so, inhibit the downstream signalling pathway [22].

The biochemical demonstration of the interaction between I329L and TRIF was a key observation, predicting that I329L could inhibit an NF-κB dependent induction of IFN-β in cells stimulated by ectopic expression of TRIF. This indeed proved to be so and prompted the testing of the individual intra- and extracellular domains of I329L on the activation of NF-κB and IFN-β in cells stimulated with Poly (I:C) or by ectopic expression of TRIF. Interestingly, the extracellular domain inhibited activation in a dose dependent manner, an observation consistent with either direct competition of I329L for the Poly (I:C) ligand or the formation of a non-functional TLR3-I329L heterodimer. The intracellular domain similarly inhibited the NF-κB dependent activation of IFN-β stimulated by the Poly (I:C) ligand. On repeating similar assays, but this time with TRIF mediated ectopic stimulation, the extracellular domain was significantly without impact, whereas the intracellular domain continued to inhibit activation of IFN-β. We may conclude that the I329L mediated inhibition of TLR signalling pursues a dual strategy, with its extra- and intracellular domains evolved for interfering with the initiation and subsequent intracellular transmission of the dsRNA stimulus, respectively.

TLR3, TLR7, and TLR9 localize to and exclusively signal from endolysosomal compartments. Recent studies have demonstrated that TLR9 is processed in endolysosomal compartments by resident proteases and that this processing is required to generate a functional receptor [12,14,28]. It has been demonstrated that TLR9 cleavage

occurs through a multistep process: the first step is mediated either by asparagine endopeptidase or by some members of the cathepsin family of proteases, particularly cathepsin L and S; the second processing event is an exclusively cathepsin–mediated N-terminal trimming, which is also required for optimal receptor function. It was also shown that TLR7 and TLR3 are processed in a similar manner, implying that receptor proteolysis is a conserved mode of regulating all nucleic acid–sensing TLRs that may have evolved alongside the ability to recognize nucleic acids as a signature of infection [11].

The dual strategy of I329L, in particular the inhibitory activity of the isolated intracellular domain is intriguing and, stimulated by these recent observations of proteolytic processing of TLRs, the possibility of a similar processing for I329L was pursued. As I329L is totally stable in transfected cells, the only rational possibility was to assume that proteolytic processing would be an event subsequent to viral activation of the TLR3 pathway. Thus I329L, in virus infected cells, was seen to be processed from its full size of 50KDa to a fragment of 25KDa. Following this, the next step was to test the stability of I329L in transfected cells, simultaneously stimulating the TLR3 pathway either with Poly (I:C) or through ectopic expression of TRIF. As described, a similar proteolytic processing of I329L occurred when the TLR3 pathway was activated either by Poly (I:C) or ectopic expression of TRIF.

Considering the requirement for endosomal TLR proteolytic processing and our observations of I329L processing, the presence of a cathepsin L sensitive site in the pI329L ectodomain sequence is an interesting feature, and a pI329L.G108A mutant was constructed in order to check its functional relevance. Interestingly, the mutant pI329L.G108A had no effect on the activation of either IFN-β or NF-κB luciferase reporters

by ectopic expression of TRIF, although it resembled the wild type I329L in inhibiting activation stimulated by Poly (I:C). Additionally, pI329L.G108A is also unable to inhibit IFN-β secretion in cells activated by ectopic expression of TRIF. Although it remains to be verified that this mutant I329L protein is not proteolytically processed in TLR3-stimulated cells, it is apparent that proteolytic processing of I329L is a necessary requirement for its inhibition of the TLR3 signalling at the level of TRIF, but not for signalling through Poly (I:C).

In conclusion, we suggest that the I329L molecule provides a dual viral strategy for inhibition of the TLR response: inhibition of its initiation via dsRNA, through formation of an I329L-TLR3 heterodimer, followed by proteolytic processing, resulting in an inhibition of the interaction between the intracellular domain of I329L and its target TRIF. This model is open to experimental confirmation, as are the precise details of the proteolytic processing.

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Chapter 3

The MGF360-18R gene of ASFV inhibits the induction of the IFN-β

3.1. Summary

A previous screening performed in this laboratory (Correia, S.M., unpublished work) identified a member of the ASFV multigene family (MGF)-360, the DP148R gene (recently renamed MGF360-18R), as an inhibitor of the induction of IFN-β. Sequence comparison of the genomes of ASFV pathogenic and non-pathogenic strains revealed that MGF360-18R is a non-conserved open-reading frame (ORF). Two variants of this viral gene were studied: a 148a.a. variant, encoded by the non-pathogenic Ba71V ASFV isolate and a 254a.a. variant, encoded by the pathogenic Benin97/1 isolate, the virus responsible for a recent outbreak of ASF in Africa.

Using luciferase reporter assays, both MGF360-18R variants were demonstrated to inhibit the induction of IFN-β in a NF-κB dependent manner. Similarly, an inhibition of IFN-β secretion was demonstrated by ELISA.

Ectopic expression of IFN signalling intermediates of both Toll-like receptor (TLR) and RIG-I-like receptor (RLR) pathways allowed the identification of potential molecular targets. These experiments, and the intracellular colocalization of this virus gene with mitochondrial MAVS, indicated that both MGF360-18R variants inhibited IFN-β induction by targeting MAVS, a key adaptor protein of the RLR pathway. Additional luciferase reporter assays demonstrated that IRF-3 is also targeted by the MGF360-18R variant from the Benin97/1 isolate, but not by the Ba71V variant.

In conclusion, the two variants of MGF360-18R were shown to inhibit IFN-β induction by an NF-κB dependent mechanism, both at the level of transcriptional activation and protein secretion. Molecular targets for

both variants of MGF360-18R have been identified, and the mechanism these two genes evolved for manipulation of the IFN response is discussed.

3.2. Introduction

Large DNA viruses, such as the ASFV, encode many proteins involved in the evasion of host immune responses. The ASFV contains a number of open reading frames ranging from 160 to 175, depending on the isolate. Approximately 90 of the proteins encoded by the ASFV genome are predicted to be structural proteins or involved in virus replication [1]. Many of the remaining 70 to 85 proteins most probably will have evolved for host evasion.

The modulation of the interferon response by ASFV has only been described in the comparison of transcriptional profiles of macrophage infected with wild type virus and a deletion mutant virus lacking six MGF360 and two MGF530 genes. These results suggest 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]. However, precisely which genes within the multigene families are responsible for this ability and their mechanism of action remains unknown. In addition, the MGF360 and 530 members were reported to be swine macrophage host range determinants that function by promoting the survival of infected cells [3]. More recently, and indeed very interestingly, MGF360 genes were proposed to be significant tick host range determinants, being required for efficient virus replication and generalization of infection in ticks [4].

The activation of the different routes of induction of IFN depends upon the specific virus, the stage of infection and, particularly, on the ability of the host cell to detect the viral infection. Two major receptor systems recognize most viral pathogen-associated molecular patterns (PAMPs): endosomal TLRs detect viral nucleic acids in endosomes of specialized cell types, whilst RLRs detect cytoplasmically located viral nucleic acids produced upon- infection [5,6]. These two systems work in concert to fight infection, converging on the activation of common transcription factors, such as NF-κB, IRF-3 and IRF-7, to promote the production of type I IFN, in addition to inflammatory cytokines.

The interferons (IFNs) are a group of secreted cytokines that are recognized as critical regulatory mediators of the immune response. The functions of IFNs are represented by three major biological activities: antiviral activity, antitumor activity and immunoregulatory activity [7]. Upon recognition of a viral infection, cells activate signal transduction pathways that culminate in establishment of an antiviral state and induction of type I IFN. The secreted cytokine then stimulates an antiviral state in neighbouring cells and induces the expression of proteins that interfere with viral processes, thus blocking viral replication [6,8]. Type II IFN, in turn, is produced by activated lymphocytes, further amplifies the IFN response to infection [9], and plays a key role in the orchestration of both the innate and acquired immune responses.

Induction of the IFN-β gene requires the activation of two families of transcription factors: the family of Nuclear Factors κB (NF-κB) and the family of Interferon Regulatory Factors (IRFs). Each of the transcription factors bind to the IFN-β promoter with limited affinity. Thus optimal induction requires cooperativity between these two factors. Together with a c-jun/ATF-2 heterodimer, IRF-3, IRF-7 and NF-κB form the

enhanceosome, a complex that binds to the IFN-β promoter, inducing gene expression [10]. Several studies revealed that binding of IRF-3 and/or IRF-7 is indispensable for induction, but activation of NF-κB and c-jun/ATF-2 may not be essential (reviewed by Versteeg & García-Sastre, 2010) [6]. Positive feedback models propose that IRF-3 alone may directly induce expression of the IFN-β gene, which feeds back onto cells and induces the synthesis of IRF-7. In the presence of a continued infection, IRF-7 enhances the transcription of the IFN-β gene and allows transcription of the IFN-α genes [8]. Subsequently, type I IFNs are produced and secreted, acting both in an autocrine and paracrine manner by binding to cell surface type I IFN receptors, activating the Jak-Stat signalling pathway and ultimately leading to the expression of hundreds of genes and inducing an “anti-viral” state in adjacent cells [11].

Finally, this brief summary would not be complete without the remark that this is only what we know today and there is still much to be learned about the organisation and function of the interferon system. As the interferon system plays a major role as an early host defence system against virus infections [12], viruses have evolved a number of counter strategies to antagonise this response. The final objective of any viral evasion strategy is to prevent upregulation of type I IFN by host cells, thereby avoiding the antiviral activity of ISGs [13].

In a previous study performed in this laboratory (Correia, S.M., unpublished results), 17 early genes with unassigned functions were tested for their capability to inhibit the expression of IFN-β (7 from MGF360 and 1 from MGF530). The genes were selected for early expression as this is a predicted feature for host evasion genes that have evolved for manipulation of IFN responses. Of the 17 genes that were tested, four inhibited the induction of IFN-β. Two of these (A276R

and MGF360-18R) are from MGF360 and one (A528R) is from MGF530.

The non-conserved ORF MGF360-18R, a member of MGF360, was selected as the focus for this work and investigated in detail in order to define how it inhibits the induction of IFN-β following the stimulation of cells with an analogue of dsRNA, Poly (I:C). Two variants of this viral gene were studied: a 148a.a. variant, encoded by the tissue culture-adapted non-pathogenic ASFV isolate (Ba71V) and a 254a.a. variant, encoded by the pathogenic Benin97/1 isolate. The results indicate that, in both isolates, this inhibition is occurring at the level of MAVS, a key adaptor protein of the RLR pathway, thereby preventing activation of the transcription factors NF-κB, IRF-3 and IRF-7. Of note, is the fact that the 'pathogenic' MGF360-18R variant (254a.a.) is also inhibiting IFN-β induction at the level of IRF-3, which may give the virus an extra advantage, as a result of a more efficient abrogation of the IFN response.

3.3. Materials and methods

3.3.1. Bioinformatic analysis

Full-length DNA sequences of MGF360-18R ORF from different ASFV isolates were aligned and compared to the known DNA sequence of MGF360-18R ORF from ASFV Benin97/1 isolate, using the *nucleotide-nucleotide* BLAST tool from the National Center for Biotechnology Information (NCBI). Screening for patterns was performed using Prosite 20.78 database [14]. The transmembrane region prediction was performed by TransMembrane Helix prediction using Hidden Markov Models 2.0 (TMHMM) program [15]. Secondary structure prediction was performed using the PSIPRED server [16].

3.3.2. Cell culture

African green monkey Vero and COS-1 cells were cultured in Dulbecco´s Modified Eagle´s Medium (DMEM) (Gibco) supplemented with 100U/ml penicillin G sodium /100µg/ml streptomycin sulfate (Gibco), 2mM L-Glutamine (Gibco) and 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco).

Immature porcine alveolar macrophage cell line (IPAM) was cultured in RPMI 1640 + GlutaMAX (Sigma) supplemented 100U/ml penicillin G sodium/ 100µg/ml streptomycin sulfate (Gibco) and 10%(v/v) heat-inactivated fetal bovine serum (FBS) (Gibco). Cells were incubated at 37ºC, in 5%CO_2.

3.3.3. Plasmids

The two variants of the ASFV MGF360-18R ORF were amplified by PCR from either BA71V or Benin-97/1 isolate genomic DNA and cloned into the pcDNA3 plasmid, in frame with an amino-terminal influenza haemaglutinin (HA) tag.

For construction of a recombinant lentivirus vector (pHR-CMV-HA-18R-eGFP), the MGF360-18R (Ba71V) or MGF360-18R (Benin97/1) coding sequences were excised from pcDNA3, together with the amino-terminal HA tag, and cloned into the vector pHR-CMV-eGFP (kindly provided by Dr. Y. Ikeda), upstream of an internal ribosome entry site (IRES)-driven enhanced green fluorescent protein gene (eGFP).

The luciferase reporter plasmids containing the sequences of the IFN-β promoter [pIFΔ(-125/+72)lucter], the NF-κB binding site of the IFN-β promoter [p(PRD2)5tkΔ(-39)lucter], the IRF-3-binding site of the ISG15

promoter [p(ISG15ISRE)4tkΔ(-39)lucter], as well as the expression vectors for IKKε, TBK1 and MAVS, were gifts of Dr. S. Goodbourn. The expression plasmid containing the human IRF-3 fused with the green fluorescence protein (GFP) was provided by Dr. J. Hiscott.

The pCMVβ plasmid contains a β-galactosidase gene under the control of human cytomegalovirus immediate early promoter, and serves as an internal control for culture to culture variations in transfection efficiency.

3.3.4. Lentivirus production

Lentivirus was produced by transient co-transfection of HEK-293T cells with the packaging and envelope plasmid together with the empty pHR-CMV-eGFP plasmid or the recombinant MGF360-18R (either the Ba71V or Benin97/1 variant) plasmid at a weight ratio of 1:1:3, respectively, using FuGENE 6 (Roche) according to the manufacturer's instructions. Supernatants containing the lentivirus were collected at 48h and 72h post-transfection, clarified by filtration, and lentivirus were collected by ultracentrifugation (125,000xg, 3h, 4°C). Virus pellets were resuspended in fresh DMEM and frozen at −80°C.

3.3.5. Lentivirus transduction of COS1 cells

The COS-18R (Ba71V), COS-18R (Benin97/1) and COS-eGFP stable cell lines were produced by lentivirus infection of COS-1 cells with either one of the recombinant plasmids or the empty pHR-CMV-eGFP, respectively, using a multiplicity of infection (MOI) of 10 in DMEM. Confirmation of lentivirus infection was done by detecting eGFP-

positive cells by optical microscopy at 48 h post-infection (p.i.), and recombinant protein expression was confirmed by Western blot.

3.3.6. Luciferase reporter gene assay

Vero cells ($6x10^4$ cells/well, in a 24 well plate) were co-transfected with 100ng of the indicated luciferase reporter plasmid, 25 ng of the β-galactosidase internal control plasmid (pCMVβ) and 300ng of either pcDNA3HA-18R (Ba71V), pcDNA3HA-18R (Benin97/1) or non-recombinant pcDNA3HA, according to the Lipofectamine 2000 (Invitrogen) protocol. Seventy two hours post-transfection, the cells were either stimulated with 35μg/ml Poly (I:C) (Amersham Biosciences) for five hours, or left untreated. After the treatment, the cells were lysed.

In an alternative protocol, the cells were co-transfected with 100ng of the indicated luciferase reporter plasmid, 25ng of pCMVβ, the indicated amounts of plasmids expressing the different components of the IFN-β induction pathway and increasing amounts of the indicated pcDNA3HA-18R (Ba71V or Benin97/1) plasmid. The quantity of DNA in each transfection was kept constant by supplementation with the non-recombinant pcDNA3HA. Forty eight hours post-transfection the cells were lysed. The luciferase activity was measured using the luciferase assay system (Promega) according to the manufacturer`s protocol. The β-galactosidase activity was measured using the Galacton-Plus kit from Tropix (Bedford, MA). The luciferase activity was normalized relatively to the β-galactosidase activity of each sample, as to correct transfection efficiency variations between different cells.

