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EVALUATION OF THE I329L AS A CANDIDATE TO DELETE FOR PRODUCTION OF A VIRAL ATTENUATED VACCINE AGAINST ASFV

ANA CATARINA MOTA CORREIA

MESTRADO EM MICROBIOLOGIA CLÍNICA

4ª EDIÇÃO

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DISSERTAÇÃO ORIENTADA PELO PROF. DOUTOR MICHAEL PARKHOUSE

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ABBREVIATIONS

ASFV – African swine fever virus

BSA – Bovine serum albumine

CD – Cluster of differentiation

cDNA – Complementary DNA

cPPT - central polypurine tract

DNA – Deoxirribonucleic acid

dsRNA - Double-stranded RNA

EIAV - Equine infectious anaemia virus

FCS – Fetal calf serum

FIV - Feline immunodeficiency virus

HEK - Human embryonic kidney

HIV - Human immunodeficiency virus

IFN – Interferon

Ig - Immunoglobulin

IKKepsilon - Iκ B kinase epsilon

IL - Interleukin

IRAK1 - Interleukin-1R-associated kinase 1

IRAK4 - Interleukin-1R-associated kinase 4

IRES - internal ribosome entry site

IRF3 - Interferon-regulatory factor 3

LPS - Lipopolysaccharide

LRRs - Leucine-rich repeats

LTR - Long terminal repeat

mAB – Monoclonal antibody

miRNA – MicroRNA

MOI - Multiplicity of infection

mRNA – Messenger ribonucleic acid

NBT – Nitroblue tetrazolium

NF-κB - Nuclear factor-κB

NGS – Normal goat serum

NO – Nitric oxide

O_2^- - Superoxide

O.D. – Optical density

O/N – Overnight

ORF – Open reading frame

PAMPs - Pathogen-associated molecular patterns

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PFA – Paraformaldehyde

PMA - Phorbol myristate

Poly(I:C) – Polyinosinic-polycytidylic acid

PRRs - Pattern recognition receptors

RISC - RNA-induced silencing complex

RNA – Ribonucleic acid

RNAi - RNA interference

ROS - Reactive oxygen species

RNS – reactive nitrogen species

RRE - Rev-responsive element

RT – Room Temperature

RT-PCR – Reverse-transcriptase polymerase chain reaction

SCR – Scramble

SFFV – Spleen focus forming virus

siRNA - Short interfering RNA

TBK - TRAF family member-associated NF-κB activator binding kinase

TLR – Toll-like receptor

TNF – Tumor necrosis factor

TRAF6 - TNFR-associated factor 6

TRIF - Toll/IL-1R domain-containing adaptor-inducing IFN-beta

WPRE – Woodchuck hepatitis virus post-transcriptional regulation element

ABSTRACT

Viruses are obligate intracellular parasites and, as a consequence of many years of co-evolution with their hosts, have evolved genes/strategies to manipulate and/or evade host cell biology and immune responses. Many of these viral evasion genes code for proteins that are non-essential for virus replication *in vitro*. They may, however, be considered as valuable "ready made tools" to extend, explore and manipulate the regulation of the basic cellular processes that they manipulate.

African swine fever virus (ASFV) is a devastative acute pathogen of domestic pigs, principally in Africa where due to progressive urbanization and informal pig rearing, endemicity is growing.

In its wild life hosts, both vertebrate (warthog and bush pig) and invertebrate (soft tick), however, the virus has evolved many genes to escape the full ferocity of the host immune response and is non-pathogenic. This project has focused on one such strategy, specifically, the ASFV gene I329L, which has been demonstrated to inhibit TLR signaling. Two important future possibilities have been defined by this work:

1) The demonstration that I329L is a non-essential virus gene will permit construction of an I329L deletion-mutant virus and work towards this has commenced with the successful subcloning of the flanking regions of I329L into the necessary transfer vector. The I329L deletion virus will further our understanding of the role of I329L in the pathogenesis of the ASFV infection and may justify the testing of an I329L deletion mutant as a vaccine.

2) The demonstration that I329L functions in mouse macrophages and the subsequent construction of a macrophage restricted I329L transgenic mouse will provide an *in vivo* system to determine the role of I329L in healthy and infected macrophages.

Key words: African Swine Fever virus, I329L, macrophages, TLR, immune evasion.

SUMÁRIO

Os Vírus são parasitas intracelulares que como consequência de evoluírem em conjunto com os seus hospedeiros, desenvolveram estratégias/genes de manipulação e/ou evasão das células e resposta imunitária do hospedeiro. Muitos destes genes de evasão codificam proteínas que são não essenciais para a replicação do vírus *in vitro*. Podem, no entanto ser consideradas ferramentas úteis no estudo e manipulação da regulação dos processos celulares que manipulam. O vírus da Peste Suína Africana (PSA) é um patogénio que causa doença aguda em porcos domésticos, principalmente em África, onde o seu estado endémico é cada vez maior devido principalmente a uma urbanização progressiva e também à criação doméstica de porcos. No entanto, nos seus hospedeiros selvagens tanto vertebrados como invertebrados, o vírus desenvolveu estratégias e genes que lhe permitem evadir fortes respostas imunitárias, sendo não-patogénico. Este projecto foca-se no estudo de um gene do ASFV, o I329L, no qual já foram previamente feitos estudos no nosso laboratório que demonstraram que inibe a via de sinalização TLR. Foram definidas duas abordagens para o estudo deste gene neste projecto: 1) A demonstração de que o I329L é um gene viral não essencial que permite a construção de um vírus de delecção mutante, tendo o trabalho neste sentido começado pela clonagem bem sucedida das regiões que flanqueiam o gene no vector de transferência apropriado. O vírus mutante para o I329L permitirá a melhor compreensão das funções deste gene na patogenicidade da infecção e poderá justificar a construção de uma vacina mutante por delecção. 2) A demonstração de que o I329L mantém a sua função em macrófagos de ratinho e a subsequente construção de um ratinho transgénico com expressão de I329L restrita a macrófagos, proporcionam um sistema *in vivo* que permitirá avaliar o papel do I329L em macrófagos infectados e não infectados.

Palavras chave: Peste Suína Africana, I329L, macrófagos, TLR, evasão imune.

1) GENERAL INTRODUCTION

1.1) INTRODUCTION

Viruses are among the most common and numerous microorganisms on Earth, having the ability to infect all types of cellular organisms. They are obligate intracellular parasites that can replicate independently of cell's chromosomes but not of the cells themselves, which means they need a host. Their replication may be destructive to the host cell, an aspect that accounts for the fact that some viruses are agents of disease. Viruses co-evolve with their hosts which allows them to develop an intimate relationship with intracellular processes and, in turn, provide them with successful replication, assembly and transmission to new susceptible hosts. Viruses can also provide important new properties to host cells that are inherited when the host cells divide. These changes may be deleterious to the hosts, for example cervical cancer caused by the human papillomavirus, HPV. [60].

1.1.1) Virus inhibition of the Innate immune response

The immediate, or innate, immune response is multicomponent and serves to isolate and contain an initial virus infection.

1.1.1.1) Virus inhibition of the complement system

Viruses have evolved strategies for protection against complement activation and these can be classified in three general categories: 1) virus proteins which are homologous to mammalian complement regulatory proteins, *2*) virus proteins which have no sequence homology, but

share functional characteristics with complement regulatory proteins and 3) viruses that incorporate host complement regulatory proteins into their envelope during virus maturation.

Some examples come from inflammation modulatory protein (IMP), a complement inhibitor of cowpox virus (CPV) that inhibits the production of macrophage chemoattractant factors C3a and C5a [53]. In doing so, no tissue damage occurs at the site of infection.

Other viruses, like herpes simplex virus (HSV) -1 and -2 and mouse cytomegalovirus (MCMV) encode Fc receptors; antibodies that bind either the virus particle or the infected cell might do it at the Fc region and will not activate phagocytes and complement [27].

1.1.1.2) Virus inhibition of interferon responses

The interferon response to virus infection is complex and multicomponent and begins almost as a virus enters a cell, where its induction activates the infected cell to an "anti-virus" state. Subsequent induction of secretion of interferons (IFNs) then alerts surrounding cells to the virus infection and these then, in turn, become activated to an anti-virus state. In addition, IFNs also play a part in the homeotasis of immune responses. The importance of the IFN response in resistance to viruses is emphasised by 1) The large number of virus genes that have been evolved for subversion of both the type I (IFNα and IFNβ) and the type II (IFNγ) IFNs and 2) The fact that mice with IFN responses deleted by knock-out of IFN receptors succumb to normally non-fatal virus infections [17, 80, 51].

1.1.1.3) Virus inhibition of apoptosis

Apoptosis is a process that is a prominent feature of all multicellular organisms and of some unicellular organisms such as yeasts, and as been highly conserved throughout evolution. Apoptosis can be triggered by a variety of inducers, including irradiation, cell-cycle

inhibitors, infectious agents like viruses and ligands of the TNF family. It is important in development and normal cell turnover processes, and also plays a key role in the innate response to and control of viral infections. Although one can consider apoptosis as an innate cellular response to limit viral propagation, later in the replicative cycle of the virus, apoptosis might facilitate virus dissemination. and that is why viral apoptosis regulators fall into two types: 1) Inhibitor proteins that function by blocking or delaying the onset of apoptosis to allow the viral replication cycle to be completed, and 2) Inducer proteins that stimulate apoptosis either by inappropriately interfering with cell cycle progression or by assisting virus release and dissemination.

The Bcl-2 proteins regulate ion permeability and membrane potential of mitochondria and inhibit the release of cytochrome c, which powerfully activates effector caspases, a family of cytosolic aspartate-specific cysteine proteases (CASPASES), which are crucial participants in the apoptotic cascade. Numerous viruses modulate the activity of bcl-2 proteins in the cell, either encoding viral homologues or changing protein levels. For example, Epstein-Barr virus (EBV), Human herpes virus-8 (HHV-8), African swine fever virus (ASFV), HSV, Murine herpes virus-68 (MHV-68), alcelaphine herpesvirus-1, adenovirus, and Bovine herpesvirus type 4 (BHV-4) code bcl-2 family homologues.

Some viruses have developed strategies to counteract hyperreactive oxides before they can exert their toxic effects on viral molecules or trigger a deleterious apoptotic response. These strategies protect viruses against attack by oxidative stress [18].

1.1.1.4) Virus inhibition of Natural Killer cells

NK cells are important effector components of the innate immune system and participate in the initial defence against viral infection, both by cellular cytotoxicity and by the production

of inflammatory cytokines. These cells are not MHC restricted and do not express T cell receptors. They are usually prevented from killing their targets by inhibitory signals provided through interaction of receptors on NK cells with self-MHC molecules on the surface of potential target cells [42]. The missing-self hypothesis proposes that NK cells recognize the absence of cell surface-expressed self-MHC class I product as a signal for attack and destruction of a target cell [59].

Some viruses might escape detection by NK cells through the production of surface-expressed MHC class I homologs that do not bind peptides, as indeed happens in both mouse and human CMV.

1.1.1.5) Virus inhibition of cytokines and chemokines

Cytokines are secreted polypeptides that coordinate inflammation, cellular activation, proliferation, differentiation and chemotaxis, and orchestrate the induction and maintenance of innate and adaptive antiviral responses. Therefore, targeting their function is of immediate value and contributes to virulence of viral pathogens. Several cytokines are of particular importance and are frequently targeted by viruses; these include IL-1, IL-12, TNF, IFN-α and -β, IFN-γ, and a number of chemokines.

Chemokines (CKs) are small disulfide-linked cytokines that are chemoattractants to leukocytes. They are divided in four different subfamilies according to conserved structural features, and their effects on leukocytes are mediated by changes in intracellular calcium and activation of second messenger systems, following binding of chemokines to G protein coupled receptors (GCRs) with seven-transmembrane regions [45].

One interesting mechanism some viruses have developed, namely large DNA viruses, is the mimicry of cytokines and their receptors. Herpesviruses secrete homologues of IL-16 and Il-

17, which could increase proliferation of the cells that are the targets of viral replication [4]. Poxviruses secrete cytokine receptors or cytokine binding proteins and these proteins were originally identified as homologues of TNFR, IL-1R and IFN-γR. Since then, secreted proteins that bind IFN-α and -β, CKs and IL-2 without any sequence homology have been described [4, 82].

1.1.2) Virus inhibition of Acquired humoral and cellular immunity

Antiviral immunity results from a combination of both cell mediated and antibody and complement immune responses.

The humoral immune, or antibody (Ab), responses will impede viruses from establishing an infection. Typically, protective immunoglobulins (Igs) will be secreted, bind to the virus surface and block interaction with cellular receptors. These Ab-virus complexes can then be recognized and eliminated by cells of the phagocytic lineage, which express Fc receptors. Also, Fc receptors will instruct NK cells to lyse Ab coated infected cells (Ab-dependent cellular cytotoxicity).

1.1.2.1) Virus inhibition of antibody responses

Receptors for the constant part of the immunoglobulin G (IgG), known as Fc receptors (FcγRs) are present on many mammalian hamatopoietic cells, such as monocytes, macrophages, neutrophils, eosinophils, platelets, B cells and certain classes of T cells [6]. FcγRs constitute an important bridge between the humoral and cellular immune systems because interaction of the IgG Fc domain with cell surface FcγRs results in the triggering of many effector functions, such as phagocytosis, cell proliferation, cell activation or inhibition, Ab

dependent cellular cytotoxicity (ADCC) and release of cytokines and inflammatory mediators. This is why some viruses express IgG Fc receptor homologues to bypass the effector consequences of Ab binding.

1.1.2.2) Virus inhibition of MHC class I presentation

Once inside a cell, viral mRNAs are translated using the cellular translation machinery. Like other proteins, cytosolic viral gene products are degraded by cellular proteases, as part of normal protein turnover among them, a multi-subunit catalytic protease complex known as the proteosome [26]. Once degraded, the viral antigenic peptides are transferred to the lumen of the endoplasmic reticulum (ER) by the transporter associated with antigen presentation (TAP) and "loaded" into the peptide binding groove of the MHC I complex, a molecule composed of a MHC I heavy chain and a β_2m microglobulin light chain [40]. Molecules of MHC I loaded with viral peptide are transported from the ER, through the Golgi apparatus to the plasma membrane, where they become available for surveillance by antigen-specific receptor $CD8^+$ T cells, or CTLs, [90, 47]. Upon recognition of the MHC I-peptide complex on the plasma membrane of the virally infected cell or of a professional APC, the interaction between the T cell receptor complex and the class I molecule-peptide complex, activates the T cell to a host antiviral immunologic response.

Once the CTL precursors are activated, they proliferate and differentiate into effector cells. Viruses that attenuate or block viral peptide presentation on the surface of the infected cell could escape detection by CTLs. Several viruses use this strategy, both transcriptionally and post-transcriptionally, and viruses have evolved multiple mechanisms to interfere with antigen presentation, emphasising the importance of CTL responses in controlling virus infections.

1.1.2.3) Viral inhibition of MHC class II presentation

When pathogens enter the endocytic pathway by phagocytosis or receptor-mediated endocytosis, many of their proteins are degraded into antigenic peptides, mostly by endosomal proteases. These antigenic peptides are presented and integrated into MHC class II molecules, typically expressed by professional APCs, the macrophages, dendritic cells (DCs), and also B cells. Once transported to the cell membrane, they are presented to $CD4^+$ T cells, which then become activated [104, 74]). Expression of MHC class II molecules can also be induced in a variety of other cell types. The activated $CD4^+$ T cells will then stimulate the development of CTLs and also help mount a serological antiviral response against the invader [46].

1.1.3) The African Swine Fever Virus

1.1.3.1) Pathogenesis and epidemiology

The African Swine Fever Virus (ASFV) was first observed in domestic pigs (*Sus scrofa*), in Kenya [67, 68] causing spleen enlargement and lethal haemorrhagic disease accompanied by extensive lymphoid tissue destruction due to apoptosis [33, 50, 72, 73, 85]. The mortality rates due to the disease, were at this point of 100% [67, 68]. Other immunological changes include hypergammaglobulinaemia, as well as reduced responses to lectins by T cells from infected animals [81, 63].

The virus persists in sub-Saharan Africa in its natural hosts, the bush pig, *Potamochoerus porcus*, and the warthog, *Phacochoerus aethiopicus*, but with total absence of clinical symptoms [29, 52]. It also infects an invertebrate, the soft tick, *Ornithodoros moubata* and

Ornithodoros erraticus, that inhabits warthog burrows (Figure 1). The soft tick parasites the warthog, thus creating a cycle of infection that maintains the virus in Africa. The virus is well adapted to these hosts producing infections that can persist for months or years. The soft tick can transmit the virus both to wildlife host and domestic pigs; porcine hosts can pass the virus between themselves through direct contact [52, 102, 105]. The bush pig and warthog are thought to be the natural reservoir of ASFV since they show no clinical signs of disease once infected with virulent and haemorrhagic isolates of the virus, that kill domestic pigs within 5-7 days post infection [52, 72, 73].

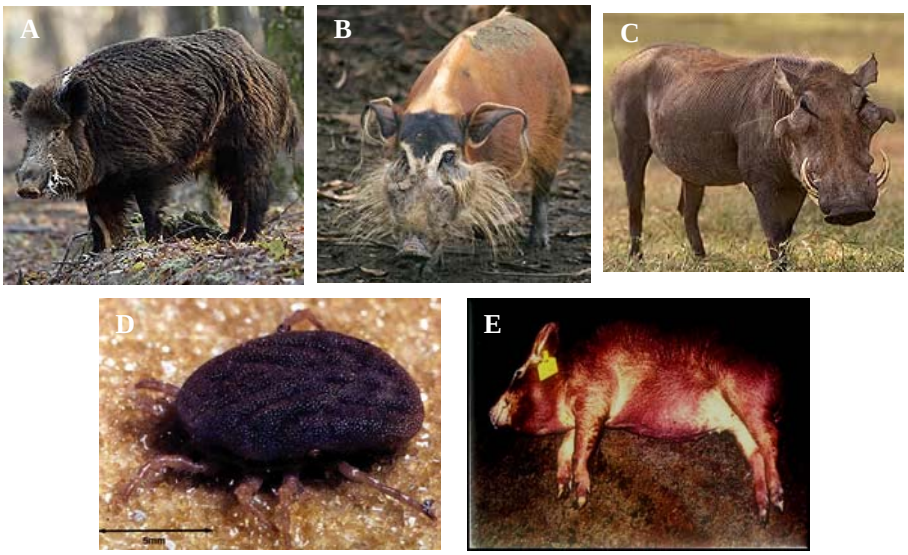

Figure 1 – Swine hosts of ASFV and vectors of infection.

