Universitat Autònoma de Barcelona

Unmasking African swine fever virus antigens inducing CD8+ T-cell responses with protective potential

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Tesi doctoral presentada per **Laia Bosch Camós** per accedir al grau de Doctora en el marc del programa de Doctorat en Medicina i Sanitat Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona, sota la direcció del Dr. **Fernando Rodríguez González**, el Dr. **Javier A. Collado Miguens** i el Dr. **Veljko Nikolin**, i la tutoria del Dr. **Francesc Accensi i Alemany**.

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Certifiquen:

Que la memòria titulada "**Unmasking African swine fever virus antigens inducing CD8$^+$ T-cell responses with protective potential**" presentada per **Laia Bosch Camós** per a l'obtenció del grau de Doctora en Medicina i Sanitat Animals, s'ha realitzat sota la seva supervisió i tutoria, i autoritzen la seva presentació per tal de que sigui valorada per la comissió establerta.

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LIST OF ABBREVIATIONS

APC	Antigen presenting cell
ASF	African swine fever
ASFV	African swine fever virus
bp	Base pair
CpG	Cytosine guanine dinucleotide
CSF	Classical swine fever
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Desoxiribonucleic acid
dpb	Days post-boost
dpc	Days post-challenge
dpi	Days post-infection
dpp	Days post-prime
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunosorbent spot
ER	Endoplasmic reticulum
EU	European Union
FBS	Fetal bovine serum
GEC	Genomic equivalent copies
GFP	Green fluorescent protein
HAD	Hemadsorption dose
HLA	Human leukocyte antigen

HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IC50	Median inhibition concentration
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LAV	Live attenuated virus
LC-MS/MS	Liquid chromatography-MS/MS
Log	Logarithmic
MGF	Multigene family
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
MS	Mass spectrometry
NF-κB	Nuclear factor kappa B
NK	Natural killer
OD	Optical density
OIE	World Organization for Animal Health
ORF	Open reading frame
PAMs	Porcine alveolar macrophages
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
pCMV	Citomegalovirus promoter
PCR	Polymerase chain reaction
PFU	Plaque forming units
PHA	Phytohemagglutinin
qPCR	Quantitative real-time PCR
RNA	Ribonucleic acid

RPMI	Roswell Park Memorial Institute
RT	Room temperature
SC	Secreting cells
SLA	Swine leukocyte antigen
TAP	Transporter associated with antigen processing
TBS	Tris-buffered saline
TCR	T-cell receptor
TGF	Transforming growth factor
Th	T helper
TMB	3,3′,5,5′-tetramethylbenzidine
TNF	Tumor necrosis factor
tRNA	Transfer RNA
Ub	Ubiquitin
VV	Vaccinia virus

ABSTRACT

The continuous spread of African swine fever (ASF) through Continental Europe after its introduction in Georgia in 2007, and its subsequent expansion in Asia from 2018, evidence this disease as a major threat to swine industry worldwide. ASF is a pig hemorrhagic disease of obligatory declaration to the World Organization for Animal Health (OIE) and causes enormous economic losses to the affected countries. The causative agent, African swine fever virus (ASFV), is a large, enveloped, icosahedral virus with a dsDNA genome of about 180 kbp in length. There is currently no commercial vaccine against ASFV. Early and efficient diagnosis followed by slaughtering of infected and in contact animals are the only control methods today recommended by the OIE, measures unfortunately not affordable by less favored regions.

ASF vaccine development is largely hindered by lack of knowledge about critical aspects of ASFV infection and protective immunity. In this regard, $CD8^+$ T lymphocytes have been widely shown to play a critical role in protective response against ASFV. However, the identity of the ASFV antigens capable of inducing protective $CD8^+$ T-cell responses remains largely unknown. Identification of such protective antigens could lead to rationale vaccine design as well as better understanding the mechanisms underlying ASFV immunity. Therefore, the present thesis aimed to determine ASFV proteins containing $CD8^+$ T-cell epitopes with potential to elicit protective responses against the Georgia2007/1 ASFV, the isolate currently circulating in Continental Europe and Asia.

Different methodologies and strategies have been explored aiming to identify both ASFV-specific $CD8^+$ T-cell epitopes and full-length proteins encoding promiscuously recognized $CD8^+$ T-cell epitopes with protective potential.

In order to identify ASFV-specific $CD8^+$ T-cell epitopes, a double strategy was employed: i) a multiparametric bioinformatics analysis using the Georgia2007/1 proteome as template; and ii) an immunopeptidomic approach based on the analysis of SLA I-bound peptides found in porcine alveolar macrophages *in vitro* infected with ASFV. The results observed when evaluating the *in vitro* recognition of the peptides by ASFV-specific PBMCs obtained from ASF-recovered pigs, suggested immunopeptidomics analysis as a more reliable strategy than *in silico* predictions for the identification of ASFV $CD8^+$ T-cell epitopes. As expected, peptides were not promiscuously recognized by PBMCs from all animals, confirming their marked restriction for specific SLA I alleles.

Further analysis using full-length proteins allowed determining few ASFV antigens promiscuously recognized by ASFV-specific PBMCs. Thus, stimulation of ASFV-specific PBMCs with autologous fibroblasts transiently transfected with plasmids encoding full-length ASFV ORFs fused to ubiquitin to improve SLA I antigen presentation, led to the identification of ASFV proteins MGF505-7R, M448R, CP312R and D1133L as immunodominant and promiscuously recognized antigens by ASFV-specific $CD8^+$ T cells. Finally, DNA immunization experiments demonstrated the protective potential of the ASFV antigens here identified against the Georgia2007/1 ASFV challenge.

Sterilizing protection against ASFV most likely will require a broad repertoire of B and T-cell specificities and thus, further investigations will be needed to determine other ASFV antigens eliciting protective responses. Likewise, it will most likely be indispensable the use of alternative expression platforms to encode the potential vaccine antigens, aiming to induce more solid immune response than that afforded by DNA vaccines, an ideal tool for antigen discovery but far from optimal for final veterinary vaccine formulations.

RESUM

La contínua propagació de la pesta porcina africana (PPA) a l'Europa continental després de la seva introducció a Georgia l'any 2007, així com la subseqüent expansió a Àsia a partir del 2018, posa en evidència la gran amenaça que aquesta malaltia representa per la indústria porcina arreu del món. La PPA és una malaltia hemorràgica porcina de declaració obligatòria a l'Organització Mundial de Sanitat Animal (OIE) que provoca enormes pèrdues econòmiques als països afectats. L'agent causant, el virus de la pesta porcina africana (VPPA), és un virus de grans dimensions, embolcallat i amb simetria icosaèdrica, amb un genoma d'ADN de doble cadena d'unes 180 kpb de longitud. Actualment no es disposa de vacuna comercial contra el VPPA. El sistema de control recomanat per la OIE es basa en un diagnòstic primerenc i eficient, seguit pel sacrifici dels animals infectats o potencialment en contacte amb el virus, mesures poc assequibles en regions menys afavorides.

El desenvolupament de vacunes contra la PPA es veu dificultat en gran part per la manca de coneixement sobre aspectes crítics de la infecció i de la immunitat protectora contra el VPPA. En aquest sentit, s'ha demostrat clarament que els limfòcits T $CD8^+$ juguen un paper clau en la resposta protectora contra el VPPA. No obstant això, els antígens del virus capaços d'induir respostes T $CD8^+$ protectores segueixen majoritàriament sense haver estat descrits. La identificació d'aquests antígens protectors podria facilitar el disseny racional de vacunes, així com l'enteniment dels mecanismes subjacents a la immunitat contra el VPPA. Així doncs, l'objectiu principal de la present tesi era la caracterització de proteïnes del VPPA que continguessin

epítops T CD8$^+$ amb potencial protector contra la soca Georgia2007/1 del VPPA, actualment circulant per Europa continental i Àsia.

S'han explorat diferents metodologies i estratègies amb l'objectiu d'identificar tant epítops T CD8$^+$ com proteïnes enteres del VPPA codificant epítops T CD8$^+$ promíscuament reconeguts i amb potencial protector.

Per a la identificació d'epítops T CD8$^+$ específics contra el VPPA, es va utilitzar una estratègia doble: i) una anàlisi bioinformàtica multiparamètrica emprant el proteoma de la soca Georgia2007/1 com a patró; i ii) un estudi immunopeptidòmic basat en l'anàlisi de pèptids units a les molècules de SLA I en macròfags alveolars porcins infectats *in vitro* amb VPPA. Els resultats obtinguts a partir d'assajos *in vitro* realitzats amb PBMCs de porcs recuperats de la infecció amb VPPA han permès definir els estudis immunopeptidòmics com a una estratègia més fiable que les prediccions *in silico* per a la identificació d'epítops T CD8$^+$ del VPPA. Tal com s'esperava, els pèptids no van ser promíscuament reconeguts per PBMCs de tots els animals, confirmant la seva marcada restricció per al·lels específics de SLA I.

Anàlisis posteriors utilitzant proteïnes completes van permetre la caracterització d'una sèrie d'antígens del VPPA capaços de ser reconeguts promíscuament per PBMCs amb resposta específica contra el VPPA. Així, l'estimulació d'aquestes cèl·lules amb fibroblasts autòlegs transitòriament transfectats amb plasmidis codificant ORFs completes del VPPA fusionades a ubiquitina, per millorar la presentació antigènica per classe I, va permetre la identificació de les proteïnes MGF505-7R, M448R, CP312R i D1133L del VPPA com a antígens immunodominants i promíscuament reconeguts per cèl·lules T CD8$^+$ específiques del VPPA. Finalment, experiments

d'immunització amb ADN van demostrar el potencial protector dels antígens identificats contra la infecció experimental amb una dosi letal de la soca Georgia2007/1 del VPPA.

La protecció total contra el VPPA molt probablement requereixi un ampli repertori d'especificitats de cèl·lules B i T, i per tant caldrà seguir aprofundint en l'estudi i caracterització d'antígens del VPPA amb potencial protector. De la mateixa manera, serà necessari explorar plataformes d'expressió alternatives que permetin obtenir respostes immunitàries més robustes que les conferides per les vacunes d'ADN, una eina ideal per a la investigació bàsica però que resta lluny de ser òptima com a formulació vacunal final d'ús veterinari.

RESUMEN

La continua propagación de la peste porcina africana (PPA) en la Europa continental desde su introducción en Georgia en el año 2007, así como su subsecuente expansión a Asia a partir del 2018, pone en evidencia la gran amenaza que esta enfermedad representa para la industria porcina a nivel mundial. La PPA es una enfermedad hemorrágica porcina de declaración obligatoria a la Organización Mundial de Sanidad Animal (OIE) que provoca enormes pérdidas económicas en los países afectados. El agente causante, el virus de la peste porcina africana (VPPA), es un virus de gran tamaño, envuelto y con simetría icosaédrica, con un genoma de ADN de doble cadena de unas 180 kpb de longitud. Actualmente no se dispone de vacuna comercial contra el VPPA. El sistema de control recomendado por la OIE se basa en un diagnóstico temprano y eficaz, seguido por el sacrificio de los animales infectados o potencialmente en contacto con el virus, medidas poco asequibles en regiones menos favorecidas.

El desarrollo de vacunas contra la PPA se ve dificultado en gran parte por la falta de conocimiento sobre aspectos críticos de la infección y de la inmunidad protectora contra el virus. En este sentido, se ha demostrado claramente que los linfocitos T $CD8^+$ juegan un papel clave en la respuesta protectora contra el VPPA. No obstante, los antígenos del virus capaces de inducir respuestas T $CD8^+$ protectoras siguen en gran parte sin haber sido descritods. La identificación de estos antígenos protectores podría facilitar el diseño racional de vacunas, así como el entendimiento de los mecanismos subyacentes a la inmunidad contra el VPPA. Así pues, el objetivo principal de la presente tesis fue la caracterización de proteínas del VPPA que contuvieran epítopos T $CD8^+$ con potencial de protector contra la cepa

Georgia2007/1 del VPPA, actualmente circulando en Europa continental y Asia.

Distintas metodologías y estrategias se han explorado con el objetivo de identificar tanto epítopos T $CD8^+$ como proteínas enteras del VPPA que codificaran epítopos T $CD8^+$ promiscuamente reconocidos y con potencial protector.

Para la identificación de epítopos T $CD8^+$ específicos contra el VPPA, se empleó una estrategia doble: i) un análisis bioinformático multiparamétrico utilizando el proteoma de la cepa Georgia2007/1 como patrón; y ii) un estudio inmunopeptidómico basado en el análisis de péptidos unidos a las moléculas de SLA I en macrófagos alveolares porcinos infectados *in vitro* con VPPA. Los resultados obtenidos a partir de ensayos *in vitro* realizados con PBMCs de cerdos recuperados de la infección por VPPA han permitido definir a los estudios immunopeptidómicos como una una estrategia más fiable que las predicciones *in silico* para la identificación de epítopos T $CD8^+$ del VPPA. Como era esperado, los péptidos no fueron promiscuamente reconocidos por PBMCs de todos los animales, confirmando su marcada restricción por alelos específicos de SLA I.

Análisis posteriores usando proteínas enteras permitieron la caracterización de una serie de antígenos del VPPA con capacidad para ser reconocidos promiscuamente por PBMCs con respuesta específica contra el VPPA. Así, la estimulación de estas células con fibroblastos autólogos transitoriamente transfectados con plásmidos codificando ORFs completas del VPPA fusionadas a ubiquitina, para mejorar su presentación antigénica por clase I, permitió la caracterización de las proteínas MGF505-7R, M448R, CP312R y

D1133L del VPPA como antígenos inmunodominantes y promiscuamente reconocidos por células T $CD8^+$ específicas del VPPA. Finalmente, experimentos de inmunización con ADN demostraron el potencial protector de los antígenos identificados, contra la infección experimental con una dosis letal de la cepa Georgia2007/1 del VPPA.

La protección total contra el VPPA muy probablemente requiera un amplio repertorio de especificidades de células B y T, por lo que será fundamental seguir profundizando en el estudio y caracterización de antígenos del VPPA con potencial protector. Del mismo modo, será necesario explorar plataformas de expresión alternativas que permitan obtener respuestas inmunitarias más robustas que las conferidas por las vacunas de ADN, una herramienta ideal para la investigación básica, pero lejos de ser óptima como formulación vacunal final de uso veterinario.

CHAPTER 1

General Introduction

1.1. AFRICAN SWINE FEVER (ASF)

1.1.1. History and epidemiology of ASF

African swine fever (ASF) is a contagious hemorrhagic disease of pigs with lethality rates up to 100% in its acute forms. Due to the devastating impact of the disease, ASF is included in the *Terrestrial Animal Health Code* of the World Organization for Animal Health (OIE) as a notifiable disease. To date, no safe and effective vaccine against ASF is available and there is no control strategy other than early diagnosis, animal quarantine and slaughter.

R. Eustace Montgomery released the first publication about ASF in 1921, describing outbreaks of a hemorrhagic disease affecting European domestic pigs introduced to Kenya between 1910 and 1917. The disease showed a mortality of 98.9% and at first, it was described as a hyperacute form of classical swine fever (CSF), due to the similar lesions found in both diseases. Pigs recovered from CSF were however still vulnerable to the new disease, thus evidencing that another pathogen was responsible for it (Montgomery, 1921). The causative agent of the disease, African swine fever virus (ASFV), was first isolated from infected wild suids in 1932 (Steyn, 1932). Although the first ASF outbreaks were reported in Eastern and Southern Africa, spreading of the disease to Central and West Africa occurred, and it remains enzootic in many sub-Saharan countries.