3.3.7. Enzyme-linked Immunoabsorbent Assay (ELISA)

IPAM cells ($6x10^4$ cells/well, in a 24 well plate) were transfected with 300ng of the indicated pcDNA3HA-18R (Ba71V or Benin97/1) or the non-recombinant pcDNA3HA plasmid, according to the Lipofectamine 2000 (Invitrogen) protocol. Forty-eight hours post-transfection, the cells were either stimulated with 100µg/ml Poly (I:C) (Amersham Biosciences) for 16 hours, or left untreated. The medium of each well was collected and centrifuged for 15 minutes at 1000xg. Quantitative determination of porcine IFN-β concentration in the supernatants was performed using the Porcine Interferon β ELISA Kit (Cusabio), according to the manufacturers' instructions. Absorbance at 450nm was measured using a BioRad ELISA reader (BioRad) and concentration of IFN-β was determined by comparison to a standard curve.

3.3.8. Western blot

Lentivirus infected COS-1 cells, stably expressing MGF360-18R (Benin97/1) ($3x10^5$ cells/well, in a 6-well plate) were transfected with either 250ng of IKKε expressing vector or 200ng of MAVS expressing vector, according to the Lipofectamine 2000 (Invitrogen) protocol. Forty-eight hours post-transfection, the cells were harvested and lysed using a non-ionic lysis buffer (15mM TrisHCl, pH 7.4, 120mM NaCl, 25mM KCl, 2mM EDTA, 2mM EGTA, 0.1mM DTT and 1% Triton X-100) containing a protease inhibitor cocktail (Sigma). Cell lysates were resolved on a 12% sodium dodecyl sulphate-polyacrilamide gel (SDS-PAGE). The separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare). Membranes were blocked with 5% non-fat milk for one hour at room temperature and incubated with the following antibodies: rabbit anti-phospho-hIRF3

(Cell Signaling), rat anti-HA-HRP conjugated (high affinity) (Roche) and anti-β-actin-HRP conjugated (Sigma) as a loading control. Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody was purchased from Invitrogen. Membranes were developed by enhanced chemiluminescence detection according to the manufacturer's instructions (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific or Luminata Forte, Millipore).

3.3.9. Immunofluorescence

Vero cells ($1.5x10^5$ cells/well, in a 6-well plate) were cultured on sterile glass coverslips and transfected with 3µg of pcDNA3HA-18R (either Ba71V or Benin97/1) or the non-recombinant pcDNA3HA plasmid, according to the Lipofectamine 2000 (Invitrogen) protocol. Forty-eight hours post-transfection, the cells were either stimulated with 100µg/ml Poly (I:C) (Amersham Biosciences) for 30 minutes, or left untreated. Mitochondria were stained incubating live cells with MitoTracker Red CMXRos (Molecular Probes) for 45 minutes, according to the manufacturers' instructions. In an alternative protocol, cells were co-transfected with 3µg of pcDNA3HA-18R (either Ba71V or Benin97/1) or the non-recombinant pcDNA3HA plasmid and 100ng of IRF3-GFP or 250ng of IKKε expressing vector. After the treatment, cells were washed with phosphate buffered saline (PBS) solution, fixed with 4% paraformaldehyde (PFA) for 20 minutes and permeabilized with PBS + 0.1% Triton-X100 for 20 minutes. After washing, the cells were blocked with PBS + 0.05% Tween-20 containing 5% normal goat serum for one hour. To visualize MGF360-18R HA tagged proteins (Ba71V or Benin97/1), coverslips were incubated with either rabbit anti-HA (Sigma) or rat-anti HA (high affinity) (Roche). Coverslips were washed and incubated with the secondary antibodies goat anti-rabbit Alexa488-conjugated (Invitrogen) or goat anti-mouse Texas Red-

conjugated (Molecular Probes), respectively. Cell nuclei were stained with DAPI. All the incubations were performed at room temperature.

Confocal fluorescent images were obtained by a *DeltaVision Core* wide-field deconvolution inverted-base microscope (Applied Precision/Olympus), with a 100x objective. Images were analyzed by ImageJ 1.43u software. The term colocalization refers to the coincidence of green and red fluorescence, as measured by the confocal microscope.

3.3.10. Immunoprecipitation

Vero cells ($5x10^5$ cells/plate, in a 60 mm Ø plate) were transfected with 6µg of either pcDNA3HA-18R (Ba71V or Benin97/1) or the non-recombinant pcDNA3HA plasmid, according to the Lipofectamine 2000 (Invitrogen) protocol. Forty-eight hours post-transfection, the cells were either stimulated with 100µg/ml Poly (I:C) (Amersham Biosciences) for 30 minutes, or left untreated.

Cells were then harvested and lysed in lysis buffer (15 mM TrisHCl, pH 7.4, 120 mM NaCl, 25 mM KCl, 2mM EDTA, 2mM EGTA, 0.1mM DTT and 1% Triton X-100) containing a protease inhibitor cocktail (Sigma). Immunoprecipitation was performed with Dynabeads protein G (Millipore), using mouse anti-human MAVS antibody (Millipore). Washes were performed using commercially available lysis buffer (Sigma). Elution was done using 2X sample buffer. Immunoprecipitation eluates were resolved on a 12% SDS-PAGE gel and proteins were transferred to a PVDF membrane (GE Healthcare). Membranes were blocked with 5% non-fat milk for one hour at room temperature and probed with mouse anti-human MAVS (Millipore),

anti-HA-HRP conjugated (Roche). Horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody was purchased from Invitrogen.

3.3.11. Statistical Analysis

Data were shown as mean values with standard deviations (SD). Differences between experimental groups were determined by a two-tailed Student *t* test, using Excel software (Microsoft).

3.4. Results

3.4.1. MGF360-18R is a non-conserved ORF of the ASFV

Several isolates of ASFV have already been fully sequenced and genome sequences are available at the Nucleotide database of the NCBI. Screening of each available ASFV genomic sequence with the nucleotide sequence of the MGF360-18R ORF (Benin97/1 isolate), revealed that this MGF360 gene is non-conserved amongst the several isolates. Both the tissue culture-attenuated and the non-pathogenic isolates of the ASFV (Ba71V and OURT88_3, respectively) code for a predicted C-terminal truncated protein of 148a.a.. Conversely, the pathogenic isolates code for predicted proteins ranging from 237 to 254a.a.. The pathogenic Benin97/1 isolate codes for the largest version of this protein, with 254a.a. (see Anex).

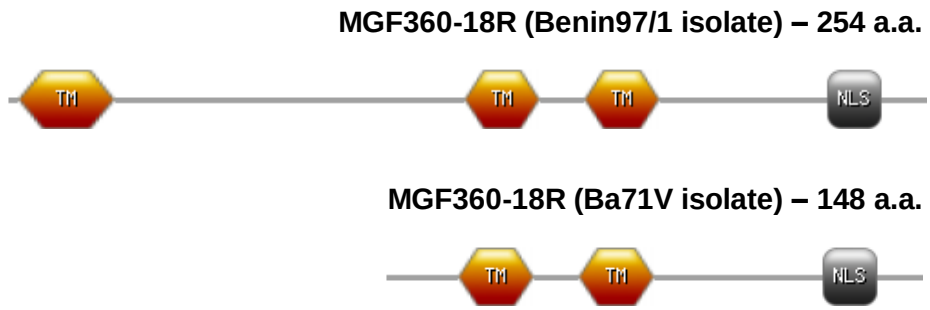


Figure 3.1 – MGF360-18R is a globular integral membrane protein.

The predicted MGF360-18R protein sequences of the Ba71V and Benin97/1 isolates were analysed for the presence of conserved patterns, using Prosite 20.78 [14] and the TMHMM program (see Fig.3.1) [15]. Secondary structure of both sequences was determined using the PSIPRED server, revealing a globular structure with two and three putative transmembrane domains, respectively [16].

To characterize the mechanism of action of this viral gene and also to define potential differences in the activity of each MGF360-18R variant, both variants (Ba71V and Benin97/1) were cloned into expression vectors and assessed for their cell localization and ability to inhibit the IFN response.

3.4.2. The MGF360-18R colocalizes with the mitochondria

The bioinformatic analysis suggested that both variants of the MGF360-18R are integral membrane proteins. In order to determine the cell compartment in which these proteins are located, immunofluorescence assays were performed.

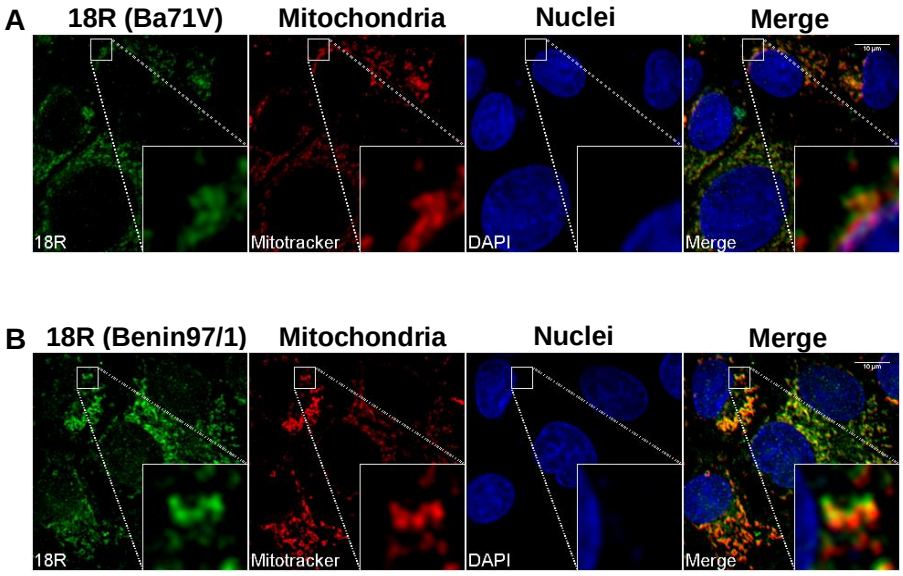


Figure 3.2 – Both variants of the ASFV MGF360-18R protein colocalize with the mitochondria.

Vero cells were transfected with pcDNA3HA-18R (either Ba71V or Benin97/1 variant). Mitochondria were visualized using MitoTracker Red CMXRos (red). The MGF360-18R proteins were visualized using a monoclonal anti-HA antibody (green). Cell nuclei were stained with DAPI (blue). Bar, 10μm.

Both ASFV ORF MGF360-18R proteins from either Ba71V or Benin97/1 isolates colocalize with the mitochondria. These cellular organelles are known to be involved in several steps of the innate immune response and, in particular, are essential for the signalling function of mitochondrial antiviral signalling protein (MAVS). This protein functions as an adaptor recruited by two cytosolic RNA sensors, the retinoic acid-inducible gene-1 (RIG-I) and the melanoma differentiation-associated gene 5 (MDA5). Signalling of these two receptors through MAVS leads to activation of IRF-3, IRF-7 and NF-κB, transcription factors responsible for IFN-β induction.

3.4.3. The inhibition of IFN-β transcription by ASFV ORF MGF360-18R is NF-κB dependent

In order to confirm and determine the effect of the two variants of the MGF360-18R ORF in the induction of IFN-β, both ORFs were screened using a luciferase reporter assay. This assay is based on transfection into Vero cells of an IFN-β promoter cloned into a luciferase reporter plasmid and its subsequent activation by the type I IFN inducer, the synthetic dsRNA analogue Poly (I:C). The Vero cell line has the additional advantage of lacking the type I IFN locus, which facilitates the interpretation of the results, since the IFN amplification loop is absent in these cells and so control background levels are low. In this screening, expression plasmids of either variant of the MGF360-18R gene (Ba71V or Benin97/1) were co-transfected with the IFN-β luciferase reporter plasmid and were found to inhibit IFN-β induction in response to Poly (I:C) (see Fig.3.3-A). The Benin97/1 variant consistently inhibited more than the Ba71V variant.

The dsRNA added to the medium can be recognized by either TLR3 or RIG-I/MDA5 (RLR pathway), resulting in the activation of both NF-κB and IRF transcription factors [17]. In order to determine if MGF360-18R mediated inhibition of IFN-β induction is also affecting NF-κB activation, a luciferase reporter plasmid containing only the NF-κB binding site of the IFN-β promoter, the positive regulatory domain (PRD)-II, was used. Again, Vero cells were transfected with the empty plasmid or the expression plasmid for either variant of MGF360-18R gene. The results obtained using this reporter indicate that both MGF360-18R variants inhibit IFN-β induction in response to Poly (I:C) through an NF-κB dependent pathway (see Fig.3.3).

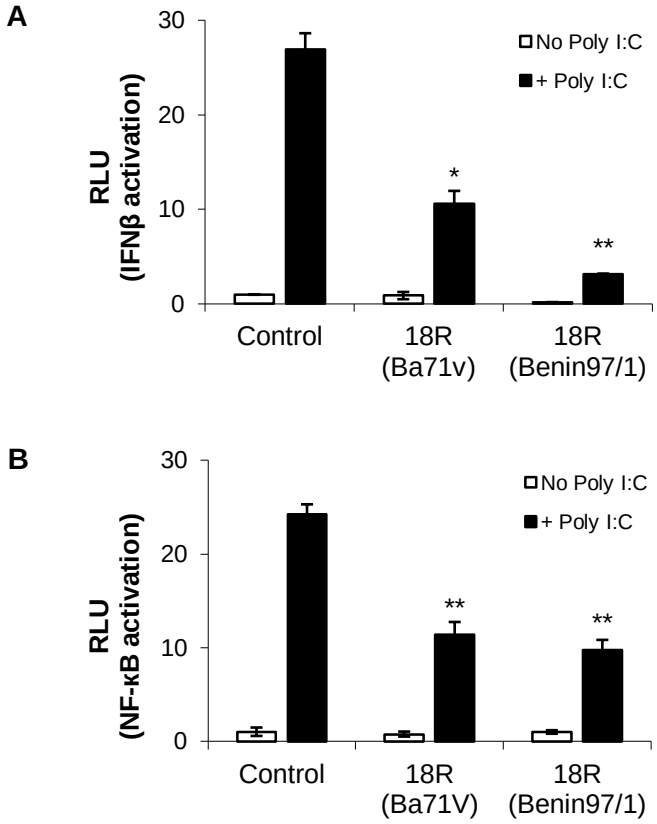


Figure 3.3 – Both variants of the ASFV MGF360-18R gene inhibit Poly (I:C) stimulated activation of IFN-β transcription in an NF-κB dependent manner.

Vero cells were co-transfected with the pcDNA3HA (Control) or pcDNA3HA-18R plasmid expressing the MGF360-18R variant from Ba71V or Benin97/1, the β-galactosidase plasmid and either the (**A**) IFN-β promoter (IFNβ) or the (**B**) NF-κB promoter (PRDII) luciferase reporter. For both assays, seventy-two hours post-transfection, the cells were either induced with 35μg/ml Poly (I:C) for five hours (■), or left untreated (□). Luciferase activity was normalized to β-galactosidase activity as a control for transfection efficiency. Data are expressed as means of Relative Luciferase Units (RLU) ± SD of triplicate well from one of three similar experiments. Statistical significance is represented as p≤0.05 (*) or ≤0.01 (**).

3.4.4. The ASFV MGF360-18R ORF inhibits induction of IFN-β secretion

To examine the inhibition of IFN-β induction at the level of protein expression, the amount of IFN-β secreted into the supernatants of cells expressing either MGF360-18R variant (Ba71V or Benin97/1) or empty control plasmid was determined by ELISA. Due to the fact that Vero cell line lacks the IFN gene, another cell line had to be used to measure IFN production. The IPAM cell line was chosen as, appropriately, it is the cell type (porcine macrophage) naturally infected by the ASFV.

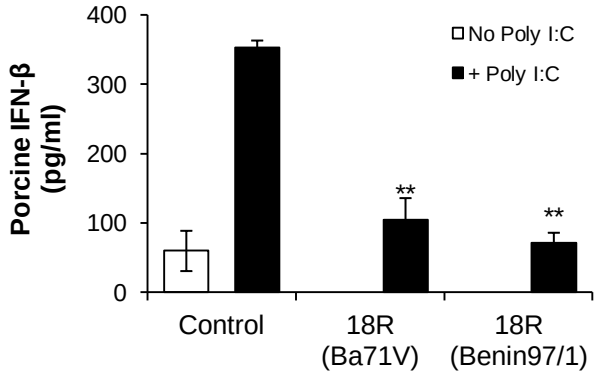


Figure 3.4 – MGF360-18R inhibits IFN-β secretion.