(A) domestic pig, *Sus scrofa*, **(B)** bush pig, *Potamochoerus porcus,* **(C)** warthog, *Phacochoerus aethiopicus*, **(D)** the soft ticks *Ornithodoros moubata* and *Ornithodoros erraticus* **(E)** domestic pig killed by ASFV with the characteristic haemorrhagic syndrome [108, 109].

After the original report of ASFV in Kenya in the 1920s, the virus was then detected in most sub-Saharan countries, where it is still endemic. It started spreading and it escaped from

Africa evolving towards less virulent strains that cause very attenuated and chronic forms of the disease [105]. Once it reached Europe through infected pork products, it was detected in Portugal and Spain in 1957, then again in 1960 where it remained endemic until the 1990s. Other European countries have been affected as well the Caribbean (Cuba, Dominican Republic and Haiti), and Brazil [102]. The disease has been eradicated from most of these countries outside Africa, with the exception of Sardinia where it still remains since it appeared in 1982. More recently, ASF has led to major economic losses after spreading through many West African countries, as is the case of Madagascar where it first appeared in 1998. Less virulent isolates have also been described in domestic pigs, as they cause a reduced mortality and, in some cases, chronic infections. Pigs that recover from this condition may remain persistently infected for long periods, thus providing a reservoir for infecting healthy pigs [55, 96, 101, 102].

The virus infects cells of the monocytic lineage, mainly the central cell of the innate immune response, the macrophage, both *in vivo* and *in vitro*. One report [94] described infection of endothelial cells *in vitro*. Depending on the viral strain, the virus codes between 100-150 polypetides. About 40 of these have been described as being incorporated into the viral particle. A further 16 have been identified as enzymes and 30-40 as possible host modification genes [35, 52]. In order to infect and replicate in macrophages, *in vivo*, the virus has evolved strategies to modulate and/or interfere with the host immune response to infection.

During disease, circulating lymphocytes and neutrophils show reduced numbers in acute infections [24, 98, 103]. This is typically accompanied by hypergammaglobulinaemia. Although as initially suggested by some studies [97, 98], there are no classical neutralizing antibodies produced during viral infection, antibodies may play a role in the protective immune response

against ASFV [16, 107, 71, 37]). For instance, serum from animals infected with a low virulence viral isolate provided protection from infection with virulent ASFV isolates and against low passage tissue culture adapted viral variants [107]. Also, the same protective or neutralizing activity for several virulent isolates was observed when testing a monoclonal antibody (mAb) that reacts against a 72 kDa ASFV protein [16]. However, neither the immune pig sera, nor the anti-72 kDa ASFV protein mAb neutralized high passage tissue culture adapted ASFV isolates. It has been suggested that the adaptation of ASFV isolates to tissue culture conditions may be associated with the loss of determinants important for virus neutralization [107].

Another important observation in reinforcing the existence of protective serological immunity was that after passive transfer of anti-ASFV serum to pigs, these animals were partially protected against a highly pathogenic virus isolate. Not only there was a delay in the appearance of viraemia and a reduction in the virus titers, but some of the animals presented no clinical symptoms after secondary challenge with the pathogenic isolate. In contrast, in the control group, symptoms and death occurred 4 days after the challenge [71].

In spite of evident need, no vaccine is yet available, and disease control relies on rapid diagnosis, implementation of sanitary measures and movement restrictions. Diagnosis of disease poses a major complication due to the varying pathogenesis caused by different isolates, the similarity to other viral haemorrhagic fevers and the need to transport highly infectious samples to labs for testing. This policy is difficult to implement in many African countries since it requires good infrastructures that are lacking in many of these countries. ASF continues to pose a major threat to pig farming worldwide. An effective vaccine would help to control a major pathogen of pigs in large endemic areas in Africa, provide an

alternative to mass slaughter of animals and protect non-endemic from accidental entry of the virus.

1.1.3.2) Virus structure, entry, replication cycle and assembly

ASFV is a large icosahedral virus and the only known member of the *Asfarviridae* family, genus *Asfivirus* [28, 29]. It has a linear double-stranded DNA genome with structural features similar to iridoviruses and genomic organization similar to poxviruses [29, 52]. The virus replicates and assembles in the cytoplasm and encodes enzymes and factors that are required for replication and transcription of the virus genome. Depending on the virus isolate, the genome varies between 170 and 190 kb [15], and sequencing of the complete genome of the Spanish tissue culture adapted isolate, Ba71v [105], of ASFV suggests that this virus may encode 151 major open reading frames (ORFs) [52].

Extracellular ASFV particles have a diameter of approximately 200 nm and are formed by a DNA-containing nucleoid surrounded by three layers: an internal membrane, an icosahedric capsid, and an external membrane (Figure 2) [19]. The virus enters cells through receptor mediated endocytosis and is released from endocytic vesicles into the cytoplasm. This occurs through a mechanism of fusion of the virus envelope with the endocytic vesicles [2, 14, 93]. Studies involving the use of antibodies against virus proteins such as p12, p54, p72 and p30 have shown that these have important roles in the process of binding of the virus (p12, p72, p54) or internalisation (p30) [16, 35, 36, 37, 38].

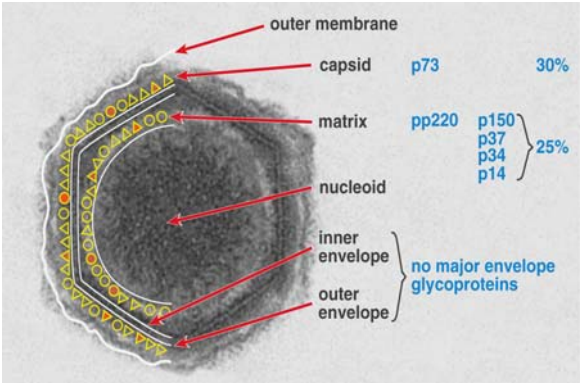

Figure 2 – African Swine Fever virus structure. ASFV particles have a complex multi-layered structure. The nucleoprotein core structure is 70 to 100 nm in diameter. The nucleoprotein core is surrounded by a core shell and an internal envelope onto which the icosahedral capsid is assembled. The capsid is 170 to 190 nm in diameter. Although earlier reports suggested that this internal membrane consisted of a collapsed double membrane layer it has also been suggested that only one membrane layer is present. (Photograph from Linda Dixon, Institute for Animal Health, Pirbright Laboratory, UK)

Early virus mRNA transcription begins in the cytoplasm and is followed by virus replication through the use of host enzymes that are packaged in virions. These early transcripts encode proteins that include important enzymes such as DNA polymerase required for virus genome replication that occurs in the cytoplasm as early as 6 hours post infection. Late transcripts, on the other hand, largely encode structural proteins.

1.1.3.3) TLR signalling pathway

In vertebrates, the innate immune system is essential for host defence, as it constitutes the first line of defence against invading and potentially pathogenic microorganisms.

The host cells express various pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs). For example, many pathogens contain carbohydrates and peptide derivatives, as well as methylated DNA, which the host can recognize as "non-self". Once there is recognition of PAMPs by PRRs, an activation of intracellular pathways occurs, that leads to the induction of inflammatory cytokines, chemokynes, interferons (IFNs) and also upregulation of co-stimulatory molecules [49].

Innate immune receptors may be transmembrane signalling receptors such as Toll-like receptors (TLRs), or soluble components that opsonize microorganisms labelling them for recognition by complement receptors, Fc receptors or integrins. These receptors may trigger a variety of responses depending on the receptor and cell type: 1) through the mediation of microbe internalisation by phagocytic cells; 2) activation of antimicrobial mechanisms such as production of reactive nitrogen and oxygen molecules or species; and 3) stimulation of the production of inflammatory cytokines and chemokines that activate other immune cells [92].

In mammals, TLRs serve as key PRRs and play a crucial role in the induction of innate immune responses and consequent development of adaptative immune responses [49].

The Toll receptor was originally identified in *Drosophila* as an essential receptor for the establishment of the dorso-ventral pattern in developing embryos. In fact, Hoffmann and colleagues demonstrated, in 1996, that Toll-mutant flies were highly susceptible to fungal infection [56].

TLRs are abundantly expressed on antigen presenting cells such as dendritic cells and macrophages [49]. The cytoplasmic portion of TLRs shows high similarity to that of the interleukin (IL)-1 receptor family, and is now called the Toll/IL-1 receptor (TIR) domain.

Despite this similarity, the extracellular portions of both types of receptors are structurally unrelated. The IL-1 receptors possess an Ig-like domain, whereas TLRs bear leucine-rich repeats (LRRs) in the extracellular domain [87].

TLRs signal through two major pathways: the MyD88-dependent pathway and the TRIF (Toll/IL-1R domain-containing adaptor-inducing IFN-beta)-dependent pathway. The MyD88-dependent pathway triggers the release of pro-inflammatory cytokines, such as TNF-alpha, IL-6, and IL-12p40. MyD88 recruits various molecules that include IRAK4 (IL-1R-associated kinase 4), IRAK1 and TRAF6 (TNFR-associated factor 6) which in turn activate the nuclear factor-κB (NF-κB) that induces pro-inflammatory cytokine genes. The TRIF-dependent pathway is unique to TLR3 and TLR4 and contributes to pro-inflammatory cytokine responses as well as to the induction of type I IFN responses, particularly IFN-beta. To achieve this, TRIF recruits TRAF3, TBK (TRAF family member-associated NF-κB activator binding kinase) and IKKepsilon (Iκ B kinase epsilon), that activate IRF3 (IFN-regulatory factor 3) (Figure 3) [9, 25, 86].

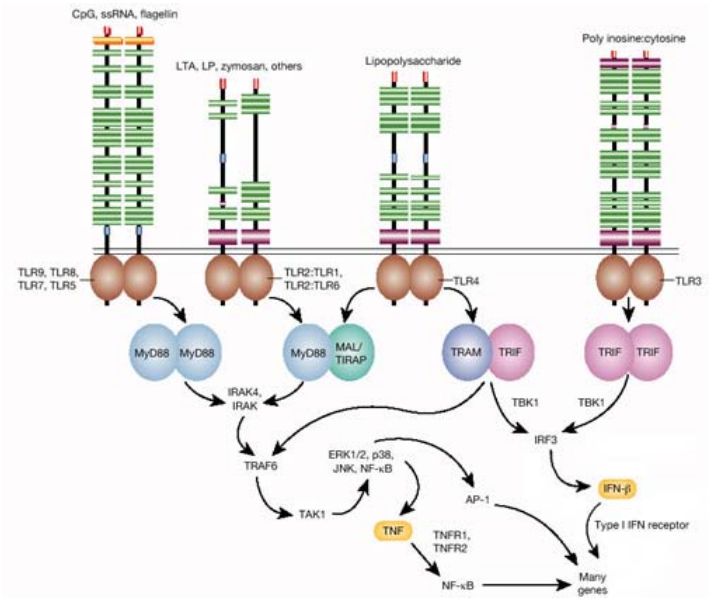

Figure 3 – TLR signalling pathway [110]

The Toll family of proteins seems to be conserved, since homologous proteins have been described not only in *Drosophila* but also in other organisms, including plants. Thirteen TLRs (TLR1 to TLR13) have been identified in humans and mice together, and equivalent forms of many of these have been found in other mammalian species. However, equivalents of certain TLR found in humans are not present in other mammals [31, 21, 86].

Different TLRs exhibit specific responses. In fact, they have their own signalling molecules to manifest these specific responses and can be classified according to the types of PAMPs they are able to recognize (Figure 4) [87]. For instance, TLR2, 4 and 6 recognize lipids. In addition, TLR4 together with its extracellular components, MD-2 and CD14, can also recognize lipopolysaccharide (LPS) from Gram-negative bacteria. TLR2, in turn, forms heterodimers with TLR1, TLR6 and CD36 to discriminate molecules such as peptidoglycan, lipopeptides and lipoproteins of Gram-positive bacteria, mycoplasma lipopeptides and fungal zymosan. TLR5 recognizes protein ligands such as bacterial flagellin. Human TLR10 is thought to heterodimerize with TLR2 and TLR1, but the target ligand remains unknown [5, 49, 95].

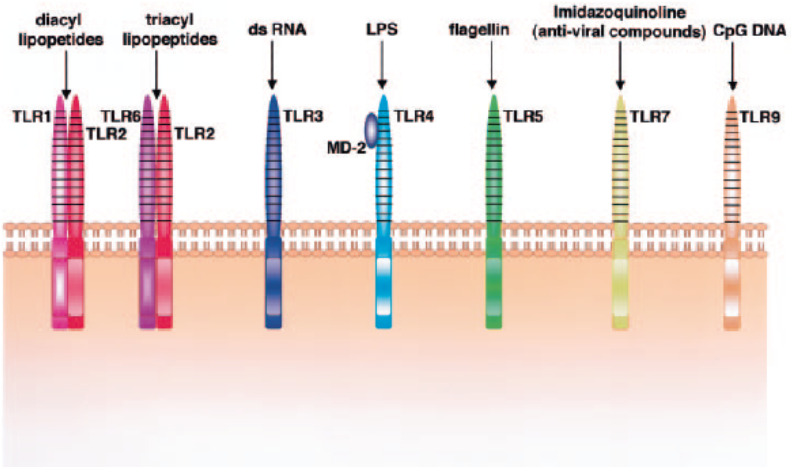

Figure 4 – Ligands recognized by the TLR family [1]

One of the best ways to understand the central role for TLR in inflammatory responses is through mice lacking specific TLR or signalling molecules. For instance, mice lacking TLR4 are non responsive to LPS and, consequently, more susceptible to infection by Gram-negative bacteria such as *Salmonella typhimurium*, and mice deficient in TLR2 show a profoundly diminished response to Gram-positive organisms such as *Staphylococcus aureus*. In spite of these results, it is important to note that not all TLR deletions lead to severe infection phenotypes. This suggests that there may be a redundancy in recognition since probably all microorganisms are most likely to be recognized by multiple TLRs and not just one. In addition to being recognized by TLRs, microbes are also recognized by other innate recognition systems, as a way to prevent pathogens from easily subverting recognition and in consequence generating a controlled inflammatory response [92].

1.1.3.4) The I329L gene of the ASFV

ASFV is adapted to survive in vertebrate and invertebrate host cells. Both types of cells have defence mechanisms that are regulated by TLRs. In the porcine host, the virus mainly infects the macrophage, a key cell in innate immunity, as described earlier. For these reasons, the ASFV genome has been analysed using bioinformatic tools, and through this analysis the I329L gene was identified.

ORF I329L is a putative transmembrane protein of the ASFV of unknown function, with an extracellular domain of 222 amminoacids, a central transmembrane region formed by 21 residues, and 40% homology with the highly conserved 69 amminoacid intracellular C-terminal domain of mammalian Toll like receptors (TLRs). The protein coded by this gene shows a variety of potential glycosylation sites (Glc NAc) in the following amminoacids: 32, 39, 44, 76, 82, 101, 105, 219. These features suggest that I329L may be a candidate viral TLR

homologue. In fact, searches for similarities with other proteins in databases, in particular after alignment with TLR proteins, have shown a significant similarity between I329L and TLR3 from *Drosophila melanogaster*, specifically in a repetition site rich in leucine residues.

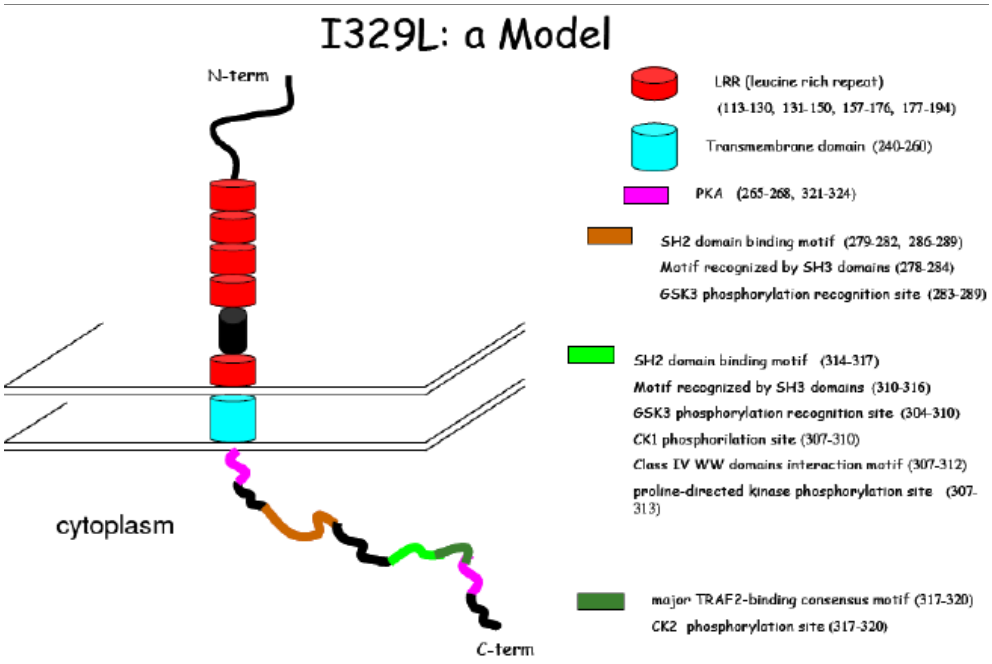

Figure 5 – Hypothetical model for I329L based of bioinformatic tools. TLRs are extremely rich in leucine residues as well as the conserved region of this protein. The extracellular portion of the molecule is formed by LRR repeats, an important motif for the interaction of these protein wit other molecules. Even though it is not clear whether I329L has a TIR domain, it resembles TRIFs in its cytoplasmatic domain. This suggests that this gene may modulate signalling through its interaction with TRIF. (Unpublished model developed by Vívian Oliveiraat the IGC)

The demonstration of interference of this gene with the activation of NF-κB, through a TLR-NF-κB reporter gene assay, has been performed in our lab by Vívian Oliveira (unpublished

data) and constitutes the stimulus for my work. The reporter gene assay consists on transfecting HEK-293T cells with three plasmids: one, containing a NF-κB-driven luciferase reporter, a second containing a CD4 extra-cellular domain co-ligated upstream of a set of TLR intracellular domains comprising TLR-1-6. As CD4 domains spontaneously dimerize when incorporated into the 293T cell membrane, the TLR intracellular domains are automatically brought into apposition. Therefore, there is constitutive signalling from the dimerized TLRs, and the resulting activation of NF-κB is detected by synthesis of luciferase. The effect of the co-transfection of the candidate gene cloned into the third plasmid provides an assay for interference of the viral protein with the TLR signalling pathway. Empty pcDNA3 serves as the negative control. It was observed that I329L inhibits NF-κB activation stimulated by multiple TLRs. This is of great importance since the NF-κB family of eukaryotic transcription factors is expressed in virtually all cell types and is involved in the regulation of expression of various genes important in immune response, inflammation and apoptosis, namely cytokines and its receptors, chemokines, growth factors and cell adhesion molecules [10, 11]. Later unpublished experiments by Vívian Oliveira showed that stimulating cells with the TLR3 ligand, polyinosinic-polycytidylic acid (poly(I:C)), a double stranded RNA analog, confirmed that I329L inhibited the TLR signaling transduction pathway.