First identification of ASFV in non-African countries was described in 1957 in Portugal. Although this first introduction was controlled, in 1960 the virus re-entered Portugal and this time, the disease spread to the whole Iberian Peninsula, remaining enzootic in the area for more than 30 years (Arias and Sánchez-Vizcaíno, 2002). That period gave rise to many sporadic outbreaks

in other European countries including France (1964, 1967, 1977), Italy (1967, 1980), Malta (1978), Belgium (1985) and the Netherlands (1986). Several countries in the Caribbean region also reported ASF (reviewed in Costard et al., 2009), Cuba being the first country affected in the area in 1971 to spread later on to Dominican Republic (1978) and Haiti (1979). In 1978, ASF was reported in Brazil, where it was probably introduced from the Iberian Peninsula through infected food waste carried by transcontinental flights and/or animal products imported by tourists (Lyra, 2006). Eradication of ASF in all these regions was achieved by exhaustive and expensive control programs consisting on early and efficient diagnosis, followed by massive slaughtering of the infected and potentially exposed animals, along with reasonable compensation policies to the affected farmers (Arias and Sánchez-Vizcaíno, 2002; Lyra, 2006).

After its eradication from Continental Europe in 1995, ASF remained endemic in the Italian island of Sardinia since 1978 up to today (Costard et al., 2013) and in sub-Saharan Africa, where the disease has remained endemic, causing enormous economic losses since it was described in 1921. The epidemiologic pressure of ASFV in sub-Saharan countries led to the spread of ASF to previously uninfected East-African countries and, from there, to Europe again, this time entering from Eastern Europe. Thus, in 2007, ASFV was confirmed in pigs from Georgia, presumably fed with ASFV-contaminated pork products through improper disposal of waste from shipping. The Georgian ASFV was closely related to isolates circulating in Mozambique, Madagascar and Zambia at that time (Rowlands et al., 2008). Within months, ASFV spread to neighboring states causing outbreaks in Armenia, Azerbaijan and the Russian Federation (Beltran-Alcrudo et al., 2008). Expansion of the disease continued with reported cases in Ukraine

(2012) and Belarus (2013), and finally reaching the European Union (EU) in 2014, when affected wild boars were found in Lithuania and Poland. Since then, ASFV has also been diagnosed in Estonia and Latvia (2014), and Moldova confirmed the first ASF-positive samples in 2016. In 2017, ASF was reported in backyard farms in Romania, and in wild pig carcasses found in the Czech Republic. Infected wild boar carcasses were declared in Hungary in April 2018, and also in Belgium in September 2018, 20 km away from both Luxemburg and France.

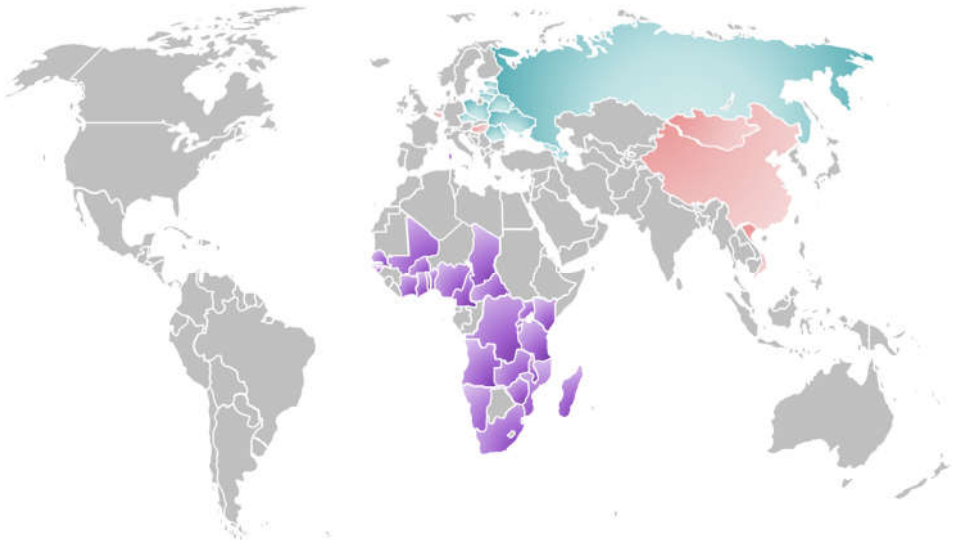


Figure 1-1. ASF-affected regions as of February 2019. Colored in purple, enzootic regions; in blue, countries that declared outbreaks after 2007; in red, countries reporting the first ASF cases in 2018 or 2019.

ASF has recently reached China, the world's largest pork producer. In August 2018, the first ASF outbreak was reported in the northeastern city of Shenyang, and it rapidly spread throughout the country (Wang et al., 2018; Zhou et al., 2018). ASFV has likewise been declared in Mongolia (January

2019) and Vietnam (February 2019). The sequence of the ASFV circulating in Asia is closely related to the Georgia2007/1 isolate (Ge et al., 2018), thereby pointing an ASF-affected Eastern European country as a potential source for the initial infection, although how the virus was introduced in China has not been elucidated yet (Li and Tian, 2018).

Despite the homology of the ASFV isolates circulating in Continental Europe and Asia, the epidemiologic situation of the disease in both areas is very different. In Continental Europe, the spread of ASFV is mainly wild boar-mediated, while most of the outbreaks declared in Asia so far affected domestic pigs, resulting in massive culling in the affected farms. Around one million pigs have been sacrificed up to today, and some estimations consider that half of the pork production in China will be lost due to ASFV.

As observed in the EU in the late 1990s, eradication programs based on efficient diagnosis and culling infected and potentially infected animals could be successfully implemented. Notwithstanding, a key aspect of that eradication strategy was an immediate and adequate economic compensation to the farmers (Arias and Sánchez-Vizcaíno, 2002). The inherent drawback of this strategy is the impossibility of implementation in resource-poor countries, where such a compensation policy in the event of culling is not affordable. In these cases, enhanced biosecurity remains the main ASF control measure.

ASFV is currently the most threatening virus for the swine industry worldwide. Serious socio-economic repercussions are associated to an ASF outbreak, especially in regions where a large proportion of pigs and pork products are exported. An ASF outbreak directly affects the economy of

affected countries due to the loss of production and profit as well as high costs of compensation for animal slaughtering, but also indirectly, due to national and international trade restrictions. Moreover, disruption of the pig trade network indirectly affects other commercial activities such as seed and grain industries. Development of an effective and safe vaccine against ASF would allow for a better control of the disease and eventually lead to the mitigation of the severe socio-economic consequences that an ASF outbreak currently represents.

1.1.2. Clinical presentation of ASF

ASF clinical signs and lesions vary depending on the virulence of the virus isolate, the route and dose of infection, and the host characteristics (Sánchez-Vizcaíno et al., 2015).

In the peracute form of the disease, generally induced by highly virulent virus strains, symptoms include high fever (41-42ºC), loss of appetite, inactivity, hyperpnoea and cutaneous hyperaemia. Animal death occur 1-4 days post-infection (dpi), with not enough time for development of evident lesions in organs (Sánchez-Vizcaíno et al., 2015).

Acute ASF, which is caused by highly or moderately virulent isolates and develops over a 7-day period, is the most usual form of the disease. It is characterized by fever (40-42ºC), manifested by loss of appetite, depression, apathy, and a tendency to huddling. Typical lesions of affected pigs include erythema, cyanosis, and pulmonary oedema, as well as hyperaemic splenomegaly, and hemorrhages in different organs including lymph nodes and kidneys (Gómez-Villamandos et al., 1995).

A subacute form of the disease is associated with moderately virulent isolates. Clinical signs are similar to those in the acute form of the disease albeit less marked, and affected animals die after 7-20 dpi. In this case, mortality rate ranges from 30 to 70% and recovered pigs can still excrete the virus up to 6 weeks after infection (Gómez-Villamandos et al., 2013, 1998).

Low virulence strains are responsible for chronic forms of ASF. No specific clinical signs are associated to this form, although affected animals display necrotic lesions of the skin and arthritis (Sánchez Botija, 1982). Infected pigs act as carrier individuals able to spread the virus for long periods of time, contributing to the persistence of the disease (Arias and Sánchez-Vizcaíno, 2002).

1.1.3. Transmission of ASFV

Wild African suids including warthogs, bush pigs, and giant forest hogs are susceptible to ASFV infection. In these wildlife populations ASF pathogenesis is characterized by an unapparent infection with transient, low viremic titers (Costard et al., 2013). Horizontal or vertical transmission is thought not to efficiently occur between these hosts, serving soft ticks of the genus *Ornithodoros* as vectors for virus transmission. Ticks remain infected for long periods of time, and transstadial, transovarial, and sexual transmission have been reported within them (Plowright et al., 1969; Sánchez Botija, 1963). Thus, they can play a main role in maintaining ASFV even without the presence of viremic hosts (EFSA Panel on Animal and Welfare, 2010).

ASFV is transmitted to domestic pigs by tick vectors, indirect contact through infected fomites, or direct contact with other infected swine (Costard et al.,

2013). After its reintroduction into Europe in 2007, the disease circulates among domestic pigs and European wild boars. The susceptibility of the European wild boar population to the disease, whose symptomatology is similar to those in domestic pigs, complicates controlling the transboundary spread of ASF (Gogin et al., 2013). Gaining knowledge on wild boars populations is therefore crucial for risk assessment on ASF in Europe and rational preventive measures. Additionally, it has been recently demonstrated that ingestion of flies previously fed on blood from ASFV-infected swine can lead to development of ASF in farm pigs (Olesen et al., 2018), albeit the relevance of this route of infection in natural conditions has not been confirmed.

Despite the fact that some recent outbreaks within Europe can be explained by transmission of the virus through the wild boar population, human-mediated transmission is presumed to have an active role in the spread of ASFV. Awareness should be promoted on ASFV transmission mechanisms as well as the socio-economic consequences of the disease in all the people potentially in contact with infected swine in order to diminish the human activities contributing to ASF spread (Depner et al., 2017).

1.2. AFRICAN SWINE FEVER VIRUS (ASFV)

African swine fever virus (ASFV) is a large enveloped, icosahedral, double-stranded DNA-virus. ASFV is classified as an asfarvirus (family *Asfarviridae*, genus *Asfivirus*), being the sole member of this family and included in the nucleocytoplasmic large DNA virus superfamily (Alonso et al., 2018). The

icosahedral symmetry of the ASFV is similar to that of iridoviruses, while the genomic organization shows a clear analogy to poxviruses.

ASFV is the only known DNA arbovirus, being able to infect soft ticks of the genus *Ornithodoros*. It is stable at a wide range of temperatures and pH and is capable of remaining infectious in feces, tissue and environment for long periods (reviewed in Bellini, Rutili and Guberti, 2016).

To date, 24 different ASFV field genotypes have been identified based on sequencing of the variable C-terminus of the B646L gene, coding for the major capsid protein p72 (Achenbach et al., 2017; Quembo et al., 2018). Genotype I is mainly localized in West Africa, and was the one introduced into Europe in the 1990s, while all the other genotypes have been reported to be present in Southeastern Africa (Brown et al., 2018). The ASFV currently circulating within Continental Europe after its reintroduction through Georgia in the 2007, and more recently reported in Asia, belongs to the genotype II (Costard et al., 2009; Ge et al., 2018). Additionally, eight serogroups have been described based on the hemadsorption inhibition activity of the isolates. While the conventional ASFV phylogenetic analysis cannot be correlated with different degrees of virulence or with cross-protection, the hemadsorption inhibition test seems to better discern biologically relevant phenotypes (Malogolovkin et al., 2015). Anyway, at present there is no analysis for accurately define virus virulence among ASFV isolates or anticipate the protection elicited by an experimental vaccine.

1.2.1. Structure of the ASFV virion

Electron microscope examinations showed that ASFV particles are about 200 nm in diameter and have a complex structure with an overall icosahedral shape. Accurate inspection of the virions identifies several concentric layers with distinct electron densities (Figure 1-2). In the center, an electron-dense nucleocapsid core of 110 nm is distinguished. This structure consists of a thick protein layer of about 30 nm designated core shell enwrapping the nucleoid, which contains not only the viral genome but also enzymes and other proteins essential during the initial phase of the infection. Surrounding the nucleocapsid, there is an internal lipid envelope coming from the endoplasmic reticulum (ER) (Andrés et al., 1998; Suárez et al., 2015), and a protein capsid (Andrés et al., 1997), which is the outermost shell of intracellular viruses. The capsid displays an hexagonal pattern and is composed by 1892 to 2172 capsomers with the appearance of hexagonal prisms with a length of 13 nm, a wide of 5-6 nm and a central hole (Carrascosa et al., 1984). Extracellular virions acquire an external lipid envelope during the budding process for virus exit from the cell (Breese and DeBoer, 1966), ending up as a particle of 175-215 nm of diameter (Almeida et al., 1967; Carrascosa et al., 1984). This outer envelope surrounds loosely the capsid (Arzuza et al., 1992).

By two-dimensional analysis of purified extracellular virus, 54 polypeptides were identified (Esteves et al., 1986), and the localization of some of them has been settled by immunoelectron microscopy (Salas and Andrés, 2013). More recently, mass spectrometry (MS)-based techniques have allowed the characterization of novel proteins in the ASFV particle, including host proteins (Alejo et al., 2018).

Two important viral proteins have been reported to be located in the outer envelope: protein p12 involved in attachment to the Vero cells (Carrascosa et al., 1991) and protein pEP402R or CD2v, which is the virus homologue of cellular T-cell adhesion receptor CD2 and mediates the hemadsorption of infected cells (Rodríguez et al., 1993). In accordance with its addition as intracellular particles bud in order to exit the cell, the morphology of the external envelope is similar to the plasma membrane and even a host protein named p24 with a putative role in the budding event is incorporated in the virus particles (Sanz et al., 1985).

The major structural protein of ASFV is the capsid protein p72, which is encoded by the B646L gene and represents 35% of the protein mass of the virus particle (García-Escudero et al., 1998). Protein pE120R is also part of the capsid and it is implicated in the movement of mature virions from the assembly sites to the plasma membrane (Andrés et al., 2001b). Another structural protein included in the capsid is pB438L (p49), which is thought to play a role in the construction or stabilization of the icosahedral vertices of the virus particle (Alejo et al., 2018; Epifano et al., 2006a).

The inner envelope seems to be derived from an ER cisterna by a wrapping mechanism (Andrés et al., 1998; Salas and Andrés, 2013), though it remains a controversial issue. Membrane proteins of this layer include p54 (pE183R), p17 (ORF D117L), pE248R, pH108R, pE199L, p22 (pKP177L) and the attachment protein p12 (pO61R) (Alejo et al., 2018; Brookes et al., 1998; Hernáez et al., 2016; Suárez et al., 2010). These proteins are involved in the assembly process or virus entry to the host cell.

Beneath the inner viral envelope, there is the core shell, a thick protein layer of about 30 nm (Andrés et al., 1997). Besides its structural function, the core shell might serve as a barrier to protect the viral genome from potentially destructive agents, such as extracellular or endosomal nucleases. This domain is basically constituted by the eight major structural proteins derived from polyproteins pp220 and pp62 processing, catalyzed by the viral cysteine proteinase pS273R, which is likewise a component of the core shell (Alejo et al., 2003; Andrés et al., 2001a). After proteolytic processing, polyprotein pp220 gives rise to proteins p150, p37, p34, p14 and p5, and pp62 is processed into p35, p15 and p8 (Alejo et al., 2018; Simón-Mateo et al., 1997, 1993). All these products are present in equimolecular amounts in the virus particle and account for 32% of the virion protein mass (Alejo et al., 2018; Andrés et al., 2002a). Polyprotein processing is an unusual feature of ASFV, since it is a common strategy of gene expression in many positive-strand RNA viruses and retroviruses.

In the DNA-containing nucleoid, two major DNA-binding proteins have been identified: protein p10 (Alejo et al., 2018; Andrés et al., 2002b; Muñoz et al., 1993) and bacterial-histone like protein pA104R (Andrés et al., 2002b; Borca et al., 1996; Frouco et al., 2017). In addition, the nucleoid contains the transcriptional machinery for the synthesis and modification of early RNAs, including the DNA-dependent RNA polymerase, the poly(A) polymerase (pC475L), capping enzymes and early transcription factors (Alejo et al., 2018; Salas, 1999). Enzymes presumably involved in maintenance of genome integrity and modulating host response have also been localized within the nucleoid (Alejo et al., 2018).