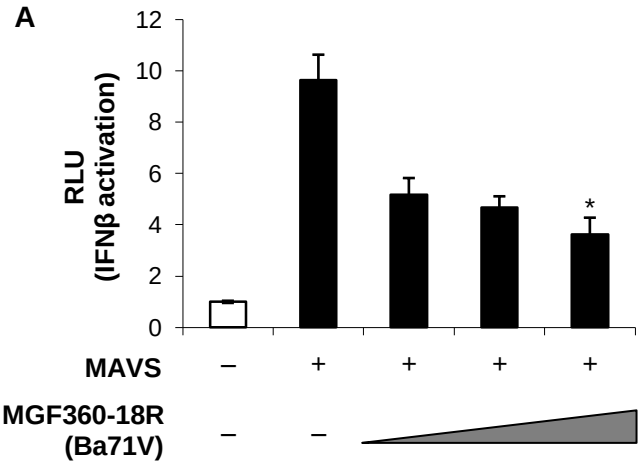
IPAM cells were transfected with pcDNA3HA (Control) or pcDNA3HA-18R plasmid expressing the MGF360-18R variant from Ba71V or Benin97/1. Forty-eight hours post-transfection, the cells were stimulated with 100μg/ml Poly (I:C) for 16 hours (■), or left untreated (□). Supernatants were collected and IFN-β concentration (pg/ml) was measured by ELISA. Data are expressed as means ± SD of triplicate well from one of two similar experiments. ** Statistically significant when compared to control vector-expressing cells ($p \leq 0.01$).

Control cells stimulated with Poly (I:C) expressed significantly higher levels of IFN-β when compared to non stimulated control cells, as expected. The expression of IFN-β protein by Poly (I:C) stimulated cells expressing either variant of MGF360-18R (Ba71V or Benin97/1), on the other hand, was almost completely inhibited (see Fig.3.4). These results are consistent with the MGF360-18R mediated inhibition of IFN-β transcription previously observed. In conclusion, MGF360-18R inhibits IFN-β induction both at the level of transcriptional activation and protein secretion.

3.4.5. The ASFV MGF360-18R ORF inhibits activation of IFN-β transcription by targeting MAVS

The cytosolic dsRNA recognition receptors (RLRs) signal through a common adaptor protein, MAVS, which is anchored to the outer mitochondrial membrane, a location known to be essential for its function. Signalling through MAVS activates two pathways: a TRAF6-dependent pathway that culminates in the activation of NF-κB, and a pathway through TBK1 and IKKε, two IκB kinase-related kinases responsible for the phosphorylation of both IRF3 and IRF-7. Following activation, these transcription factors translocate into the nucleus and initiate transcription of IFN genes. Considering the mitochondrial localization of both MGF360-18R variants, as well as their inhibitory effect on both the induction of IFN-β promoter and NF-κB activation, MAVS is an immediately plausible candidate as a possible target for the viral protein.

In order to determine if both MGF360-18R variants are able to inhibit IFN induction signalling mediated by MAVS, Vero cells were co-transfected with the MAVS expression plasmid and the IFN-β luciferase reporter plasmid, in the presence of increasing quantities of MGF360-18R (either Ba71V or Benin97/1). In the positive control, ectopic expression of MAVS significantly increased the IFN-β promoter-mediated luciferase activity. As presented here, the expression of both variants of the MGF360-18R gene inhibits the activation of IFN-β gene transcription by MAVS (see Fig.3.5). Once again, the Benin97/1 variant was consistently a more potent inhibitor than the Ba71V variant.



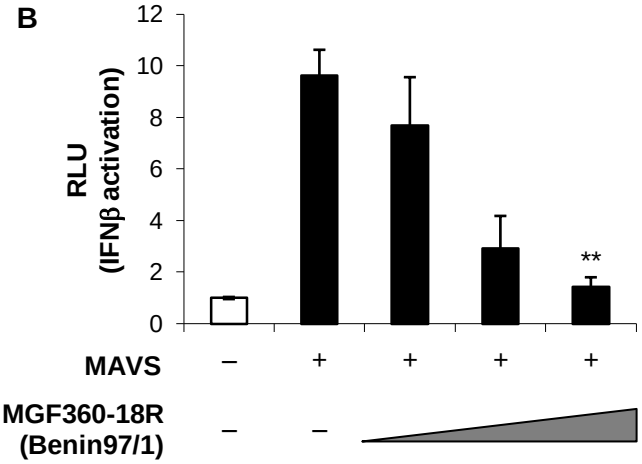


Figure 3.5 – Both variants of the ASFV MGF360-18R gene inhibit activation of IFN-β transcription by MAVS.

Vero cells were co-transfected with the IFN-β promoter luciferase reporter, the β-galactosidase control plasmid, 40ng of MAVS (■) and increasing amounts (200-600ng) of pcDNA3HA-18R plasmid expressing the MGF360-18R variant from Ba71V (**A**) or Benin97/1 (**B**). Luciferase activity was normalized to β-galactosidase activity as a control for transfection efficiency. Data are expressed as means of RLU ± SD of triplicate well from one of three similar experiments. Statistical significance, compared to control vector-expressing cells, is represented as p≤0.05 (*) or ≤0.01 (**).

To further investigate the impact of both MGF360-18R variants on MAVS signalling, increasing amounts of the adaptor protein were expressed in cells transfected with MGF360-18R expression plasmid (either Ba71V or Benin97/1 variant) and stimulated with Poly (I:C), or left untreated. The impact of the viral gene on the IFN-β luciferase reporter activity was measured.

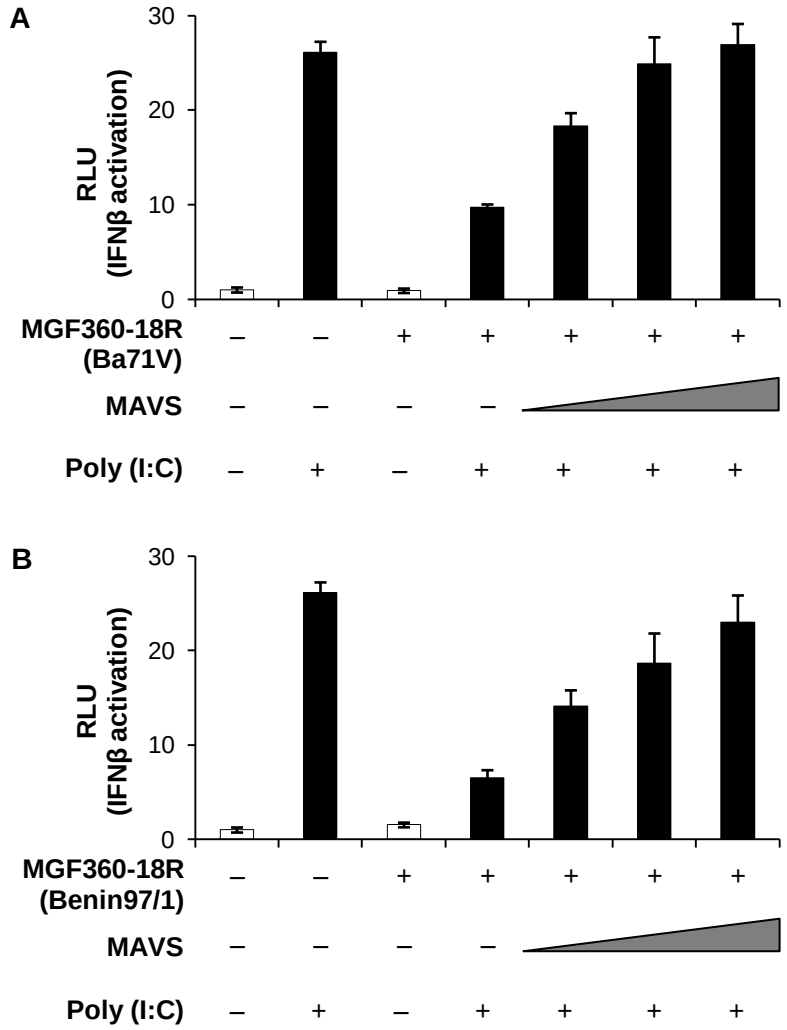


Figure 3.6 – Overexpression of MAVS reverses MGF360-18R mediated inhibition of the IFN-β transcription.

Vero cells were co-transfected with increasing amounts (40-100ng) of MAVS plasmid in the presence of either pcDNA3HA (Control) or pcDNA3HA-18R plasmid expressing the MGF360-18R variant from Ba71V (**A**) or 18R-Benin97/1 (**B**), the IFN-β promoter luciferase reporter and the β-galactosidase control plasmid. Seventy-two hours post-transfection, the cells were stimulated with 35μg/ml Poly(I:C) for five hours (■), or left untreated (□). Luciferase activity was normalized to β-galactosidase activity as a control for transfection efficiency. Data are expressed as means of RLU ± SD of triplicate

well from one of three similar experiments. ** Statistically significant when compared to control vector-expressing cells (p≤0.01).

As can be seen (Fig. 3.6), overexpression of MAVS reversed the MGF360-18R mediated inhibition of the Poly (I:C) activated IFN-β reporter, in a dose-dependent manner. This result is consistent with the hypothesis that both variants of the ASFV MGF360-18R ORF target MAVS signalling.

A potential target for evading antiviral responses is the adaptor protein MAVS itself. Immunofluorescence assays were performed to verify if both variants of MGF360-18R and MAVS colocalize to the same location.

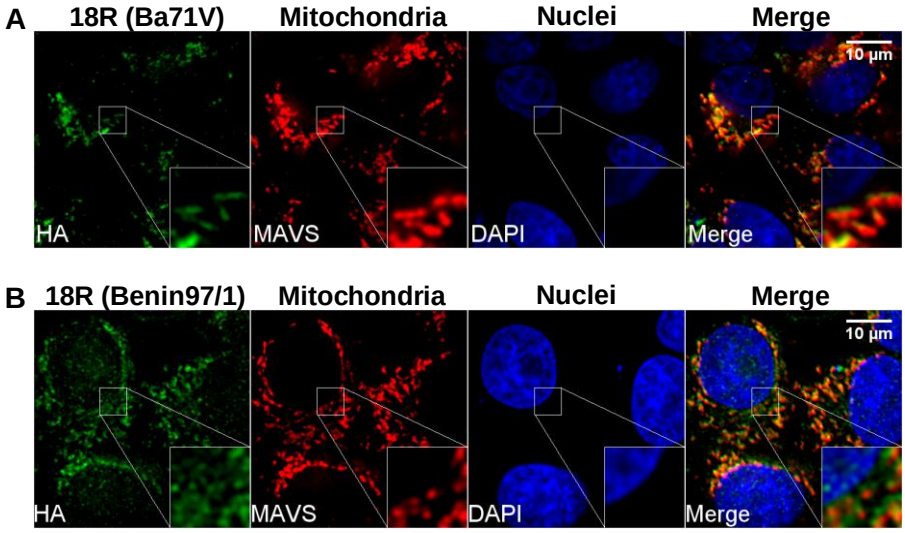


Figure 3.7 – Both variants of the MGF360-18R colocalize with MAVS.

Vero cells were transfected with pcDNA3HA-18R (either Ba71V or Benin97/1 variant). MAVS protein was visualized using mouse anti-MAVS antibody (Santo Cruz) (red). The MGF360-18R proteins were visualized using a monoclonal anti-HA antibody (green). Cell nuclei were stained with DAPI (blue). Bar, 10μm.

As can be seen, Ba71V variant of MGF360-18R protein is being expressed to the same location of MAVS, while the Benin97/1 variant shows only a partial colocalization. The MAVS adaptor has been reported to be present in both mitochondria and peroxisome. The results presented in both Fig.3.2 and Fig.3.7, suggest that both variants of MGF360-18R colocalize to mitochondrial MAVS.

The colocalization of MGF360-18R and MAVS indicate that there may be an interaction between these two proteins. An immunoprecipitation assay was performed, in order to verify this assumption.

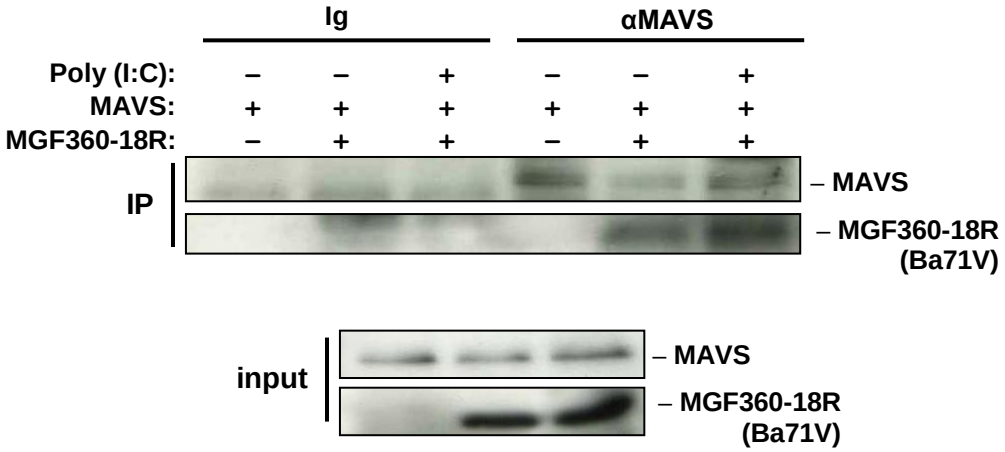


Figure 3.8 – The ASFV ORF MGF360-18R (Ba71V) co-immunoprecipitates with MAVS.

Vero cells were transfected with either non-recombinant pcDNA3HA or pcDNA3HA-18R plasmid expressing the MGF360-18R variant from Ba71V. Forty-eight hours post-transfection, the cells were stimulated with 100μg/ml Poly (I:C) for 30 minutes, or left untreated. Cell lysates were immunoprecipitated with control mouse IgG (Ig) or anti-MAVS (αMAVS). The immunoprecipitates (IP) were analyzed by immunoblot with anti-HA or anti-MAVS (upper panel). Expression of MAVS and MGF360-18R (Ba81V) in the lysates was detected by immunoblot analysis with anti-MAVS and anti-HA (bottom panel).

The results presented here (see Fig.3.8) confirm that MGF360-18R (Ba71V variant) directly interacts with MAVS, possibly interfering with its function as an essential adaptor for the RLR signalling pathway. This same assay is to be performed using cells expressing the Benin97/1 variant of MGF360-18R protein, in order to confirm if both variants of this viral gene directly interact with MAVS.

3.4.6. The MGF360-18R (Benin97/1) ASFV gene, but not the Ba71V variant, inhibits both TLR and cytosolic IFN-β induction pathways, acting at the level of IRF-3

To verify if the action of both variants of the MGF360-18R gene over the IFN-β induction in response to Poly (I:C) is limited to interfering with the MAVS adaptor protein, we assayed for impact of the viral genes on the activation of downstream signalling intermediates of the RLR pathway. Vero cells were co-transfected with IRF-3 expression plasmid and the ISG15 luciferase reporter plasmid, in the presence of increasing quantities of MGF360-18R (either Ba71V or Benin97/1). In the positive control, ectopic expression of IRF-3 significantly increased the ISG15 promoter-mediated luciferase activity. As presented here, the expression of the Benin97/1 variant of the MGF360-18R gene also inhibits the activation of the IRF-3 transcription factor. In contrast, the Ba71V variant did not (see Fig 3.9).

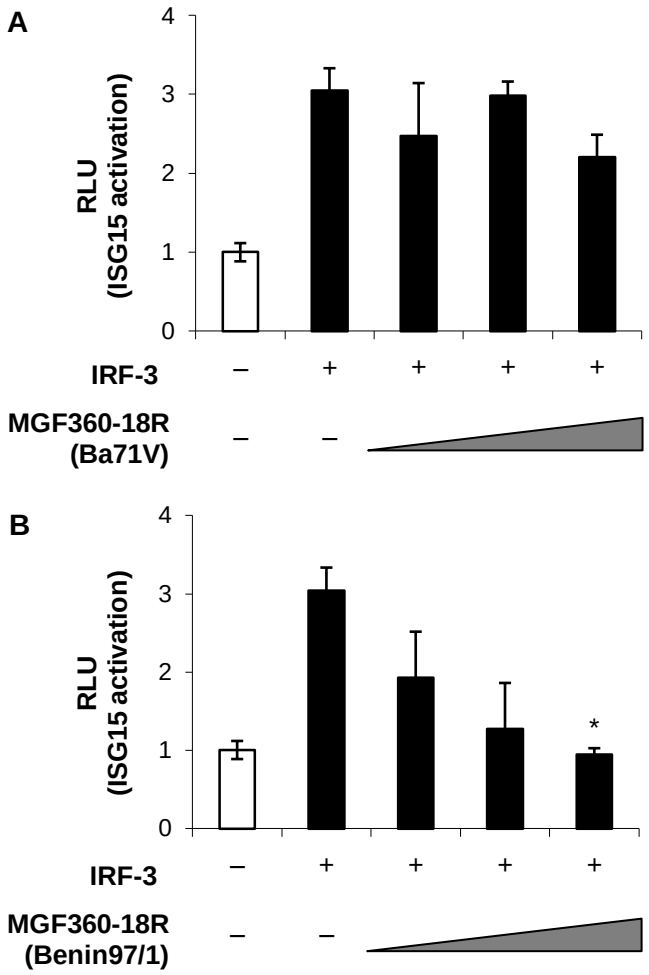


Figure 3.9 – Expression of Benin97/1 variant of MGF360-18R, but not Ba71V variant, inhibits the activation of IRF-3

Vero cells were co-transfected with the ISG15 promoter luciferase reporter, the β-galactosidase control plasmid, 100ng of IRF-3 (■) and increasing amounts (200-600ng) of pcDNA3HA-18R plasmid expressing the MGF360-18R variant from Ba71V (**A**) or Benin97/1 (**B**). Luciferase activity was normalized to β-galactosidase activity as a control for transfection efficiency. Data are expressed as means of RLU ± SD of triplicate well from one of three similar experiments. * Statistically significant when compared to control vector-expressing cells ($p \leq 0.05$).