These results strongly suggest that I329L is a host modification gene and thus a suitable candidate for the construction of a mutant deletion virus vaccine. Work towards such a vaccine and a deeper understanding of the role of I329L in host-pathogen interaction constitutes the objective of this project.

1.2) OBJECTIVE

This project is focused on exploring the function and utility of a host modification gene from the African Swine Fever Virus (I329L gene) manipulating toll receptor like (TLR) signalling. The goals of this project were to (1) determine whether I329L is an essential gene, (2) gain insights into function of the gene in the pig macrophage cell, and (3) assess of the impact of I329L *in vivo* by constructing a transgenic mouse selectively expressing I329L in macrophages. Such a transgenic mouse would be a model to understand how interference with TLR signalling might manipulate macrophage function in health and disease.

2) I329L IS A NON ESSENTIAL GENE OF THE ASFV

2.1) INTRODUCTION

One of the possible approaches to produce an attenuated ASFV is through the deletion of genes known to be involved in evasion of the host immune system. As described earlier in this thesis, the gene I329L is a candidate for the construction of a mutant deletion virus vaccine. In order to test this possibility, it is first necessary to demonstrate that this gene is non-essential for the virus growth and replication *in vitro*. If the gene was essential to the virus, then its deletion would not yield a viable virus.

There are two possible approaches to test if this gene is essential to ASFV: 1) the knockdown of I329L through siRNA technology and 2) the construction of an I329L mutant deletion virus.

2.1.1) Inhibition of expression of I329L through siRNA technology

RNA interference (RNAi) is the process of mRNA degradation that is induced by double-stranded RNA (dsRNA) in a sequence specific manner. This process has been observed in all eukaryotes, from yeast to mammals. The RNAi pathway is thought to be an ancient mechanism for protecting the host and its genome against viruses that use dsRNA in their life cycles. This process plays a role not only in RNA and dsRNA stability/degradation but also in the regulation of translation, transcription, chromatin structure, and genome integrity. In the RNAi pathway, the dsRNA is processed to short interfering RNA (siRNA) which consists in 21-25 bp dsRNA with dinucleotide 3' overhangs. Specifically, the guide strand of the siRNA

is assembled into an RNA-induced silencing complex (RISC) that cleaves the target mRNA [79, 84].

Due to its efficiency in specifically silencing the expression of any gene for which a sequence is available, it has been widely used and has become the cornerstone of many research projects.

The production of infectious virus by appropriately siRNA transfected cells will prove that I329L is a non-essential gene and thus a candidate for generation of an attenuated I329L-deleted ASFV vaccine.

Conventional transfection was used for transferring siRNA to Vero cells for gene silencing. The conditions for I329L specific siRNA delivery were optimized to avoid cytotoxicity. Two RNA regions were selected and the corresponding siRNA oligonucleotides were purchased. At first, these were tested separately, but later on both were combined, as this was more effective than a single siRNA, to achieve knock down. 24 hours post-transfection of siRNA, Vero cells were then infected with Ba71v. To confirm gene silencing, RT-PCR to detect I329L mRNA was performed on transfected Vero cells collected 18 hours post-infection. As a control, amplification of another ASFV gene (A238L) was also performed on the same samples in order to confirm the specificity of the silencing. The A238L gene of the ASFV is an inhibitor of transcription that encodes a bifunctional protein that inhibits both NF-κB and NFAT activation [65, 66, 75, 76]. We chose this gene as a control, since it is expressed throughout the cycle of the virus, including the time point that we were studying. A non-specific siRNA sequence from Dharmacon served as the negative control. Non-targeting, negative controls provide a baseline for measuring the effects of experimental siRNAs. Negative siRNA controls lack sequence complementarity to gene products and should have no effect in mRNA stability, mRNA expression, or protein expression. These controls are important for

distinguishing sequence-specific silencing from non-specific effects in the RNAi experiment. There are a variety of negative controls available, but we used a "scramble" control. This strategy consists in using a siRNA that has the same nucleotide composition, but not the same sequence, as the test siRNA. This is achieved in two ways: randomizing (also known as scrambling) the nucleotides in the siRNA or reversing the sequence of the siRNA. When their sequences fail to target a known gene or have a miRNA seed region match with a known gene, these siRNAs are appropriate as negative control siRNAs.

2.1.2) Construction of the I329L mutant deletion virus

The genome of ASFV can be manipulated through a process of homologous recombination between the virus genome and plasmid vectors transfected into the infected cell. Even though this kind of approach has been limited by the lack of suitable transfer vectors, García et al. described the construction of plasmid vectors with applications permitting the generation of recombinant viruses containing multiple deletions as well as viruses expressing foreign genes. In this work, the plasmid p72GUS10T (5.1 kb) was selected. This contains an ASFV chimeric gene formed by the fusion of the coding sequence of the *gusA* gene to the virus promoter p72.4 which was cloned into the polylinker region of plasmid pUC118 [34].

2.2) METHODS AND MATERIALS

2.2.1) Cell lines

Vero cells (African green monkey epithelial cells) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Calf Serum, FCS, (heat inactivated), 2mM L-Glutamine, 100 Units/mL Penicillin, 100 μg/mL Streptomycin, all purchased from Gibco, Invitrogen Corporation.

Cells were routinely checked for mycoplasm contamination by performing a specific PCR.

2.2.2) siRNA Transfection and Ba71v infection

2.2.2.1) Treatment with siRNA oligos

siRNA targets were used to knock down expression of I329L in Vero cell lines. siRNA target sequences were purchased from Dharmacon. We chose two target sequences to be transfected into Vero cells: target 1, 5'-CAACCTACCTATATTACAA-3' and target 2, 5'-TTATGCTCCTGTATATCAT-3'. These primers were kept at a 75 μM stock concentration. We first transfected each target separately but afterwards we had to combine the two in transfection in order to see an effect.

$3x10^5$ cells/well were seeded on 6 well plates and left overnight. On the following day, cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions and left overnight. On day 3, 24 hours post-transfection, cells were infected with Ba71v using a multiplicity of infection (MOI) of 5. Ba71v is a tissue culture adapted Spanish isolate of the ASFV that encodes 151 major ORFs [105]. All procedures regarding the use of

Ba71v were carried out at the P3 laboratory facility of the Instituto Gulbenkian de Ciência (Oeiras, Portugal) according to the in-house security rules.

To perform the siRNA experiment we used the appropriate controls and also tested the minimum quantity of siRNA needed to observe gene knockdown. It is important to use as little quantity of siRNA possible as too much siRNA transfected into the cells may lead to off-target or cytotoxic effects. On the other hand, if too little siRNA is used, reduction of target-gene expression may be undetectable. The experimental scheme was according to figure 6.

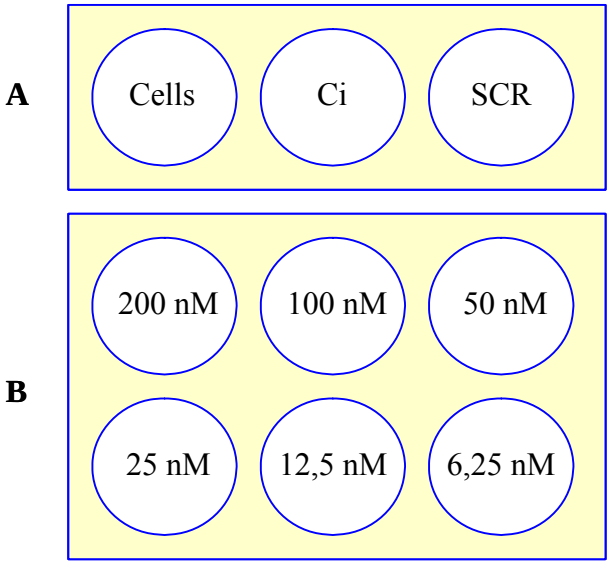

Figure 6 – siRNA experimental design scheme. A) We used three controls: Vero cells only, Ba71v infected cells (Ci) and negative control scramble transfected and Ba71v infected Vero cells (SCR). **B)** Vero cells were transfected with different quantities of siRNA (200nM-6.25nM) as well as infected with Ba71v (MOI 5).

2.2.2.2) Demonstration of siRNA inhibition of I329L expression by RT-PCR

Total RNA was extracted from siRNA transfected and Ba71v infected Vero cells, using Trizol Reagent (Sigma). Samples of RNA were digested with DNase I (Invitrogen) and cDNA synthesis was performed using MMLV-Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. I329L expression in the cDNA was detected by PCR, using Taq DNA Polymerase, on a PTC-100 Peltier-Effect Cycling apparatus. Primer sequences were I329Lup, 5'-GCTACTTCTTCTTGAACATGA-3' and I329Llow, 5'-GCTTAGGAAGTG GCTTAACAGG-3' and PCR conditions were dNTPs 200 µM, primers 1 µM, $MgCl_2$ 2 mM, 94ºC – 4', 40X (94ºC – 1', 50ºC – 1', 72ºC – 2'), 72ºC – 10'. As a quantitative and qualitative control, tubulin was amplified using Taq DNA Polymerase with dNTPs 200 µM, primers 1 µM, $MgCl_2$ 2 mM, 94ºC – 4', 40X (94ºC – 1', 55ºC – 1', 72ºC – 2'), 72ºC – 10'. Tubulin primer sequences were the following: TubulinUp, 5'-GGTGGATCTAGAACCTGGG-3' and TubulinLow, 5'-CCCAGTGAGTGGGTCAGC-3'. To control that only I329L expression was being targeted and not any other viral gene, the cDNA production for another ASFV gene, A238L, was also tested. A238L PCR was performed with Taq DNA Polymerase with dNTPs 200 µM, primers 1 µM, $MgCl_2$ 2 mM, 94ºC – 3', 40X (94ºC – 1', 54ºC – 1', 72ºC – 1'), 72ºC – 3' and the primer sequences were A238Lup 5'-CTAGAATTCATGGAACACATGTTTCC A-3' and A238Llow 5'-CTACTCGAGTTACTTTTCATACTTGTT-3'.

2.2.2.3) Detection of intracellular and extracellular virus

After knocking down the expression of I329L through siRNA we wanted to know if this gene was essential for the ASFV. With that purpose we 1) stained Ba71v infected Vero cells as well as siRNA transfected and Ba71v infected Vero cells, by immunofluorescence, using a

p73 specific monoclonal antibody; and 2) infected freshly plated Vero cells with the supernatants of each of the siRNA wells experiments, in order to count viral plaques.

For immunofluorescence, sterilized glass coverslips were put in the wells as the cells were being seeded for the siRNA assays. As some of the cells adhered to the coverslip surface, we used them for a staining with an antibody against p73, a major capsid protein of ASFV. Cells were fixed with 4% paraformaldehyde (PFA) in PBS for 20 minutes at room temperature (RT). Blocking was performed at RT for 30 minutes with PBS containing 0.05% Tween-20 and 5% Normal Goat Serum (NGS). The cells were permealized using PBS containing 0.1% Triton X-100. The staining with the primary monoclonal antibody 4H3 specific for p73 was performed at RT for 1 hour in blocking solution with a previously titrated concentration of the antibody. The secondary staining was also performed at RT for 1 hour with a goat anti-mouse IgG Alexa 568 antibody, purchased from Molecular Probes. To stain the nucleus of the cells, they were incubated in a DAPI solution (20 ng/µl) for 2 minutes. All washes between stainings were performed at RT in PBS containing 0.05% Tween-20.

Viruses were recovered from the supernatants of siRNA transfected and infected cells. These, were then titrated with the purpose of counting viral plaques, to determine if infectious progeny was being produced after knocking-down I329L.

In order to titrate the supernatants we used freshly plated Vero cells and infected them with a series of dilutions of the supernatants from pure supernatant to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilutions. DMEM medium with 0.5% agarose was added to the infected cells and left for five days. Afterwards, the cells were washed with PBS 1X and fixed with 4% PFA for 20 minutes

at RT. The cells were then stained with 0.1% toluidine blue for 30 minutes at RT and washed with tap water. After drying, the viral plaques were counted.

2.2.3) Cloning of the mutant deletion virus construct

In order to delete I329L from Ba71v the left and right flanking regions (~700 bp each) of the ASFV gene were cloned on either side of the p72.4 Gus 10T cassette. For instance, the amplification of the left flank of the gene was performed by inserting an EcoRI site at the 5' end and a KpnI or SmaI site at the 3'end (in this case we used a KpnI site). These sites were cloned upstream of p72.4 Gus 10T. The right flank of the gene was also amplified by inserting a PstI site at the 5'end and a HindIII site at the 3'end, in order to clone it downstream of the p72.4 Gus 10T cassette.

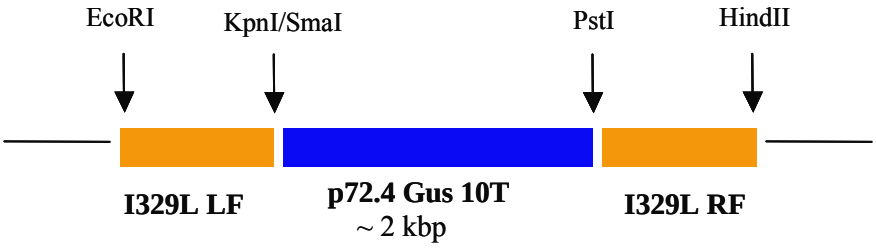

Figure 7 - Cloning strategy of I329L left (LF) and right flanks (RF) into p72.4 Gus 10T plasmid.

The right and left flanks (RF and LF, respectively) were amplified by PCR using Pfu DNA Polymerase, on a PTC-100 Peltier-Effect Cycling apparatus. Primer sequences were I329LRFup, 5'- GCCTGCAGCCTGTATATACTATTAAAAATT -3', I329LRFlow, 5'- GCAAGCTTACCCTGATGGAAGACTATGTAT-3', I329LLFup, 5'- GCGAATTCCATAA CTAGATCGGTGGTAAT-3', and I329LLFlow, 5'- GCGGTACCATGTGTGGTTTATTTT

AGTATG -3'. PCR conditions were dNTPs 200 µM, primers 1 µM, $MgSO_4$ 2 mM, 94ºC – 4', 30X (94ºC – 1', 55ºC – 1', 72ºC – 2'), 72ºC – 10'. These fragments were restriction digested and cloned into p72.4 Gus 10T according to the scheme in figure 7.

After infection of Vero cells with Ba71v, followed by transfection of the I329LLF-Gus-I329LRF, progeny virus will be harvested, and the recombinants will be identified and purified by isolating viruses/plaques expressing the Gus gene (blue on X-Gluc substrate). Through the use of this strategy the Gus gene can be stably inserted into the Ba71v genome, replacing the I329L gene through recombinant homology.

The cells will then be infected with the mutant deletion virus to check that there is virus production and thus confirm that I329L is a non-essential gene. Even though the construct is already cloned and verified by sequencing, the remaining part in the construction of the deletion virus is ongoing work in our lab.

2.3) RESULTS

2.3.1) Expression of I329L during Ba71v infection in Vero cells

The ASFV ORF I329L has been described as a late expression gene [77]. In order to know the exact time point of expression of I329L during virus infection, we infected Vero cells with Ba71v and recovered the cells at different time points of infection: 8h, 10h, 12h, 14h, 16h, 18h and 20h and the expression of this I329L gene was determined by RT-PCR. As can be seen, I329L is indeed a late gene, its mRNA first being detected at 14h, 16h and 18h (Figure 8).

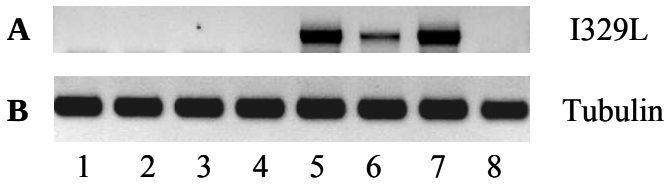

Figure 8 – Expression of I329L during Ba71v infection. A) RT-PCR for the I329L gene is negative for non infected cells (lane 1) as well as for time points 8h, 10h and 12h after infection (lanes 2, 3 and 4). I329L was expressed at 14h, 16h and 18h post infection (lanes 5, 6 and 7), but not at 20h post infection (lane 8). **B)** PCR for tubulin, as a control of the quantity and quality of the cDNAs.

After demonstrating transcription of I329L at 14h, 16h and 18h post infection, we decided to use the 18h time point for the next steps of the siRNA experiment.

All the following procedures regarding siRNA described in this thesis were performed at this time point of infection.

2.3.2) Transfection of siRNA targets in Ba71v infected Vero cells

We first decided to test the transfection of each siRNA target (1 and 2) separately in order to see if one target sequence was able to induce the knockdown of the gene. However, under these conditions, I329L expression was not suppressed (Figure 9A, 9B). On the other hand, when we combined both targets and transfected them into the cells, an inhibition of expression of I329L was observed with a concentration of siRNAs of 200 nM (Figure 9C). The inhibition was specific and the combined treatment with two siRNA probes did not inhibit transcription of A238L (Figure 9D).