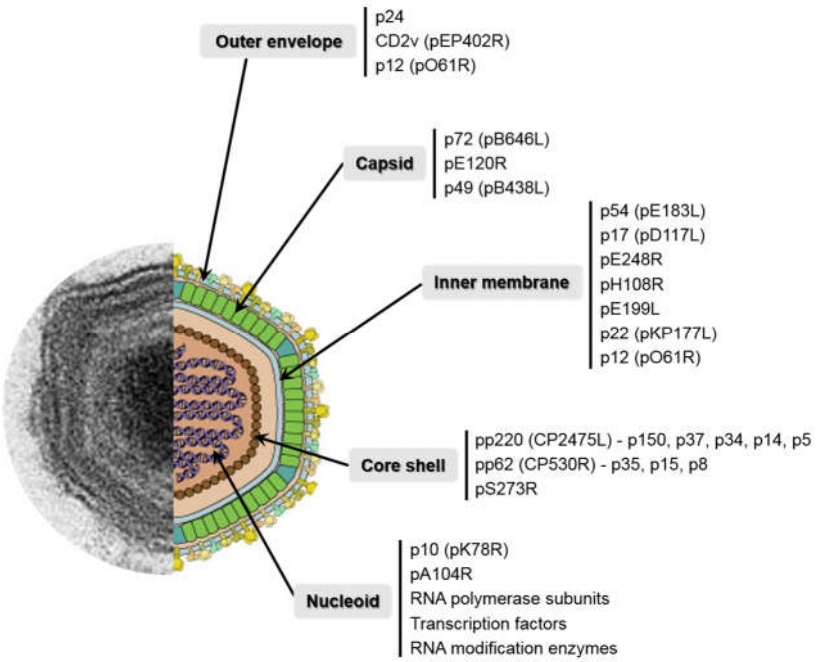


Figure 1-2. Electron microscopy image and schematic representation of the ASFV virion.

ASFV genome is constituted by a single molecule of linear double-stranded DNA that varies in length from about 170 to 193 kbp depending on the isolate. ASFV codes for more than 150 open reading frames (ORFs), which are separated by short intergenic regions and read from both DNA strands (Yáñez et al., 1995). The virus genome termini are covalently closed by imperfectly base-paired hairpin loops which are inverted and complementary, as in the case of poxviruses (González et al., 1986). Adjacent to the hairpins there are terminal inverted repeats, identical at both DNA ends (de la Vega et al., 1994), and multigene families (MGF), which are believed to have arisen by duplication and variation of a single ancestral gene (Sogo et al., 1984). Five MGFs have been described in AFSV: MGF100, 110, 300, 360, and 505/530, being located within the left terminal 40 kbp and right terminal 20 kbp (Sumption et al., 1990). The number of genes which belong

to each family varies considerably among isolates, thus accounting for the variability in the genome size (Almazán et al., 1992; de la Vega et al., 1990).

1.2.2. ASFV infection in pigs

When ticks are not involved, ASFV infects pigs using the oronasal route, replicating first in tonsils or dorsal pharyngeal mucosa, and spreading to the mandibular or retropharyngeal lymph nodes, provoking the subsequent viremia (Plowright et al., 1994). Newborn piglets experimentally infected with acute ASFV strains can show an accelerated course of infection and primary viremia can be detected as early as 8 hours post-infection, while secondary viremia can occur from 15 to 24 hours post-infection. After 30 hours, virus is found in almost all organs (Colgrove et al., 1969), and ASFV titer in the blood may reach 10^8 HAD_{50}/mL in acute cases.

The main target of ASFV is the mononuclear phagocytic system (Oura et al., 1998a; Pan, 1987), being those cells of advanced maturation stages (expressing higher levels of macrophage-specific markers and SLA-II antigens) most susceptible to infection (Sánchez-Torres et al., 2003). Macrophages act in the host as antigen presenting cells (APCs), thus their extensive destruction compromise the immune system of the host and may be a key event of the disease pathogenesis (Penrith et al., 2004). Severe lymphopenia is also seen in infected animals due to bystander apoptosis of uninfected lymphocytes, which is associated with production of pro-inflammatory cytokines by infected macrophages (Gómez-Villamandos et al., 2003, 1995; Oura et al., 1998b). Some ASFV isolates present hemadsorbing activity and hence during viremia are found associated with erythrocytes

(Quintero et al., 1986; Wardley and Wilkinson, 1977), but also with lymphocytes and neutrophils (Plowright et al., 1994).

Lymphoid organs are the first place where infection and depletion of monocytes/macrophages is found, especially lymph nodes and spleen (Ramiro-Ibáñez et al., 1997; F. Rodríguez et al., 1996). In final phases of the disease, other cell types have been seen to support virus replication, including megakaryocytes (Edwards et al., 1984), platelets (Gómez-Villamandos et al., 1996), tonsilar epithelial cells (Gómez-Villamandos et al., 1997), hepatocytes, kidney cells, endothelial cells (Gómez-Villamandos et al., 1995), neutrophils (Carrasco et al., 1996b) and lymphocytes (Ballester et al., 2010; Carrasco et al., 1996a).

1.2.3. ASFV infection cycle in susceptible cells

The mechanism by which ASFV enters susceptible cells has not yet been unmasked. Many studies have been performed in stable cell lines with adapted ASFV strains (instead of using porcine macrophages, the ASFV natural target cell), opening the floor for the discussion about their validity. Early studies stated a receptor-mediated endocytosis mechanism for ASFV entry in Vero cells (Alcamí et al., 1990, 1989), yet recent studies have provided contradictory evidences for an alternative micropinocytosis-like mechanism (Sánchez et al., 2012), or a clathrin-dependent endocytosis mechanism (Hernáez and Alonso, 2010). More recently, ASFV entrance by both constitutive macropinocytosis and clathrin-mediated endocytosis has been described (Hernáez et al., 2016). After crossing the plasma membrane, ASFV takes advantage of the endocytic pathway. Gradual acidification of the endosomes lumen is required for successful infection, since disruption of the

outer membrane and desencapsidation depend on the acid pH of late endosomes compartments (Alonso et al., 2013). Desencapsidation leads to exposition of the inner viral envelope, which can fuse with the membrane of late multivesicular endosomes and deliver the genome-containing naked cores into the cytosol (Hernáez et al., 2016).

ASFV core particles are charged with all the enzymes and factors needed for early gene transcription (Salas, 1999), and enzymes required for DNA replication are synthesized just after virus entry into the cytoplasm (Dixon et al., 2013). Thus, ASFV particles carry all the necessary for self-replication and transcription, theoretically without requiring cellular machinery. Nevertheless, there is proof of viral DNA replication within the host cell nucleus during an initial stage of the infection (García-Beato et al., 1992; Tabares and Sánchez Botija, 1979). During this early phase, small DNA fragments are synthesized and accumulated in the nucleus, and they are believed to eventually serve as precursors of the mature cross-linked viral DNA that assemble in discrete perinuclear structures, where the rest of DNA replication and virus morphogenesis occurs (Rojo et al., 1999). Supporting the existence of early nuclear interactions, a later study by our group described the rearrangement and modification of several nuclear host components early after ASFV infection (Ballester et al., 2011). Disruption of the nuclear envelope and degradation of the RNA polymerase II were observed, perhaps being a viral strategy to control the cell cycle.

Leaving aside this early nuclear stage, ASFV replication and morphogenesis occur predominantly in the cytoplasm in specialized perinuclear areas called viral factories (Breese and DeBoer, 1966), beginning about 6 hours post-infection (Dixon et al., 2013). Virion assembly is thought to begin with the

recruitment of ER cisternae to viral factories, a process dependent on the presence of the structural protein p54 (Rodríguez et al., 2004), and probably the MGF 110 member pXP124L (Netherton et al., 2004). These modified ER-derived membranes will be included as an inner viral envelope, over which the icosahedral capsid, mainly composed of protein p72, is assembled (Cobbold and Wileman, 1998; García-Escudero et al., 1998). Correct folding and aggregation of p72 require the action of the viral chaperone pB602L (Cobbold et al., 2001; Epifano et al., 2006a), as well as protein pB438L, crucial for the formation of the icosahedral vertices of the capsid (Epifano et al., 2006). The newly formed virions are finally transported from the viral factories to the cell membrane in a process dependent on the microtubule network (Hernáez et al., 2006; Jouvenet et al., 2004), despite the mechanisms are not known with certainty. During viral egress, virions acquire their outer envelope, which comes from the cytoplasmic membrane of the infected cell (Andrés et al., 2001b).

1.3. INTERACTION OF ASFV WITH THE HOST IMMUNE SYSTEM

Evidence that protective immunity can be developed against ASFV is reflected in warthogs and bush pigs, the natural hosts, which do not suffer from the disease. Moreover, pigs surviving infection with low or moderate virulent strains are protected from challenge with closely related virulent viruses, thus supporting the theory that protective immunity against ASF can be achieved.

Typically, protection against viruses and clearance of virus-infected cells rely on a synergistic effect of both cellular and humoral immune responses. Humoral responses against viruses include type I interferon (IFN) as part of innate immunity and (neutralizing) antibodies in the case of adaptive immunity, whereas cellular responses are mainly attributed to NK cells and cytotoxic T lymphocytes (CTLs). Nevertheless, the exact mechanisms involved in protection against ASF remain poorly understood, and the little that is known mainly comes from experimental infections using attenuated field isolates (King et al., 2011; Onisk et al., 1994; Oura et al., 2005) or tissue cultured-adapted viruses (Lacasta et al., 2015; Ruiz Gonzalvo et al., 1983).

In vitro studies on the effects of ASFV infection on the functionality of macrophages, its main target cell, have been performed, aiming to understand the early events of the innate immune system leading to immunity or pathogenesis (Franzoni et al., 2017; Gil et al., 2008, 2003; Zhang et al., 2006). Moreover, studies on host-virus interaction have highlighted the remarkable capacity of ASFV to evade the host immune response (Dixon *et al*., 2004; Reis et al., 2017). Likewise, efforts have been focused on the study of ASFV-specific antibody responses (Escribano et al., 2013), as well as on the characterization of T helper and CTLs lymphocytes responding to ASFV (Takamatsu et al., 2013). Sure enough, other cell subsets must play important roles in fighting ASFV. In this regard, a few studies have centered their attention on NK cells (Leitão et al., 2001; Martins and Leitão, 1994; Mendoza et al., 1991; Norley and Wardley, 1983), NK T cells (Takamatsu et al., 2013) and γδ T cells (Takamatsu et al., 2006), but their significance requires further investigation.

1.3.1. Evasion of the host immune response

The obligate parasitism exhibited by viruses compels them to employ strategies to take over cellular metabolism and modulate the host response to infection for their own benefit. In the case of ASFV, one-third to half of the ORFs described encode structural proteins or proteins directly involved in virus replication, while the rest is thought to aid virus survival and transmission by manipulating host machinery (Dixon et al., 2004).

The fact that macrophages are the main target cells for ASFV replication is *per se* both a challenge and an advantage for achieving a productive infection. Macrophages play a central role in triggering and orchestrating the host's immune responses, thus infecting them comes with the risk for the virus of being early sensed and eliminated, but also might allow for manipulation of immune competent cells at a very early stage. Many ASFV-encoded proteins have been described to interfere signaling pathways in infected macrophages, thereby transcriptionally altering the expression of immunomodulatory genes. A well-known inhibitor of transcriptional activation in ASFV-infected macrophages is protein A238L, which suppresses the activation of the NF-κB transcription factor, most probably down-regulating pro-inflammatory cytokine and chemokine responses important for recruiting inflammatory cells to sites of infection (Powell et al., 1996; Revilla et al., 1998). Another clear example is the suppressed expression of type I IFN in macrophages infected with virulent isolates of ASFV. Members of the MGF360 and MGF530 mediate evasion of the IFN I system (Golding et al., 2016), and the importance of this effect is reflected in the fact that ASFV deletion mutants lacking genes from MGF360 and MGF530 are attenuated in pigs (Afonso et al., 2004; O'Donnell et al., 2015; Reis et al., 2016).

Inhibition of IFN induction is also mediated by proteins A528R and I329L (Correia et al., 2013). With the purpose of limiting virus proliferation, host innate defenses are capable of inactivating protein synthesis. ASFV avoids the fully shut down of the host translation machinery, and DP71L protein has been confirmed to be involved in this evasion mechanism. However, evidence suggests the existence of complementary ASFV factors interfering with this process (Zhang et al., 2010).

Several studies have analyzed the effect of ASFV infection on the expression of surface markers in macrophages, including MHC expression. It is thought that the levels of SLA class II are not altered by ASFV infection (Franzoni et al., 2017; Lithgow et al., 2014; Sánchez-Torres et al., 2003). Conversely, down-regulation of SLA I expression was observed in infected macrophages with the non-pathogenic BA71V and the attenuated NH/P68 isolates, but not with the virulent 22653/14 ASFV (Franzoni et al., 2017). This could be a mechanism of virulent strains to evade early recognition by NK cells, thus hindering the development of an effective immune response (Franzoni et al., 2018a; Leitão et al., 2001).

Apoptosis is another cellular process modulated by ASFV. Three ASFV-encoded proteins have been determined to inhibit programmed cell death: A179L (Banjara et al., 2017; Revilla et al., 1997), A224L (Nogal et al., 2001), and EP153R (Hurtado et al., 2004), hence contributing to maintain the host cell functional to ensure enough time for viral replication. On the other hand, ASFV proteins E183L and EP153R induce caspase-3 activity and apoptosis (Hernáez et al., 2004), which might be advantageous after replication to induce cell lysis, and permit both virus egress and recruitment of phagocytic cells that could be further infected. Additionally, ASFV infection induces

apoptosis in bystander lymphocytes (Gómez-Villamandos et al., 1995), a mechanism also documented during infection with other hemorrhagic viruses, such as CSF virus (Sato et al., 2000) and Ebola virus (Geisbert et al., 2000). Lymphocyte depletion might contribute to evade the host's immune response, facilitating virus persistence and leading to the lethal pathology observed after infection with virulent isolates.

One more interesting protein from the point of view of manipulating the host response is the virus CD2-like protein (CD2v), encoded by the EP402R gene. This protein causes the characteristic hemadsorption of red blood cells around ASFV-infected cells (Borca et al., 1994; Rodríguez et al., 1993), which is also enhanced by EP153R, a C-type lectin homologue (Galindo et al., 2000). CD2v also facilitates virus dissemination by mediating binding of extracellular virions to red blood cells (Borca et al., 1998; Ruiz-Gonzalvo et al., 1996), and there is *in vitro* evidence of the immunosuppressive capabilities of CD2v by inhibiting lymphocyte proliferation (Borca et al., 1998).

1.3.2. Innate immune response: macrophage-derived mediators

Innate immunity provides the early line of defense against pathogens. Macrophages play central roles in innate responses, but are also involved in activation of the adaptive immune system. Replication of ASFV in mononuclear phagocytes interferes with these functions, thus altering the immune response of the host (Dixon et al., 2004; Powell et al., 1996). Alterations provoked by ASFV infection are primarily reflected in variations on macrophage-derived mediators, which determine the development of innate inflammatory responses to viral infection. Although some authors described differences between virulent and attenuated isolates, contradictory

results have also been reported (Gil et al., 2008, 2003; Lacasta et al., 2015; Powell et al., 1996; Whittall and Parkhouse, 1997; Zhang et al., 2006), thus giving rise to controversy regarding the cellular signaling pathways altered in infected macrophages. The use of ASFV isolates with different virulence and phylogenetically distant, distinct primary cell cultures and stimulation protocols might explain the inconsistent results obtained.