In order to verify if the MGF360-18R (Ba71V variant) protein is interfering with any other signalling intermediates in the IFN-β induction pathway, the IFN-β luciferase reporter activity was measured in cells ectopically expressing the upstream signalling intermediates TBK1 or IKKε.

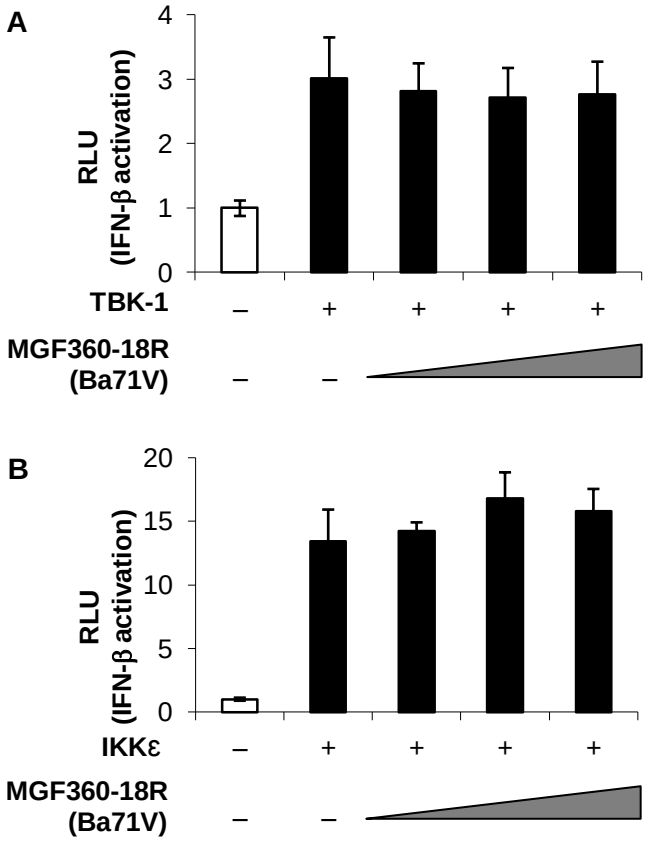
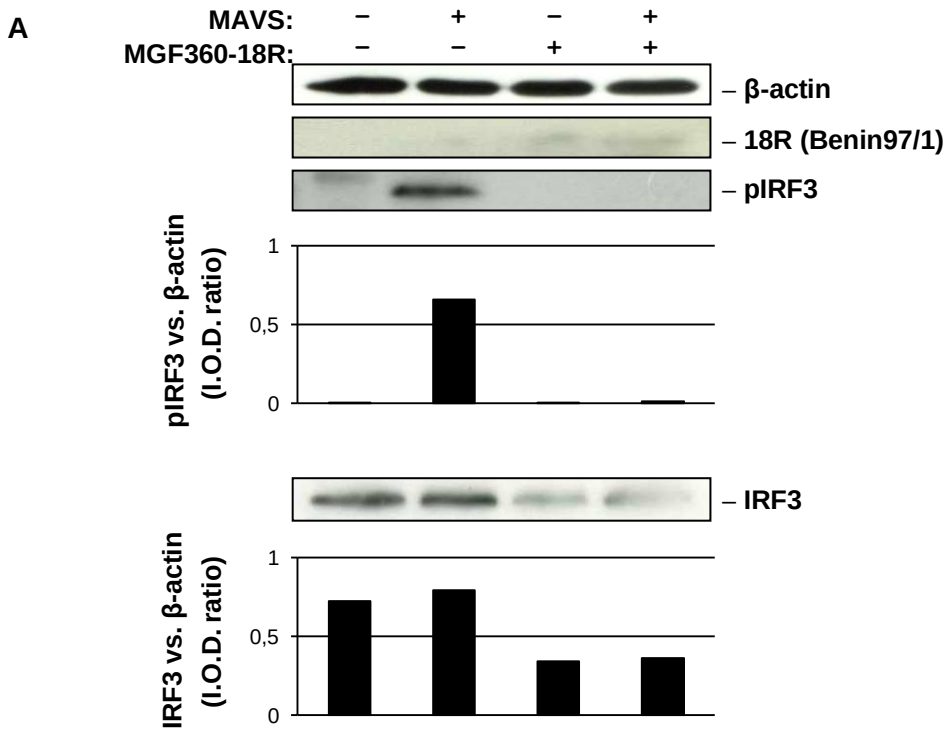


Figure 3.10 – ASFV ORF MGF360-18R (Ba71V variant) does not inhibit IFN-β induction by ectopic expression of TBK1 (A) or IKKε (B).

Vero cells were co-transfected with the IFN-β promoter luciferase reporter, the β-galactosidase control plasmid, 100ng of TBK1 (**A**) or IKKε (**B**) (■) and increasing amounts (200-600ng) of pcDNA3HA-18R plasmid expressing the MGF360-18R variant from Ba71V. Luciferase activity was normalized to β-galactosidase activity as a control for transfection efficiency. Data are expressed as means of RLU ± SD of triplicate well from one of three similar experiments.

As presented here, the Ba71V variant of the MGF360-18R gene had no effect over the activation of IFN-β gene transcription by ectopic expression of either TBK1 (Fig.3.10-A) or IKKε (Fig.3.10-B). This confirms that the inhibitory action of the Ba71V variant of MGF360-18R over the signalling mechanism leading to induction of IFN-β is limited to interfering with the MAVS adaptor.

The Benin97/1 variant of MGF360-18R, however, has an additional inhibitory role over the activity of IRF-3, a critical transcription factor necessary for induction of type I IFN. To further understand the mechanism by which this viral protein inhibits the activation of IRF-3, Vero cells were transfected with MGF360-18R (Benin97/1 variant) expression plasmid, and the levels of phosphorylated and total IRF-3 were estimated by immunoblot analysis using specific antibodies.



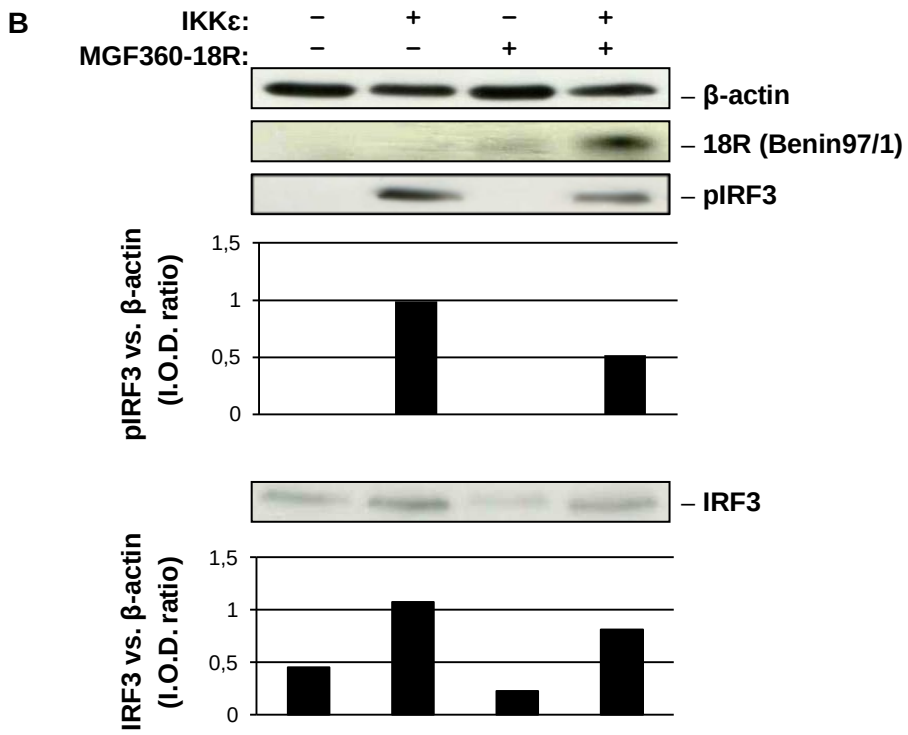


Figure 3.11 – Expression of the ASFV MGF360-18R (Benin97/1 variant) protein results in a reduction of the levels of phosphorylated IRF3 and total IRF3, on activated cells.

COS-1 cells stably expressing MGF360-18R (Benin97/1) were stimulated by either ectopic expression of MAVS (A) or IKKε (B). Forty-eight hours post-transfection, the cells were lysed and total cell lysates were immunobloted with anti-phosphorylated IRF-3 and anti-IRF-3. Anti-β-actin antibody was used as loading control. Expression of MGF360-18R (Benin97/1) was detected using an anti-HA-HRP conjugated antibody. WB signal was quantified by determining the integrated optical density (I.O.D.) of a given band, normalized to the corresponding loading control, using ImageJ 1.43u software.

This assay revealed that expression of the Benin97/1 variant of MGF360-18R is able to effectively reduce the cellular levels of IRF-3 on activated cells. This can be seen on cells activated by ectopic expression of either MAVS or IKKε, two proteins acting at different

levels of the RLR signalling pathway. These observations are in accordance to the results obtained by luciferase reporter assays (see Fig.3.5-B and Fig.3.9-B).

3.5. Discussion

ASFV inhibits production of IFN

African swine fever virus (ASFV) is an economically important cytoplasmically replicating DNA virus of pigs, endemic in many African countries, and which has entered Portugal with a disastrous economic impact [18]. There is no vaccine and, although ASFV is known to interfere with signalling pathways controlling the transcription of a large number of immunomodulatory genes, such as cytokines [19,20,21], no individual virus gene manipulating the IFN response has been reported. This is all the more surprising as ASFV not only results in an acute phase, but may also persist. It is hard to imagine that a virus could persist in the face of an efficient IFN response.

The ORF MGF360-18R, which has been identified in a previous screening performed in our laboratory (Correia, S.M., unpublished work) as an inhibitor of the IFN-β induction, belongs to the ASFV MGF360 family. Several studies have established that proteins encoded by the MGF360 and MGF530 genes are involved in the modulation of the interferon response by ASFV [2]. The individual family members, however, have not been investigated as possible inhibitors of the IFN response.

A sequence comparison of the genomic sequence of different ASFV isolates revealed that MGF360-18R is a non-conserved ORF. The pathogenic isolates code for significantly larger proteins, relatively to the attenuated and non-pathogenic isolates. The Benin97/1 isolate

codes for the largest variant of this protein, with 254a.a., while the attenuated variant of this protein encompass only 148a.a. (see Anex).

Both variants of ORF MGF360-360 were shown to inhibit the induction, expression and secretion of IFN-β. To determine the intracellular target of the MGF36018R gene, luciferase assays were performed, in which different components of the IFN induction pathway were ectopically expressed. It was possible to determine that both variants inhibited the RLR pathway at the level of MAVS adaptor, thereby interfering with both NF-κB activation and induction of type I IFN. Both variants were also shown to inhibit the Poly (I:C) stimulated secretion of the IFN-β protein by the porcine cell line (IPAM), thus emphasising the biological significance of this viral gene in the inhibition of IFN response by ASFV. Interaction of MGF360-18R with MAVS was confirmed by intracellular colocalization of the two proteins and by co-immunoprecipitation. Immunofluorescence analysis revealed a clear co-localization with mitochondria, but not peroxisome, thus indicating that MGF360-18R protein acts upon the more sustained antiviral response, inhibiting the RLR mitochondria-dependent pathway that leads to the induction of IFN-β expression through the activation of IRF-3 and NF-κB.

Moreover, MGF360-18R variant from Benin97/1 was shown to inhibit the induction of ISG15 promoter on cells ectopically induced by overexpression of IRF3. This indicates that, in addition to its inhibitory role in the RLR pathway by interfering with MAVS, this variant of the MGF360-18R is able to inhibit the activity of the IRF3 transcription factor, hence directly affecting IRF-3 stimulated ISG transcription. The results indicate that expression of MGF360-18R (Benin97/1) protein results in diminished quantities of IRF-3 in the cell, which might

indicate an increased degradation of this transcription factor. The exact mechanism, however, remains to be completely elucidated.

MAVS as a target for virus strategies to inhibit the IFN response

The Melanoma Differentiation-Associated Gene (MDA)-5 and the Retinoic Acid Inducible Gene (RIG)-I are RLR family members involved in viral recognition and induction of type I IFN. Both have a caspase recruitment domain (CARD) that allows for interaction with their common adaptor, Mitochondrial Antiviral Signalling (MAVS), and the subsequent antiviral responses. Recently, these cytosolic RNA receptors were described to be involved in antiviral signalling in response to viruses containing a dsDNA genome, such as Epstein–Barr virus (EBV) [22] and vaccinia virus [23], respectively. Some viruses attack RLRs themselves, either through cleavage or direct inhibition of the receptor. Another potential target for evading antiviral responses is the adaptor protein MAVS.

The C-terminal transmembrane domain of the adaptor protein MAVS anchors it to the mitochondrial outer membrane, suggesting a crucial role for mitochondria as a platform for the signalling pathways leading to type I IFN induction [24]. Recent studies showed that, in response to viral infection, MAVS redistributes in the mitochondria, forming large aggregates that are potent activators of IRF-3 [25]. A mitochondrial membrane protein, Mitofusin (MFN)-1, is known to be involved in the redistribution of MAVS along the mitochondria, following RLR activation, as well as in the fusion of the mitochondrial network. This fusion promotes the interaction between MAVS and the STimulator of Interferon Genes (STING), an antiviral signalling adaptor localized in the endoplasmic reticulum membrane, thereby amplifying the antiviral response. Another protein also involved in the regulation of mitochondrial fusion, MFN-2, is a direct MAVS inhibitor, counteracting

the action of MFN-1 and possibly contributing to the fine-tuning of MAVS-mediated signalling [24]. These observations support the view that basic mitochondrial functions are integrated with innate immunity, and that innate immune signalling based on the mitochondria largely depends on this organelle's activity and fitness.

Very recently, MAVS was also identified on the peroxisome membrane, and it was proposed that peroxisomal MAVS is required for the rapid induction of antiviral effectors, mediated by the transcription factors IRF-1 and IRF-3. Mitochondrial MAVS is necessary for a more sustained response, inducing IFN-β expression through the activation of IRF-3 [26]. Antiviral immunity through the RLR pathway requires MAVS signalling from both organelles, an indication that both pathways are interconnected, cooperating for the establishment of an antiviral state. Interaction of both variants of MGF360-18R with peroxisomal MAVS was excluded by immunofluorescence assays. The clear colocalization of the viral proteins with the mitochondria and MAVS, are suggestive of an interaction with mitochondrial MAVS. Both variants of MGF360-18R most possibly interfere with RLR signalling through the mitochondria, thus preventing the establishment of a more sustained antiviral response. Immunoprecipitation of mitochondrial fractions with MAVS antibody could help to clarify if MGF360-18R indeed interacts with mitochondrial MAVS.