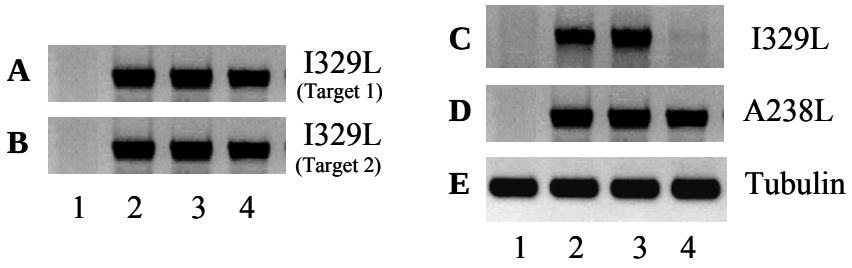

Figure 9 – Expression of I329L after transfection of siRNA targets and infection with Ba71v. A, B) Transfection of targets 1 and 2 respectively, non combined. RT-PCR for the I329L gene is negative for non transfected and non infected cells (lane 1), but positive for infected cells (lane 2) as well as for cells transfected with siRNA negative control Scramble (lane 3). Cells transfected with only one of the siRNA targets do not inhibit I329L expression (lane 4). **C)** RT-PCR for the I329L gene is negative for non transfected and non infected cells (lane 1), and positive for infected cells (lane 2) as well as for infected cells transfected with siRNA negative control Scramble (lane 3). Lane 4 demonstrates that I329L expression was knocked down by transfection of a combination of

both siRNA targets at a final concentration of 200 nM. **D)** RT-PCR for the A238L gene (lanes 2, 3 and 4) not only demonstrates that the cells are infected, but also that the siRNA target sequences are specific for I329L and have no effect in the expression of other viral genes. **E)** Tubulin amplification served as a control for the quantity and quality of the cDNAs.

2.3.3) siRNA titration in Ba71v infected Vero cells

It is important to use the minimum quantity of siRNA as possible, as too high concentrations of siRNA transfected into the cells may lead to off-target or cytotoxic effects. On the other hand, if too little siRNA is used, the reduction of target-gene expression may be insufficient. Thus the amounts of siRNA tested in the transfection step were titrated (200 nM-6.25 nM). Effective knockdown was observed at oligonucleotide concentrations of 100 nM or 200 nM (lanes 5 and 4 respectively, Figure 10A). Lower quantities did not result in the inhibition of I329L expression (lanes 6-9, Figure 10A). Once again, there was no impact on transcription of the positive control, A238L.

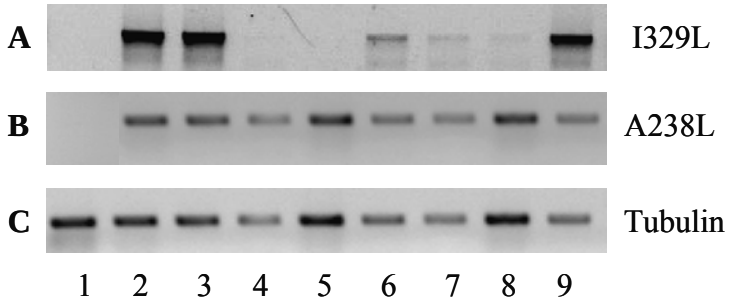

Figure 10 – Titration of the quantity of siRNA targets in infected Vero cells. **A)** RT-PCR for the I329L gene is negative for non transfected and non infected cells (lane 1), and positive for infected cells (lane 2), as well as for cells

transfected with siRNA negative control Scramble (lane 3). Lanes 4-9 correspond to 200 nM, 100 nM, 50 nM, 25 nM, 12.5 nM and 6.25 nM of siRNA target transfection titration respectively. I329L expression is knocked down at 200 nM and 100 nM concentrations of siRNA targets (lanes 4 and 5). Lower concentrations of siRNA target were unable to knockdown expression of I329L (Lanes 6-9). **B)** RT-PCR for the A238L gene not only demonstrates that the cells are infected, but also that the siRNA target sequences are specific for I329L and have no effect in the expression of other viral genes. **C)** PCR for tubulin, as a control of the quantity and quality of the cDNAs.

2.3.4) Analysis of virus production from infected Vero cells transfected with I329L siRNA targets

After confirming the specific knock-down of I329L through siRNA technology, we next investigated 1) the impact of knockdown of I329L on virus production and 2) the production of infectious virus by cells by cells with synthesis of I329L knocked down.

In order to achieve these objectives, we 1) stained the cells with an antibody against p73, a major capsid protein, to check that the cells were being infected and 2) recovered the supernatants from the siRNA transfected and for infection of Vero cells followed by counting the number of viral plaques.

2.3.4.1) Staining of p73, the major capsid protein of ASF virus

The major structural protein of ASFV is a capsid protein, p73. This protein is thought to associate with the membranes that form the envelope of the virus [23]. Cobbold et al. generated

a mouse monoclonal antibody 4H3 to determine the onset of expression of p73 and the time course of packaging of capsid proteins into virions.

We used this antibody followed by a Goat anti-mouse IgG Alexa 568 secondary antibody in cells infected with the culture supernatants from the control and I329L specific knocked down Ba71v virus infected Vero cells.

As can be seen, there was no difference in the expression of p73 with or without siRNA knockdown of the I329L gene at all the concentrations of siRNA (200nM-6.25nM) tested. Only two of the images of the staining are presented in this thesis, one with just infected cells and one with both siRNA transfected (200nM) and infected cells (Figure 11).

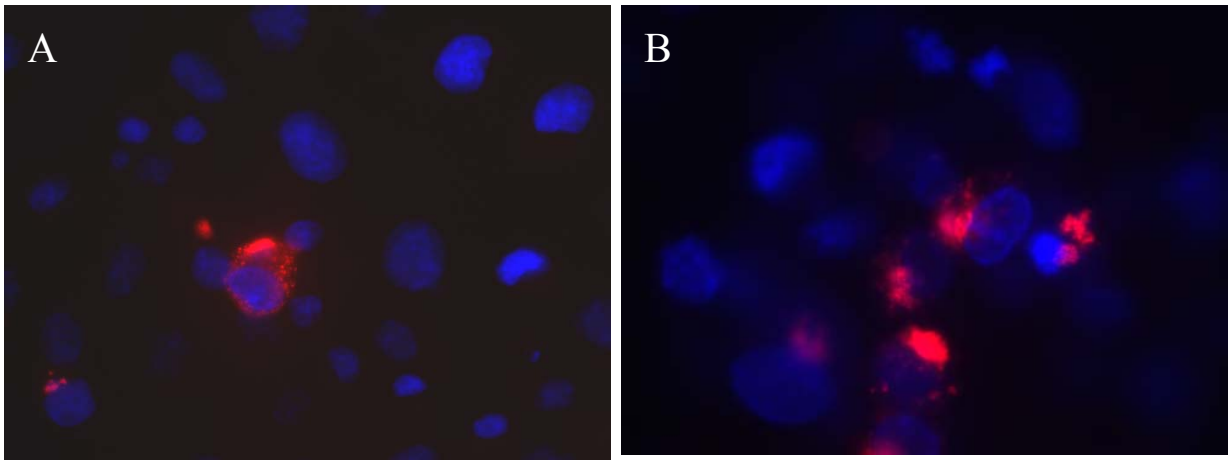

Figure 11 – Staining of Vero cells with 4H3 monoclonal antibody. p73 can be observed in red and DAPI (blue) stains the nucleus. The staining was identical both in **A)** Ba71v infected cells and in **B)** siRNA transfected and Ba71v infected Vero cells (Magnification 40X).

2.3.4.2) Analysis of viable virus production from siRNA recovered supernatants

After confirming the specific knockdown of I329L through siRNA technology we used the supernatants of the Ba71v virus infected cells with and without siRNA mediated knockdown of I329L.

By comparing the number of viral plaques of Ba71v infected cells and Ba71v infected and transfected cells (with siRNA in the concentrations range defined) we could conclude that there was no impact in the production of infectious virus. Thus knocking-down I329L did not affect the production of infectious ASFV virus, and therefore I329L is truly a non-essential gene.

Figure 12 – Counting of viral plaques after siRNA supernatant titration. We observed no significant difference in the number of viral plaques by comparing the number of plaques counted both after infection with the supernatant of Ba71v infected cells (non siRNA transfected, sample 2) and the supernatant of Ba71v infected and scramble siRNA transfected cells (sample 3), with the number of

plaques counted after infection with the supernatants of both I329L siRNA transfected and Ba71v infected cells (samples 4-9).

2.4) DISCUSSION

RNAi is an effective method of gene silencing, and is a very important tool in the study of genetics, molecular biology and physiology. It has been yielding considerable insights into the innate and adaptive immune systems, namely into the mechanisms that regulate the development, activation and function of cells that mediate immunity. Even though this technique may in the future be tested for clinical purposes, its limitations such as inefficient delivery *in vivo*, incomplete silencing of target genes, non-specific immune responses and off-target effects, must first be overcome. Nevertheless, it seems to be a powerful and promising tool applicable to vaccination and immunotherapy [61].

The I329L ORF is a host modification gene of the ASFV, as suggested by previous results in our lab, and therefore a suitable candidate for the construction of a mutant deletion virus vaccine. We decided to take two approaches to investigate whether this gene was essential to the virus or not.

In the first one, we used siRNA oligos in order to specifically knockdown the expression of I329L in Ba71v Vero infected cells. The I329L gene, already described as a late expression gene [77], was knocked down at the time of its expression at 18 hours post infection. Upon siRNA oligo transfection we were able to see an inhibition of expression when using a combination of two oligos at 100 and 200 nM. Furthermore, supernatants recovered from cells infected with virus, but with expression of I329L knocked down, contained similar numbers of effective virus as control non-knocked down infected cultures. To further confirm that the cells had been infected, we performed immunofluorescence using a monoclonal antibody against p73, a major capsid protein of the virus. Once again, we observed no difference between the staining of control infected cells and cells that were both infected and

treated with siRNA to knockdown the expression of I329L. In addition, these results show that p73 expression is not affected by the knocking down of I329L and, in consequence, virus envelope formation is not altered.

The fact that the knockdown of expression of this gene does not affect assembly and production of infectious virus, not only shows RNA technology can be used to inhibit I329L synthesis in ASFV infected cells, but also constitutes the key finding of this study, namely that I329L is a non essential gene of the ASFV. Thus, the data presented in this chapter, together with the fact that I329L inhibits TLR3, makes this host manipulation gene a possible candidate to delete for the production of a viral attenuated vaccine.

Another approach to show that this gene was not essential to the virus was through the construction of an I329L mutant deletion virus. To this end, the necessary recombinant transfer plasmid for construction of the I329L deletion mutant has been constructed and the remaining work towards construction of the I329L deletion mutant is ongoing in our lab. Whether such a mutant will indeed prove to be a vaccine candidate will depend on its pathogenicity in the pig. If still pathogenic, one approach to achieve the vaccine would be to sequentially delete other genes known to be involved in evasion of the host immune system, such as I329L, or genes required for virus virulence or for efficient replication in the tick vector. The deletion of these immune evasion genes might change the balance in favour of the virus towards an effective host immune response and thus an increased chance of developing an effective protective immune response in vaccinated animals. This strategy has been shown to be efficient in Herpes simplex virus, where the blocking of virus immune evasion proteins improved the potency of the vaccine [48].

3) FAILURE TO DETECT AN IMPACT OF I329L EXPRESSION IN SUPEROXIDE PRODUCTION STIMULATED BY LPS AND DSRNA (POLY I:C) IN IPAM CELLS

3.1) INTRODUCTION

Macrophages play an important role in the control of inflammation as well as in the regulation of immune response and indeed upon viral infection, macrophages can contribute to immunopathology in the host. Because of this, some viruses have evolved genes which are cytokine analogues, function as cytokine and chemokine receptors, or provide mechanisms that inhibit cytokine function [2, 64, 100].

Inflammation an important early response to infection. For example, multiple inflammatory cells such as eosinophils, neutrophils and macrophages have the ability to generate reactive oxygen species (ROS), which not only damage the pathogen but may also lead to disease [70].

In relation to macrophages, it is important to distinguish between the different cell types of monocytic origin, since there may be differences in terms of antioxidant and redox properties. For instance, alveolar macrophages, located between air and lung tissue are directly exposed to high oxygen levels and various pollutants, and therefore have different characteristics from peritoneal macrophages in terms of their ability to release TNF-α, IL-6, NO or O_2^- [32]. In addition, the effects of oxidants on redox signalling appear to be cell-specific and stimulus-dependent [32]. A better understanding of the mechanisms through which ROS/RNS (Reactive oxygen and nitrogen species respectively) may affect macrophage response could have valuable implications for the development of anti-inflammatory therapeutics since these cells

are involved in propagating inflammation, through the secretion of pro-inflammatory molecules, as well as in the resolution of the inflammatory response through phagocytosis of apoptotic and necrotic cells [32].

As previously described in this thesis, ASFV is a DNA virus that infects macrophages of domestic swine. Although the basis of the biological changes caused by this virus are not fully understood, cytokine expression could cause inflammation as well as induce changes in immune function at sites of infection. Also, once infected, macrophages may respond differently to cytokines. Studies have been carried out to test the ability of infected macrophages to produce different cytokines. This was evaluated through the production of superoxide, in response to interferon-γ (IFN-γ) and lipopolysaccharide (LPS) stimulation [100].

3.2) METHODS AND MATERIALS

3.2.1) Cell lines

IPAM cells (Immature porcine alveolar macrophages), IPAM cells infected with empty lentiviruses and IPAM cells infected with I329L recombinant lentiviruses, previously available in the lab and supplied by Vívian Oliveira, were cultured in RPMI 1640 with glutamine medium supplemented with 10% Fetal Calf Serum (FCS, heat inactivated), 100 Units/mL Penicillin, 100 μg/mL Streptomycin, and 1 mM sodium pyruvate all purchased from Gibco, Invitrogen Corporation.

Cells were routinely checked for contamination with mycoplasm by performing a specific PCR.

3.2.2) I329L expression in I329L lentivirus-transduced IPAM cells

Lentivirus-based vectors are under development in gene therapy research since, unlike oncoretrovirus vectors, these allow the delivery of genes to a variety of target cells. Human immunodeficiency virus (HIV)-based vectors have been used both *in vitro* and *in vivo*, through the removal of some accessory genes that minimize the risk from them. However there is still some concern if they are to be used in HIV-infected patients. As an alternative, lentivirus-based vectors, derived from non-primate lentiviruses not know to be pathogenic or infectious for humans, have been developed so that they have the ability to transduce non-dividing cells. Feline immunodeficiency virus (FIV) and equine infectious anaemia virus (EIAV) are two examples of lentivirus-based vectors that have a minimal sequence homology to HIV and weak transcriptional activity in human cells [44].

A scheme of the cloning vector used to produce lentivirus with our gene of study is shown on figure 13.

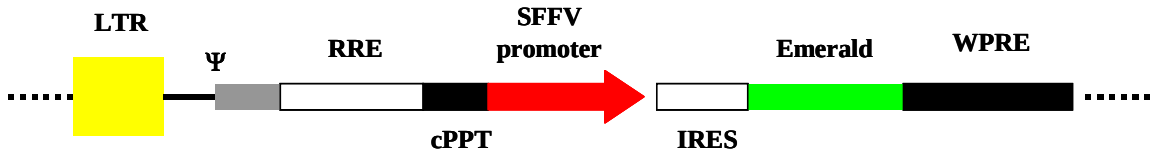

Figure 13 – Scheme of the cloning vector SIN-BX-IR/EMW. This vector has a 5' long terminal repeat (LTR), but not a 3', which makes it non-infectious. It also has a rev-responsive element (RRE), a central polypurine tract (cPPT), a spleen focus forming virus (SFFV) promoter, an internal ribosome entry site (IRES) sequence, an emerald sequence (that is similar to eGFP, but with a 4 point mutation that makes it much more brighter) and a Woodchuck hepatitis virus post-transcriptional regulation element (WPRE) sequence. To develop the lentivirus carrying our gene of interest, an HA tag was also cloned upstream the viral gene. The final construct consisted on HA-I329L being cloned upstream the IRES sequence and downstream the SFFV promoter.

In order to check if I329L was being expressed in the I329L lentivirus-transduced IPAM cell line, we used two approaches, 1) detection of I329L mRNA expression by RT-PCR and 2) detection of I329L through immunofluorescence using an antibody against the co-expressed HA tag.

3.2.2.1) Detection of I329L expression by RT-PCR

Total RNA was extracted from I329L lentivirus-transduced IPAM cells and also from IPAM cells and empty lentivirus-transduced IPAM cells (as controls), using Trizol Reagent (Sigma). Samples of RNA were digested with DNase I (Invitrogen) and cDNA synthesis was performed using MMLV-Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. I329L expression in the cDNA was detected by PCR, using Taq DNA Polymerase, on a PTC-100 Peltier-Effect Cycling apparatus. Primer sequences were I329Lup, 5'-GCTACTTCTTCTTGAACATGA-3' and I329Llow, 5'-GCTTAGGAAGTGGCTTAAC AGG-3' and PCR conditions were dNTPs 200 μM, primers 1 μM, $MgCl_2$ 2 mM, 94ºC – 4', 40X (94ºC – 1', 50ºC – 1', 72ºC – 2'), 72ºC – 10'. As a quantitative and qualitative control, tubulin was amplified using Taq DNA Polymerase with dNTPs 200 μM, primers 1 μM, $MgCl_2$ 2 mM, 94ºC – 4', 40X (94ºC – 1', 55ºC – 1', 72ºC – 2'), 72ºC – 10'. Tubulin primer sequences were the following: TubulinUp, 5'-GGTGGATCTAGAACCTGGG-3' and TubulinLow, 5'-CCCAGTGAGTGGGTCAGC-3'.

3.2.2.2) Detection of I329L through immunofluorescence

I329L lentivirus-transduced IPAM cells, already constructed by Vívian Oliveira were tested by immunofluorescence for I329L expression, using an antibody to the coexpressed Influenza haemaglutinin peptide (HA) tag, cloned upstream the viral gene.

For immunofluorescence, sterilized glass coverslips were put in the wells containing the IPAM cells until they became adherent. Afterwards, cells were fixed with 4% paraformaldehyde (PFA) for 20 minutes at room temperature (RT). Blocking was performed at RT for 30 minutes with PBS containing 0.05% Tween-20 and 3% Bovine Serum Albumine

(BSA). The cells were permealized by treatment with PBS containing 0.1% Triton X-100 for 20 minutes. The staining with the primary antibody rat anti-HA was performed at RT for 1 hour in blocking solution with a previously titrated concentration of the antibody. The secondary staining was also performed at RT for 1 hour with a goat anti-rat FITC. Rat anti-HA (high affinity) was purchased from Roche and goat anti-Rat-FITC (adsorbed against mouse Ig) was purchased from SouthernBiotech.