Studies of the transcription patterns of key immune mediators revealed differences in macrophages *in vitro* infected with highly virulent and low virulent or attenuated ASFV isolates (Gil et al., 2008, 2003; Zhang et al., 2006). Using a porcine cDNA microarray, macrophages *in vitro* infected with the highly virulent Malawi LIL20/1 showed increased expression of pro-inflammatory cytokines (TNFα, IL-6, and IFNβ), chemokines, surface proteins, and proteins involved in cell signaling, immediately after infection (4 hours post-infection). However, expression of these genes returned to a base-level after 16 hours, most probably due to the shut-off of protein synthesis induced by virus-encoded proteins or host mechanisms (Zhang et al., 2006), which would eventually lead to programmed cell death of the infected macrophages. Supporting these results, increased levels of soluble factors such as TNFα, IL-1 and IL-6 were detected in serum of piglets inoculated with the highly virulent isolate E70 (Salguero et al., 2002). Conversely, other authors described that production of pro-inflammatory cytokines such as IL-1, TNFα, IFNα and IL-8 was totally impaired in porcine alveolar macrophages (PAMs) *in vitro* infected with virulent ASFV isolates, coinciding with high levels of TGFβ, presumably due to immunosuppressive effects mediated by the virus (Powell et al., 1996; Whittall and Parkhouse, 1997).

In other studies, significantly increased levels of transcripts for pro-inflammatory cytokines, including IFNα, TNFα, IL-6, IL-12 and IL-15, were observed in macrophages infected with the low virulent ASFV/NH/P68, compared to the expression in macrophages infected with the highly virulent L60 isolate (Gil et al., 2008, 2003). Similarly, higher levels of IL-18, IL-1β and IL-1α were detected in supernatants of macrophages after interacting with the non-pathogenic BA71V isolate compared to the infection with the virulent 22653/14 ASFV (Franzoni et al., 2017). Analysis of the transcriptomic and proteomic profile in gastrohepatic lymph nodes of pigs infected with the attenuated E75CV1 ASFV and the virulent E75 uphold these results. Up-regulation of relevant mediators of the innate arm was demonstrated at day 1 post-infection in E75CV1-infected pigs, while those inoculated with E75 showed inverted cytokine dynamics (Herrera-Uribe et al., 2018; Lacasta et al., 2015).

Although contradictory, these results may indicate that virulent ASFV isolates are either capable to bypass immune detection by suppressing macrophage responses or are so aggressive that the cellular signaling of the macrophage is heavily altered in the early steps of infection. In both cases, development of an effective immune response would be impaired, leading to disease progression. Oppositely, ASFV isolates of reduced virulence could trigger a more efficient innate immune response, probably aiding in controlling the first rounds of replication, allowing sufficient time to mount a specific response, and explaining the milder ASF pathology observed *in vivo* (Franzoni et al., 2018a).

1.3.3. Antibody-mediated immune response

Anti-viral antibodies are generated during ASFV infection, but the mechanism by which they could be directly involved in protection has long been a controversial issue.

The implication of an antibody-mediated mechanism of resistance to ASFV was confirmed by passive administration of antibodies or colostrum from ASF convalescent pigs, which was seen to be sufficient for protection against lethal challenge (Onisk et al., 1994; Schlafer et al., 1984a, 1984b; Wardley et al., 1985). Low virus titers associated with complement-dependent antibody-mediated cytotoxicity were seen in passive transfer experiments, and protection seemed to be conferred by the effects of antibody-dependent cell-mediated cytotoxicity (Wardley et al., 1985). In other studies, the presence of antibodies has correlated with delayed disease onset and reduced virus titers (Knudsen et al., 1987).

Virus neutralization is supported by the observation that sera from convalescent swine as well as monoclonal antibodies can *in vitro* neutralize several tissue adapted isolates of ASFV, either by blocking the binding of the virus to Vero cells or by inducing the detachment of non-internalized virus (Escribano et al., 2013; Gómez-Puertas et al., 1996). Specifically, neutralizing antibodies directed against p72, p54 and p30 virion proteins have been described, the first ones inhibiting virus attachment and the anti-p30 blocking the internalization of already attached virions (Gómez-Puertas et al., 1996). Antibodies against ASFV protein CD2v have been described as capable of inhibiting ASFV infection *in vitro*, correlating with *in vivo* resistance to ASF (Ruiz-Gonzalvo et al., 1996). In addition, several other immunodominant

proteins have been characterized using sera from pigs recovered from ASFV infection (Kollnberger et al., 2002; Reis et al., 2007), albeit their protective role has not been demonstrated or remains controversial.

Nevertheless, certain ASFV peculiarities with respect to antibody-mediated neutralization still makes it a questionable topic in terms of its protective implications. Firstly, high-passage viruses have been seen to lose their neutralization susceptibility due to changes in phospholipid composition of viral membranes (Gómez-Puertas et al., 1997). Besides, the presence in convalescent swine sera of blocking antibodies that inhibit virus neutralization *in vitro* has been demonstrated (Escribano et al., 2013), which might partake in the existence of a persistent non-neutralized ASFV fraction of about 10% that could explain the chronic ASFV infection (Gómez-Puertas et al., 1996; Ruiz Gonzalvo et al., 1986; Zsak et al., 1993). Moreover, the detection of non-neutralizing antibodies has even been correlated with exacerbation of the disease (Argilaguet et al., 2011; Sunwoo et al., 2019). In themselves, these results demonstrate that ASFV-specific antibody response isgenerated during ASF infection, but the fine balance between its protective and detrimental role is yet to be understood.

1.3.4. T cell-mediated immune response

T lymphocytes are the main mediators of cellular immunity. They contribute to the defense of the organism leading and regulating immune responses as well as directly attacking infected or malignant cells. Two major subsets of T cells are differentiated: T helper (Th) lymphocytes expressing the CD4 co-receptor on their surface and CTLs characterized by CD8 expression.

Experimental models using attenuated viruses that confer protection against further challenge have been an important base for studying the cellular immune responses to ASFV, since they provide immune cells of ASF-convalescent animals. Using PBMCs from animals surviving infection with attenuated viruses, ASFV-specific proliferation of both $CD4^+$ and $CD8^+$ T cells has been reported, and also of double positive lymphocytes (Canals et al., 1992), which are thought to include memory/effector T cells (Zuckermann and Husmann, 1996). Nevertheless, as in the case of antibody response, the precise contribution of cell-mediated response to protective immunity to ASF is not fully characterized.

The involvement of Th lymphocytes was first shown in *in vitro* studies which demonstrated that $CD4^+$ T cells were required for the induction of ASFV-specific antibodies (Casal et al., 1987). Moreover, ASFV-specific memory Th cells have been proposed to be induced (Canals et al., 1992). Nonetheless, the T cell subset that has received the most attention has been the $CD8^+$. The crucial importance of $CD8^+$ T cells in ASFV clearance was evidenced by specific depletion of this cell subset in ASF immune pigs, which were no longer protected from challenge infection (Oura et al., 2005). Specific anti-ASFV antibodies were detected in those animals, thereby supporting the hypothesis that antibody response is not sufficient for protection. Strengthening the significance of $CD8^+$ T-cell immune response to ASFV, partial protection has been described in the absence of vaccine-induced antibodies, but correlating with antigen-specific $CD8^+$ T-cell proliferation (Argilaguet et al., 2012; Lacasta et al., 2014).

One of the major $CD8^+$ T cell subsets is the cytotoxic T lymphocyte population. CTLs are crucial in fighting intracellular pathogens such as

viruses, due to their ability to directly kill infected cells by secreting death-inducing effector molecules. Induction of ASFV-specific effector CTLs from memory CTLs by *in vitro* stimulation has been demonstrated, being signaling through the CD8 receptor essential (Martins et al., 1988, 1993; Scholl et al., 1989). Two distinct phenotypes of porcine CTLs lysing ASFV infected cells have been described: a conventional CD8 single positive, and a $CD4^+CD8\alpha\beta^+$ phenotype, both being perforin positive and having swine leukocyte antigen I (SLA I)-restricted cytotoxicity. Increasing levels of circulating $CD8^+$ conventional lymphocytes have been correlated with the onset of ASF clinical signs, while such increase was not seen in completely protected animals. In accordance with the *in vivo* observation, *in vitro* stimulation with ASFV of PBMCs from immune pigs showed that CD4/CD8$\alpha\beta$ double positive CTLs were significantly increased in a protected pig in comparison to a diseased one, whose proliferating CTLs were virtually conventional $CD8^+$ (Denyer et al., 2006). These results may be reflecting a critical role of double positive CTLs in ASFV clearance, despite no more published data is available regarding this presumable correlation.

Differentiation of CTLs from naïve $CD8^+$ T cells is triggered by the interaction with an APC presenting their cognate antigen (Figure 1-3). Through the T-cell receptor (TCR), T cells interact with APCs, which are capable of capturing and processing antigens and finally present them in the form of small peptides (epitopes) on the cell surface by molecules of the major histocompatibility complex (MHC). CD8 acts as a co-receptor in this interaction, binding to MHC class I molecules, thereby being induction of CTLs dependent on the epitope sequence presented by MHC I molecules. Although the SLA I-dependency of ASFV-specific CTLs has been demonstrated (Alonso et al., 1997; Jenson et al., 2000; Leitão et al., 2001, 1998;

Martins et al., 1993), the ASFV antigenic determinants leading to the activation of $CD8^+$ T cells remain largely unknown.

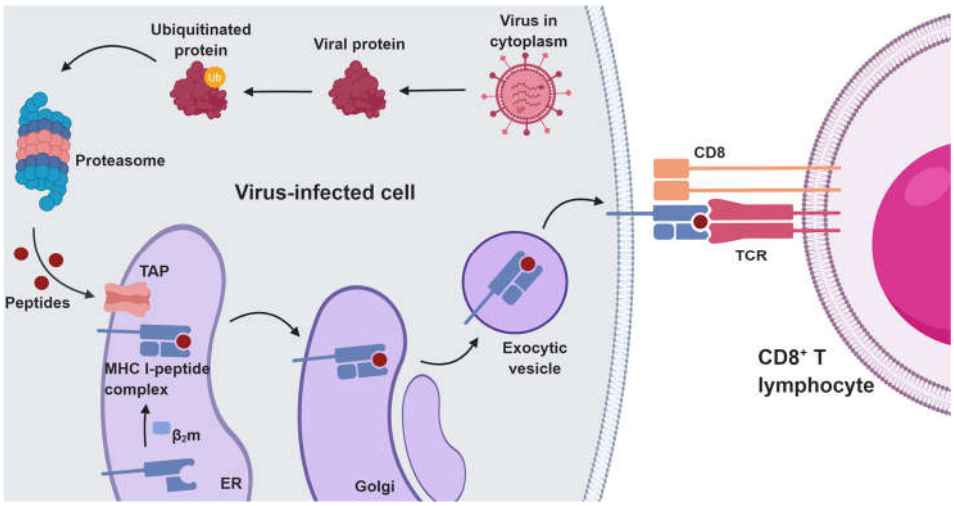


Figure 1-3. MHC I antigen processing and presentation pathway. ER: endoplasmic reticulum; b_2m: b_2-microglobulin; TAP: transporter associated with antigen processing; TCR: T-cell receptor; Ub: ubiquitin.

Screening of potential antigenic determinants within the ASFV is a challenging task due to the complex nature of this virus, coding for more than 150 ORFs and from which many of them there is no expression nor functional data available. A few *in vitro* studies have centered their attention on identification of ASFV proteins inducing $CD8^+$ T-cell responses. Alonso and colleagues used recombinant vaccinia virus (VV) expressing ASFV protein p32 to infect alveolar macrophages and those were used as target cells for ASFV-specific CTLs, showing a higher level of lysis compared to cells infected with wild type VV (Alonso et al., 1997). Another strategy explored was the fusion of ASFV antigens with lipoproteins, which were supposed to be taken-up by blood-derived macrophages, and used as target cells for CTLs. Using this methodology, cytotoxic activity was found against proteins

p72 and G1340L (Leitão et al., 2000, 1998). Aiming to maximize the coverage of antigens screened, a random plasmid library of ASFV genomic DNA was generated and expressed in pig-derived fibroblasts. Sequencing of the clones that induced $CD8^+$ T cell proliferation revealed that one of the clones encoded a segment of I329L, a putative ASFV membrane protein. Notably, sequences that were in different reading frames than any of the known ASFV ORFs were identified in this study (Jenson et al., 2000).

1.4. EXPERIMENTAL VACCINES AGAINST ASFV

As previously mentioned, there is currently no commercial prophylactic treatment against ASF, being biosecurity measures, efficient diagnosis and slaughter of affected and potentially exposed animals the only control methods. Albeit the many efforts to experimentally induce protective immunity against ASF, all the strategies tested so far resulted unsuccessful or harboring inherent drawbacks to be used in field conditions.

In this section, an overview is given about the main vaccine strategies against ASFV studied to date, but also about immunization strategies explored aiming to characterize antigens with protective potential. Although no safe and cost-effective approach has been achieved, there is no doubt that immunization and challenge protection experiments have contributed to a better understanding of the immune mechanisms leading to protection against ASFV.

1.4.1. Inactivated and live attenuated viruses

First attempts to obtain an ASF vaccine were focused on inactivated viruses, consisting on intact non-proliferating viruses obtained by chemical or physical treatment. Although ASFV-specific antibody response was induced in animals immunized with inactivated preparations of the virus, no protective effect was ever achieved (Forman et al., 1982; Mebus, 1988; Montgomery, 1921; Stone and Hess, 1967), thus supporting the idea that antibody-mediated responses are not sufficient for protection. More recently, modern adjuvants have been used to enhance the efficacy of inactivated ASFV vaccines, again with no success in conferring protective immunity (Blome et al., 2014).

The use of classical attenuated viruses, which are non-virulent live forms of the pathogen, has also been studied. Either generated by successive passages in tissue culture or isolated from areas where the disease is endemic, ASFV live attenuated viruses (LAVs) conferred long-term protection against homologous virulent viruses (Boinas et al., 2004; Lacasta et al., 2015; Leitão et al., 2001; Oura et al., 2005). Attenuated viruses can likewise be created by genetic modification. This methodology is based on the deletion of concrete non-essential genes, generally involved in virulence or in evasion of the host's immune response, leading to virus attenuation (Lewis et al., 2000; Monteagudo et al., 2017; O'Donnell et al., 2015; Reis et al., 2016). The main disadvantages of LAVs are related to biosafety issues: risk of residual virulence and possibility of reversion to pathogenic wild types. In spite of the safety concerns with LAVs, they represent the most feasible approach for an effective ASF vaccine in the short/medium term, according to the European Commision (https://ec.europa.eu/food/animals/animal-diseases/control-

measures_en). Furthermore, their use in experimental conditions has been pivotal for the study of the immunological mechanisms underlying protection against ASF.

In the field of ASFV deletion mutants, the latest relevant progress is the LAV BA71ΔCD2, a successful experimental vaccine developed by our group. This attenuated virus not only confers protection against the parental BA71 isolate, but also against a lethal challenge with heterologous viruses as the E75 isolate (both genotype I strains), and the highly virulent Georgia2007/1 (genotype II) currently circulating in Continental Europe and Asia. The unique cross-protection afforded by BA71ΔCD2 correlated with its ability to induce cross-reactive $CD8^+$ T cells, and it is thought that the absence of CD2v could have led to the induction of broader T-cell responses (Monteagudo et al., 2017). Although biosafety concerns still hamper the use of BA71ΔCD2 in the field, its exceptional capabilities make it an ideal tool to explore aspects of the immune response responsible for protection against ASFV. The work described in this thesis relies on the cross-protective capabilities of the LAV BA71ΔCD2 to identify ASFV antigens with protective potential against the Georgia2007/1 isolate.