Proteolytic cleavage of MAVS is a frequent strategy used by viruses to inhibit RIG-I dependent type I IFN expression. The NS3/4A protease of hepatitis C virus [27], the 3ABC protease of hepatitis A virus [28] or the 3Cpro cysteine protease of coxsackievirus B3 [29] are known examples of such a strategy. Poliovirus infection triggers a caspase-dependent cleavage of MAVS, whereas rhinovirus degrades MAVS in a caspase independent manner. In fact, overexpression of caspase

inhibitors or the anti-apoptotic factor Bcl-xL prevents MAVS cleavage during viral infection, suggesting that MAVS may be involved in apoptosis of virus-infected cells [30]. The murine gamma herpesvirus 68 (γHV68) exploits innate antiviral signalling pathways to evade the innate immune response. It hijacks MAVS and IKK-β in order to promote site-specific (Ser468) phosphorylation of RelA, a crucial subunit of the transcriptionally active NF-κB dimer. In result of this phosphorylation, RelA is primed for ubiquitination and proteasome-mediated degradation. As such, γHV68 efficiently abrogates NF-κB activation and inflammatory cytokine expression [31].

Although the results presented here point out MAVS as a target for MGF360-18R (both Ba71V and Benin97/1 variants), the exact mechanism of action of these viral proteins remains to be clarified. There is no indication that MAVS is being degraded or processed by proteolysis. The direct interaction of MGF360-18R with MAVS may impede its association with the RLR receptors, thereby blocking the signalling mechanism. Another possibility is that the viral protein prevents MAVS redistribution and aggregation in the mitochondria, an event that is crucial for the activation and propagation of the antiviral signalling cascade.

IRF3 as a target for virus strategies to inhibit the IFN response

Significantly, the MGF360-18R (Benin97/1 variant) gene impaired the activation of both the IFN-β promoter and the ISG15 promoter (ISRE element) through ectopic expression of IRF-3. While this is indicative that the Benin97/1 variant of MGF360-18R, in addition to interfering with MAVS, also targets IRF-3, the precise mechanism being used to modulate this transcription factor is yet to be completely elucidated.

The induction of Type I IFN is primarily regulated at the level of transcription and involves the formation of a large, multi-subunit complex called the “enhanceosome”, which comprises the binding of promoter-specific transcription factors, associated structural elements and basal transcriptional machinery to the enhancer DNA sequence [32]. The IRF-3 transcription factor is a critical player in the induction of IFN-β. It is expressed constitutively and in the absence of infection it localizes in the cytoplasm as an inactive monomer [33], which has been described to constitutively shuttle in and out of the nucleus [34].

Many viruses have evolved efficient ways of subverting the host immune system by targeting IRF-3 activity. The different mechanisms described include its targeting for degradation, and the inhibition of its phosphorylation and thus its subsequent nuclear translocation and binding to the promoter region of IFN-β gene. For instance, E3 ubiquitin ligase RBCC protein interacting with PKC1 (RBCK1) binds to IRF3 and targets it for ubiquitination and subsequent degradation through a proteasome-dependent pathway [35]. Our results are indicative that the MGF360-18R gene might be targeting IRF-3 for degradation. A luciferase assay in the presence of the proteasomal inhibitor MG132 should be performed, in order to determine if this variant of the MGF360-18R gene would be capable of inhibiting the luciferase activity of the IFN-β promoter reporter gene in such conditions.

In conclusion, both variants of the ASFV MGF360-18R protein impair activation of IFN-β induction through targeting MAVS, a key adaptor protein of the RLR pathway. The MGF360-18R variant from the Benin97/1 isolate, also targets IRF-3, which might give the virus an extra advantage. The full details of the mechanism of action of both variants of this viral gene remain to be elucidated.

3.6. References

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Chapter 4

The MGF360-18R gene of ASFV inhibits the impact of both type I and type II IFN

4.1. Summary

The IFN system is an important first line of defence against virus infections. Viruses have evolved defence strategies, not only to inhibit the expression of IFN, but also to modulate the establishment of the antiviral state, thereby inhibiting the antigen presentation through increased MHC class I expression.

The main focus of this work is MGF360-18R, a non-conserved protein of the ASFV that evolved for the inhibition of IFN responses. Here, its ability to modulate IFN-mediated signalling is demonstrated. The results presented show that the ASFV MGF360-18R protein is able to impair the host cell response to both IFN-α and IFN-γ (Jak-STAT pathway) inducing STAT1 degradation by the 26S proteasome.

4.2. Introduction

In order to establish an effective antiviral response, the response to IFNs released by infected or activated cells must be rapid and efficient. These cytokines can stimulate an antiviral state in an autocrine or paracrine manner by binding to distinct receptors on the surface of infected or neighbouring target cells. One of the major signalling cascades activated by IFNs is the Jak-STAT signalling pathway, which leads to induction of ISGs and the resulting synthesis of proteins that are able to interfere with several cellular and viral processes. As a result, replication of the virus can be blocked or impaired, and both infected and neighbouring cells are made more susceptible to apoptosis, limiting virus spread. In addition, interferons also have a role in the priming and activation of several cell types of both the innate and adaptive immune system.

Type I and type II IFNs bind to specific cell surface receptors, activating distinct but related signalling pathways, known as the Jak-STAT pathways. Binding of type I IFN to its specific receptor results in the activation of the Janus tyrosine kinases, Jak1 and Tyk2, which will phosphorylate STAT1 (at Tyr^{701}) and STAT2 (at Tyr^{690}), respectively. The activated STATs dissociate from the type I IFN receptor, forming a stable heterodimer that associates with IRF-9, forming the ISGF3 tertiary complex. This transcription factor translocates to the nucleus and bind to IFN-stimulated response elements (ISRE) present in the promoter region of IFN-stimulated genes (ISGs) [1,2,3], thereby resulting in the transcription of several genes associated with this arm of the IFN response. Type III IFNs have type I IFN-like biological functions and, although binding to a distinct receptor, are able to activate the same Jak-STAT pathway [4].

Type II IFN acts by a separate Jak-STAT pathway. The binding of IFN-γ to the Type II IFN receptor activates both Jak1 and Jak2, leading to the phosphorylation of STAT1 (at Tyr^{701}) and posterior homodimerization. STAT1 homodimers translocate to the nucleus and bind to unique elements of IFN-γ stimulated genes, the gamma-activation sequence (GAS), and stimulate transcription of genes characteristic of responses to IFN type II. Of note is the fact that type I IFN stimulation can also lead to formation of STAT1-homodimers and leads to the induction of genes containing GAS elements in their promoter region [1].

The response to IFNs must be tightly regulated and terminated once the viral threat is over, in order to avoid damage to the host. A common mechanism for the regulation of several cellular processes is proteolysis following the conjugation of ubiquitin to proteins. Ubiquitination of STAT proteins results in their degradation by the 26S

proteasome-dependent pathway. This is the only mechanism that reduces the levels of STAT proteins in the cell, helping to regulate STAT signalling and restrain the inflammatory response. Regulation of IFN-activated STAT1 levels by the ubiquitin-proteasome pathway was demonstrated in 1996, by Kim and Maniatis [5] and, some years later, PDLIM2 protein was identified as a STAT1 ubiquitin E3 ligase [6]. It was later reported that phosphorylation of PDLIM2 Ser-137 is required for Ub-P-STAT1 formation and degradation by the 26 S proteasome system [7].

Given that interferon-mediated signalling plays an important role in anti-viral immunity, it is not surprising that viruses have evolved multiple means for its downregulation. Type I and type II IFN activate downstream components that can be either unique or common to both signalling pathways. Thus, viruses can block the impact of IFN at several levels, inhibiting only one of these two pathways or both. Modulation of STAT activity, either directly or indirectly, is a very common viral strategy. Viral proteins can inhibit the Jak kinases, preventing STAT activation [8], act by binding to STAT proteins, inducing their degradation [9], or prevent nuclear accumulation of STAT [10]. Other viruses induce expression of cellular inhibitors of the Jak-STAT pathways [11]. All these strategies share the common objective of inhibiting the signal transduction pathways triggered upon binding of IFN to its specific receptor.

Deletion of members of the ASFV multigene families (MGF) 360 and 530 has been shown to increase production of type I IFN and activation of IFN induced genes in infected macrophages. This suggests that these genes may have a role in inhibiting transcription of ISGs, although this remains to be demonstrated [12].

The non-conserved ORF MGF360-18R, a member of MGF360, was investigated in detail in order to understand the mechanism by which it inhibits the response to both type I and type II IFN. Two variants of this viral gene, the pathogenic Benin97/1 isolate variant and the shorter tissue culture-adapted Ba71V variant, characterized in the previous chapter, were studied. Both inhibit the induction of IFN-β and in this chapter we define how the same two variants inhibit the impact of secreted Type I and Type II IFN. The results indicate that both ASFV MGF360-18R proteins inhibit the impact of type I and type II IFN (Jak-STAT pathway) by inducing STAT1 degradation by the 26S proteasome.

4.3. Materials and methods

4.3.1. Cell culture

African green monkey Vero cells were cultured in Dulbecco´s Modified Eagle´s Medium (DMEM) (Gibco) supplemented with 100U/ml penicillin G sodium /100µg/ml streptomycin sulfate (Gibco), 2mM L-Glutamine (Gibco) and 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco). Cells were incubated at 37ºC, in 5%CO2.

4.3.2. Plasmids

The two variants of the ASFV open reading frame MGF360-18R were amplified by PCR from either BA71V or Benin-97/1 isolate DNA and cloned into the pcDNA3 plasmid in frame with an amino-terminal influenza haemaglutinin (HA) tag.

The IFN-α/β responsive plasmid [p(9-27ISRE)4tkΔ(-39)lucter] and the IFN-γ responsive plasmid [p(IRF-1*GAS)6tkΔ(-39)lucter] were gifts of Dr. S. Goodbourn. The first contain four tandem copies of the 9-27

ISRE sequence (AGGAAATAGAAACTG) fused to the firefly luciferase gene and the latter contain six tandem copies of the IRF-1 GAS site (TTTCCCCGAAA) also fused to the firefly luciferase gene.

The pCMVβ plasmid contains a β-galactosidase gene under the control of human cytomegalovirus immediate early promoter, and serves as an internal control for culture to culture variations in transfection efficiency.

4.3.3. Luciferase reporter gene assay

Vero cells ($6x10^4$ cells/well, in a 24 well plate) were co-transfected with 100ng of the indicated luciferase reporter plasmid, 25ng of the β-galactosidase internal control plasmid (pCMVβ) and 300ng of either pcDNA3HA-18R (Ba71V), pcDNA3HA-18R (Benin97/1) or non-recombinant pcDNA3HA, according to the Lipofectamine 2000 (Invitrogen) protocol. Seventy two hours post-transfection the cells were either stimulated with 1U/μl human IFN-γ (cells transfected with GAS reporter) or 1U/μl human IFN-α (cells transfected with the ISRE reporter) for five hours, or left untreated.

In an alternative protocol using the proteasome inhibitor MG132 (Calbiochem), the cells were either treated with 10μM MG132, or with DMSO, together with 1U/μl human IFN-γ.

After the treatment, the cells were lysed. The luciferase activity was measured using the luciferase assay system (Promega) according to the manufacturer`s protocol. The β-galactosidase activity was measured using the Galacton-Plus kit from Tropix (Bedford, MA). The luciferase activity was normalized relatively to the β-galactosidase activity of each sample, as to correct transfection efficiency variations between different cells.

4.3.4. Western blot

Vero cells ($3x10^5$ cells/well, in a 6 well plate) were transfected with 3µg of either pcDNA3HA-18R (Ba71V) or the non-recombinant pcDNA3HA, according to the Lipofectamine 2000 (Invitrogen) protocol. Alternatively, lentivirus infected COS-1 cells, stably expressing MGF360-18R (Benin97/1), were used. Forty-eight hours post-transfection, the cells were stimulated with 1U/µl human IFN-γ (ImmunoTools) or 1U/µl human IFN-α/β (ImmunoTools), during the indicated amounts of time, or left untreated. In an alternative protocol using the proteasome inhibitor MG132 (Calbiochem), the cells were either treated with 10µM MG132, or with DMSO, together with 1U/µl human IFN-γ, for the indicated amounts of time.

Cells were then harvested and lysed using a non-ionic lysis buffer (15mM Tris-HCl, pH 7.4, 120mM NaCl, 25mM KCl, 2mM EDTA, 2mM EGTA, 0.1mM DTT and 1%Triton X-100) containing a protease inhibitor cocktail (Sigma). Cell lysates were resolved on a 12% sodium dodecyl sulphate-polyacrilamide gel (SDS-PAGE). The separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare). Membranes were blocked with 5% non-fat milk for one hour at room temperature and probed with the following antibodies: rabbit anti-human phospho-STAT1(Tyr^{701}) (Cell Signaling), rabbit anti-human STAT1 (CT) (Millipore), rabbit anti-human phospho-STAT2(Tyr^{690}) (Cell Signaling), rabbit anti-human STAT2(CT) (Santa Cruz), rat anti-HA-HRP conjugated (high affinity) (Roche) and anti-β-actin-HRP conjugated (Sigma) as a loading control. Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody was purchased from Invitrogen. Membranes were developed by enhanced chemiluminescence detection according to the manufacturer's

instructions (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific or Luminata Forte, Millipore).

4.3.5. Immunoprecipitation

Vero cells ($5x10^5$ cells/plate, in a 60 mm Ø plate) were transfected with 6µg of either pcDNA3HA-18R (Ba71V) or the non-recombinant pcDNA3HA. Forty-eight hours post-transfection, the cells were either stimulated or not stimulated with 1U/µl human IFN-γ during 45 minutes, in the presence of either 10µM MG132 or DMSO. Cells were then harvested and lysed in lysis buffer (15 mM TrisHCl, pH 7.4, 120 mM NaCl, 25 mM KCl, 2mM EDTA, 2mM EGTA, 0.1mM DTT and 1% Triton X-100) containing a protease inhibitor cocktail (Sigma). Immunoprecipitation was performed with Dynabeads protein G (Millipore), using rabbit anti-human STAT1(CT) antibody (Millipore). Washes were performed using commercially available lysis buffer (Sigma). Elution was done using 2X sample buffer. Immunoprecipitation eluates were resolved on a 12% SDS-PAGE gel and proteins were transferred to a PVDF membrane (GE Healthcare). Membranes were blocked with 5% non-fat milk for one hour at room temperature and probed with the following antibodies: rabbit anti-human STAT1(CT) (Millipore), anti-HA-HRP conjugated (Roche) and rabbit anti-ubiquitin (Cell Signaling). Detection of native antibodies on immunoblot membranes was performed using Clean-Blot IP Detection Reagent (HRP) (Thermo Scientific).

4.3.6. Statistical Analysis

Data were shown as mean values with standard deviations (SD). Differences between experimental groups were determined by a two-tailed Student *t* test, using Excel software (Microsoft).

4.4. Results

4.4.1. The ASFV MGF360-18R protein inhibits response to both type I and type II IFN

As interferon-mediated signalling plays an important role in anti-viral immunity, not only in the induction of cellular, anti-viral proteins but also in stimulating antigen presentation through increased MHC class I expression, there is an obvious advantage for viruses to block IFN signalling. The objective of this work was to determine whether the MGF360-18R protein, already described as an inhibitor of the induction of IFNs in the previous chapter, was capable of impacting on the cellular response to both type I and type II IFNs. In addition, both variants of this ASFV ORF were tested and compared, regarding their inhibitory effect on the impact of type I and type II IFN.

Reporter plasmids containing the luciferase gene under the control of ISRE or GAS elements were used to quantify the response of cells to IFN-α and IFN-γ, respectively. As can be observed, the luciferase activities of both reporter plasmids were strongly induced after stimulation with IFN-α and IFN-γ (Fig.4.1). In cells expressing either variants of the MGF-18R protein, however, the induction of both reporters was clearly and similarly reduced. This indicates that the two variants of MGF360-18R are able to inhibit type I (Fig.4.1-A) and type II (Fig.4.1-B) IFN signalling pathways.

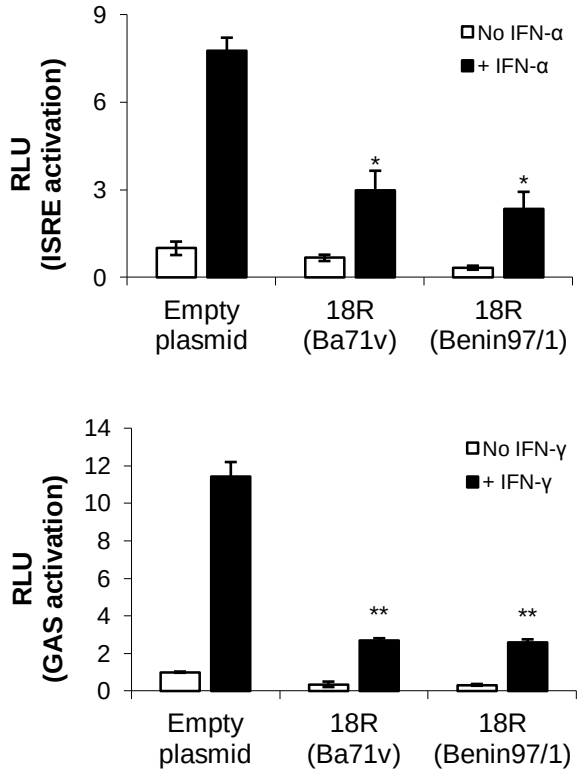


Figure 4.1 – The two MGF360-18R variants (Ba71V and Benin97/1) inhibit signalling by both type I and type II IFN receptors.