To stain the nucleus of the cells, coverslips were incubated in a DAPI solution (20 ng/µl) for 2 minutes. All washes between stainings were performed at RT in PBS containing 0.05% Tween-20.

3.2.3) Assays of macrophage function

Whittall and Parkhouse described a protocol in pigs in which alveolar and peritoneal macrophages were obtained by lavage of lungs and lavage of peritoneum respectively. Afterwards, they performed assays of macrophage function through the evaluation of responses to IFN-γ (interferon gamma) and LPS (lipopolysaccharide) by measuring the ability of macrophages to produce superoxide after stimulation with phorbol myristate (PMA), using nitroblue tetrazolium (NBT) for detection [100].

The NBT test has been widely used to measure superoxide release by macrophages during an oxidative burst. This method allows the measurement of reduced NBT formazan, through its solubilization [78].

We followed an adaptation of the protocol described, using IPAM cells. After checking the ability of LPS to activate these cells, we repeated the protocol on empty lentivirus-transduced IPAM cells and on I329L lentivirus-transduced IPAM cells.

3.2.3.1) Measurement of nitric oxide

$1x10^5$ IPAM cells/well were plated on a 96 well plate and left to adhere overnight. On the following day a series of LPS dilutions were prepared (10 000 ng/mL, 1000 ng/mL, 100 ng/mL, 10 ng/mL and 1 ng/mL), added to the plates and incubated for 48 hours at 37ºC. Then, the medium was removed and replaced with RPMI medium with the same supplements described above, but without phenol red, and containing 1 mg/mL NBT, purchased from Sigma. PMA, also purchased from Sigma, was also added at a concentration of 1 μg/mL, in order to stimulate the cells. Plates were incubated at 37ºC for 30 minutes. Afterwards, the medium was removed and IPAM cells were fixed with 70% methanol for 20 minutes at RT. Finally, formazan dye was solubilized by adding 120 μl of 2M potassium hydroxide and 180 μl of dimethyl sulphoxide. The plate was read at 595 nm on a Microplate Manager 4.0 Bio-Rad apparatus.

3.2.3.2) Measurement of nitric oxide in control and I329L -transduced IPAM cells

After checking the background levels in IPAM cells, we then proceeded to repeat the assay on I329L lentivirus-transduced IPAM cells and in empty lentivirus-transduced IPAM cells as the negative control. Based on the results we obtained on IPAM cells, we decided to use only LPS and poly(I:C) to stimulate the cells, and not PMA.

$1x10^5$ IPAM cells/well were plated on a 96 well plate and left to adhere overnight. On the following day a series of LPS dilutions were prepared (10 000 ng/mL, 1000 ng/ mL, 100 ng/ mL, 10 ng/ mL and 1 ng/ mL), added to the plates and incubated for 48 hours at 37ºC. Then, the medium was removed and replaced with RPMI medium with the same supplements described above, but without phenol red, and containing 1 mg/mL NBT, purchased from

Sigma, and the plates were incubated at 37ºC for 30 minutes. Afterwards, the medium was removed and the cells were fixed with 70% methanol for 20 minutes at RT. Finally, formazan dye was solubilized by adding 120 µl of 2M potassium hydroxide and 180 µl dimethyl sulphoxide. The plate was read at 595 nm on a Microplate Manager 4.0 Bio-Rad apparatus.

This assay was also done using a polyinosinic-polycytidylic acid (poly(I:C)) stimulus instead of an LPS one. The concentrations tested for poly(I:C) were 125 µg/ml, 25 µg/ml, 5 µg/ml and 1 µg/ml.

3.2.3.3) Analysis of porcine cytokine gene expression through RT-PCR

To assess cytokine expression upon poly(I:C) stimulation of IPAM cells, empty lentivirus-transduced cells and I329L lentivirus-transduced cells, were plated in a 6-well plate, $6x10^5$ cells/well. On the following day they were stimulated with 5 µg/ml poly(I:C) and left for 48h. Then, total RNA was collected from stimulated and non-stimulated cells (controls), using Trizol Reagent (Sigma). RNA samples were digested with DNase I (Invitrogen) and cDNA synthesis was performed using MMLV-Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. IFN-α, IFN-β, IFN-γ and control β-actin expression in the cDNA was detected by PCR, using Taq DNA Polymerase, on a PTC-100 Peltier-Effect Cycling apparatus. Primer sequences were IFN-αUp, 5'-GGCTCTGGTGCATGAGATGC-3' and IFN-αLow, 5'-CAGCCAGGATGGAGTCCTCC-3' and PCR conditions were dNTPs 200 µM, primers 1 µM, $MgCl_2$ 2 mM, 95ºC – 2', 40X (95ºC – 30'', 50ºC – 10'', 72ºC – 30''), 72ºC – 10' [20]. Primer sequences for IFN-βUp, 5'-GGCCATGGCTAACAAGTGCATCC-3' and IFN-βLow, 5'-CCGGTCAGTTCCGGAGGTAATC-3' and PCR conditions were dNTPs 200 µM, primers 1 µM, $MgCl_2$ 2 mM, 94ºC – 4', 40X (94ºC – 1', 50ºC – 1', 72ºC – 2'), 72ºC – 10' [83]. Primer sequences for IFN-γUp, 5'-GTTTTTCTGGCTCTTACTGC-3' and IFN-

γLow, 5'-CTTCCGCTTTCTTAGGTTAG -3' and PCR conditions were dNTPs 200 μM, primers 1 μM, $MgCl_2$ 1.5 mM, 94ºC – 3', 33X (94ºC – 45'', 54ºC – 45'', 72ºC – 45''), 72ºC – 10' [88]. As a quantitative and qualitative control, β-actin was amplified using Taq DNA Polymerase with dNTPs 200 μM, primers 1 μM, $MgCl_2$ 2 mM, 94ºC – 3', 28X (94ºC – 45'', 54ºC – 45'', 72ºC – 45''), 72ºC – 10'. β-actin primer sequences were the following: β-actinUp, 5'-GGACTTCGAGCAGGAGATGG-3' and β-actinLow, 5'-GCACCGTGTTGGCGTAGAGG-3' [30].

3.3) RESULTS

3.3.1) I329L expression in I329L lentivirus-transduced IPAM cells

The expression of I329L on I329L lentivirus-transduced IPAM cells was confirmed at the RNA level by RT-PCR, and at the protein level by immunofluorescence using an antibody specific for the HA immunotag.

3.3.1.1) Detection of I329L expression by RT-PCR

We collected RNA and consequently synthesized cDNA from IPAM cells, empty lentivirus-transduced IPAM cells, and I329L lentivirus-transduced IPAM cells. Then, we checked for expression of I329L, by RT-PCR. We observed that only I329L lentivirus-transduced IPAM cells cDNA expressed I329L, as expected. Tubulin amplification was used as a control for the quality and quantity of the RNA (Figure 14B, bottom panel).

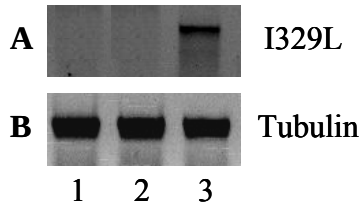

Figure 14 – Expression of I329L cDNA in I329L lentivirus-transduced IPAM cells. A) RT-PCR for the I329L gene is negative for IPAM cells (lane 1) as well as for empty lentivirus-transduced IPAM cells (lane 2). I329L expression was detected in I329L lentivirus-transduced IPAM cells. **B)** PCR for tubulin, as a control of the quantity and quality of the cDNAs.

3.3.1.2) Detection of I329L expression by immunofluorescence

An immunofluorescence was performed against the HA immunotag. We observed I329L expression on I329L lentivirus-transduced IPAM cells (Figure 15C), but not in control untreated IPAM cells (Figure 15A) or empty lentivirus-transduced IPAM cells (Figure 15B). Interestingly, the staining of I329L was confined to the perinuclear area of the cells indicating its selective localization in the Golgi apparatus.

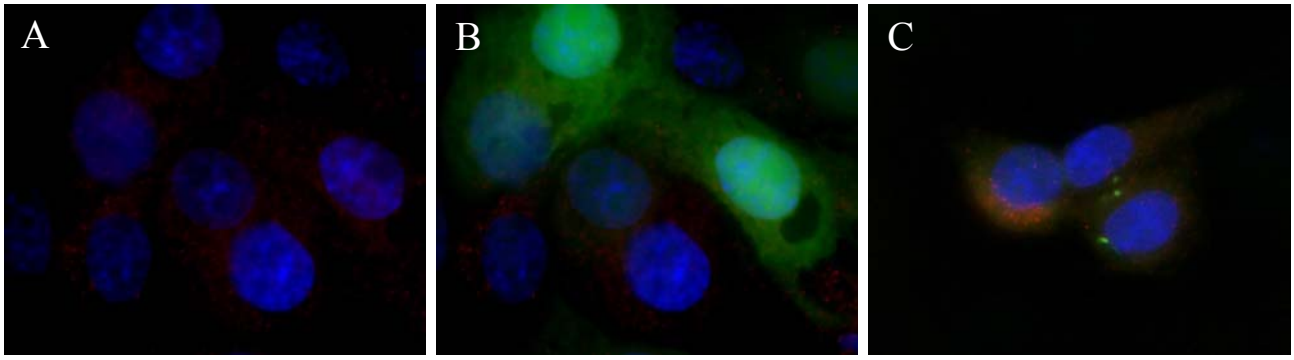

Figure 15 – Staining of I329L lentivirus-transduced IPAM cells, with and anti-HA antibody. A) IPAM cells showing a DAPI (blue) stained nucleus **B)** empty lentivirus-transduced IPAM cells **C)** I329L lentivirus-transduced IPAM cells, showing anti-HA specific staining in the perinuclear area (red). (Magnification 60X)

3.3.2) Superoxide production measurement on IPAM cells after LPS and PMA stimulation

Having demonstrated expression of the viral protein in the recombinant retrovirus transduced IPAM cells, the next step was to determine the impact of the virus protein. First, we

confirmed that IPAM cells are respondive to LPS stimulation, using a series of dilutions of LPS and PMA, and measured superoxide production using NBT (Table 1), as described by Whittall and Parkhouse [100]. As negative controls, we also measured the responses of IPAM cells alone and IPAM cells+NBT.

Table 1 – Absorbance readings on LPS and PMA triggered IPAM cells

	[LPS]	Absorbance
IPAM Cells	-	0.192
IPAM cells + NBT	-	0.181
IPAM cells + LPS + NBT	1 ng/ mL	**0.524**
	10 ng/ mL	**0.559**
	100 ng/ mL	**0.394**
	1000 ng/ mL	**0.441**
	10 000 ng/ mL	**0.420**
IPAM cells + LPS + PMA + NBT	1 ng/ mL	**0.438**
	10 ng/ mL	**0.581**
	100 ng/ mL	**0.490**
	1000 ng/ mL	**0.534**
	10 000 ng/ mL	**0.459**

We observed that IPAM cells can be triggered through stimulation with LPS alone as well as with a combination of both LPS and PMA. In fact, the addition of PMA did not augment superoxide production values, compared with IPAM cells that were only stimulated with LPS. In the negative controls, superoxide production values were virtually the same in both controls of IPAM cells (0.192) alone and IPAM cells with NBT (0.181), suggesting that NBT does not stimulate macrophages.

Next, we repeated this assay on control and I329L recombinant lentivirus-transduced IPAM cells, using only LPS as the stimulus.

3.3.3) Superoxide production measurement on empty lentivirus-transduced IPAM cells and I329L lentivirus-transduced IPAM cells after LPS stimulation

A series of dilutions of LPS were used to stimulate untreated IPAM cells, empty lentivirus-transduced IPAM cells and recombinant I329L lentivirus-transduced IPAM cells. The assay was performed this assay in triplicates with unstimulated IPAM cells as the negative control.
We observed a stimulation of superoxide production by IPAM cells upon stimulation with LPS, as mentioned above (4.3.2). The differences in superoxide production are not significant in the various LPS concentrations. Regarding superoxide production in empty lentivirus-transduced IPAM cells and I329L lentivirus-transduced IPAM cells upon stimulation with LPS, we observed no difference, when comparing with the results on IPAM cells. The stimulation with LPS was not titratable, similar responses being observed with all concentrations of LPS tested (Figure 16).

The most important result, disappointing however, was the failure of ASFV gene I329L to modify the response of IPAM cells to LPS.

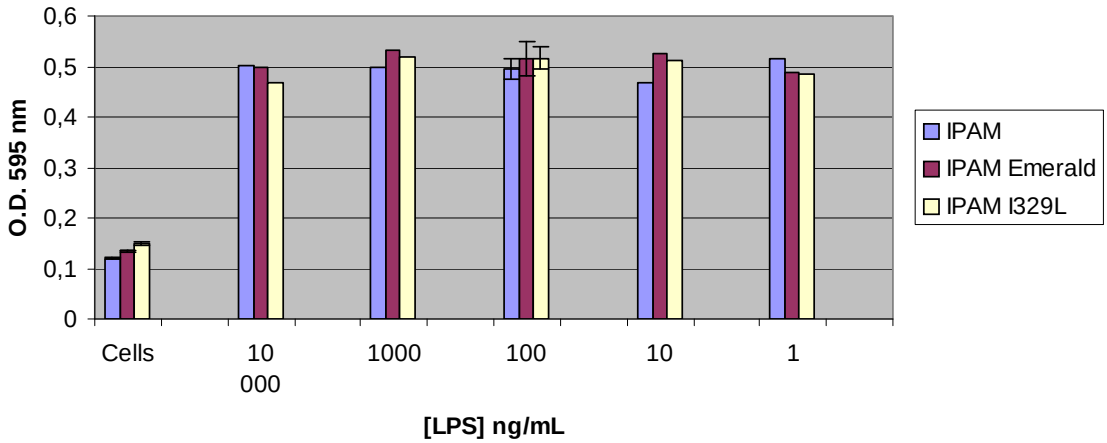

Figure 16 – Superoxide production of IPAM cells, empty lentivirus-transduced IPAM cells and I329L lentivirus-transduced IPAM cells, upon LPS stimulation. The graph shows that superoxide production is higher, once IPAM cells are stimulated with LPS, when compared with non stimulated cells (blue bars). It can also be observed that there is no significant difference between IPAM cells, empty lentivirus-transduced IPAM cells (pink bars) and I329L lentivirus-transduced IPAM cells (yellow bars) in terms of superoxide production, even with a wide range of stimulation by LPS.

3.3.4) Superoxide production measurement on empty lentivirus-transduced IPAM cells and I329L lentivirus-transduced IPAM cells after poly(I:C) stimulation

Previous results regarding the I329L gene have shown that this gene inhibits TLR3 signaling. We have used LPS as a stimulus, in order to repeat the experiment described by Whittall and Parkhouse. However, LPS is a ligand to TLR4. In order to mimic the recognition of a dsRNA by TLR3, we decided to use a different stimulus, with poly(I:C).

We tested a series of dilutions of poly(I:C), 125 μg/mL, 25 μg/mL, 5 μg/mL and 1 μg/mL, to trigger 3 cell lines: on IPAM cells, empty lentivirus-transduced IPAM cells and I329L lentivirus-transduced IPAM cells. We performed this assay in triplicates and used IPAM cells without poly(I:C) stimulus, as a control.

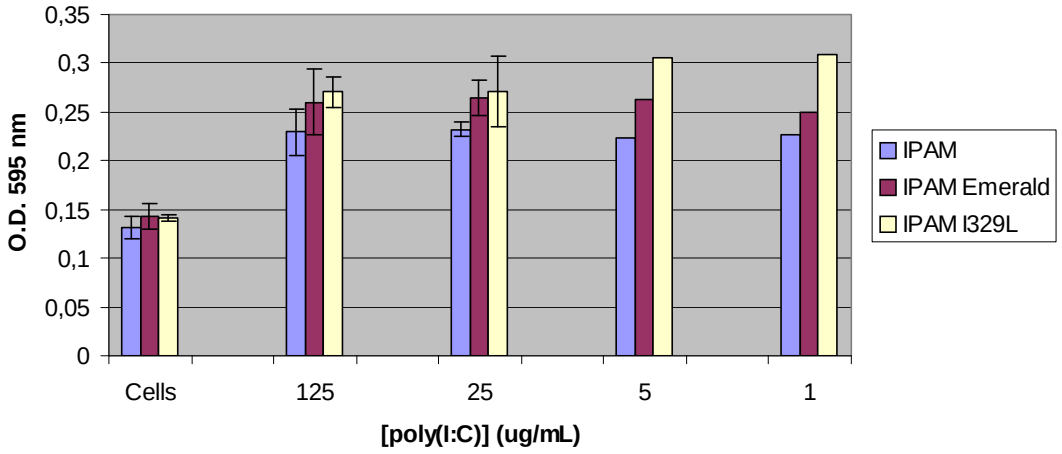

Figure 17 – Superoxide production of IPAM cells, empty lentivirus-transduced IPAM cells and I329L lentivirus-transduced IPAM cells, upon poly(I:C) stimulation. The graph shows that superoxide production is higher, once IPAM cells are stimulated with poly(I:C), when compared with non stimulated cells (blue bars). When cells were stimulated with 125 μg/mL and 25 μg/mL of poly(I:C), no difference was observed, in terms of superoxide production, between the three cell lines. This suggests that these concentrations may be toxic to the cells. Once the cells were stimulated with poly(I:C) at concentrations of 5 μg/mL and 1 μg/mL, we were able to observe a slight augment in superoxide production in I329L lentivirus-transduced IPAM cells (yellow bars). However, this increase is not statistically significant because, even though there is a difference between stimulated I329L

lentivirus-transduced IPAM cells and stimulated IPAM cells, this difference is lower in relation to empty lentivirus-transduced IPAM cells (pink bars).

3.3.5) Detection of cytokine expression by RT-PCR

According to previous luciferase assay results in our lab, I329L inhibits IFN-β.

In order to see if the cytokine levels were altered in this type of assay, we decided to assess cytokine expression in IPAM, empty lentivirus-transduced IPAM cells and I329L lentivirus-transduced IPAM cells after these were stimulated with poly(I:C). IFN-α, IFN-β and IFN-γ expression was detected by RT-PCR. As can be seen (Figure 18) there was no impact of I329L expression on the transcription of IFN α, β, and γ. Interestingly, the IPAM cells synthesized IFN-γ.