1.4.2. Protein-based subunit vaccines

In order to avoid, or at least diminish, the unwanted side effects of traditional inactivated and attenuated vaccines, subunit vaccines arise as an ideal option for the future development of ASF-vaccines. Discovering that in occasions the presence of the whole microorganism is not strictly necessary to induce protection led to a huge revolution in the vaccine field, allowing the design of successful vaccines with only given molecules of the pathogen, generally

proteins. This strategy provides the advantage of no risk of infection, but in general protein-based subunit vaccines do not elicit strong and long-term immunity. Moreover, their success is highly dependent on identification of relevant antigens inducing protective immune responses. Considering the complexity of ASFV, encoding more than 150 ORFs, selection of such antigenic determinants is a major issue, and protein-based subunit vaccines have not shown consistent positive outcomes in ASF protective studies. Promising results were first obtained using recombinant ASFV proteins expressed in the baculovirus system. Thus, immunization with baculovirus-expressed ASFV structural proteins p30 and p54 yielded partial protection against E75, correlating with a specific antibody response (Barderas et al., 2001; Gómez-Puertas et al., 1998). However, these results were not reproducible using a combination of p54, p30, p72 and p22, as well as a different ASFV isolate as a challenge model (Neilan et al., 2004). The CD2v expressed in the baculovirus system has also been used in protection trials, conferring a dose-dependent protection (Ruiz-Gonzalvo et al., 1996). Unfortunately, the cost-effectiveness scale up of such protein subunit vaccine made its use in the field unsustainable.

1.4.3. DNA-based immunization approaches: previous results from our laboratory

DNA vaccination consists on the *in vivo* administration of a plasmid vector coding for antigens of the pathogen of interest under the control of an eukaryotic promoter (Davis, 1997). After its administration, the plasmid is capable of entering the host cells, both locally at the site of inoculation and into resident APCs (Hung et al., 2006), where the codified antigen is expressed, processed and displayed on the cell surface so that immune cells can recognize it.

DNA vaccines have been successfully used in numerous animal models of infection and cancer, generating both humoral and cellular immune responses. Additionally, DNA plasmids have intrinsic adjuvant properties due to the presence of unmethylated CpG motifs, which has been described to rapidly trigger an innate immune response (Klinman et al., 1997). Despite these advantages, the major drawback of DNA vaccines is that they are poorly immunogenic compared to other immunization strategies, especially in large animals and humans (Babiuk et al., 2003; Kutzler and Weiner, 2008; van Drunen Littel-van den Hurk et al., 2004). DNA vaccines frequently require repeated boost immunizations in order to enhance the potency of the protective response induced. Other recurrent strategies for improving immunogenicity of DNA vaccines include: optimization of the expression of the genes included in the DNA construct, targeting of the antigenic determinants to a specific cell subset or cellular processing pathway, improvement of the delivery systems used and use of adjuvants (Barouch et al., 2004; Jilek et al., 2005; Kutzler and Weiner, 2008; Rodriguez and Whitton, 2000; van Drunen Littel-van den Hurk et al., 2004).

First attempts to use DNA vaccines in the lab were focused on three ASFV structural proteins: p30 (CP204L), p54 (E193L) and CD2v (EP402R), which were previously described to be immunogenic and have protective potential using the baculovirus expression system (Barderas et al., 2001; Gómez-Puertas et al., 1998; Ruiz-Gonzalvo et al., 1996). In our hands, the protection achieved were dramatically dependent on the plasmid used. Immunization with a pCMV plasmid encoding a fusion of p54, p30 and the extracellular domain of the CD2v did not confer protection against an E75 lethal challenge, although it did induce specific cellular and antibody responses (Argilaguet et al., 2011). On the contrary, when the same antigens were administered as

a fusion with the ubiquitin gene, partial protection (33%) was achieved, correlating with proliferation of $CD8^+$ T cells recognizing 9-mer peptides from the ASFV CD2v protein (Argilaguet et al., 2012). Besides confirming the important role of $CD8^+$ T cells in protection against ASFV, these results highlighted the convenience of targeting the antigens to the appropriate antigen presentation route. The ubiquitination strategy is based on promoting the delivery of the proteins to the proteasome (Rodriguez and Whitton, 2000), which normally degrades misfolded intracellular proteins that had been targeted with a poly-ubiquitin chain. In the proteasome, proteins are chopped into small peptides, which are transferred to the ER though transmembrane transporters associated with antigen processing (TAP transporters). Once in the ER, the peptides with the highest affinity fit in the binding groove of the MHC class I molecules, and the MHC-peptide complexes are finally directed to the plasma membrane, where they can be recognized by a matching $CD8^+$ T cell.

Using this physiological mechanism, the ubiquitination strategy was then expanded to an expression library immunization of more than 4000 clones encoding random ASFV genome fragments (excluding the sequences encoding p30, p54 and CD2v to avoid competition). Immunization with the ubiquitinated expression library yielded a 60% survival against a lethal challenge with the E75 ASFV isolate, again correlating with the detection of ASFV-specific T-cell responses (Lacasta et al., 2014). These results clearly demonstrated the presence of additional protective $CD8^+$ T-cell determinants within the ASFV genome. Sequencing of the clones within the random expression library to determine which ASFV genes were in frame with ubiquitin, and thus could be associated with the protection observed, determined protein CP312R as a potential protective antigen (Lacasta, 2012).

Aiming to extend these results to the ASFV isolate currently circulating in Continental Europe and Asia, DNA constructs based on the Georgia2007/1 sequence of previously identified antigens in E75 were tested for their protective potential. Unexpectedly, all of the tested immunization formulations failed to confer protection against a Georgia2007/1 lethal challenge, and the course of infection in immunized pigs were indistinguishable from that found in control animals (Rodríguez et al., unpublished results). The lack of protection observed contrasted with our previous results using the E75 model and, far from being completely understood, these results might be explained by differences in the ASFV strain used, including differential virulence (Georgia2007/1 is more virulent than E75) and/or by the existence of relevant antigenic differences between viruses (the protective CTL epitopes described in the E75 CD2v protein are absent in Georgia2007/1).

Regardless of the protection conferred, DNA immunization has proven a worthy tool for the elucidation of ASFV-relevant antigenic determinants as well as for providing valuable insights into the mechanisms involved in the protective immune response against ASF.

1.4.4. Prime-boost immunization strategies

As previously mentioned, subunit vaccines do not elicit strong immunogenic responses, and several approaches have been tested aiming to increase the potency of the protective immune responses induced. In this regard, the more recent strategies are based on prime-boost immunization protocols, especially heterologous regimens. Conversely to homologous prime-boost strategies, in which the same formulation is used in both the prime and boost,

heterologous prime-boost approaches are based on administration of the antigens with different delivery systems and in more than one injection (Nascimento and Leite, 2012). There is evidence for heterologous prime-boost immunization to be more effective in terms of immunogenicity and protection than homologous prime-boost approaches (Lu, 2009). Thus, 4- to 10-fold increase in T-cell responses has been demonstrated when using DNA immunization followed by a boost with an heterologous vector (Fioretti et al., 2010).

A few studies have explored prime-boost immunization protocols against ASF. Combinations of ASFV recombinant proteins and DNA, albeit inducing strong immune responses, conferred no protection against challenge with an Armenian ASFV strain (Revilla, 2016). Viral-vector based vaccines encoding individual ASFV proteins have also been tested for immunogenicity, including immunization with recombinant adenoviruses followed by a boost with the same vectors (Lokhandwala et al., 2017) or with recombinant modified vaccinia Ankara vectors (Lopera-Madrid et al., 2017). Both strategies elicited robust cellular and antibody responses, but their protective capacity was not assessed. In another study attempting to identify ASFV proteins with protective potential, 47 ASFV putative antigens were administered to pigs by DNA prime and recombinant VV boost. After immunization, specific cellular and antibody responses were induced against many individual antigens as well as whole live ASFV. Nevertheless, none of the pigs survived Georgia2007/1 lethal challenge, although a significant reduction of virus titers in blood and lymph nodes was reported (Jancovich et al., 2018). Finally, in the study of Murgia and collaborators a prime with an alphavirus replicon system expressing ASFV proteins targeting DCs was used, followed by a boost with the naturally attenuated OURT88/3 ASFV

strain. In this case, consequences on antigen-specific antibody responses were addressed, concluding that such a prime-boost approach could broaden the pattern of recognized epitopes (Murgia et al., 2019), but again the protective potential of the immune response induced in the immunized animals was not tested using a challenge infection with a virulent ASFV strain.

In summary, the enhanced immunogenicity of prime-boost regimens has been observed in terms of the ASFV-specific cellular and humoral responses induced. Nevertheless, as in the case of protein- and DNA-based subunit vaccines, the little knowledge about the relevant ASFV antigens inducing protective responses as well as the nature of the optimal immune response to be generated are major drawbacks for the rational design of such immunization approaches.

CHAPTER 2

Objectives

Developing a safe and effective vaccine against ASFV would definitely represent a key step forward in ASF disease control and eradication. Nonetheless, rational vaccine design is hindered by the lack of knowledge about the mechanisms underlying protective immunity against ASFV. In this respect, $CD8^+$ T cells have been shown to play a critical role in protective response against ASFV, but the relevant antigens specifically stimulating this cell subset remain largely unknown. Henceforth, the general objective of this thesis was to identify ASFV antigens eliciting immunodominant $CD8^+$ T-cell responses with protective against the Georgia2007/1 ASFV, the strain currently circulating in Continental Europe and Asia. Accordingly, the specific aims of the present thesis were:

1. To perform *in silico* $CD8^+$ T-cell epitope predictions using the Georgia2007/1 proteome as template and test their *in vitro* recognition by ASFV-specific PBMCs.

2. To characterize SLA I-restricted peptides presented by porcine alveolar macrophages infected *in vitro* with ASFV by means of SLA I immunoprecipitation and MS sequencing of the bound peptides and evaluate their *in vitro* recognition by ASFV-specific PBMCs.

3. To identify ASFV full-length proteins promiscuously recognized by ASFV-specific $CD8^+$ T cells. To perform these studies, PBMCs from ASFV-recovered animals were stimulated with autologous fibroblasts transfected with expression plasmids encoding individual ORFs fused to ubiquitin, aiming to improve SLA I antigen presentation.

4. To perform DNA immunization experiments with plasmids encoding ASFV full-length proteins with potential to induce ASFV-specific $CD8^+$ T cells (selected in Objectives 1 to 3) and characterize their immunogenicity and protective potential against a Georgia2007/1 challenge.

5. To design DNA plasmids encoding multiple epitopes (from ASFV proteins containing peptides previously characterized in Objectives 1 and 2) in frame with ubiquitin and to determine their capacity to elicit ASFV-specific cellular responses upon immunization, as well as their potential to confer protection against a lethal challenge with the Georgia2007/1 ASFV.

CHAPTER 3

Materials and Methods

3.1. CELLS AND VIRUSES

3.1.1. Porcine alveolar macrophages (PAMs)

PAMs from healthy conventional pigs (Landrace x Large White) were obtained by lung lavage with PBS 1X supplemented with 1 μg/ml gentamicin (Sigma-Aldrich). The PBS solution was administered through the trachea using a sterile funnel, pulmonary lobes were gently massaged for 5 minutes, and the volume was collected into a sterile container. After three washes with 250 ml of PBS solution, the recovered fluid was centrifuged at 400 xg for 10 minutes. Cell pellets were washed once with PBS 1X, and suspended in RPMI 1640 medium (Gibco) supplemented with 2 mM L-glutamine (Invitrogen), 100 IU/mL of penicillin (Invitrogen), 100 μg/ml of streptomycin (Invitrogen) and 10% heat-inactivated porcine serum (Gibco). PAMs were maintained in cell culture at 37°C, 5% CO2, or were frozen in FBS 10% DMSO (Sigma-Aldrich) and stored at -150°C.

3.1.2. Primary pig fibroblasts

Establishment of primary fibroblasts cultures was done from a 2 cm^2 piece of ear tissue sample following previously described protocols (Takashima, 1998). Briefly, tissue was cut into small sections and incubated o/n at 37°C with a 0.5% trypsin solution in PBS. Cells were filtered through a 40 μm cell strainer (Corning) to discard the remaining tissue lumps, and centrifuged at 150 xg for 10 minutes. Supernatant was discarded and cells were suspended in complete DMEM supplemented with 10% FBS (HyClone, GE HealthCare), 100 IU/ml of penicillin (Invitrogen), 100 μg/ml of streptomycin (Invitrogen), 2 mM L-glutamine (Invitrogen), and and 50 IU/ml Nystatin (Sigma-Aldrich).

Primary fibroblasts were seeded in T-flasks, and maintained their viability after multiple serial passages. Cell passaging was done by trypsinization.

3.1.3. Peripheral blood mononuclear cells (PBMCs)

Porcine PBMCs were isolated from whole blood using Histopaque-1077 (Sigma-Aldrich) density gradient solution. Blood samples drawn from the jugular vein of the pigs into 10 ml EDTA vacutainer tubes (Becton Dickinson), were diluted 1:1 in PBS. Diluted blood was gently layered on the top of 10 ml Histopaque-1077 in a 50 ml conical tube and centrifuged at 400 xg for 30 minutes at 20°C, without acceleration nor break. The whitish buffy coat formed in the interphase containing the mononuclear cells was aspirated and transferred to a clean 15 ml conical tube, filled with PBS, and centrifuged at 400 xg for 10 minutes at 20°C. Supernatant was discarded, and red blood cells were lysed by hypotonic shock using 9 ml sterile distilled water for 30 seconds followed by addition of 3.5 mL of 3.5% NaCl solution. Afterwards cells were centrifuged at 400 xg for 10 minutes at 20°C, washed with PBS, and suspended in RPMI 1640 medium (Gibco) supplemented with 2 mM L-glutamine (Invitrogen), 100 IU/mL of penicillin (Invitrogen), 100 μg/ml of streptomycin (Invitrogen) and 10% heat-inactivated FBS (HyClone, GE HealthCare). For their use in the ELISpot assays, 50 μM β-mercaptoethanol (Sigma-Aldrich) was added to the medium to help maintain a reducing environment.

3.1.4. Rabbit kidney RK13 cells

Rabbit kidney epithelial RK13 cell line was cultured at 37ºC, 5% CO_2 in DMEM supplemented with 10% FBS (HyClone, GE HealthCare), 100 IU/ml

of penicillin (Invitrogen), 100 μg/ml of streptomycin (Invitrogen), and 2 mM L-glutamine (Invitrogen).

3.1.5. African swine fever viruses

Two different ASFV virulent field isolates were used: BA71 and Georgia2007/1, kindly provided by Dr. María Luisa Salas (Centro Biología Molecular Severo Ochoa, Madrid, Spain) and Dr. Linda Dixon (The Pirbright Research Institute, UK), respectively. The live attenuated BA71ΔCD2 virus, a deletion mutant from BA71 lacking the CD2v gene (EP402R) was previously obtained in the laboratory (Monteagudo et al., 2017).

3.2. MULTIPARAMETRIC *IN SILICO* PREDICTIONS OF CD8$^+$ T-CELL EPITOPES

Georgia2007/1 proteome was retrieved from Uniprot (UP000141072) for *in silico* CD8$^+$ T-cell epitope prediction. Predictions were made using the NetMHCpan 3.0 software (Nielsen and Andreatta, 2016). 42 SLA class I alleles were considered, and peptides ranging from 8 to 11 amino acid residues, with an IC50 (concentration of peptide inhibiting binding of a standard peptide by 50%) below 500 nM were selected. 8,648 different sequences were obtained. To further select the most promising theoretical CTL candidates, additional features were evaluated for each peptide, including:

i) Proteasome cleavage, analyzed by using the MHC-I Processing tool from IEDB (http://tools.iedb.org/processing). This program allows evaluating

how efficiently a peptide or its N-terminally prolonged precursors can be liberated from its source protein by the immunoproteasome.

ii) Promiscuity: Number of SLA I alleles predicted to bind the peptide with an affinity of 500 nM or lower.

iii) Overlap: Number of predicted peptides with a SLA binding affinity of 500 nM or lower, overlapping in at least one amino acid to a given polypeptide.

iv) Peptide immunogenicity: Prediction of the immunogenicity of a peptide taking into account its amino acid properties and their position within the sequence (Calis et al., 2013).

The values of each trait were divided in 10 intervals, so that the best values received a score of 10 and the worst ones were scored as 1. The final score consisted in the sum of all the values, and was finally used to select the best candidates.