Vero cells were co-transfected with the pcDNA3HA (empty plasmid) or pcDNA3HA-18R plasmid expressing the MGF360-18R variant from Ba71V or Benin97/1, the β-galactosidase plasmid and either the (**A**) ISRE or the (**B**) GAS promoter luciferase reporter. For both assays, seventy-two hours post-transfection, the cells were either induced with 1U/µl IFN-α or 1U/µl human IFN-γ for five hours (■), respectively, or left untreated (□). Luciferase activity was normalized to β-galactosidase activity as a control for transfection efficiency. Data are expressed as means of Relative Luciferase Units (RLU) ± SD of triplicate well from one of three similar experiments. Statistical significance is represented as p≤0.05 (*) or ≤0.01 (**).

4.4.2. The MGF360-18R protein diminishes the total amount of cellular STAT1 but has no effect on STAT2

Upon binding of type I and type II IFN to their cognate receptors, the Jak-STAT pathways are activated, culminating in the nuclear translocation of activated STATs, initiating transcription of antiviral genes. It is not surprising that viruses have evolved mechanisms to inhibit IFN signalling, particularly by interfering with the activity of STATs. These proteins are modulated by different cellular mechanisms that can be exploited by viruses to their own advantage, such as proteasomal degradation, inhibition of phosphorylation by the Jak tyrosine kinases and finally inhibition of translocation into the nucleus [1,8,13].

To further understand the mechanism by which the ASFV MGF360-18R protein inhibits the response to both type I and type II IFN, Vero cells were transfected with MGF360-18R expression plasmid, and the levels of endogenous STAT1 and STAT2 were estimated by immunoblot analysis using specific antibodies.

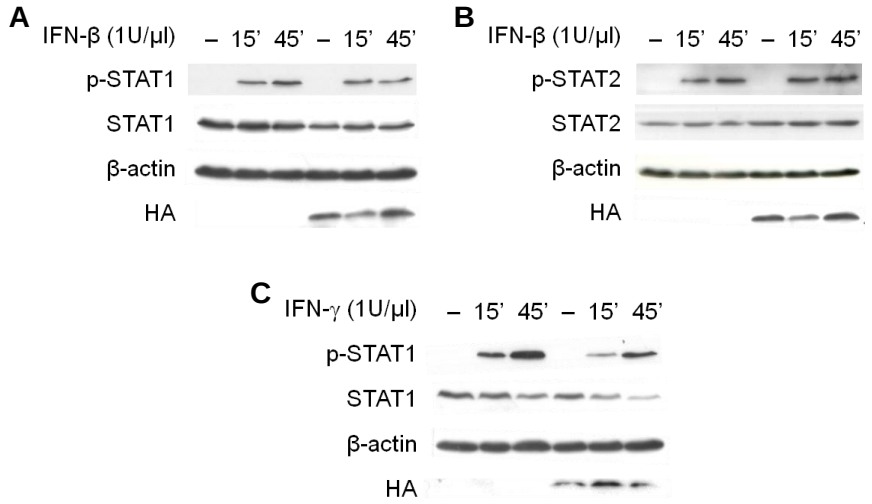


Figure 4.2 – Cells expressing MGF360-18R (Ba71V) protein show a reduction in the levels of STAT1, but not STAT2.

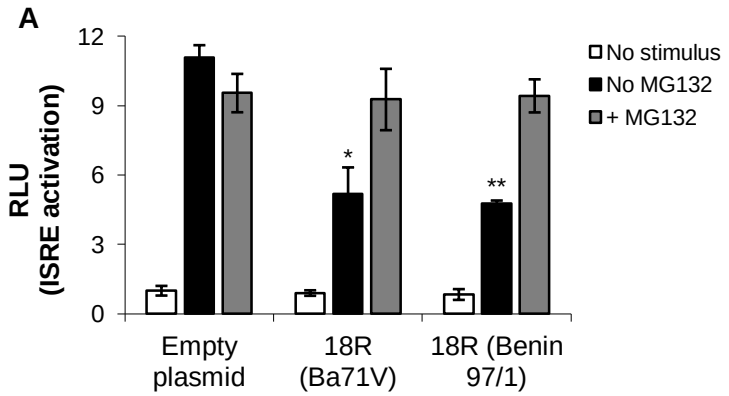
Vero cells were transfected with non-recombinant pcDNA3HA or pcDNA3HA-18R (Ba71V) expressing plasmid. Forty-eight hours post-transfection, cells were either induced with 1U/μl human IFN-β (**A**, **C**) or IFN-γ (**B**) for the indicated times, or left untreated. The cells were lysed and total cell extracts were immunobloted with anti-phosphorylated STAT1(Tyr^{701}) and anti-STAT1(CT) (**A, B**) or anti-phosphorylated STAT2(Tyr^{690}) and anti-STAT2(CT) (**C**). Anti-β-actin antibody was used as loading control. Expression of MGF360-18R (Ba71V) was detected using an anti-HA-HRP conjugated antibody.

Immunoblot analysis using antibodies against STAT1 revealed that there is less STAT1 in MGF360-18R expressing cells. When activated by either IFN-β or IFN-γ, cells expressing the viral gene also show a reduction in the levels of phosphotyrosine (701) for of STAT1, when compared to control cells (Fig.4.2-A and C). In contrast, the levels of STAT2 are not affected (Fig.4.2-B).

4.4.3. MG132, a proteasome inhibitor, reverts the inhibition of GAS reporter by MGF360-18R

No obvious degradation products of STAT1, such as breakdown intermediates originated by the action of sequence-specific endoproteases, were visible in the immunoblots. This observation suggested that the STAT1 protein is being degraded by a processive protease such as the ones acting in proteasome-mediated degradation.

To test this hypothesis, Vero cells were treated with the proteasome inhibitor MG132, and the response of cells to IFN-γ was measured using the GAS luciferase reporter plasmid, containing a STAT1 binding promoter sequence. As can be observed, the luciferase activity of this reporter plasmid, when compared to control cells, was reduced in cells expressing either variants of the MGF360-18R protein, as expected. When these cells were treated with MG132, the luciferase activity was the same as in control cells (Fig.4.3). This demonstrates that, by blocking the proteasome-mediated degradation of STAT1, there is a reversion of the MGF360-18R mediated inhibition of the impact of type II IFN.



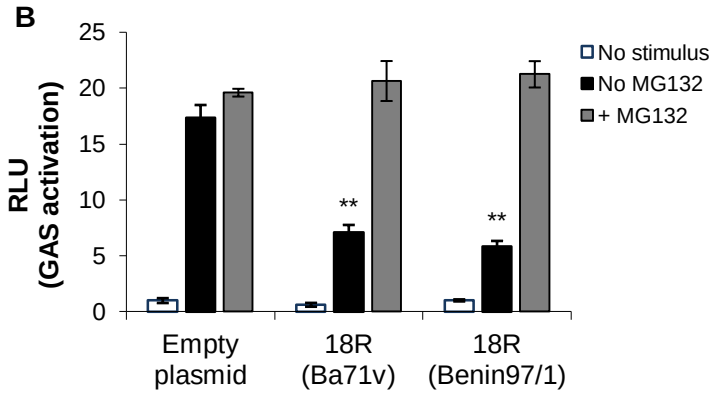


Figure 4.3 – Addition of MG132 reverts MGF360-18R-mediated inhibition of the impact of both type I and type II IFN.

Vero cells were co-transfected with the empty pcDNA3HA (Control) or pcDNA3HA-18R plasmid expressing the MGF360-18R variant from Ba71V or Benin97/1, the β-galactosidase plasmid and the (**A**) ISRE or (**B**) GAS promoter luciferase reporter. Seventy-two hours post-transfection, the cells were either induced with 1U/µl human IFN-γ in the presence of 10µM of MG132 (■) or DMSO (■) for five hours, or left untreated (□). Luciferase activity was normalized to β-galactosidase activity as a control for transfection efficiency. Data are expressed as means of Relative Luciferase Units (RLU) ± SD of triplicate well from one of three similar experiments. Statistical significance is represented as p≤0.05 (*) or ≤0.01 (**).

4.4.4. The proteasome inhibitor MG132 blocks degradation of STAT1 in cells expressing MGF360-18R

To confirm that the results obtained in Fig. 4.3 are due to the inhibitory activity of MG132 on the proteasomal degradation of STAT1, the levels of STAT1 were examined in cells treated with this proteasome inhibitor (Fig.4.4 and 4.5).

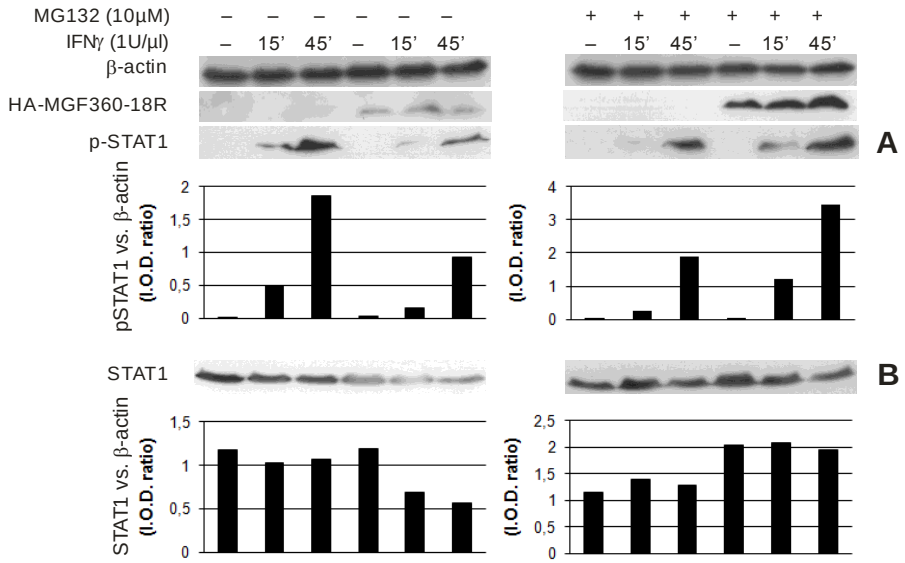


Figure 4.4 – The proteasome inhibitor MG132 blocks degradation of STAT1 in cells expressing ASFV MGF360-18R (Ba71V).

Vero cells were transfected with control pcDNA3HA or pcDNA3HA-18R (Ba71V) plasmid. Forty-eight hours post-transfection, cells were induced with 1U/μl human IFN-γ, either in the absence or presence of MG132, during the indicated amounts of time. The cells were lysed and total cell extracts were immunobloted with anti-phosphorylated STAT1(Tyr^{701}) (**A**) and anti-STAT1(CT) (**B**). Anti-β-actin antibody was used as loading control. Expression of MGF360-18R (Ba71V) was confirmed using an anti-HA-HRP conjugated antibody. WB signal was quantified by determining the integrated optical density (I.O.D.) of a given band, normalized to the corresponding loading control, using ImageJ 1.43u software.

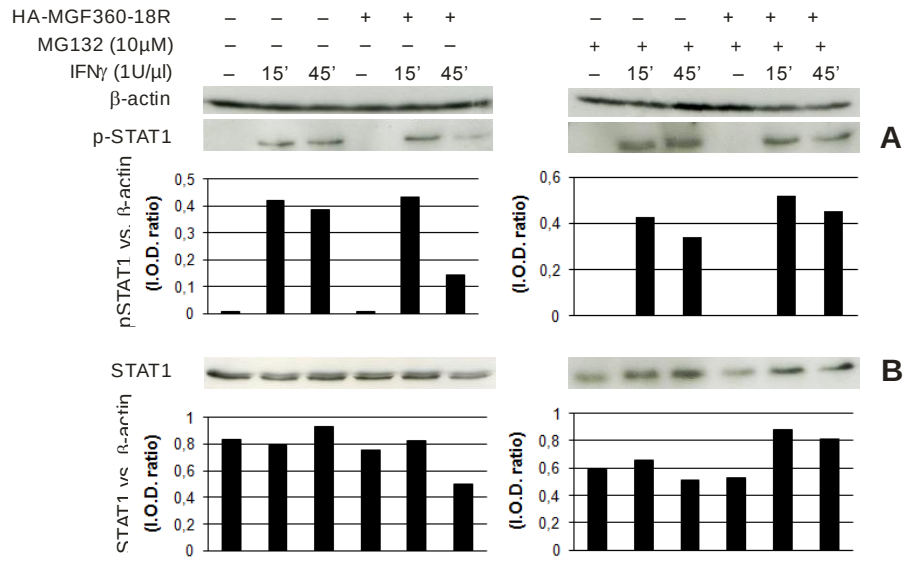


Figure 4.5 – The proteasome inhibitor MG132 blocks degradation of STAT1 in cells expressing ASFV MGF360-18R (Benin97/1).

COS-1 cells stably expressing MGF360-18R (Benin97/1) or eGFP, were induced with 1U/μl human IFN-γ, either in the absence or presence of MG132, during the indicated amounts of time. The cells were lysed and total cell extracts were immunobloted with anti-phosphorylated STAT1(Tyr^{701}) (**A**) and anti-STAT1(CT) (**B**). Anti-β-actin antibody was used as loading control. WB signal was quantified by determining the integrated optical density (I.O.D.) of a given band, normalized to the corresponding loading control, using ImageJ 1.43u software.

The results are consistent with the hypothesis that both variants of the MGF360-18R are targeting STAT1 for degradation. However, the results presented in Fig.4.5 are not conclusive, since the expression of the MGF360-18R (Benin97/1) could not be confirmed.

4.5. Discussion

African swine fever virus (ASFV) has evolved a variety of strategies to evade host innate defence mechanisms, such as inflammation, interferon and cell death [14]. Since the interferon response is one of the first lines of defence against viral infections, it would be surprising if the virus had not evolved mechanisms to inhibit the induction of IFN and, as well, the impact of the subsequent secretion of IFN, after binding to its cellular receptors [3,15]. However, nothing is known of the precise mechanisms by which the virus might downregulate the impact of IFN. Here we demonstrate that the non-conserved ASFV ORF MGF360-18R inhibits the impact of both type I and type II IFNs through inducing degradation of the critical signalling intermediate STAT1.

The Jak-STAT signalling pathway, which is able to induce the expression of specific genes, the ISGs, is the major signalling cascade activated by IFNs. In direct consequence, an antiviral state is elicited in the infected cells, which will either block or impair viral replication or make infected cells more susceptible to apoptosis, thereby limiting virus spread. In addition, interferons also have a role in the priming, activation and differentiation of several cell types of both the innate and adaptive immune system.

Afonso *et al*. reported that deletion of some members of the ASFV multigene families (MGF) 360 and 530 increases production of type I IFN and activation of IFN induced genes in infected macrophages, leading to the suggestion that these genes may have a role in inhibiting transcription of ISGs [12]. The non-conserved ORF MGF360-18R was identified, in a previous screening performed in our laboratory (Correia, S.M., unpublished work), as an inhibitor of the impact of both type I and type II IFN. Here we demonstrate that both the full length

variant of this gene from the pathogenic Benin97/1 isolate and the truncated gene from the non-pathogenic, tissue-culture adapted Ba71V virus, similarly inhibit the impact of type I and type II IFNs through degradation of the critical intracellular signalling intermediate STAT1.

Specifically, the results indicate that in cells expressing the ASFV MGF360-18R protein and stimulated with either type I or type II IFN, both phosphorylated and unphosphorylated STAT1 levels are reduced, but the levels of STAT2 remain unaffected. Modulation of STAT activity is a very common viral tactic, which can be achieved by employing several strategies, with the common objective of inhibiting the signal transduction pathways triggered upon binding of IFN to its specific receptor. By reducing the levels of STAT1 protein present in IFN activated cells, the ASFV protein MGF360-18R is able to inhibit the impact of both type I and type II IFN, given that this transcription factor is common to both signalling pathways (see Fig.1.4).