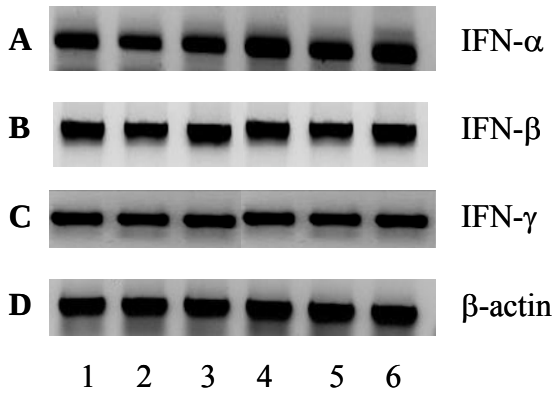

Figure 18 – Expression of IFN-α, IFN-β and IFN-γ cDNA in IPAM cells, empty lentivirus-transduced IPAM cells, and I329L lentivirus-transduced IPAM cells, upon poly(I:C) stimulation. Lanes 1, 3 and 5 correspond to non stimulated cells, and lanes 2, 4 and 6 correspond to poly(I:C) stimulated cells. **A)** RT-PCR for the IFN-α is positive in all cells **B)** RT-PCR for the IFN-β is positive for all cell types **C)** RT-PCR

for the IFN-γ is positive in all cells **D)** PCR for β-actin, as a control of the quantity and quality of the cDNAs.

3.4) DISCUSSION

Innate immune responses are crucial in the control of viral infection. Once viral proteins or nucleic acids are recognized by pattern recognition receptors (PRRs) of the Toll-like receptor family, a triggering of the downstream signalling cascades occur. This may lead both to cell activation and cytokine production [62].

Studies regarding the responsiveness of ASFV infected macrophages to IFN-γ and LPS have been described by Whittall and Parkhouse. This method comprised the measurement of superoxide production through the NBT method. The NBT test has been widely used in several areas, as a measurement of oxidative burst, based on the observation that leukocytes from patients with chronic granulomatous disease lack the ability to give an oxidative burst [7]. It has also been widely used in order to measure macrophage activation [69].

Whittall and Parkhouse observed that the infected cells were less responsive to IFN-γ and LPS than the cells infected with UV-inactivated virus.

Although it is not the same assay, we decided to test IPAM cells (swine macrophages) in relation to their ability to respond to a range of concentrations of LPS. Upon LPS stimulation, we observed that these cells showed signs similar to infection, which reflected that LPS could function as stimulus for these cells.

Afterwards, instead of using infected cells, as described in the original protocol, we decided to test the same conditions in IPAM cells that had previously been transduced with lentivirus expressing our study gene, I329L (tagged with an HA immunotag). As an additional control, we also used IPAM cells transduced with the empty lentivirus (empty lentivirus-transduced IPAM cells). These cells lines were tested not only for RNA expression of the gene, by RT-

PCR, but also for protein production, by immunofluorescence. This assay could provide us clues on how the expression of this gene could affect the way in which the cells are able to be stimulated by LPS, in terms of superoxide production. The results showed us that there was no significant difference between the three cell lines tested (IPAM, IPAM emerald and IPAM I329L), after stimulation with LPS.

Previous studies in our lab, have shown that I329L may be inhibiting Toll-like signalling pathway through TLR3 (unpublished data by Vívian Oliveira). Different TLRs, recognize different molecules, exhibiting different responses accordingly. While LPS is recognized by TLR4, TLR3 recognizes dsRNA molecules. Considering the interaction of I329L with TLR3, and not TLR4, the expression of this gene might not be expected to alter the levels of superoxide production in comparison with the control cells. To test this possibility, we decided to use poly(I:C) as a stimulus instead of LPS, since this molecule had previously been described as being able to activate macrophages, as well as B lymphocytes and dendritic cells [62]. Also, these responses are dependent on the expression of both TLR3 and TRIF [62]. Poly(I:C), a double stranded RNA analog, is recognized by TLR3, that in turn seems to be inhibited by I329L. Surprisingly, upon stimulation of I329L lentivirus-transduced cells with poly(I:C) (5 μg/ mL and 1 μg/ mL) we noted a slight augmentation, that did not happen with higher doses of poly(I:C) that are probably toxic to the cells. This augmentation cannot be considered statistically significant, since even though there was a slight difference between stimulated I329L lentivirus-transduced IPAM cells and stimulated IPAM cells, this difference is insignificant once we compare the levels of superoxide production in I329L lentivirus-transduced IPAM cells and empty lentivirus-transduced IPAM cells.

Since I329L has been demonstrated to inhibit TLR3 responses, it could be expected that its expression would suppress the recognition of poly(I:C) by this receptor. This would lead to

the observation of a reduced or even an absence of response, reflected by a decrease in superoxide production, once cells expressing the gene were stimulated with poly(I:C). Thus, the results obtained cannot at the moment be explained.

Also, in terms of cytokine expression we observed no difference between the three cell lines upon poly(I:C) stimulation. Even though results in our lab have shown that I329L inhibits production of IFN-β as measured by an IFN-β reporter luciferase assay, we still observed IFN-β expression in I329L lentivirus-transduced cells stimulated with poly(I:C). Despite these results, one must take into account that, this system is not the same as a luciferase assay, where only transfected cells are analysed. In contrast, in lentivirus infection as the green protein is expressed independently and not as a fusion protein to I329L, all the cells are being analysed whether they do or do not express I329L. Finally, it may be that IPAM cells are not appropriate for this investigation as they correspond to immature rather than mature macrophages.

4) CONSTRUCTION OF A TRANSGENIC MOUSE SELECTIVELY EXPRESSING I329L IN MACROPHAGES

4.1) INTRODUCTION

The construction of transgenic mice is considered a powerful strategy for the better understanding of the functions of proteins involved in health and disease. As an added refinement, the use of specific promoters for a restricted cellular expression may overcome undesirable effects such as lethality or whole body transgenesis.

For millions of years viruses have been co-evolving with their hosts as they develop strategies that allow them to manipulate host cell biology, for example cellular processes such as cell division, apoptosis, inflammation, interferon and acquired immune responses [3, 4, 13, 54, 89, 106].

For this reason, virus host evasion genes provide ready made tools to explore and manipulate the regulation of these basic cellular processes. Based on this idea, we have constructed a transgenic mouse with a macrophage restricted expression of a viral gene that shares 40% homology with the highly conserved C terminal domain of mammalian Toll like receptors (TLRs), the I329L gene of the African Swine Fever Virus (ASFV), and which has been demonstrated to inhibit signalling via TLR3 (our unpublished work).

We considered that the construction of this transgenic mouse would be useful to observe the impact of the expression of I329L specifically in the target cell of the African swine fever virus, the macrophage, and might in addition serve as a model for gene therapy. Critical to this objective is the requirement for I329L to function in murine cells as well as those of the natural host, the pig. Several studies have been performed in our lab, which suggest that the

ASFV gene I329L should be functional at the level of a mouse cell. For example,the original demonstration of inhibition of TLR signalling done in our laboratory by Vívian Oliveira, was performed in Vero (monkey) cells. In addition, Sílvia Almeida has successfully constructed a T cell restricted transgenic mouse, expressing A238L, an ASFV gene that inhibits transcription mediated by NF-κB and NFAT [65, 66, 75, 76]. This mouse developed a transplantable, angiogenic thymic tumour, consisting of $CD4^{+}CD8^{+}CD69^{-}$ (mono-) oligoclonal lymphoblasts, with uncontrolled growth in the thymus and metastasis to both the secondary lymphoid organs (spleen and lymph nodes) and non-lymphoid tissues, such as kidney, lung and liver. In addition, inhibition of TLR signalling I329L has been observed in Vero (monkey) cells and as demonstrated in this thesis, mouse macrophages.

Moreover, the TLRs are evolutionarily conserved molecules reflecting their extreme importance in the innate immune system and raising the probability that virus counter measures will be equally conserved, a property which is emphasized by the fact that I329L function is not limited to pig cells, thus permitting the possible application of this ASFV gene to other species.

4.1.1) Lenti-SP-EGFP, a synthetic promoter for Macrophage gene therapy

Macrophages differentiate from hematopoietic stem cells in bone marrow and are recruited to most tissues of the body. There are several factors that make macrophages suitable vehicles for the delivery of therapeutic genes: (1) the accessibility of hematopoietic stem cells (2) the range of locations to where macrophages are recruited and (3) their intrinsic biosynthetic capacity [22].

For macrophage gene therapy, hematopoietic stem cells are mobilized from bone marrow, isolated by apheresis, and transduced *ex vivo* to establish the stable integration into a

chromosome of an expression cassette with the gene(s) of choice. After being autologously reinfused, these transduced hematopoietic stem cells form various lineages of blood cells, including macrophages [8, 91]. Because they are under the control of macrophage-specific promoters, the therapeutic genes should only be expressed at high levels in macrophages. So, strong macrophage promoters are valuable tools for this potentially therapeutic strategy [39].

Several macrophage promoters have been characterized such as the sheep visna virus long terminal repeat [12], the microsialin (CD68) promoter [57], the chicken lysozyme minigene construct [99], and the scavenger receptor-A (SA) promoter [43]. However, these native macrophage promoters may not be strong and/or specific enough for the purpose of gene therapy. For this reason efforts have been made to create synthetic promoters with high levels of activity in skeletal muscle for potential therapeutic advantage [58]. The development of a synthetic myeloid-specific promoter has been described. This promoter is small in size, has high-level macrophage selective activity, and can be used successfully in lentiviral vectors to transduce hematopoietic stem cells, leading to sustained expression of the transgene in transplanted animals [39].

4.2) METHODS AND MATERIALS

4.2.1) Cell lines

RAW 264.7 cells (Mouse leukaemic monocyte macrophage cell line) were cultured in RPMI 1640 with glutamine medium supplemented with 10% Fetal Calf Serum (FCS, heat inactivated), 100 Units/mL Penicillin, 100 μg/mL Streptomycin, and 1 mM sodium pyruvate all purchased from Gibco, Invitrogen Corporation.

Cells were routinely checked for contamination with mycoplasm by performing a specific PCR.

4.2.2) Luciferase reporter Assays

The reporter plasmid for the IFN-β promoter [pIFΔ(-125/+72)lucter], the reporter plasmid for NF-κB [p(PRD2)5tkΔ(-39)lucter], and the TK TATA only reporter [ptkΔ(-39)lucter] were gifts of Dr. S. Goodbourn. 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.

A mouse macrophage cell line, RAW 264.7 cells, ($6x10^4$ cells/well, in a 24 well plate) was co-transfected with 100ng of reporter plasmid, 25 ng of the β-galactosidase internal control plasmid (pCMVβ) and 300ng of either pcDNA3HA-I329L, or the empty pcDNA3HA, according to the FuGENE HD protocol (Roche). Forty eight hours post-transfection the cells were either stimulated or not stimulated with 25μg/ml Poly (I:C) for five hours. After the 5 hour stimulus the cells were lysed. The luciferase activity was measured using the luciferase assay system (Promega) according to the manufacturer`s protocol. The variations in the

transfection efficiency between different cells were corrected by dividing luciferase values by β-galactosidase values.

4.2.3) Mice

FVB/N mice were kept at the Animal House facility of the Instituto Gulbenkian de Ciência (Oeiras, Portugal), and all experimental procedures involving animal manipulation were according to the Direcção Geral de Veterinária de Portugal.

4.2.4) Gene amplication and Construction of the Lenti-SP-I329L-myc plasmid

The I329L gene was amplified by PCR from DNA of Ba71v, a non-pathogenic strain of ASFV that is adapted to grow in Vero cells. We used Pfu DNA Polymerase and the following three sets of primers/oligonucleotides: I329LupEcoRIAgeI, 5'-GCGAATTCACCGG TATGCTAAGGGTTTTCATA-3', I329LlowXhoI, 5'-GCCTCGAGCTTTCTTCTTGAAC ATGA-3', and I329LlowAgeIBsrGI, 5'-GCACCGGTTGTACATCACAGATCCTCTTCTGA GATGAGTTT-3'. PCR was performed on a PTC-100 Peltier-Effect Cycling apparatus using the following conditions: dNTPs 200 μM, primers 1 μM, $MgSO_4$ 2 mM, 94ºC – 4', 30X (94ºC–1', 50ºC–1', 72ºC–2'), 72ºC–10'. I329LupEcoRIAgeI and I329LlowXhoI primers were used to amplify I329L and clone it as a 5'EcoRI-3'XhoI fragment into pcDNA4/Myc-His Version A (Invitrogen), in order to get a Myc tag. Then, pcDNA4-I329L-Myc-His was used on a second PCR, using I329LupEcoRIAgeI and I329LlowAgeIBsrGI primers. The construct was sequenced to confirm sequence fidelity and then, I329L-myc was removed from pcDNA4 and sucloned as a 5'AgeI-3'BsrgI fragment into Lenti-SP. Lenti-SP-EGFP plasmid was kindly sent by Dr. Senlin Li (University of Texas Health Science Center at San

Antonio). This plasmid contains synthetic promoter elements, known to be highly active in myeloid cells, along with a minipromoter (SP+), and has been reported to drive high levels of expression in human and mouse myeloid/macrophage cell lines [39].

I329L was cloned into this plasmid in order to be under the control of this synthetic macrophage specific promoter.

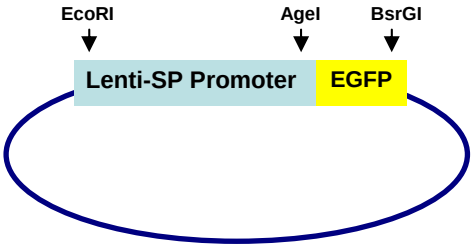

Figure 19 - Schematic view of the Lenti-SP-EGFP synthetic plasmid, with the known restriction sites

The recombinant vector was digested with EcoRI and BsrGI to release the expression cassette from the bacterial backbone pGL3-Basic vector (Promega, Madison, WI). The final construct of the transgenic cassette was according to Figure scheme 20.

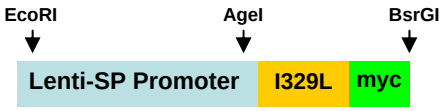

Figure 20 - Schematic view of the microinjected Lenti-SP-I329L-myc construct

After purification, the cassette was delivered to the Transgenic Unit of the IGC where it was microinjected into FVB/N fertilized eggs, which were then transferred into pseudo-pregnant NMRI foster females.

Founder mice were crossed with wild-type FVB/N mice to obtain hemizygous mice.

4.2.5) Sequencing

Sequencing was done to confirm the fidelity of the amplified gene sequence, using primers that comprised part of the gene sequence: I329LseqMiddleUp, 5'-TACCTATATTAC AATAATCTAA-3' and I329LseqMiddleLow, 5'-ACTGCGGAAGACGCTTTAGGT-3'. The kit used was BigDye terminator v1.1, Part Nr 4336776, Applied Biosystems and the PCR conditions were 96ºC-1' and 25X (96ºC-10', 50ºC-5'', 60ºC-4'). The PCR products were analysed on a 377 DNA Sequencer and 3130xl Genetic Analyser, Applied Biosystems.

4.2.6) Mouse genotyping by PCR

Mice were routinely genotyped by PCR using DNA from tail biopsies obtained at the time of weaning (3 weeks old). Tails were digested in Laird´s buffer (100 mM Tris.Cl pH 8.5, 5 mM EDTA, 0.2 % SDS, 200 mM NaCl) with Proteinase K (100 μg/ml) and then DNA was precipitated with isopropanol (0.7 volumes) at RT.

Detection of the complete I329L gene in the DNA by PCR was performed using Taq DNA Polymerase and the following primers: I329Lup, 5'-GCTACTTCTTCTTGAACATGA-3' and I329Llow, 5'-GCTTAGGAAGTGGCTTAACAGG-3' and PCR conditions were dNTPs 200 μM, primers 1 μM, $MgCl_2$ 2 mM, 94ºC – 4', 30X (94ºC–1', 50ºC–1', 72ºC–2'), 72ºC–10'.

4.2.7) Culture of transgenic macrophage cells

Macrophage cells were obtained through peritoneal lavage of transgenic and control mice. The mice were sacrificed and PBS was injected into their peritoneal cavity. Afterwards, the

PBS containing peritoneum cells was collected and the cells were recovered by centrifugation at 1200 rpm for 5 minutes. The cell pellet was resuspended in RPMI 1640 with glutamine medium supplemented with 10% Fetal Calf Serum (FCS, heat inactivated), 100 Units/mL Penicillin, 100 μg/mL Streptomycin, and 1 mM sodium pyruvate all purchased from Gibco, Invitrogen Corporation. The cells were cultured for 24 hours, and the macrophages were easily separated from the other cells by their adherence to the tissue culture plastic plates and the removal of non-adherent cells by washing, as described by Whitthall and Parkhouse, 1997.

4.2.8) Demonstration of transgene expression by RT-PCR

Total RNA was extracted from brain, thymus, heart, lung, liver, spleen, kidney and macrophages (obtained through peritoneal lavage) of transgenic and control mice using Trizol Reagent (Sigma). Samples of RNA were digested with DNaseI (Invitrogen) and cDNA synthesis was performed with MMLV-Reverse Transcriptase (Invitrogen), according to the manufacturer's instructions. Detection of the complete I329L gene in the cDNA was performed by PCR using Taq DNA Polymerase, on a PTC-100 Peltier-Effect Cycling apparatus. Primer sequences were I329Lup, 5'-GCTACTTCTTCTTGAACATGA-3' and I329Llow, 5'-GCTTAGGAAGTGGCTTAACAGG-3' and PCR conditions were dNTPs 200 μM, primers 1 μM, $MgCl_2$ 2 mM, 94ºC – 4', 30X (94ºC – 1', 50ºC – 1', 72ºC – 2'), 72ºC – 10'. As a quantitative and qualitative control, tubulin was amplified using Taq DNA Polymerase with dNTPs 200 μM, primers 1 μM, $MgCl_2$ 2 mM, 94ºC – 4', 40X (94ºC – 1', 55ºC – 1', 72ºC – 2'), 72ºC – 10'. Tubulin primer sequences were the following: TubulinUp, 5'-GGTGGATCTAGAACCTGGG-3' and TubulinLow, 5'-CCCAGTGAGTGGGTCAGC-3'.