Aiming to compare the repertoire of peptides selected, an additional list was made, incorporating TAPREG score as a new parameter, previously used in the laboratory to identify the CD2v CTL peptides from the E75 ASFV strain (Argilaguet et al., 2012). TAPREG server computes binding affinity of peptides to TAP using a Support Vector Machine Regression (Diez-Rivero et al., 2010). The addition of TAPREG score provided an alternative list, and the final peptide selection was obtained combining both peptide lists. When overlapping peptides were found in both lists, both the one with the best score and the larger peptide were selected. Additionally, larger peptides (15-27 amino acids) were selected according to the presence of ten or more overlapping peptides in a given hot spot.

3.3. MASS SPECTROMETRY-BASED IMMUNOPEPTIDOMICS

3.3.1. *In vitro* infection of PAMs with ASFV

5 x 10^6 PAMs/well were seeded in 6-well plates for ASFV infection, using the indicated multiplicity of infection (MOI). The virus inoculum (0.5 ml) was diluted in complete RPMI without serum and applied to the PAMs monolayers. Following a 2-hour incubation at 37°C, 5% CO_2, the inoculum was discarded, and cells were replenished with complete RPMI supplemented with 10% porcine serum (Gibco). A parallel plate subjected to same conditions was used to monitor the ASFV infection. In this case, cell supernatants were harvested and the virus kinetics was analyzed by qPCR, as previously described (Lacasta et al., 2014). Cells were incubated at 37°C and 5% CO_2, and harvested by scrapping when cytopathic effect was evident (dependent on the MOI used). PAMs were centrifuged at 350 xg for 5 minutes at 4°C and washed with PBS. Supernatant was discarded and pellets frozen at -80°C until used.

3.3.2. Typing of SLA I genes

Typing of SLA 1, SLA 2 and SLA 3 classical SLA I genes was performed from PAMs used in the immunopeptidomics studies in order to discard limiting the identification of peptides to a single pig haplotype. Genomic DNA was isolated using NucleoSpin Blood kit (Macherey-Nagel) and typing was kindly performed by Dr. Chankyu Park (Konkuk University, Seoul, Republic of Korea) (Choi et al., 2015; Le et al., 2019; Lee et al., 2019).

3.3.3. Affinity purification of SLA I molecules

SLA I-peptide complexes were immunoprecipitated using 4B7/8 α-SLA I antibody-conjugated CNBr Sepharose beads (GE Healthcare). Hybridome culture supernatant of mAb α-SLA I was kindly provided by Dr. Javier Domínguez (Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Madrid). Coupling of the antibody to CNBr-activated sepharose was performed following manufacturer's instructions. A D-tube dialyzer maxi with a molecular weight cut-off of 12-14 kDa (Novagen) was used to dialyze the antibody-containing supernatant at 4°C against 0.1 M sodium carbonate buffer pH 8.3 containing 0.5 M NaCl (coupling buffer). Lyophilized sepharose was suspended in 1 mM HCl pH 3 and incubated at RT for 20 minutes in end-over-end rotation to wash away the lyophilization additives, centrifuged at 500 xg for 2 minutes at RT, and washed once with coupling buffer. The antibody in coupling solution was added to the washed sepharose at an optimal coupling concentration of 0.8-1.2 mg/ml, and rotated end-over-end o/n at 4°C. The optical density (OD) of the antibody solution at 280 nm was measured before and after coupling to determine the coupling efficiency and incubate longer if necessary. The sepharose was spun down at 500 xg for 2 minutes at RT, and the coupling buffer discarded. Any remaining active groups were blocked for 2 hours at 4°C in end-over-end rotation with 0.1 M Tris-HCl pH 8. The antibody-coupled sepharose was washed with three cycles of alternating pH using 0.1 M acetic acid, pH 4 containing 0.5 M NaCl (acidic wash buffer), and 0.1 M Tris-HCl, pH 8 containing 0.5 M NaCl (basic wash buffer). The coupled sepharose was finally suspended in 50 mM Tris-HCl, pH 8 containing 150 mM NaCl (immunoprecipitation buffer) for the immunoprecipitation. PBS 0.1% (w/v) sodium azide was used for long-term storage of the coupled sepharose at 4°C.

Cell pellets were thawed on ice and lysed with 500 µl of 1% n-Dodecyl β-D-Maltoside (Thermo Fisher Scientific) in immunoprecipitation buffer and 1X complete protease inhibitor cocktail (Thermo Fisher Scientific), and incubated for 8 hours at 4°C with end-over-end rotation. Cell lysates were clarified by centrifugation at 20000 xg for 20 minutes at 4°C, and incubated 2 hours at 4°C end-over-end with sepharose without antibody attached to remove any protein non-specifically interacting with the sepharose. The 500 µl of clarified lysate were then added to an equal volume of 4B7/8 α-SLA I antibody-conjugated CNBr sepharose in immunoprecipitation buffer (approximately 250 µl of sepharose in 250 µl of buffer) an incubated at 4°C o/n with end-over-end rotation. Non-specifically bound molecules were removed by washing with 15-20 sepharose volumes of 150 mM NaCl, 50 mM ammonium bicarbonate. SLA I-peptides complexes were eluted in 4-5 sepharose volumes of 50% acetonitrile, 5% formic acid, and stored at -80°C until analysis.

3.3.4. Western blot to detect immunoprecipitated SLA I-peptide complexes

Five per cent of the volume of each sample was evaporated to dryness using a Concentrator 5301 (Eppendorf), suspended in 25 µl of 1X NuPAGE LDS sample buffer (Invitrogen) with 10% β-mercaptoethanol, and heated at 100°C for 5 minutes. Half of the sample volume (2.5% of the total eluted volume) was run in a 4-12% gradient NuPAGE Bis-Tris acrylamide SDS-PAGE (Invitrogen) at 200 V during 1.5 hours in 1X NuPAGE MES SDS running buffer (Invitrogen) containing NuPAGE antioxidant (Thermofisher). His-tagged protein ladder (Thermofisher) was used as molecular weight marker. The gel was transferred to a nitrocellulose membrane (Amersham, Protran Premium) using a XCell SureLock™ Mini-Cell with a blot module

(Thermofisher) during 4 hours at 50 V in transfer buffer made of 12 mM Tris-HCl (pH 8) containing 96 mM glycine, and 20% methanol (v/v). Following transfer, the nitrocellulose membrane was stained with ATX Ponceau S red staining solution (Biochemika Fluka) and destained in distilled water to confirm the protein transfer. Thereafter, the nitrocellulose membrane was blocked in 3% non-fat milk (w/v) dissolved in wash buffer (TBS 0.1% Tween-20) for 1 hour at RT with gentle agitation on an orbital shaker). 4B7/8 α-SLA I antibody in blocking buffer at a concentration of 4 μg/ml was added to the membrane, and incubated for 1 hour at RT with gentle agitation, following 3 washes for 20 minutes with wash buffer. The membrane was then incubated with anti-mouse IgG HRP-conjugated (Sigma-Aldrich) diluted 1:10000 in blocking buffer for 1 hour at RT with agitation. For the His-tag marker, mouse anti-His tag HRP-conjugated (Novex) 1:100000 was used. After extensive washing as described above, the specific signal on the membrane was developed by using Western Lightning Ultra chemiluminescence substrate (PerkinElmer) for 5 minutes at RT in the dark. A Fluorchem HD2 (Alpha Innotech) was used for imaging.

3.3.5. On-tip desalting and LC-MS/MS analysis

Desalting and analysis of the samples by liquid chromatography coupled to mass spectrometry (LC-MS/MS) was performed at the Proteomics Laboratory of CSIC-UAB (Cerdanyola del Vallès, Barcelona, Spain). Samples were desalted with TopTips C18 (PolyLC Inc), following the standard procedure. The eluates obtained from the desalting process were evaporated to dryness and reconstituted in 20 ml of 5% MeOH, 1% HCOOH for analysis by LC-MS/MS. The MS system used was an LTQ XL Orbitrap (ThermoFisher) equipped with a nanoESI ion source. The total amount of each sample (20 μl)

was loaded into the chromatographic system consisting in a C18 preconcentration cartridge (Agilent Technologies) connected to a 15 cm long, 100 μm i.d. C18 column (Nikkyo Technos Co Ltd). The separation was done at 0.4 μL/min in a 120-minute acetonitrile gradient from 3 to 40% (solvent A: 0.1% formic acid, solvent B: acetonitrile 0.1% formic acid). The HPLC system was composed of an Agilent 1200 capillary nano pump, a binary pump, a thermostated micro injector and a micro switch valve. The LTQ XL Orbitrap was operated in the positive ion mode with a spray voltage of 1.8 kV. The spectrometric analysis was performed in a data dependent mode, acquiring a full scan followed by 10 MS/MS scans of the 10 most intense signals detected in the MS scan from the global list. The full MS (range 400-1800) was acquired in the Orbitrap with a resolution of 60000. The MS/MS spectra were done in the linear ion-trap.

3.3.6. Database search and peptide identification

All LC-MS/MS spectra were searched using SEQUEST (Proteome Discoverer v1.4, ThermoFisher) using a combined database including Sus Scrofa, BA71 and Georgia2007/1 ASFV, and the 6-frame translation of each virus genome (in order to identify peptides in and out of known ORFs). The following parameters were fixed: peptide confidence = High, peptide rank = 1, Xcorr > 2. Additionally, pig-specific 9-mers identified were used to create a sequence logo for each PAMs batch using WebLogo (Crooks et al., 2004). Each logo consists of stacks of symbols, one stack for each position in the sequence. The overall height of the stack indicates the sequence conservation at that position, while the height of symbols within the stack indicates the relative frequency of each amino acid at that position. The binding site description given by the sequence logo was used to select or discard dubious sequences.

3.4. ASFV GENE EXPRESSION PLASMIDS

3.4.1. Plasmids encoding full-length ASFV proteins

The ASFV gene expression library used in Chapter 4 was already available in the lab, and was built based on the E75 ASFV isolate (GenBank accession number FN557520.1). E75 ORFs were cloned in frame with ubiquitin into the pCMV-Ub plasmid (Rodriguez et al., 2001). Additional construction of plasmids based on the Georgia2007/1 sequence (GenBank accession number FR682468) was done following the same strategy. A FLAG-tag sequence was added before the stop codon of the Georgia2007/1 gene in order to confirm the protein expression by immunofluorescence.

3.4.2. Anti-FLAG-tag immunofluorescence to check protein expression

Protein expression of Georgia2007/1 plasmids was checked by anti-FLAG-tag immunofluorescence in transfected RK13 cells. Transfection of RK13 cells was done using Lipofectamine 3000 transfection kit (Invitrogen) according to the manufacturers' instructions. Mock-transfected cells served as negative control. After 2 days of incubation at 37°C and 5% CO_2, cells were fixed with 3% PFA 1 hour at 4°C followed by permeabilization with 0.2% Tween20 in PBS 1X 30 minutes at 37°C. AlexaFluor 488-conjugated anti-FLAG-tag monoclonal antibody (MA1-142-A488, Invitrogen) was diluted 1:100 and added to the cells for 1 hour at RT. Hoechst 33342 (Life Technologies) was used for nucleus staining. Cells were finally examined by fluorescence microscopy.

3.4.3. ASFV multiepitope-encoding plasmids

CTL epitope prediction of the ASFV Georgia2007/1 proteins selected to be included in the multiepitopes constructs was performed using the NetMHCpan 3.0 software. Protein sequences were retrieved from the Georgia2007/1 proteome (Uniprot accession number UP000141072). The 42 SLA I alleles available in NetMHCpan 3.0 were considered, and peptides ranging from 8 to 11 amino acid residues with an IC50 below 500 nM were selected for further analysis. Protein regions containing a high density of predicted epitopes were selected. In the multiepitope-II (ME-II), each domain also included a peptide identified by previous MS-based immunopeptidomics assays. A single DNA construct was designed with the domains, linked by optimal proteasomal cleavage sites (AAY) (Velders et al., 2001) and with ubiquitin as a leader sequence (Figure 6-1) aiming to enhance their SLA I processing and presentation (Argilaguet et al., 2012; Lacasta et al., 2014; Rodriguez and Whitton, 2000). Plasmids encoding the multiepitopes were synthesized by GenScript (New Jersey, USA).

3.5. *IN VIVO* EXPERIMENTS

3.5.1. Animals and animal safety

Male Landrace x Large White piglets were used in all the *in vivo* experiments described. Pigs were fed *ad libitum* and identified by numbered ear tags, and a seven-day acclimation period was established before manipulation of the animals. Animal care and procedures were carried out in accordance with the guidelines of the Good Experimental Practices and under the supervision of

the Ethical and Animal Welfare Committee of the Universitat Autònoma de Barcelona (Spain).

3.5.2. Peptide immunization

Three- to four-week-old piglets were used for peptide immunization experiments, which were carried out at the IRTA Monells pig experimental farm (Girona, Spain). Pigs received two intramuscular administrations in the hindquarters three-week apart. Peptide cocktails (1 ml) included 20 nM of each peptide with complete Freund's adjuvant (Thermo Fisher Scientific) in the first immunization and incomplete Freund's adjuvant (Thermo Fisher Scientific) in the second. EDTA-blood samples were drawn from the jugular vein two weeks after the second peptide administration for PBMCs isolation.

3.5.3. Source of PBMCs to be used as effector cells aiming to quantify ASFV-specific T-cell response

Pigs experimentally infected with Georgia2007/1 yields a 100% mortality, before they are capable to induce ASFV-specific T cells. Therefore, an alternative route was followed to obtain ASFV-specific T cells. For the isolation of PBMCs from ASF-convalescent animals, BA71ΔCD2 immunization-Georgia2007/1 challenge *in vivo* experiments were performed at the biosafety level 3 facilities at the Centre de Recerca en Sanitat Animal (IRTA-CReSA, Barcelona, Spain). Six- to eight-week-old piglets were used, and either 10^6 plaque forming units (PFU) or 3.3 x 10^4 PFU of BA71ΔCD2 in 1 ml of PBS were administered intramuscularly to the animals as previously described (Monteagudo et al., 2017). Three weeks later, pigs were challenged intramuscularly with a lethal dose of 10^3 genomic equivalent copies (GEC) of the Georgia2007/1 ASFV isolate. Three weeks after the BA71ΔCD2

immunization (before Georgia2007/1 challenge), and two to three weeks after the challenge, EDTA-blood samples were drawn and the isolated PBMCs were used in different assays to quantify ASFV-specific T-cell response.

3.5.4. Heterologous DNA prime-BA71ΔCD2 boost and Georgia2007/1 challenge

The high virulence of Georgia2007/1 hampers finding a lethal challenge dose allowing pig survival for more than a week, thus impeding monitoring of the immune response. To increase the chances to unmask the protective potential of specific ASFV antigens, a prime-boost protocol was established, priming animals with DNA vaccines (encoding specific ASFV antigens) and boosting with a low dose of BA71ΔCD2. Three- to four-week-old male Landrace x Large White piglets were housed together in an experimental box (12 m^2) of the biosafety level 3 facilities at IRTA-CReSA (Barcelona, Spain). Pigs were immunized with two doses of 0.6 mg in 1.5 ml saline of the correspondent endotoxin-free DNA plasmid or plasmid cocktail (Qiagen) two weeks apart. One-third of each vaccine dose was intramuscularly injected into the femoral quadriceps, one-third into the trapezius muscle of the neck, and the last third was subcutaneously injected into the ear, according to an optimized protocol previously described by our group (Argilaguet et al., 2011). Control pigs received the pCMV-Ub empty plasmid following the same administration protocol. Two weeks after the second DNA dose, a suboptimal dose (partially protective) of 10^3 PFU of the BA71ΔCD2 live attenuated ASFV was administered to the pigs as a boost. Three weeks later, pigs were challenged intramuscularly with a lethal dose of 10^3 GEC of the Georgia2007/1 ASFV isolate. Blood samples were drawn from the jugular vein of the pigs and nasal swabs were taken before and after each of the following time points: DNA prime (0, 4 and 7 dpp), BA71ΔCD2 boost (0, 4, 7 and 14 dpb), and

Georgia2007/1 challenge (0, 4, 7, 14 and 21 dpc). Post-mortem examinations were carried out to confirm or discard the presence of ASFV-compatible pathological lesions.