The observed reduction of STAT1 levels could be explained by an interference of the viral gene at either the level of transcription, translation or reduction of the protein half-life. The fact that no breakdown intermediates of STAT1 were observed in the immunoblots performed with lysates containing protease inhibitors is consistent with degradation via the proteasome rather than a result of the action of sequence-specific endoproteases.

Previous studies have demonstrated that the levels of activated STAT1 are regulated by the ubiquitin-proteasome pathway [5]. This raised the possibility that the polyubiquitination pathways of the cell could have been highjacked by the MGF360-18R protein, inducing polyubiquitination of STAT1 and its concomitant degradation by the proteasome. The assays performed using MG132, a proteasome

inhibitor, confirmed this hypothesis. In cells treated with MG132, a 26S proteasome inhibitor, both variants of the MGF360-18R protein no longer inhibited the expression of genes controlled by ISRE and GAS sequences in their promoter regions upon stimulation with IFN-α and IFN-γ, respectively. Additionally, the expression of either variants of the MGF360-18R protein no longer downregulate STAT1 levels. In cells where the 26S proteasome was blocked by MG132, the viral protein loses its ability to inhibit the impact of both type I and type II IFN. This indicates that the Jak-STAT pathway is being inhibited as a result of an increased proteasomal degradation of STAT1 induced by expression of MGF360-18R. It is possible that the MGF360-18R is hijacking the polyubiquitination pathways, inducing the ubiquitination of STAT1, and targeting it for degradation by the 26S proteasome. In order to test this hypothesis, lysates of cells expressing MGF360-18R can be immunoprecipitated with anti-STAT1 antibody and immunoblotted with anti-ubiquitin antibody, to verify if there is an accumulation of ubiquitinated STAT1 in IFN-γ activated cells treated with MG132, when compared to control cells.

Further studies are needed to clarify the role of the ASFV protein in the induction of STAT1 polyubiquitination and define its mechanism of action. Protein ubiquitination is a common form of post-translational modification that regulates a broad spectrum of protein substrates in diverse cellular pathways. The attachment of ubiquitin to proteins occurs through a three-enzyme (E1–E2–E3) cascade. The E3 ubiquitin ligase, which is best represented by the superfamily of the Cullin-RING complexes, catalyses the ubiquitin attachment to its specific target. Previous studies on molecular mechanisms governing the stability of activated/phosphorylated STAT1 suggest that different E3 ligases target STAT1 for proteasomal breakdown, depending on its phosphorylation status [6,7,16]. The Cullin4A (Cul4A)-RING ubiquitin

E3-ligases (CRL4) are protein complexes that include the DNA-Damage-Binding protein 1 (DDB1), a protein that functions as an adaptor protein to link CUL4A and CUL4-associated factors (DCAFs). The Cul4A-DDB1 core complex maintains efficient and timely assembly with different DCAFs to target distinct cellular substrates for ubiquitination, forming more than 90 E3–ligase complexes, responsible for the regulation of a broad spectrum of cellular processes(reviewed by Iovine B. *et al.*, 2011) [17]. It was demonstrated that the V protein of simian virus 5 (SV5) is able to hijack this ubiquitin ligase machinery through interaction with DDB1, recruiting STAT1 and targeting it for degradation [18]. It is now known that RNA viruses of the *Paramyxoviridae* family (*Mononegavirales* order) code for V proteins whose expression greatly reduces the half life of STAT1 and/or STAT2, thereby inhibiting the Jak-STAT IFN signalling pathway (reviewed by Naijar & Fagard, 2010) [19].

Regarding the role of SV5 V protein in the ubiquitination of STAT1, a model has been proposed in which, after infection with SV5, the V protein acts as a linker bringing DDB1/Cul4A complexes into a close and stable association with STAT1/STAT2 complexes. An E3 ligase complex is formed and STAT1 becomes polyubiquitinated, being subsequently degraded by the proteasome. The DDB1/SV5-V/STAT2 complex then becomes destabilized and STAT2 either captures another STAT1 to form a new degradation complex or dissociates from the complex and binds a free STAT1 molecule, with the resulting STAT1/STAT2 complex being recaptured by the DDB1/SV5 V-containing E3 ligase. In uninfected cells DDB1/Cul4A complexes do not associate with STAT1 and STAT2, which can associate together in the absence of IFN stimulated phosphorylation [20].

SV5 can only target STAT1 in cells that express STAT2, the same being true for mumps virus. For instance, SV5 does not replicate efficiently or cause STAT1 degradation in the mouse, where the murine STAT2 protein is unusually divergent in amino acid sequence. STAT2 can thus act as a host range determinant for this virus. (reviewed by Horvath C.M., 2004) [21]. Considering that STAT2 is required for STAT1 turnover, the fact that HEK-293T cells have low levels of endogenous STAT2 was pointed as a possible explanation of why it is not possible to see a loss of STAT1 in HEK-293T cells transiently transfected with the SV5 V protein [18].

It is tempting to speculate whether MGF360-18R could be inducing STAT1 ubiquitination and proteasomal degradation by highjacking the DDB1/Cul4A-RING ubiquitin ligase machinery. An obvious experiment would be to attempt the co-immunoprecipitation of DDB1 with the MGF360-18R protein. This viral protein fails to inhibit type I and type II IFN signalling in HEK-293T cells (data not shown), and it would be worth to set up a luciferase assay using GAS and ISRE luciferase reporters in HEK-293T cells transfected with STAT2 expression plasmid, and see if this could rescue the inhibitory activity of MGF360-18R. In addition, it is known that most DCAFs possess an α-helical motif with a conserved DxR sequence, responsible for binding to DDB1 [22]. MGF360-18R does have an α-helical motif with identical characteristics, which could be further characterized through bioinformatic analysis. The construction of a deletion mutant protein lacking this motif could prove useful to verify if this motif is necessary for the MGF360-18R mediated inhibition of the Jak-STAT pathway.

Up to now, no ASFV gene has been described that interferes with the Jak-STAT signalling pathway. The work described in this chapter demonstrates that the MGF360-18R protein of ASFV inhibits both type

I and type II IFN signalling cascades as well as the previously described inhibition of IFN-β induction (Chapter 4). Thus MGF360-18R is a multifunctional virus host evasion molecule which, through its impact at both the induction and impact of IFN responses, may affect virus pathogenesis through its activity at different stages of the virus replication cycle.

4.6. References

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Chapter 5

Final Considerations

5.1. Final Considerations

Viral genomes encode essential proteins, such as structural proteins, enzymes, and a number of proteins, not necessarily essential, that have evolved for host cell evasion. The latter, as they modulate the host-pathogen interaction, contribute to the pathogenesis of a viral infection and may have practical applications for the manipulation of cell biology and immune responses.

For ASFV, most of the structural proteins, and the enzymes required for virus replication, and some of the proteins that are involved in viral host evasion have been already identified by sequence homology and biochemical studies. There are, in addition, a large number of genes without homology to cellular genes (reviewed by Dixon, 2008) [1], that have most likely evolved for host cell manipulation. The function of such “unassigned” virus genes can only be revealed through functional approaches. Being an early innate host defence mechanism, the interferon system is a key player against virus infections and, as such, viruses have evolved a number of counter strategies to antagonise this response [2].

Two ASFV genes subverting IFN responses with entirely different strategies have been pursued in the work presented in this thesis; one with no homology (MGF360-18R) and the other (I329L) with a marginal homology only revealed after extensive bioinformatic analysis. The results and implications have been extensively discussed in each of the three experimental chapters, and so the following discussion will be a brief recapitulation of the key points.

Modulation of IFN-β expression by the MGF360-18R protein of ASFV

The comparison of the transcriptional profiles of macrophage infected with wild type ASFV and a deletion mutant virus lacking six MGF360 and two MGF530 genes, resulted in the observation that some of the genes belonging to any of these two multigene families might have evolved to manipulate interferon responses [3]. A functional screening of 17 non-homologous ASFV genes, previously performed at our laboratory (Correia SM, unpublished work), identified four genes that are able to inhibit the induction of the IFN-β in response to Poly (I:C). One of these, the MGF360-18R gene, a member of MGF360, was investigated in detail in order to understand its mechanism for inhibiting the induction and impact of IFN-β.

Sequence comparison of the genomes of several ASFV isolates revealed that MGF360-18R is a non-conserved open-reading frame (ORF). For this work, we selected two variants of this viral gene: the Benin97/1 variant, with 254a.a., which the largest known variant of this protein, and the Ba71V variant, with 148a.a., encoded by the tissue culture attenuated ASFV strain (see Anex).

Both MGF360-18R variants were demonstrated to inhibit the induction, expression and secretion of IFN-β in a NF-κB dependent manner. Moreover, both variants were found to colocalize to mitochondria and to target MAVS, a key adaptor protein of the RIG-I-like receptors (RLR) pathway. All viruses, even those replicating within the nucleus (e.g. herpes viruses), include a cytoplasmic phase in their replication strategy, and the RLRs specifically recognize viral dsRNA in the cytoplasm. Interestingly, these cytosolic receptors were recently described to be involved in antiviral signalling in response to viruses containing a dsDNA genome, such as Epstein–Barr virus [4] and

vaccinia virus [5]. By interfering with MAVS, MGF360-18R is able to block the cytosolic antiviral recognition mechanism, inhibiting both NF-κB activation and induction of type I IFN. The interaction between MGF360-18R (Ba71V variant) and MAVS was demonstrated by biochemical evidence, but this same assay was not yet performed for the Benin97/1 variant of MGF360-18R. On the other hand, the exact mechanism by which this viral protein is interfering with MAVS function remains to be clarified. Several observations support that basic mitochondrial functions are integrated with innate immunity, and that innate immune signalling based on the mitochondria largely depend on this organelle's activity and fitness (reviewed by Arnoult, 2011) [6]. Considering its mitochondrial location, it should be interesting to verify if MGF360-18R has any effect on the mitochondrial potential, thus affecting this organelle's fitness.

Moreover, MGF360-18R variant from Benin97/1 was shown to inhibit the activity of the IRF3 transcription factor, hence directly affecting IRF-3 stimulated ISG transcription, in addition to its inhibitory role in the RLR pathway by interfering with MAVS. Several possibilities for the mechanism by which this variant of the MGF360-18R protein inhibits the activity of IRF3 were extensively discussed in the respective experimental chapter. This additional inhibitory action of this MGF360-18R variant is particularly interesting as it corresponds to the variant found in pathogenic isolates of the ASFV. Comparatively to the tissue culture adapted, non-pathogenic variant, the MGF360-18R protein encoded by the Benin97/1 ASFV isolate is able to block IFN response in a more effective manner, by interfering at two distinct and crucial stages of mechanisms leading to induction of type I IFN. The transcription factor IRF3 is common to most mechanisms leading to induction of IFN, thus inhibition of IRF-3 efficiently blocks induction of IFN by all remaining anti-viral recognition mechanisms.

In conclusion, both variants of the ASFV MGF360-18R protein impair activation of IFN-β induction through targeting MAVS, a key adaptor protein of the RLR pathway. The MGF360-18R variant from the Benin97/1 isolate, also targets IRF-3, which may give the virus an extra advantage.

Modulation of the impact of both type I and type II IFN by the MGF360-18R protein of ASFV

Deletion of members of the ASFV multigene families (MGF) 360 and 530 has also been shown to increase activation of IFN induced genes in infected macrophages. This suggests that these genes may have a role in inhibiting transcription of ISGs, although this remains to be demonstrated [3].

The non-conserved ORF MGF360-18R was investigated in detail in order to understand the mechanism by which it modulates IFN-mediated signalling. The results indicated that the impact of both type I and type II IFN on the Jak-STAT signalling cascades is impaired by the expression of both variants of ASFV MGF360-18R. As the STAT transcription factors are key regulators in this signalling pathway, their activation was examined on cells expressing either variants of MGF360-18R. The results presented show that both variants of ASFV MGF360-18R protein are able to reduce the cellular levels of both phosphorylated and unphosphorylated STAT1, but the levels of STAT2 remain unaffected.

The response to IFNs is a tightly regulated cellular process, as it must be terminated once the viral threat is over, in order to avoid damage to the host. Ubiquitination of STAT proteins results in their degradation by the 26S proteasome-dependent pathway, and this is the only known mechanism to reduce the levels of STAT proteins in the cell, helping to regulate STAT signalling and restrain the inflammatory response [7].

Given that interferon-mediated signalling plays an important role in anti-viral immunity, it is not surprising that viruses have evolved multiple means for its downregulation. Type I and type II IFN activate downstream components that can be either unique or common to both signalling pathways. Thus, viruses can block the impact of IFN at several levels, inhibiting only one of these two pathways or both. In this case, we describe an ASFV non-conserved gene, MGF360-18R, that impairs the host cell response to both type I and type II IFN (Jak-STAT pathway) inducing STAT1 degradation by the 26S proteasome.

STAT1 not only plays important roles in the response to type I and type II IFN, but is also involved in the response to various stressful stimuli that induce cell cycle arrest and apoptosis. Indeed, STAT1 has been reported to regulate the transcription of several genes involved in cell cycle control and is able to upregulate the expression of procaspases, the latent forms of the caspases, which are proteases that transmit the apoptotic pathway in the cytoplasm by sequential cleavage in response to external or internal stimuli (reviewed by Naijar & Fagard, 2010) [8]. Programmed cell death during ASFV infection is a tightly regulated process in which the action of inducers is balanced by the expression of antiapoptotic genes. Although ASFV induces apoptosis in the cell in a postbinding step, during or after virus uncoating, this cellular process is delayed up to 13h after the infection, a time at which viral morphogenesis is well under way [9]. Several ASFV genes have been shown to be involved in the inhibition of apoptosis using different mechanisms [10,11,12]. Here we studied another ASFV gene, MGF360-18R, which induces the degradation of STAT1, with loss of its pro-apoptotic and cell cycle regulation functions.

An interesting observation, when comparing the effect of the expression of either variant of the ASFV ORF MGF360-18R in the anti-

viral mechanisms studied in this work, is their distinct roles regarding the inhibition of IFN-β induction pathway. On the other hand, in the Jak-Stat pathway (STAT1), both MGF360-18R variants target the same cellular protein and the results indicate that the mechanism of action of either MGF360-18R variant, in this pathway, is the same. Regarding the IFN-β induction pathway, however, the Benin97/1 variant of MGF360-18R clearly has a greater impact, and is able to target two distinct and crucial proteins involved in the induction of IFN-β subsequently to viral infection. The peptide region that is truncated in the tissue culture adapted strain (Ba71V) should be more extensively studied, as it possibly accounts for the extra inhibitory role of the Benin97/1 variant. Phylogenetic analysis of the available ASFV genome sequences revealed considerable genetic diversity at the genome level, in particularly at the level of the composition of the variable regions [13]. The most variable genes belong to the multigene families, and ASFV MGF360/530 genes have been reported to affect viral growth in macrophage cell cultures and virulence in pigs [14]. Comparison of pathogenic and non-pathogenic strains could prove useful in identifying new ASFV genes involved in the virus pathogenesis.

Mechanism of ASFV ORF I329L-mediated inhibition of type I IFN induction

In a recent report, the conserved ASFV ORF I329L was described as an inhibitor of the TLR3 pathway, downmodulating both IFN-β secretion and NF-κB activation. Several observations raised the possibility that I329L might exert its inhibitory activity through interaction with TRIF, the TLR3 adaptor protein [15].

Here we present formal biochemical proof of an interaction between the I329L protein and TRIF. Functional analyses of I329L truncated

mutants were performed, to further define the role of each I329L domain. The expression of the transmembrane + intracellular domain (TM/IC) fragment of I329L inhibited induction of IFN-β and NF-κB activation by ectopically expressed TRIF, indicating a disruption of signal transduction through the impact of I329L intracellular domain on TRIF. Intriguingly, the extracellular + transmembrane domain (EC/TM) fragment of I329L inhibited Poly (I:C)-mediated, but not TRIF-mediated, induction of IFN-β. This observation suggests that I329L also inhibits dsRNA-mediated activation of the TLR3 pathway via its EC/TM domain, perhaps through formation of a non-signalling I329L-TLR3 heterodimer. In conclusion, the I329L mediated inhibition of TLR signalling pursues a dual strategy, with its extra- and intracellular domains evolved for interfering with the initiation and subsequent intracellular transmission of the dsRNA stimulus, respectively.