4.3) RESULTS

4.3.1) Confirmation of the impact of I329L on a mouse macrophage cell line

Due to previous results in our lab regarding this gene and other genes of ASFV, it was our prediction that I329L would be functional at the level of the mouse cell. In order to test this prediction, we used a mouse macrophage cell line (RAW 264.7) focusing on two relevant transcription factors (IFN-β and NF-κB (PRD2)) and performed luciferase reporter assays on I329L transfected RAW 264.7 cells given a poly(I:C) stimulus.

A clear response of these cells to poly(I:C) was observed in control cultures. When I329L was transfected into the cells, however, this response decreased dramatically (Figure 21). Thus the ASFV gene I329L functions at the level of the mouse cell and so the construction of the I329L transgenic mouse was commenced.

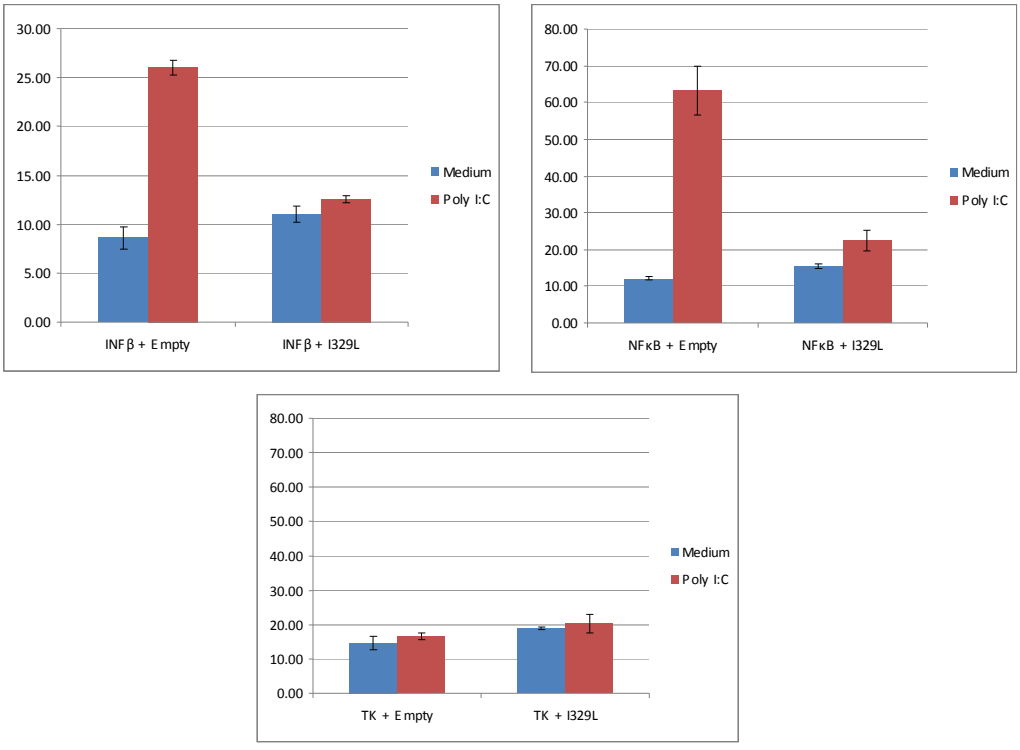

Figure 21 – I329L inhibits RAW 264.7 cell response to poly(I:C).

Figure 21 – I329L inhibits RAW 264.7 cell response to poly(I:C). The graphs show that I329L transfected cells have a decreased response to poly(I:C) stimulation, in comparison with empty plasmid transfected cells. This decrease is observed either using IFN-β and NF-κB (PRD2) luciferase reporter genes. TK minimal promoter was used as a negative control for luciferase.

4.3.2) PCR analysis of transgenic mice

One transgenic mouse, a male, was obtained from a litter of a total of eleven mice (five males and six females), after injection of fertilized eggs with the expression cassette of the Lenti-SP plasmid containing the I329L transgene and a downstream Myc immunotag.

The founder mouse was crossed with wild type FVB/N females in order to obtain F1 litters. Transgene expression was determined by PCR (Figure 22A). We observed that the founder mouse transmitted the transgene, since 7/10 animals were positive by PCR (Figure 22B).

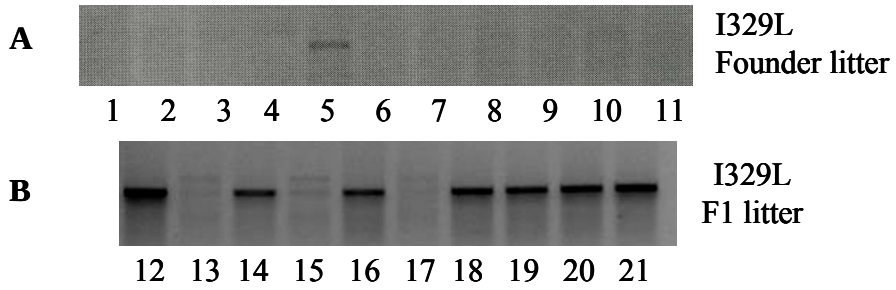

Figure 22 – PCR analysis of the founder and F1 litter mice of the I329L macrophage restricted transgenic line. A) After microinjection of the expression cassette, the litter was analysed by PCR, and one transgenic mouse was detected (number 5). **B)** The founder transgenic mouse (number 5) was then crossed with wild type females to obtain an F1 litter, that was also genotyped. In

the F1 litter we obtained 7 transgenic mice (numbers 12, 14, 16, 18, 19, 20 and 21) out of 10.

4.3.3) Proof of macrophage restricted transgene expression

Wild type and transgenic mice were sacrificed and their peritoneal cavities were washed with PBS in order to obtain macrophages. After purifying the macrophages, as described, RNA was extracted from them and a cDNA copy was prepared corresponding to the sequence of the I329L transgene.

I329L expression was only observed in macrophages of transgenic mice, and not in wild type mice (Figure 23A).

To confirm that I329L expression was specific to macrophages, wild type and transgenic mice were sacrificed and their brain, thymus, heart, lung, liver, spleen and kidneys were dissected. RNA was extracted and cDNA was synthesized for each sample. We observed no I329L expression in any of the wild type or transgenic organs. (Figure 23C).

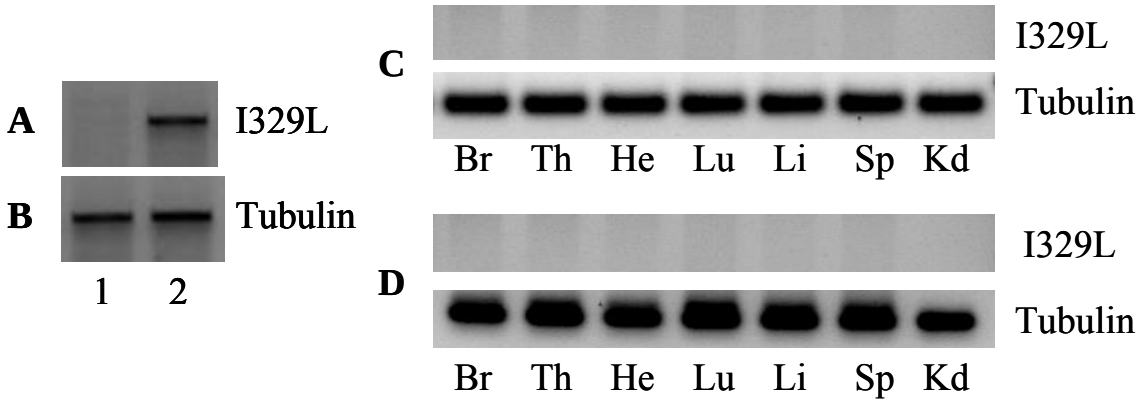

Figure 23 – Expression of I329L is specific in macrophages collected from the I329L restricted transgenic mouse line. A) RT-PCR for the I329L gene is negative for wild type macrophages (lane 1), I329L specific expression was

detected in macrophages of I329L transgenic mice (lane 2). **B)** PCR for tubulin, as a control of the quantity and quality of the cDNAs. **C)** RT-PCR is negative for the I329L gene in brain (Br), thymus (Th), heart (He), lungs (Lu), liver (Li), spleen (Sp) and kidneys (Kd) of wild type mice. **D)** RT-PCR is negative for the I329L gene in brain (Br), thymus (Th), heart (He), lungs (Lu), liver (Li), spleen (Sp) and kidneys (Kd) of I329L transgenic mice.

Tubulin was amplified as a control of the quantity and quality of the cDNAs.

4.4) DISCUSSION

Viruses have been exploiting the cell biology of their hosts for millions of years. The strategies so evolved not only provide new insight into our systems and into the pathogenesis of infection, but also provide ready made tools for genetic manipulation and provide an alternative to the established procedures for constructing conventional transgenic and gene deletion mutant eukaryotic organisms, for probing the properties of virus host evasion genes, and for manipulating the genetic programme of mammalian cells *in vivo*. This novel strategy to modify and/or manipulate gene expression in mouse models using virus host evasion genes constitutes the basis of the construction of an I329L macrophage restricted expression mouse, an African swine fever virus (ASFV) gene manipulating signaling via TLRs.

Prior to developing the macrophage restricted I329L transgenic mouse, we proved that this gene was functional at the level of a mouse macrophage. A mouse macrophage cell line (RAW 264.7 cells), was transfected with a plasmid containing the I329L gene and, after a poly(I:C) stimulus, luciferase reporter assays were done using IFN-β and NF-κB-driven luciferase reporters. The RAW 264.7 cells were observed to respond to poly(I:C) stimulus with a stimulation of both reporters. Significantly, when a plasmid containing I329L was transfected into cells, there was a clear decrease in the response with both reporters. These results conclusively demonstrated that I329L is functional in a mouse macrophage and emphasized the conserved nature of the TLR system and the corresponding virus strategy for its subversion.

In order to construct this transgenic mouse we used a plasmid constructed by Dr. Senlin Li (University of Texas Health Science Center at San Antonio), that has a synthetic promoter specific for macrophages. Since ASFV infects macrophages and the I329L gene inhibits

signal transduction for TLR3, the development of this transgenic mouse cell line is a rational approach to determine the impact of this individual gene on macrophage cell biology. The observation of the effect of the gene *in vivo* together with the findings *in* vitro, may contribute to our understanding of both the function of I329L and the innate immune response.

After I329L was cloned into this plasmid, the cassette was prepared and microinjected into mouse oocytes. One transgenic male founder mouse was obtained which was crossed with wild type mice in order to obtain F1 mice. As expected, the transgene was transmitted to the F1 offspring. The next logical step was to check that I329L was indeed being specifically expressed by macrophages, as predicted by the promoter construct. Thus, macrophages obtained through peritoneal lavage and other tissues of wild type and transgenic mice were tested for transgene expression at the mRNA level by RT-PCR. While brain, thymus, heart, lungs, liver, spleen and kidney from wild type and transgenic mice were negative, there was expression of the transgene by transgenic, but not wild type macrophages. As some of the organs examined do contain macrophages we may assume that they are not present in sufficient amounts to be detectable by the RT-PCR proceeding, whereas pure macrophage populations were, as stated above, positive.

The construction and analysis of a transgenic mouse is a complex process, time consuming and technically challenging. Much now still remains to be analysed in these transgenic mice, for example the integrity of the expression cassette and functional assays in the transgenic macrophages (see chapter 3) intact mice. Unfortunately, this work will now pass to another member of the group. Studies for the future will include the response of these transgenic macrophages to different stimuli *in vitro*, for example by upregulation of MHC Class II and production of NO. As an assessment of the impact of the transgene *in vivo*, infection models such as tuberculosis (a disease in which the bacteria replicates in macrophages), or the mouse

γ herpesvirus MHV68 which also infects macrophages [41], as well as B cells may be considered.

5) FINAL CONCLUSIONS

Viruses have been exploiting the cell biology of their hosts for millions of years. The strategies so evolved not only provide new insight into our systems and into the pathogenesis of infection, but also constitute ready made tools for genetic manipulation and thus an alternative to the established procedures for constructing conventional transgenic and gene deletion mutant eukaryotic organisms.

IFN and TLR responses are very important for innate immunity against viruses, and its inhibition confers and advantage to the virus, allowing it to survive and replicate. Viral evasion genes constitute suitable tools for the understanding and manipulation of host-pathogen interaction, allowing the development of new strategies for the control of viral infections, non-infectious diseases such as cancer, inflammatory diseases, and for gene therapy. Also, the elimination of viral evasion genes appears to be a suitable approach in the development of attenuated vaccines.

We have proved in this thesis, that the I329L gene is a non-essential gene of the ASFV and begun the construction of an I329L deletion-virus mutant. Also, we have demonstrated that this gene functions in mouse macrophages (a cell type which the virus infects in its natural porcine host) and constructed an I329L macrophage restricted-expression transgenic mouse.

The combined properties of the I329L deletion-mutant and the I329L restricted transgenic mouse will provide excellent systems to study the mechanism of action and possible utility of I329L through transcription profiles and infection models.

6) REFERENCES

1. **Akira, S.** 2003. Toll-like receptor Signaling. J. Biol. Chem. **278(40):** 38105-38108.

2. **Alcami, A., Carrascosa, A.L., Viñuela, E.** 1989. The entry of African swine fever virus into Vero cells. Virology **171:** 68-75.

3. **Alcami, A., Koszinowski, U.H.** 2000. Viral mechanisms of immune evasion. Immunol. Today **21(9):** 447-455.

4. **Alcami, A.** 2003. Viral mimicry of cytokines, chemokines and their receptors. Nat. Rev. Immunol. **3(1):** 36-50.

5. **Akira, S., Uematsu, S., Takeuchi, O.** 2006. Pathogen recognition and innate immunity. Cell **124:** 783-801.

6. **Anderson, C.L. and Looney, R.J.** 1986. Human leukocyte IgG Fc receptors Immunol Today **7:** 264-266.

7. **Baehner, R.L., Nathan D.G.** 1967. Leukocyte oxidase: defective activity in chronic granulomatous disease. Science **155(764):** 835-836.

8. **Bank, A.** 2003. Hematopoietic stem cell gene therapy: Selecting only the best. J. Clin. Invest. **112:** 1478–1480.

9. **Barton, G.M., Medzhitov, R.** 2003. Toll-like Receptor signalling Pathways. Science **300:**1524-1525.

10. Baeuerle, P.A. 1998a. IkappaB-NF-kappaB structures: at the interface of inflammation control. Cell **95(6):** 729-731.

11. Baeuerle, P.A. 1998b. Pro-inflammatory signaling: last pieces in the NF-kappaB puzzle? Curr Biol **8(1):** R19-22.

12. Bellosta, S., Mahley, R.W., Sanan, D.A., Murata, J., Newland, D.L., Taylor, J.M., Pitas, R.E. 1995. Macrophage-specific expression of human apolipoprotein E reduces atherosclerosis in hypercholesterolemic apolipoprotein E-null mice. J. Clin. Invest. **96:** 2170–2179.

13. Benedict, C.A., Norris, P.S., Ware, C.F. 2002. To kill or be killed: viral evasion of apoptosis. Nat. Immunol. **3(11):** 1013-1018.

14. Bernardes, C., Valdeira, M.L., Ramalhosantos, J., Delima, M.C. 1995. Study of fusion and uncoating mechanisms of African swine fever virus. Mol. Biol. Cell **6:** 1051-1051.

15. Blasco, R., de la Veja, I., Almazan, F., Aguero, M., Vinuela, E. 1989. Genetic variation of African swine fever virus: variable regions near the ends of the viral DNA. Virology **173:** 251-257.

16. Borca, M.V., Irusta, P., Carrillo, C., Afonso, C.L., Burrage, T., Rock, D.L. 1994a. African swine fever virus structural protein p72 contains a conformational neutralizing epitope. Virology **201:** 413-418.

17. Boyd, A., Fazakerley, J.K., and Bridgen, A. 2006. Pathogenesis of Dugbe virus infection in wild-type and interferon-deficient mice. J. G. V. **87(Pt 7):** 2005-2009.

18. Bugert, J.J. and Darai, G. 1997. Recent advances in *Molluscum contagiosum* virus research. Arch. Virol. **13:** 35-47.

19. Carrascosa, J.L., Carazo, J.M., Carrascosa, A.L., García, N., Santisteban, A., Viñuela, E. 1984. General morphology and capsid fine structure of African swine fever virus particles. Virology **132:** 160-172.

20. Cheng, G., Chen, W., Li, Z., Yan, W., Zhao, X., Xie, J., Liu, M., Zhang, H., Zhong, Y., Zheng, Z. 2006. Characterization of the porcine alpha interferon multigene family. Gene **382:** 28-38.

21. Chuang, T.H., Ulevitch, R.J. 2000. Cloning and characterization of a sub-family of human toll-like receptors: hTLR7, hTLR8 and hTLR9. Eur. Cytokine Netw. **11:** 372-378.

22. Clarke, S., Gordon, S. 1998. Myeloid-specific gene expression. J. Leuk. Biol. **63:** 153-168.

23. Cobbold, C., Whittle, J.T., Wileman, T. 1996. Involvement of the Endoplasmic Reticulum in the Assembly and Envelopment of African Swine Fever Virus. J. Virol. **70:** 8382-8390.

24. Coggins, L. .1974. African swine fever virus. Pathogenesis. Prog. Med. Virol. **18:** 48-63.

25. Colonna, M. 2007. TLR pathways and IFN-regulatory factors: To each its own. Eur. J. Immunol. **37:** 306-309.

26. Coux, O., Tanaka, K., and Goldberg, A.L. 1996. Structure and functions of the 20S and 26S proteasomes. Annu Rev Biochem **65:** 801-847.

27. Crnkovic-Mertens, I., Messerle, M., Milotic, I., Szepan, U., Kucic, N., Krmpotic, A., Jonjic, S., and Koszinowski, U.H. 1998. Virus attenuation after deletion of the cytomegalovirus Fc receptor gene is not due to antibody control. J. V. **72(2):** 1377-1382.

28. Dixon, L.K., Rock, D.L., Vinuela, E. 1995. African swine fever like viruses In Virus Taxonomy Sixth Report of the International Committee on Taxonomy of Viruses (ed. C.M.F. F.A. Murphy, D.H.L. Bishop, S.A. Ghabrial. A.W. Jarvis, G.P. Martelli, M. A. Mayo and M.D. Summers), pp 92-94, Vienna & New York.

29. Dixon, L.K., Abrams, C.C., Bowick, G., Goatley, L.C., Kay-Jackson, P.C., Chapman, D., Liverani, E., Nix, R., Silk, R., Zhang, F. 2004. African swine fever virus proteins involved in evading host defence systems. Vet. Immunol Immunopathol. **100:** 117–134.