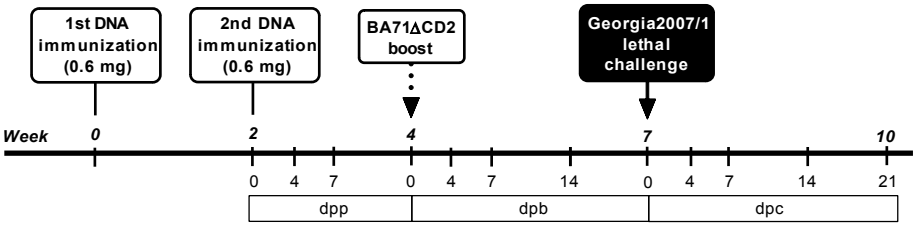


Figure 3-1. Chronogram of the experiment showing the days of immunization and challenge, and the sampling days.

3.5.5. Monitoring of ASF-compatible clinical signs

Animals were observed daily according to a welfare schedule to monitor their health status and to record the clinical signs after the infection with ASFV. Clinical evaluation included rectal temperature, behavior, body condition (prominence of vertebrae and ribs), cyanosis, digestive signs and respiratory signs. Each parameter was scored from 0 to 3 according to the severity (0: normal, 1: mild, 2: moderate, 3: severe), as described by Galindo-Cardiel and collaborators. The humane endpoint was reached when progression of the disease led to an unacceptable loss of general welfare (Galindo-Cardiel et al., 2013).

3.5.6. Quantification of virus titers in serum and nasal swabs by qPCR

Viral DNA from sera and nasal swab-PBS suspensions was quantified using a SYBR Green real-time PCR (qPCR) method previously described in our laboratory (Lacasta et al., 2014). Briefly, the viral genomic DNA was obtained

from 200 µl of sera or swab-PBS suspensions using the NucleoSpin Blood kit (Macherey-Nagel), and then employed as template to amplify a 85 bp-long fragment from the ASFV serine protein kinase gene (*R298L*) using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). Results were expressed as log_{10} numbers of GEC per ml of sera or nasal swab, and the limit of detection of the assay was established at 10^3 GEC/ml.

3.5.7. Immunological readouts

3.5.7.1. Porcine IFNγ ELISpot

IFNγ response was assessed by ELISpot assay using purified mouse anti-pig IFNγ Clone P2G10 (BD Pharmingen) as capture antibody and biotinylated mouse anti-porcine IFNγ antibody P2C11 (BD Pharmingen) as detection antibody, following a previously reported method (Lacasta et al., 2014). Briefly, 96-well plates (Costar 3590, Corning) were coated o/n at 4°C with 5 µg/ml capture antibody in carbonate-bicarbonate buffer, pH 9.6. Plates were washed 3x with PBS, and blocked for 1 hour at 37°C with complete RPMI with 10% FBS. 5 x 10^5 PBMCs/well were used in a final volume of 200 µl with the correspondent stimuli. Peptides were added as a stimulus at a final concentration of 4 µg/ml, and RPMI and 10 µg/ml phytohemagglutinin-M (PHA-M, Sigma-Aldrich) were used as negative and positive controls, respectively. When the LAV BA71ΔCD2 were used as stimulus, 10^5 PFU were added per well. After o/n incubation at 37°C, 5% CO_2, cells were washed out with PBS 0.05% Tween20, and IFNγ was detected using 0.5 µg/ml of biotinylated anti-porcine IFNγ antibody 1 hour at 37°C. After washing, the ELISpot was developed by adding 50 µl of insoluble 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Calbiochem) and stopped by washing with water. The frequency of specific IFNγ-secreting cells (IFNγ-SC)

represented in the graphs is the mean of two replicates, subtracting the counts in the negative control wells. 300 spots/well was considered the limit of our assay resolution (wells with more than 300 spots received a score of 300).

For the use of fibroblasts as APCs in the ELISpot assay, the ratio used was 1 APC:5 autologous PBMCs. Plasmid transfection of the fibroblasts was done by electroporation using the Neon Transfection System 10 μl Kit (Invitrogen). Fibroblasts were collected by trypsinization, centrifuged at 250 xg for 5 minutes at RT and washed with PBS. The appropriate number of cells (100000 fibroblasts/condition) were placed into a clean Eppendorf tube, and suspended in 10 μl of Neon Resuspension Buffer R, and mixed with 500 ng of the corresponding plasmid or plasmid cocktail. Electroporation was done with the following pulse conditions: pulse voltage = 1700 V, pulse width = 20 ms, pulse number = 1. Fibroblasts electroporated with the empty pCMV-Ub plasmid were used as a negative control. Finally, electroporated cells were placed in the corresponding well of a 96-well plate with the autologous PBMCs and proceeded as described above. When working with transfected fibroblasts, no replicates were made. The number of spots in a control well using fibroblasts transfected with the empty pCMV-Ub plasmid, which never exceeded 10, was subtracted from the specific IFNγ-SC represented in the graphs.

3.5.7.2. Detection of ASFV-specific antibodies by ELISA

ASFV-specific antibodies in pig sera were detected by the OIE-approved indirect ELISA assay based on the use of soluble extracts from ASFV-infected cells (Gallardo et al., 2013). The presence of positive sera was detected using peroxidase-conjugated anti-pig IgG at 1/20000 dilution (Sigma-Aldrich) as

secondary antibody and soluble TMB as specific peroxidase substrate (Sigma-Aldrich). Reactions were stopped with 1 N H_2SO_4 (Sigma-Aldrich). Plates were read at a wavelength of 450 nm and results were expressed as OD values.

CHAPTER 4

In silico predictions, immunopeptidomics and gene libraries: identification of ASFV $CD8^+$ T-cell epitopes

4.1. INTRODUCTION

Experimental challenge protocols using attenuated viruses have demonstrated the feasibility of inducing protective immune response to ASFV (King et al., 2011; Lacasta et al., 2015; Monteagudo et al., 2017; Oura et al., 2005), but the molecular and immunological mechanisms eliciting this immunity are poorly understood. Although ASFV-specific antibody responses have been reported (Escribano et al., 2013), and a few studies centered their attention on the innate mechanisms contributing to ASF immunity (Franzoni et al., 2018a), their exact contribution in eliciting protective response remains elusive. On the other hand, $CD8^+$ T lymphocytes are known to play a critical role in protective response against ASFV in a SLA I-dependent manner (Argilaguet et al., 2012; Oura et al., 2005). However, the relevant ASFV $CD8^+$ T-cell antigens are largely unknown. Addressing this critical gap would be important to better understand the role that cellular immunity plays in protection against ASFV, as well as facilitate rational vaccine design.

Many antigen discovery strategies have yielded successful results in terms of identification of pathogen-relevant antigens, but there are no established guidelines for such a process, and each methodology harbor inherent drawbacks. As a result, the aim of this study was to explore the effectiveness of three different strategies for identifying ASFV $CD8^+$ T-cell epitopes with protective potential against the Georgia2007/1 ASFV isolate, the virus currently circulating in Continental Europe and Asia. The first approach here explored was a multiparametric bioinformatic analysis using the Georgia2007/1 proteome as template for the prediction of the peptide sequences more likely to be promiscuously presented by the SLA I pathway.

Computational immunology has provided powerful tools for predicting relevant T-cell epitopes of pathogens (Baratelli et al., 2017; Burgara-Estrella et al., 2013) and cancer cells (Ott et al., 2017; Sahin et al., 2017). Bioinformatic prediction methods rely on the knowledge of peptide-binding motifs of MHC molecules, which in turn depends on the amount of experimental information published. While wide databases on HLA binding motifs are available, predictive tools for swine and other farm animals are limited due to the lack of T-cell epitope data, and they generally infer the results from HLA peptide binding preferences. Therefore, care must be taken when working with non-human predictive software.

The second strategy here employed consisted on characterizing the repertoire of ASFV SLA I-bound peptides found in PAMs *in vitro* infected with the virus. MS profiling of MHC-associated peptides, referred to as immunopeptidomics studies, has emerged as a robust approach to determine pathogen peptides naturally presented during infection. However, an important disadvantage of this methodology lies on the fact that it will lead to the detection of the most abundant peptides, i.e. the most immunodominant, which do not necessarily tie in with the ones with most protective potential (reviewed in Yewdell and Bennink, 1999). Viruses influence immunodominance in many ways, e.g. temporal gene expression (Coupar et al., 1986; Rickinson et al., 1996), and interference with the antigen processing pathway (Hewitt, 2003), ultimately adjusting the peptide repertoire to the most convenient to evade the host immune response. Even so, refinement of MS instrumentation and data-processing capabilities is progressively facilitating the detection of low-abundant peptides, making immunopeptidomics studies a worthy method to precisely define MHC-bound peptides. The potential of each individual peptide from both *in silico*

predictions and immunopeptidomics assays to stimulate ASFV-specific T cells was assessed by IFNγ ELISpot, using as effector cells PBMCs from animals inoculated with the LAV BA71ΔCD2, a successful experimental vaccine developed in our laboratory. Since BA71ΔCD2 is capable to confer protection against the heterologous Georgia2007/1 strain, it can be assumed that protective antigens will be shared between both isolates. In this *in vitro* stimulation assay, peptides directly bind to the SLA I molecules exposed on the cell surface and are capable to stimulate specific $CD8^+$ T cells, albeit they are limited to their specific SLA I molecule match.

Therefore, the third strategy here tested sought to overcome the haplotype specificity of peptide-based assays by using as *in vitro* stimulators full-length proteins, which might contain epitopes with multiple SLA I specificities. Aiming to enhance the SLA I processing and presentation of the antigens, we used gene expression plasmids each encoding individual full-length ASFV ORFs fused to ubiquitin (Rodriguez et al., 2001; Rodriguez and Whitton, 2000) as a source of ASFV antigens for the assay. Individual plasmids were transfected into pig skin fibroblasts thus, serving as APCs in an IFNγ ELISpot assay using autologous PBMCs of ASF-convalescent pigs as effector cells.

4.2. RESULTS

4.2.1. Evaluation of Georgia2007/1 CD8$^+$ T-cell epitope predictions

The sequences scoring the best theoretical ratings in the multiparametric bioinformatic analysis using the Georgia2007/1 proteome as template were synthesized. The final selected set included 330 peptides from 110 ASFV proteins. 266 peptides were a direct output of the prediction software (Appendix I), thus ranging from 8 to 11 amino acids in length, and 64 longer sequences (12-27 amino acids in length) were selected due to the presence of multiple overlapping predicted epitopes (Appendix II).

Interestingly, 22 single proteins accounted for almost the 50% of the selected peptides (Table 4-2). The highest percentage of these proteins corresponded to enzymes involved in nucleic acid metabolism (22.2%), followed by proteins encoded by multigene families (9.9%) and those with uncharacterized function (9.6%). On the contrary, structural and host cell interaction proteins were less represented in this set, accounting for a 3.6 and 1.8%, respectively. With regard to temporal expression, proteins of unknown expression were predominant (63.6%), and both early and late proteins were represented.

Out of the 330 predicted peptides, only one induced an IFNγ response in PBMCs from Georgia2007/1 survivors previously immunized with BA71ΔCD2, thereby *in silico* predictions yielding a percentage of 0.3% of recognized peptides. The immunogenic peptide corresponded to residues 68-86 of the MGF100-1L, and 11 out of the 20 (55%) animals tested showed a specific IFNγ secretion. It has to be taken into account that the peptide is a 19-mer, and was therefore not a direct outcome of the software used, but it

was selected because peptides within that sequence had ten or more predicted $CD8^+$ T-cell overlapping epitopes (Table 4-1).

Table 4-1. Predicted epitopes within the peptide MGF100-1L$_{68-86}$ (LQMAPGGSYFITDNMTEEF). The table summarizes the number of predictions that overlap with each sequence, and the SLA I alleles predicted to present it.

68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	Overlapping peptides
L	**Q**	**M**	**A**	**P**	**G**	**G**	**S**	**Y**											8
SLA-1*0801, SLA-2*1001																			
L	**Q**	**M**	**A**	**P**	**G**	**G**	**S**	**Y**	**F**										9
SLA-1*0801, SLA-2*0601, SLA-2*1001																			
L	**Q**	**M**	**A**	**P**	**G**	**G**	**S**	**Y**	**F**	**I**									**12**
SLA-2*0601, SLA-2*1001																			
	Q	**M**	**A**	**P**	**G**	**G**	**S**	**Y**											8
SLA-1*0201, SLA-1*0202, SLA-1*0401, SLA-1*0701, SLA-1*0702, SLA-1*0801, SLA-1*1301, SLA-2*1001																			
	Q	**M**	**A**	**P**	**G**	**G**	**S**	**Y**	**F**										9
SLA-1*0201, SLA-1*0202, SLA-1*0401, SLA-1*0801, SLA-1*1301, SLA-2*1001																			
		M	**A**	**P**	**G**	**G**	**S**	**Y**	**F**	**I**									**12**
SLA-2*0502																			
								Y	**F**	**I**	**T**	**D**	**N**	**M**	**T**	**E**	**E**	**F**	**12**
SLA-1*1301																			
									F	**I**	**T**	**D**	**N**	**M**	**T**	**E**	**E**	**F**	9
SLA-1*1301																			
										I	**T**	**D**	**N**	**M**	**T**	**E**	**E**	**F**	6
SLA-1*0201, SLA-1*0401, SLA-1*0601, SLA-1*1301																			

Table 4-2. Georgia2007/1 proteins accounting for 49.5% of the predicted CTL epitopes, with their predicted or experimental activity and temporal expression.

Function/protein	Selected peptides (%)		Similarity/activity	Temporal expression
Multigene families	**33**	**9.9**		
MGF110-9L	7	2.1		Unknown
MGF360-1L	5	1.5		Early
MGF360-9L	6	1.8		Unknown
MGF360-21R	6	1.8		Unknown
MGF505-11L	9	2.7		Early
Nucleotide metabolism, transcription, replication and repair	**74**	**22.2**		
C475L	8	2.4	Poly(A) polymerase large subunit	Unknown
C962R	8	2.4	DNA primase	Unknown
D1133L	6	1.8	Helicase superfamily II	Late
D205R	5	1.5	RNA polymerase subunit 5	Unknown
EP424R	5	1.5	FtsJ-like methyl transferase domain	Unknown
F1055L	5	1.5	Helicase superfamily II	Unknown
F778R	5	1.5	Ribonucleotide reductase (large subunit)	Unknown
G1211R	6	1.8	DNA polymerase family B	Early/Late
G1340L	8	2.4	VV A8L-like transcription factor	Unknown
NP1450L	8	2.4	RNA polymerase subunit 1	Late
P1192R	10	3.0	DNA topoisomerase type II	Late

Table 4-2 (continued).

Function/protein	Selected peptides (%)		Similarity/activity	Temporal expression
Other enzymes	**8**	**2.4**		
R298L	8	2.4	Serine protein kinase	Unknown
Morphogenesis	**12**	**3.6**		
CP2475L	12	3.6	Polyprotein pp220	Late
Host cell itneraction	**6**	**1.8**		
EP402R	6	1.8	CD2 homolog	Late
Uncharacterized	**32**	**9.6**		
B475L	5	1.5		Unknown
C717R	11	3.3		Unknown
M1249L	16	4.8		Unknown
TOTAL	**165**	**49.5**		

4.2.2. Evaluation of SLA I-restricted peptides identified by mass spectrometry-based immunopeptidomics

PAMs infected with either Georgia2007/1, BA71 or the LAV BA71ΔCD2 were used for the MS-based immunopeptidomics analysis. The increase of virus titers in the supernatants assessed by qPCR evidenced the replication of the viruses in the cells (Figure 4-1A). After anti-SLA I immunoprecipitation and elution, the presence of SLA I-peptide complexes was confirmed by western blot (Figure 4-1B). The band located between 40 and 50 kDa coincides with the expected molecular weight of about 45 kDa of the SLA class I heavy chain. The slightly heavier band and the 25 kDa band most probably correspond to the heavy and light chains of the anti-SLA I antibody used for immunoprecipitation, which have detached from the sepharose beads. Samples from non-infected PAMs were also analyzed by western blot to discard the possibility of an unspecific interaction of the anti-SLA I antibody (data not shown).