Considering the recent observations of proteolytic processing of endosomal TLRs, we tested the possibility of a similar processing for I329L, as this could help understanding the dual strategy of I329L, in particular the inhibitory activity of the TM/IC fragment [16]. Evidence was obtained indicating proteolytic processing of I329L as a consequence of viral infection or activation of TLR3 signalling, either by Poly (I:C) or TRIF. Consistent with the requirement of this processing for the full inhibitory activity of I329L, a mutant I329L protein lacking a cathepsin L sensitive site continued to inhibit Poly (I:C)-mediated induction of IFN-β, but was no longer able to inhibit IFN-β induction and secretion, nor NF-κB activation, through ectopically expressed TRIF.

Structural studies of the TLR3 ectodomain bound to Poly (I:C) indicate that a C-terminal cleavage product of TLR3 would contain residues implicated in direct interaction between the two TLR3 molecules within the dimer as well as the leucine-rich repeats [LRR] 19–21, required for

ligand binding [17,18]. The finding that TLR3 is proteolytically processed does not explain how this event results in TLR3 activation and optimal response to Poly (I:C). Proteolysis may be required for a conformational shift in dimer structure, enhancing binding to TRIF and intracellular signalling. Alternatively, receptor cleavage may lead to altered affinity for ligand, which could also increase the likelihood of receptor activation. It would be interesting to find if proteolytic cleavage of TLR3 is affected by expression of the ASFV I329L protein.

The I329L proteolysis mechanism was not clarified in this work. Proteolytic processing as a mechanism of protein maturation is a well known feature of ASFV [19]. The ASFV late protein pS273R belongs to the family of SUMO-1-processing cysteine proteinases, and is capable of cleaving viral proteins at specific Gly-Gly-X sites. Such a site is present in the I329L protein sequence and is coincident with the cathepsin L sensitive site. We must consider the possibility of I329L proteolysis resulting in two smaller, mature proteins, which will have distinct roles in the inhibition of type I IFN induction. Functional studies with the extracellular fragment of I329L, corresponding to the N-terminal residues being removed upon proteolysis, could elucidate if this fragment has any inhibitory activity.

Based in the results presented here, a model was suggested in which the I329L molecule inhibits the TLR response using a dual strategy: I329L forms a non-functional heterodimer with TLR3, thereby inhibiting the activation of the pathway via dsRNA binding, and, following proteolytic processing, the resulting TM/IC fragment of I329L is able to target TRIF, inhibiting the intracellular signalling initiation. This model is open to experimental confirmation, as are the precise details of the proteolytic processing.

In conclusion, in this work, we were able to demonstrate how two ASFV genes, MGF360-18R and I329L, use different strategies to modulate the IFN response. The deletion of virus “evasion” genes offers a rational strategy for the development of non-pathogenic, deletion mutant viruses. Specifically, mutant viruses unable to counteract the IFN response are in fact excellent candidates for live attenuated vaccines as they can be produced at high titers in IFN-deficient cultures. Finally, the work does support the suspicion that the non-homologous, non assigned portion of the ASFV (and hence other virus) genomes may be considered as a repository of the host evasion genes.

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ANEX

Screening of different ASFV isolates for the MGF360-18R sequence

Characterization of the ASFV isolates screened for the sequence of the MGF360-18R gene

Abbreviation	Virus designation	Host species	Country	Continent	Virulence	18R variant
ASFV-Ba71V	Ba71V	Vero cell adapted	Spain	Europe	Atenuated	A
ASFV-OurT88_3	OurT88_3	Tick	Portugal	Europe	Low	A
ASFV-Pret	Pretorisuskop-96-4	Tick	South Africa	Africa	High	B
ASFV-Ten62	Tengani62	Warthog	Malawi	Africa	High	B
ASFV-War	Warthog	Warthog	Namibia	Africa	Unknown	B
ASFV-Georgia	Georgia 2007/1	Domestic Pig	Georgia	Europe	High	B
ASFV-Ken	Kenya 1950	Domestic Pig	Kenya	Africa	High	C
ASFV-Mal	Malawi Lil-20-1 1983	Domestic Pig	Malawi	Africa	High	C
ASFV-Mku	Mkuzi 1979	Tick (1978)	Zululand	Africa	Unknown	D
ASFV-Benin97/1	Benin97	Domestic Pig	Benin	Africa	High	D
ASFV-E75	E75	Domestic pig	Spain	Europe	High	D
ASFV-Warm	Warmbaths	Tick	South Africa	Africa	Unknown	D

Variant A: 148a.a.; Variant B: 237a.a.; Variant C: 241a.a.; Variant D: 254a.a.

Alignment of putative MGF360-18R protein sequences expressed by different ASFV isolates

```
     ------------------------------------------------        ASFV-BA71V
     ------------------------------------------------        ASFV-OURT88_3
  1  MQNKIPNFNLFFFFLYRMLEIVLATLLGDLQRLRVLTPQQRAVAFFRANT    50  ASFV-E75
  1  MQNKIPNFNLFFFFLYRMLEIVLATLLGDLQRLRVLTPQQRAVAFFRANT    50  ASFV-Benin97/1
  1  MQNKIPNFNLFFFFLYRMLEIVLATLLGDLQRLRVLTPQQRAVAFFRANT    50  ASFV-Mku
  1  MQNKIPNFNLFFFFLYRMLEIVLATLLGDLQRLRVLTPQQRAVAFFRANT    50  ASFV-Warm
  1  ----------------MLEIVLATLLGDLQRLRVLTPQQRAVAFFRANT    33  ASFV-War
  1  ----------------MLEIVLATLLGDLQRLRVLTPQQRAVAFFRANT    33  ASFV-Pret
  1  ----------------MLEIVLATLLGDLQRLRVLTPQQRAVAFFRANT    33  ASFV-Teng
  1  ----------------MLEIVLATLLGDLQKLKDLTPPQRAVAFFRANT    33  ASFV-Ken
  1  ----------------MLEIVLATLLGDLQKLRDLTPMQRAVAFFRVNT    33  ASFV-Mal


     ------------------------------------------------        ASFV-BA71V
     ------------------------------------------------        ASFV-OURT88_3
 51  KELEDFLRSDGQSEEILSGPLLNRLLEPSCPLDILTGYHLFRQNPKAGQL   100  ASFV-E75
 51  KELEDFLRSDGQSEEILSGPLLNRLLEPSCPLDILTGYHLFRQNPKAGQL   100  ASFV-Benin97/1
 51  KELEDFLCSDGQSEEILSGPLLNRLLEPSGPLDILTGYHLFRQNPKAGQL   100  ASFV-Mku
 51  KELEDFLCSDGQSEEILSGPLLNRLLEPSGPLDILTGYHLFRQNPKAGQL   100  ASFV-Warm
 34  KELEDFLCSDGQSEEILSGPLLNRLLEPSGPLDILTGYHLFRQNPKAGQV    83  ASFV-War
 34  KEVEDFLCSDGQSEEVLSGPLLNRLLEPSGPLDILTGYHLFRQNPKAGQV    83  ASFV-Pret
 34  KELEDFLCPDGQSEEVLSGSLLNRLLEPSGPLDILTGYHLFRQNPKAGQL    83  ASFV-Teng
 34  KELEDFLYPDGQSEELLPGLLLNRLLEPSGSIDILTGYHLFRENPKAGRL    83  ASFV-Ken
 34  KELEDFLYPDGQSEELLPGLLLNRLLEPSGPIDILTGYHLFRENPKAGRL    83  ASFV-Mal
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  1   ------MLERLYDANIYNILSRLRPEKVRNKAIELYWVFRAIHICHAPLV    44  ASFV-BA71V
  1   ------MLERLYDANIYNILSRLRPEKVRNKAIELYWVFRAIHICHAPLV    44  ASFV-OURT88_3
101   RGLEVKMLERLYDANIYNILSRLRPEKVRNKAIELYWVFRAIHICHAPLV   150  ASFV-E75
101   RGLEVKMLERLYDANIYNILSRLRPKKVRNKAIELYWVFRAIHICHAPLV   150  ASFV-Benin97/1
101   RGLEVKMLERLYDANIYNILSRLRPEKVRNKAIELYWVFRAIHICHAPLV   150  ASFV-Mku
101   RGLEVKMLERLYDANIYNILSRLRPEKVRNKAIELYWVFRAIHICHAPLV   150  ASFV-Warm
 84   RGLEVKMLERLYDANIYNILSRLRPEKVRNKAVELYWVFRAINMCHAPLV   133  ASFV-War
 84   RGLEVKMLERLYDANIYNILSRLRPEKVRNKAVELYWVFRAINMCHAPLV   133  ASFV-Pret
 84   RGLEVKMLERLYDANIYNILSRLRPEKVRNKAVELYWVFRAINMCHAPLV   133  ASFV-Teng
 84   RGLEVKMLERLYDANIYNMLARLRPELVRDKAIELYWLFRAILMCHSPLV   133  ASFV-Ken
 84   RGLEVKLLERLYDANIYNMLAQIRPELVRIKAIELYWLFRAILMCHSPLV   133  ASFV-Mal
            :***********:*:::**: ** **:****:**** :**:***


 45   LDIVRYEEPDFAELAFICAAYFGEPQVMYLLYKYMPLTRAVLTDAIQISL    94  ASFV-BA71V
 45   LDIVRYEEPDFAELAFICAAYFGEPQVMYLLYKYMPLTRAVLTDAIQISL    94  ASFV-OURT88_3
151   LDIVRYEEPDFAELAFICAAYFGEPQVMYLLYKYMPLTRAVLTDAIQISL   200  ASFV-
151   LDIVRYEEPDFAELAFICAAYFGEPQVMYLLYKYMPLTRAVLTDAIQISL   200  ASFV-
151   LDIVRYEEPDFAELAFICAAYFGEPQVMYLLYKYMPLTRAVLTDAIRISL   200  ASFV-
151   LDIVRYEEPDFAELAFICAAYFGEPQVMYLLYKYMPLTRAVLTDAIRISL   200  ASFV-
134   LDIVRYEEPDFAELAFICAAYFGEPQVMYLLYKYMPLTRAVLTDAIQISL   183  ASFV-
134   LDIVRYEEPDFAELAFICAAYFGEPQVMYLLYKYMPLSRAVLTDAIQISL   183  ASFV-
134   LDIVRNEELDFAELAFICAAYFGEPQVMYLLYKYMPLTRAVLTDAIQISL   183  ASFV-
134   LEIVRHETMDFAETAFICAAYFSEPQVMYALYKFIPISRAVLADAIQMCL   183  ASFV-
134   LEIVRHETMDFAELAFICAAYFSEPQVMYALYKFIPISRAVLADAIEMSL   183  ASFV-
      *:*** *  **** ********.*********::*::****:***.:.**
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 95   ESNNQVGICYAYLMGGSLKGLVSAPLRKRLRAK-LRSQRKKKDVLSPHDFLLLLQ--  148  ASFV-BA71V
 95   ESNNQVGICYAYLMGGSLKGLVSAPLRKRLRAK-LRSQRKKKDVLSPHDFLLLLQ--  148  ASFV-OURT88_3
201   ESNNQVGICYAYLMGGSLKGLVSAPLRKRLRAK-LRSQRKKKDVLSPHDFLLLLQ--  254  ASFV-E75
201   ESNNQVGICYAYLMGGSLKGLVSAPLRKRLRAK-LRSQRKKKDVLSPHDFLLLLQ--  254  ASFV-Benin97/1
201   ESNNQVGICYAYLMGGSLKGLVSAPLRKRLCAK-LRSQRKKKDVLSPHDFLLLLQ--  254  ASFV-Mku
201   ESNNQVGICYAYLMGGSLKGLVSAPLRKRLRAK-LRSQRKKKDVLSPHDFLLLLQ--  254  ASFV-Warm
184   ESNSQVGICYAYLMGGSLKGLVRAPLRKRLRAK-LRSQRKKKDVLPPHDFLLLLQ--  237  ASFV-War
184   ESNSQVGICYAYLMGGSLKGLVRAPLRKRLRAK-LRSQRKKKDVLPPHDFLLLLQ--  237  ASFV-Pret
184   ESNSQVGICYAYLMGGSLKGLVRAPLRKRLRAK-LRSQRKKKDVLPPHDFLLLLQ--  237  ASFV-Teng
184   ESNSEAGICYAYLMGGSLKGKVPGSLRKRLRASPLRQERKKKNVLPPHEFLLMLHGI  240  ASFV-Ken
184   ESNSETGICYAYLMGGSLKGKVPGPLRKRLRASPLRQERKKKNVLPPHEFLLMLHGI  240  ASFV-Mal
      **.:.*************** *...***** *. **.:****:**.**:***:*:
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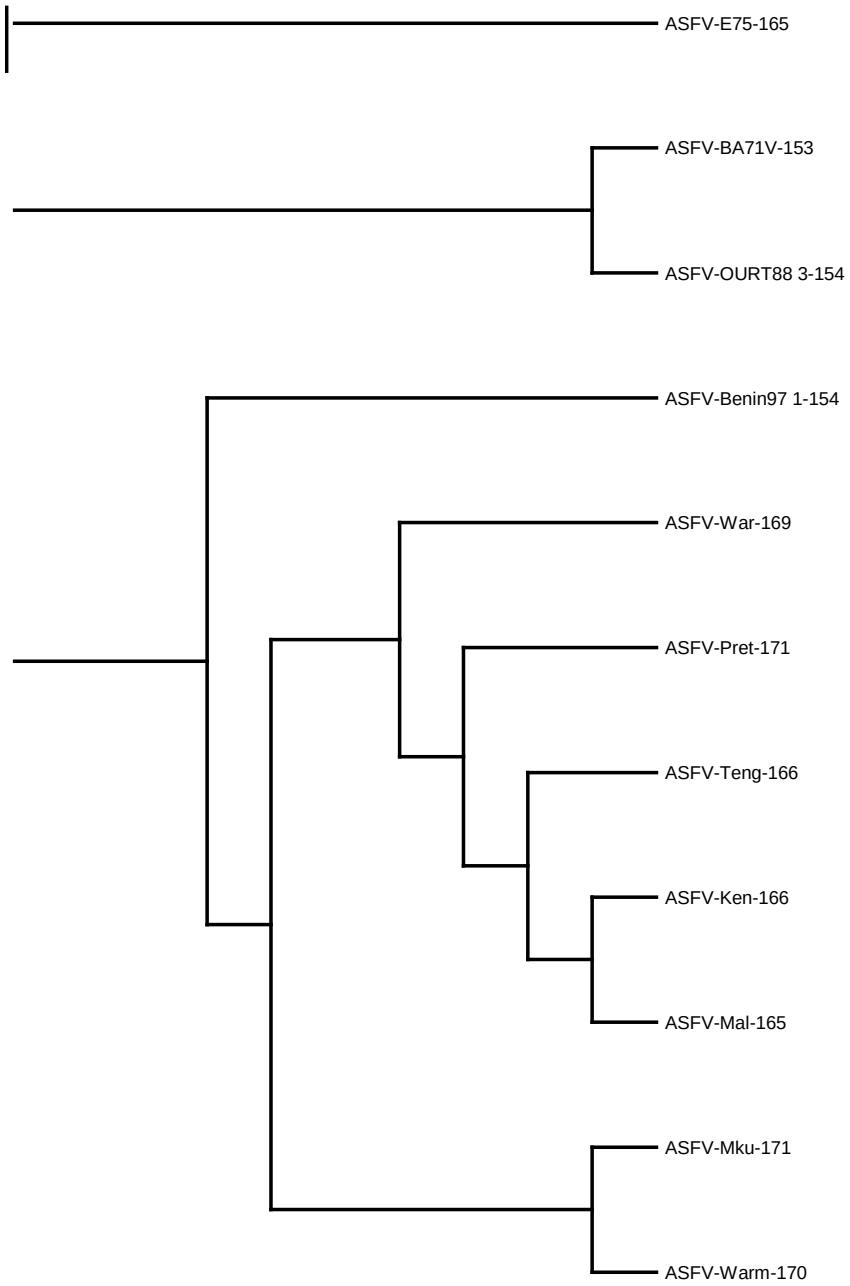
Cladogram showing the relationship between the several variants of MGF360-18R



Sequence and location of the MGF360-18R transmembrane domains predicted by the TMHMM program

Virus designation	Start	End	TM sequence
Benin97/1	4	30	KIPNFNLFFFFLYRMLEIVLATLLGDL
	127	147	KVRNKAIELYWVFRAIHICHA
	160	180	DFAELAFICAAYFGEPQVMYL
Ba71V	21	41	KVRNKAIELYWVFRAIHICHA
	54	74	DFAELAFICAAYFGEPQVMYL

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