30. Dozois, C.M., Oswald, E., Gautier, N., Serthelon, J.P., Fairbrother, J.M., Oswald, I.P. 1997. A reverse transcription-polymerase chain reaction method to analyse porcine gene expression. Vet. Immunol Immunopathol **58:** 287-300.

31. Du, X., Poltorak, A., Wei, Y., Beutler, B. 2000. Three novel mammalian toll-like receptors: gene structure, expression, and evolution. Eur. Cytokine Netw. **11:** 362-371.

32. Forman, H.J., Torres, M. 2001. Redox signaling in macrophages. Mol. Asp. Med. **22:** 189-216.

33. Fruh, K., Gruhler, A., Krishna, R.M., Schoenhals, G.J. 1999. A comparison of viral immune escape strategies targeting the MHC class I assembly pathway. Immunol. Rev. **168:** 157-166.

34. García, R., Almazán, F., Rodríguez, J.M., Alonso, M., Viñuela, E., Rodríguez, J.F. 1995. Vectors for the genetic manipulation of African swine fever vírus. J. Biotechnol. **40:** 121-131.

35. Gómez-Puertas, P., Rodríguez, F., Oviedo, J.M., Ramiro-Ibáñez, F., Ruiz-Gonzalvo, F., Alonso, C., Escribano, J.M. 1996. Neutralizing Antibodies to Different Proteins of African Swine Fever Virus Inhibit both Virus Attachment and Internalization. J. Virol. **70:** 5689-5694.

36. Gómez-Puertas, P., Escribano, J.M. 1997. Blocking antibodies inhibit complete African swine fever virus neutralization. Virus Res **49:** 115-122.

37. Gómez-Puertas, P., Oviedo, J.M., Rodriguez, F., Coll, J., and Escribano, J.M. 1997b. Neutralization susceptibility of African swine fever virus is dependent on the phospholipid composition of viral particles. Virology **228(2):** 180-189.

38. Gómez-Puertas, P., Rodríguez, F., Oviedo, J.M., Brun, A., Alonso, C., Escribano, J.M. 1998. The African swine fever virus proteins p54 and p30 are involved in two distinct steps of virus attachment and both contribute to the antibody-mediated protective immune response. Virology **243:** 461-471.

39. He, W., Qiang, M., Ma, W., Valente, A. J., Quinones, M. P., Wang, W., Reddick, R. L., Xiao, Q., Ahuja, S. S., Clark, R. A., Freeman, G. L., Li S. 2006. Development of a synthetic promoter for Macrophage Gene Therapy. Hum. Gen. Ther. **17:** 949-959.

40. Heemels, M.T. and Ploegh, H. 1995. Generation, translocation, and presentation of MHC class I-restricted peptides. Annu Rev Biochem **64:** 463-491.

41. Herskowitz, J.H., Siegel, A.M., Jacoby, M.A., Speck S.H. 2008. Systematic mutagenesis of the murine gammaherpesvirus 68 M2 protein identifies domains important for chronic infection. J. Virol. **82(7):**3295-310.

42. Hoglund, P., Olsson-Alheim, M.Y., Johansson, M., Karre, K., and Sentman, C.L. 1997. Lack of F1 anti-parental resistance in H-2b/d F1 hybrids devoid of beta2-microglobulin. Eur J Immunol **27(1):** 342-345.

43. Horvai, A., Palinski, W., Wu, H., Moulton, K.S., Kalla, K., Glass, C.K. 1995. Scavenger receptor A gene regulatory elements target gene expression to macrophages and to foam cells of atherosclerotic lesions. Proc. Natl. Acad. Sci. U.S.A. **92:** 5391–5395.

44. Ikeda, Y., Collins, M.K.L., Radcliff P.A., Mitrophanous K.A., Takeuchi, Y. 2002. Gene transduction efficiency in cells of different species by HIV and EIAV vectors. Gen. Ther. **9:**932-938.

45. Janeway, C., Travers, P., Walport, M., and Shlomchik, M. 2004. Immunobiology: The Immune System in Health and Disease. Garland Publishing, New York.

46. Jonjic, S., Mutter, W., Weiland, F., Reddehase, M.J., and Koszinowski, U.H. 1989. Site-restricted persistent cytomegalovirus infection after selective long-term depletion of CD4+ T lymphocytes. J Exp Med **169(4):** 1199-1212.

47. Jorgensen, J.L., Reay, P.A., Ehrich, E.W., and Davis, M.M. 1992. Molecular components of T-cell recognition. Annu Rev Immunol **10:** 835-873.

48. Judson, K.A., Lubinski, J.M., Jiang, M., Chang, Y., Eisenberg, R.J., Cohen, G.H., Friedman, H.M. 2003. Blocking immune evasion as a novel approach for prevention and treatment of herpes simplex virus infection. J. Virol. **148(4):** 693-706.

49. Kawai, T., Akira, S. 2007. TLR signaling. Sem. Immunol. **19:** 24-32.

50. Khanna, R., Burrows, S.R., Steigerwald-Mullen, P.M., Thomson, S.A., Kurilla, M.G., Moss, D.J. 1995. Isolation of cytotoxic T lymphocytes from healthy seropositive individuals specific for peptide epitopes from Epstein-Barr virus nuclear antigen 1: implications for viral persistence and tumor surveillance. Virology **214:** 633-637.

51. Koerner, I., Kochs, G., Kalinke, U., Weiss, S., and Staeheli, P. 2007. Protective role of beta interferon in host defense against influenza A virus. J. Virol. **81(4):** 2025-2030.

52. Kollnberger, S.D., Gutierrez-Castañeda, B., Foster-Cuevas, M., Corteyn, A., Parkhouse, R.M.E. 2002. Identification of the principal serological immunodeterminants of African swine fever virus by screening a virus cDNA library with antibody. J. Gen. Virol. **83:** 1331-1342.

53. Kotwal, G.J. 2000. Approaches of the diagnosis of hepatitis viruses. Mol Biotechnol **16(3):** 271-289.

54. Krajcsi, P., Wold, W.S. 1998. Viral proteins that regulate cellular signalling. Journal of General Virology **79:** 1323-1335.

55. Leitão, A., Cartaxeiro, C., Coelho, R., Cruz, B., Parkhouse, R.M.E., Portugal, F., Vigário, J.D., Martins, C.L. 2001. The non-haemadsorbing African swine fever virus isolate ASFV/NH/P68 provides a model for defining the protective anti-virus immune response. J. Gen. Virol. **82:** 513-523.

56. Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M., Hoffmann, J.A. 1996. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults. Cell **86:** 973-983.

57. Li, A.C., Guidez, F.R., Collier, J.G., Glass, C.K. 1998. The macrosialin promoter directs high levels of transcriptional activity in macrophages dependent on combinatorial interactions between PU.1 and c-Jun. J. Biol. Chem. **273:** 5389–5399.

58. Li, X., Eastman, E.M., Schwartz, R.J., Draghia-Akli, R. 1999. Synthetic muscle promoters: Activities exceeding naturally occurring regulatory sequences. Nat. Biotechnol. **17:** 241–245.

59. Ljunggren, H.G. and Karre, K. 1990. In search of the 'missing self': MHC molecules and NK cell recognition. Immunol Today **11(7):** 237-244.

60. Madigan, M. T., Martinko, J. M., Parker, J. 2003. Essentials of Virology, p. 231-263. *In* Madigan, M. T., Martinko, J. M., Parker, J. (ed.), Brock Biology of Microorganisms, 10th ed. Prentice Hall, Upper Saddle River, New Jersey.

61. Mao, C.P., Lin, Y.Y., Hung, C.F., Wu, T.C. 2007. Immunological research using RNA interference technology. Immunology **121(3):** 295-307.

62. Marshall-Clarke. S., Downes, J.E., Haga, I.R., Bowie, A.G., Borrow, P., Pennock, J.R., Grencis, R.K., Rothwell, P. 2007. Polyinosinic acid is a ligand for Toll-like receptor 3. J. Biol. Chem. **282(34):** 24759-24766.

63. Martins, C.L., Scholl, T., Mebus, C.A., Fisch, H., Lawman, M.J. 1988. Modulation of Porcine peripheral blood-derived macrophage functions by *in vitro* infection with African swine fever virus (ASFV) isolates of different virulence. Viral Immunol. **1:** 177.

64. McFadden, G., Lalani, A., Everett, H., Nash, P., Xu, X. 1998. Virus-encoded receptors for cytokines and chemokines. Semin Cell Dev Biol **9(3):** 359-368.

65. Miskin, J.E., Abrams, C.C., Goatley, L.C., Dixon, L.K. 1998. A viral mechanism for inhibition of the cellular phosphatase calcineurin. Science **281(5376):** 562-565.

66. Miskin, J.E., Abrams, C.C., Dixon, L.K. 2000. African swine fever virus protein A238L interacts with the cellular phosphatase calcineurin via a binding domain similar to that of NFAT. J. Virol. **74(20):** 9412-9420.

67. Montgomery, R. 1921a. On a form of swine fever occurring in British East Africa (Kenya colony). J. Comp. Pathol. **34:** 159-191.

68. Montgomery, R. 1921b. On a form of swine fever occurring in British East Africa (Kenya colony). J. Comp. Pathol. **34:** 243-262.

69. Murray, H.W., Cohn, Z.A. 1980. Macrophage oxygen-dependent antimicrobial activity. III. Enhanced oxidative metabolism as an expression of macrophage activation. J. Exp. Med. **152(6):**1596-1609.

70. Nagata, M. 2005. Inflammatory cells and oxygen radicals. Curr Drug Targets Inflamm Allergy **4(4):** 503-504.

71. Onisk, D.V., Borca, M.V., Kutish, G., Kramer, E., Irusta, P., and Rock, D.L. 1994. Passively transferred African swine fever virus antibodies protect swine against lethal infection. Virology **198(1):** 350-354.

72. Oura, C.A.L., Powell, P.P., Parkhouse, R.M.E. 1998. African Swine Fever: A disease characterized by apoptosis. J. Gen. Virol. **79:** 1427-1438.

73. Oura C.A.L., Powell P.P., Anderson E., Parkhouse R.M.E. 1998. The Pathogenesis of African Swine Fever in the resistant bushpig. J. Gen. Virol. **79:** 1439-1443.

74. Pieters, J. 1997. MHC class II restricted antigen presentation. Curr. Opin. Immunol. **9(1):** 89-96.

75. Powell, P.P., Dixon, L.K., Parkhouse, R.M.E. 1996. An IkappaB homolog encoded by African swine fever virus provides a novel mechanism for downregulation of proinflammatory cytokine responses in host macrophages. J. Virol. **70(12):** 8527-8533.

76. Revilla, Y., Callejo, M., Rodriguez, J.M., Culebras, E., Nogal, M.L., Salas, M.L., Vinuela, E., Fresno, M. 1998. Inhibition of nuclear factor kappaB activation by a virus-encoded IkappaB-like protein. J. Biol. Chem. **273(9):** 5405-5411.

77. Rodriguez, J.M., Salas, M.L., Viñuela, E. 1992a. Genes homologous to ubiquitin-conjugating proteins and eukaryotic transcription factor SII in African swine fever virus. Virology **186 (1):** 40-52.

78. Rook, G.A.W., Steele, J. Umar, S., Dockrell, H.M. 1985. A simple method for the solubilisation of reduced NBT, and its use as a colorimetric assay for activation of human macrophages by gamma-interferon. J. Immunol. Met. **82:** 161-167.

79. Sachse, C., Krausz, E., Kronke, A., Hannus, M., Walsh, A., Grabner, A., Ovcharenko, D., Dorris, D., Trudel, C., Sonnichsen, B., Echeverri, C.J. 2005. High-throughput RNA interference strategies for target discovery and validation by using synthetic short interfering RNAs: functional genomics investigations of biological pathways. Met. Enzymol. **392:** 242-277.

80. Samuel, M.A. and Diamond, M.S. 2005. Alpha/beta interferon protects against lethal West Nile virus infection by restricting cellular tropism and enhancing neuronal survival. J. Virol. **79(21):** 13350-13361.

81. Sanchez-Vizcaino, J. M., Slauson, D. O., Ruiz-Gonzalvo, F., Valero, F. 1981. Lymphocyte function and cell-mediated immunity in pigs with experimentally induced African swine fever. Am. J. Vet. Res. **42:** 1335.

82. Seth, R.B., Sun, L., and Chen, Z.J. 2006. Antiviral innate immunity pathways. Cell Res. **16(2):** 141-147.

83. Solórzano, A., Webby, R.J., Lagger, K.L., Janke, B.H., García-Sastre, A., Richt, J.A. 2005. Mutations in the NS1 protein of Swine Influenza Virus impair anti-Interferon activity and confer attenuation in pigs. J. Virol. **79(12):** 7535-7543.

84. Sontheimer, E. J. 2005. Assembly and function of RNA silencing complexes. Nat. Rev. Mol. Biol. **6(2):** 127-138.

85. Spriggs, M. K., Armitage, R. J., Comeau, M. R., Stockbine, L., Farrah, T., Macduff, B., Ulrich, D., Alderson, M. R., Mullberg, J., Cohen, J. I. 1996. The extracellular domain of the Epstein-Barr virus BZLF2 protein binds the HLA-DR beta chain and inhibits antigen presentation. J. Virol. **70:** 5557-5563.

86. Tabeta, K., Georgel, P., Janssen, E., Du, X., Hoebe, K., Crozat, K., Mudd, S., Shamel, L., Sovath, S., Goode, J. 2004. Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. Proc. Natl Acad. Sci. U. S. A **101:** 3516-3521.

87. Takeda, K., Akira, S. 2004. TLR signaling pathways. Sem. Immunol. **16:** 3-9.

88. Techau, M. E., Johansen, M. V., Aasted, B., Lind, P., Ornbjerg, N., Oswald, I. 2007. Cytokine mRNA profiles in pigs exposed prenatally and postnatally to *Shistosoma japonicum*. Vet. Res. **38:** 25-36.

89. Tortorella, D., Gewurz, B. E., Furman, M. H., Schust, D. J., Ploegh, H. L. 2000. Viral subversion of the immune system. Ann. Rev. Immunol. **18:** 861-926.

90. Townsend, A. and Bodmer, H. 1989. Antigen recognition by class I-restricted T lymphocytes. Annu Rev Immunol **7:** 601-624.

91. Trobridge, G., Beard, B.C., Kiem, H.P. 2005. Hematopoietic stem cell transduction and amplification in large animal models. Hum. Gene Ther. **16:** 1355–1366.

92. Underhill, D.M. 2003. Toll-like receptors: networking for success. Eur. J. Immunol. **33:** 1767-1775.

93. Valdeira, M.L., Bernardes, C., Cruz, B., Geraldes, A. 1998. Entry of African swine fever virus into Vero cells and uncoating. Vet. Microbiol. **60:** 131-140.

94. Vallee, I., Tait, S.W., Powell, P.P. 2001. African swine fever virus infection of porcine aortic endothelial cells leads to inhibition of inflammatory responses, activation of the thrombotic state, and apoptosis. J. Virol. **75:** 10372-10382.

95. Vasselon, T., Detmers, P.A. 2002. Toll receptors: a central element in innate immune responses. Infect. Immun. **70:** 1033-1041.

96. Villeda, C.J., Williams, S.M., Wilkinson, P.J., Vinuela, E. 1993a. Consumption coagulopathy associated with shock in acute African swine fever. Arch. Virol. **133:** 467-475.

97. Wardley, R.C., de, M.A.C., Black, D.N., de Castro Portugal, F.L., Enjuanes, L., Hess, W.R., Mebus, C., Ordas, A., Rutili, D., Sanchez Vizcaino, J., Vigario, J.D., Wilkinson, P.J., Moura Nunes, J.F., and Thomson, G. 1983. African Swine Fever virus. Brief review. Arch Virol **76(2):** 73-90.

98. Wardley, R. C., Norley, S. G., Martins, C. V., Lawman, M. J. 1987. The host response to African swine fever virus. Prog. Med. Virol. **34:** 180-192.

99. Whitman, S.C., Rateri, D.L., Szilvassy, S.J., Cornicelli, J.A., Daugherty, A. 2002. Macrophage-specific expression of class A scavenger receptors in LDL receptor $^{-/-}$ mice decreases atherosclerosis and changes spleen morphology. J. Lipid Res. **43:** 1201–1208.

100. Whittall, J. T. D., Parkhouse, R. M. E. 1997. Changes in swine macrophage phenotype after infection with African swine fever virus: cytokine production and responsiveness to interferon-γ and lipopolysaccharide. Immunology **91:** 444-449.

101. Wilkinson, P. J., Wardley, R. C., Williams, S. M. 1981. African Swine Fever Virus (Malta/78) in pigs. J. Comp. Pathol. **91:** 277-284.

102. Wilkinson, P. J. 1984. The persistence of African Swine Fever Virus in Africa and the Mediterranean. Prev. Vet. Med. **2:** 71-82.

103. Wilkinson, P.J. 1989. African swine fever virus. *In* "Virus Infections of Porcines", (M.B. Pensaert, Ed.), pp. 17-37. Elsevier Sciences Publishers B.V., Amsterdam, Holland.

104. Wolf, P.R. and Ploegh, H.L. 1995. How MHC class II molecules acquire peptide cargo: biosynthesis and trafficking through the endocytic pathway. Annu Rev Cell Dev Biol **11:** 267-306.

105. Yáñez, R. J., Rodríguez, J. M., Nogal, M. L., Yuste, L., Enríquez, C., Rodriguez, J. F., Viñuela, E. 1995. Analysis of the complete nucleotide sequence of African swine fever virus. Virology **208(1):** 249-78.

106. Yewdell, J. W., Hill, A. B. 2002. Viral interference with antigen presentation. Nat. Immunol. **3(11):** 1019-1025.

107. Zsak, L., Onisk, D.V., Afonso, C.L., and Rock, D.L. 1993. Virulent African swine fever virus isolates are neutralized by swine immune serum and by monoclonal antibodies recognizing a 72-kDa viral protein. Virology **196(2):** 596-602.

108. http://filin.vn.ua/mammels/suidae.htm

109. http://www.maladies-a-tiques.com/Argasides-et-pathologies_.htm

110. http://www.reactome.org/cgi-bin/eventbrowser?DB=test_reactome_21&ID=168898&