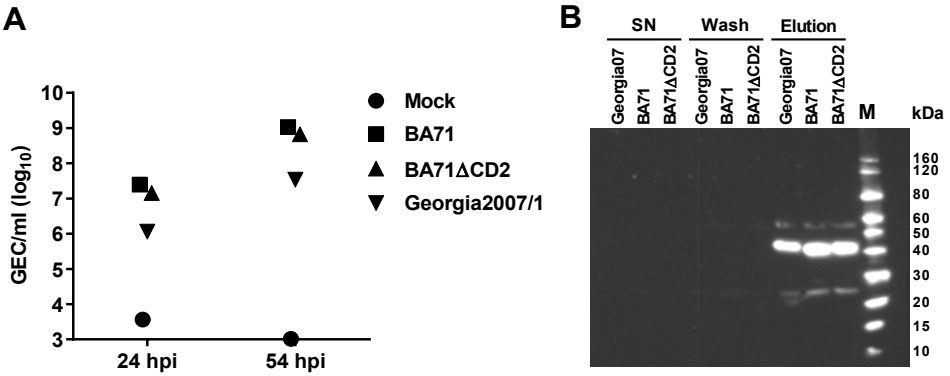


Figure 4-1. (A) ASFV detection by qPCR in supernatants of *in vitro* infected PAMs of a representative assay. The initial inoculum of this assay was 10^5 GEC/ml (MOI 0.01). (B) Western blot for the detection of SLA I molecules in supernatants (SN) of infected cell lysates after incubation with anti-SLA I-coupled sepharose beads, the last wash of the sepharose beads before elution of the SLA I-peptide complexes, and the eluted SLA I-peptide complexes. M: Molecular weight marker.

SLA 1, SLA 2 and SLA 3 constitutively expressed classical SLA I genes were determined from the PAMs used in the immunopeptidomics studies in order to discard limiting the identification of peptides to a single pig haplotype. As shown in Table 4-3, different alleles were determined in all the sequenced genes.

Table 4-3. Alleles of the SLA 1, SLA 2 and SLA 3 classical SLA I genes of the PAMs used in the immunopeptidomics studies. Allele annotation from IPD-MHC Database.

PAMs	SLA 1	SLA 2	SLA 3
1	07:02 / 14:02	02:02_1 / 11:04	04:02 / 04:02
2	11:01:02 / 08:01	05:02 / 07:01	07:01:01 / 03:02
3	04:01:01 / 15:03	07:03_4 / 04:01	ND

ND: Not determined due to low quality of the DNA sample.

Unfortunately, no peptides were found from Georgia2007/1-infected macrophages, independently of the PAMs used, or the multiplicity and time of infection. On the contrary, macrophages infected with BA71 or BA71ΔCD2 did render SLA I-specific peptides. These comparative assays allowed confirming that the lack of Georgia2007/1 SLA I-restricted peptides was strain-specific. Potential explanations for this reality will be further discussed.

Using PAMs from three animals, 135 SLA I-bound peptides (106 different sequences) from 56 different ASFV proteins were determined (Appendix III). 86% of the sequences identified were identical for both BA71 and Georgia2007/1 isolates, 12% of the peptides only differed in one or two amino acids that theoretically did not play key roles in SLA I binding, and only 1.9%

of them showed significant divergences in their sequence between both viruses. These results confirmed the usefulness of this methodology to identify highly conserved peptides between ASFV strains.

Interestingly enough, while BA71-infected PAMs led to the determination of 44 ASFV sequences, 88 peptides were profiled from the BA71ΔCD2-infected samples. As expected for SLA I ligands, the length of peptides ranged from 8 to 13 amino acids, with 50% of the peptides being 9-mers (Figure 4-2A). From the perspective of function, the biggest percentage was for proteins of unknown function, accounting for 35.6% of the total peptides, but peptides involved in transcription and replication, morphogenesis, host cell interaction, and from multigene families proteins were also identified (Figure 4-2B). Regarding the temporal expression of the proteins during the infective cycle, early, intermediate and late proteins were identified, the latter ones representing the highest percentage (35.6%), although a 45.9% of the peptides came from proteins of unknown temporal expression (Figure 4-2C).

With a total of nine peptides, the ASFV protein from which the major number of peptides were identified was the uncharacterized protein B475L, followed by the structural polyprotein pp220 encoded by the CP2475L gene (Simón-Mateo et al., 1993), and the helicase encoded by the D1133L ORF (Yáñez et al., 1993), from which eight and seven peptides were determined, respectively (Table 4-5).

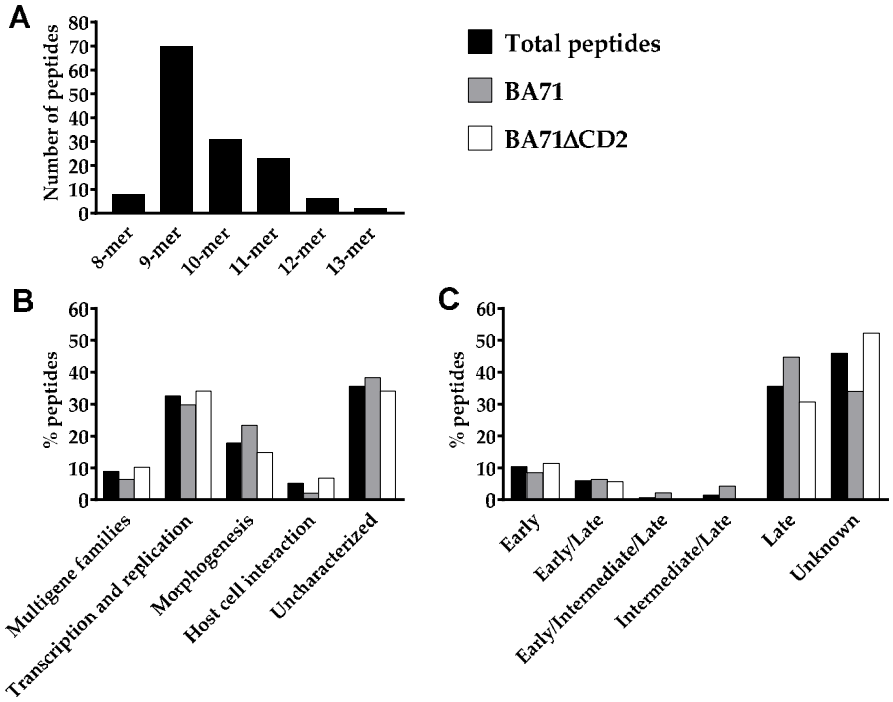


Figure 4-2. Characteristics of the ASFV peptide sequences identified by MS-based SLA I-immunopeptidome analysis regarding (A) peptide length, (B) protein function, and (C) temporal expression.

Five SLA I peptides mapped in regions out of any known ORF (Table 4-4), confirming results previously described (Jenson et al., 2000), and some of them without even having a conventional initiation codon. Strikingly, the five out of frame peptides were all identified from BA71ΔCD2-infected samples. Upholding the idea that these out of frame peptides could also be expressed and play a role in triggering protective response against the Georgia2007/1 ASFV, homologous sequences of four out of these five peptides were found in the genome of the Georgia2007/1 isolate.

Table 4-4. ASFV peptides out of any known ORF identified by MS-based immunopeptidomics of PAMs *in vitro* infected with the BA71ΔCD2 virus**.** The sequence of the peptides and their genome location in both BA71 (grey) and Georgia2007/1 (white) viruses are shown, as well as their overlap with known ORFs, and the variation observed in the reading frame.

Peptide	Genome location	Overlap	Reading frame variation
KTVRNTTPLL	47083 - 47112	A859L	Inverted sense
KTV**H**N**I**TPLL	53564 - 53593		
MKMIQILLL	169806 - 169832	DP238L	Reading frame shift
MKMI**E**ILLL	176053 - 176079		
TCQFEQHLVAI	28806 - 28838	MGF505-3R	Inverted sense
TCQFEQHL**A**AI	35269 - 35301		
HSFSIAMVY	14016 - 14042	Intergenic region	-
HSFSIAMVY	16888 - 16914		
KIDTFTNSSAL	13876 - 13908	Intergenic region	-
-	-		

Table 4-5. ASFV proteins from which peptides were identified by MS-based immunopeptidomics analysis of PAMs infected with ASFV. The table summarizes the total number of sequences identified from each protein, the number of peptides from BA71- and BA71ΔCD2-infected PAMs, the activity of the protein, and their temporal expression during the infective cycle.

Function/Protein	Total peptides	BA71	BA71ΔCD2	Activity/similarity	Temporal expression
Multigene families	**12**	**3**	**9**		
MGF110-6L	2	1	1		Early
MGF360-10L	2	1	1		Unknown
MGF360-8L	1	0	1		Early
MGF505-1R	2	0	2		Early
MGF505-2R	1	0	1		Late
MGF505-3R	1	0	1		Early
MGF505-5R	1	0	1		Early
MGF505-7R	1	0	1		Early
MGF505-9R	1	1	0		Early
Transcription, replication and repair	**44**	**14**	**30**		
C315R	1	0	1	Transcription factor IIB-like	Unknown
C475L	4	1	3	Poly(A) polymerase large subunit	Unknown
D1133L	7	3	4	Helicase superfamily II	Late
D205R	4	0	4	RNA polymerase subunit 5	Unknown
D339L	1	0	1	RNA polymerase subunit 7	Unknown
E301R	2	1	1	Proliferating cell nuclear antigen-like protein	Unknown
EP1242L	3	3	0	RNA polymerase subunit 2	Late
EP364R	1	0	1	ERCC nuclease domain	Unknown

Table 4-5 (continued).

Function/Protein	Total peptides	BA71	BA71ΔCD2	Activity/similarity	Temporal expression
Transcription, replication and repair	**44**	**14**	**30**		
EP424R	3	2	1	FtsJ-like methyl transferase domain	Unknown
F334L	2	0	2	Ribonucleotide reductase (small subunit)	Unknown
G1211R	5	1	4	DNA polymerase family B	Early/Late
G1340L	1	0	1	VV A8L-like transcription factor	Unknown
H359L	1	1	0	RNA polymerase subunit 3	Unknown
I243L	1	1	0	Transcription factor SII	Early/Intermediate/Late
M448R	1	0	1	RNA ligase	Unknown
NP1450L	1	0	1	RNA polymerase subunit 1	Late
NP419L	1	0	1	DNA ligase	Early
P1192R	4	1	3	DNA topoisomerase type II	Late
Q706L	1	0	1	Helicase superfamily II	Unknown
Morphogenesis	**24**	**11**	**13**		
A137R	1	0	1	Protein p11.5	Late
A151R	1	1	0	Protein oxidation pathway	Early/Late
B602L	3	0	3	Chaperone	Late
B646L	5	2	3	Major capsid protein p72	Late
CP2475L	8	6	2	Polyprotein pp220	Late
E120R	2	0	2	Structural protein p14.5, DNA-binding protein	Unknown
E183L	3	1	2	Structural protein p54	Late
E248R	1	1	0	Structural protein	Late

Table 4-5 (continued).

Function/Protein	Total peptides	BA71	BA71ΔCD2	Activity/similarity	Temporal expression
Host cell interaction	**7**	**1**	**6**		
A179L	2	1	1	Bcl-2 apoptosis inhibitor	Early/Late
A238L	1	0	1	IkB-like protein, inhibitor of host gene transcription	Unknown
QP383R	4	0	4	Nif-S like	Unknown
Uncharacterized	**48**	**18**	**30**		
B117L	1	0	1		Unknown
B125R	3	0	3		Unknown
B475L	9	5	4		Unknown
C129R	3	1	2		Late
C257L	1	0	1		Unknown
CP123L	2	0	2		Unknown
CP312R	2	0	2		Unknown
DP238L	2	1	1		Early
E111R	1	0	1		Unknown
F317L	3	1	2		Unknown
H233R	3	1	2		Unknown
H339R	3	1	2		Late
I226R	2	2	0		Intermediate/Late
I73R	2	1	1		Early
I9R	1	0	1		Unknown
K145R	5	2	3		Late
M1249L	5	3	2		Unknown

Out of the 111 different peptides identified by the immunopeptidomics approach, five induced an IFNγ response in PBMCs from animals surviving Georgia2007/1 challenge (Table 4-6), thus representing a 4.5% of the total number of peptides. Interestingly, the three peptides that were recognized by more than one tested animal were identified in BA71ΔCD2-infected PAMs, while the antigenic peptides profiled exclusively from BA71-infected samples induced an IFNγ response in only 10% of the animals (Table 4-6). Far from being conclusive, it suggests that the peptide repertoires of BA71ΔCD2 and BA71 are slightly different.

Table 4-6. ASFV epitopes from immunopeptidomics studies *in vitro* inducing an IFNγ response in PBMCs from ASF-convalescent animals inoculated with the LAV BA71ΔCD2. An animal was classified as responder if 20 or more spots were counted.

Peptide sequence	Protein	Responding animals	Sample	Georgia2007/1 identity
NPTIIMEQY	H339R	1 /10 (10%)	BA71	100%
KNILNTLMF	I226R	1 /10 (10%)	BA71	100%
DKDGNSALHYL	A238L	6 /20 (30%)	BA71ΔCD2	100%
AKIVEEGGEES	K145R	4 /20 (20%)	BA71/BA71ΔCD2	100%
NSTLVIRI	MGF505-7R	4 /20 (20%)	BA71ΔCD2	87.5% NSTLVIRL

As expected, peptides were not uniformly recognized by all pigs, most probably reflecting their marked restriction for specific SLA alleles. Supporting this idea, inoculation of pigs with Freund's-adjuvanted cocktails of about 25 peptides identified by MS-based immunopeptidomics showed that some of the peptides were immunogenic but, again, not consistently recognized by all the pigs (Table

4-7). The peptides here employed were identified in the first immunopeptidomics analysis perfomed. Two immunization groups were defined depending on the theoretical binding affinity of each peptide to the SLA I alleles available at NetMHCpan3.0. Those peptides having high binding affinities (IC50<1000 nM) to the majority of the alleles analyzed were classified as strong binders, while those with lower theoretical binding affinities (IC50>1000 nM) were grouped as weak binders. Two of the recognized peptides, from proteins D1133L and I226R, were classified as strong binders, while the third one, from protein G1211R, was a theoretical weak binder. Remarkably, opposed to what was expected according the theoretical predictions, the theoretical weak binder was recognized by 66.7% of the tested animals, while the two strong binders induced an IFNγ response in only one out of the six pigs (Table 4-7).

Table 4-7. ASFV-specific epitopes inducing an IFNγ response assessed by ELISpot in PBMCs of animals inoculated with Freund's-adjuvanted peptide cocktails.

Theoretical binding affinity	Peptide sequence	Protein	Responding animals	Sample	Georgia2007/1 identity
Strong	YKDETLPYL	D1133L	1 /6 (16.7%)	BA71/BA71ΔCD2	100%
	KNILNTLMF	I226R	1 /6 (16.7%)	BA71	100%
Weak	ENIAYERLETL	G1211R	4 /6 (66.7%)	BA71ΔCD2	90.9% ENIVYERLETL

4.2.3. Use of gene expression plasmids for the identification of immunodominant ASFV antigens

As reflected in the results above described, peptide-based approaches present a major drawback: limited presentation by restricted SLA haplotypes. With the aim of avoiding this restriction, we attempted to extend the studies described above, aiming to identify promiscuous $CD8^+$ T-cell determinants from ASFV, focusing on its full-length antigens. pCMV-Ub plasmids encoding full-length ASFV ORFs were transfected into primary fibroblasts and those were used as APCs in the ELISpot assay, using PBMCs from the same animal (autologous) as effector cells.

The optimal conditions for fibroblast electroporation using the Neon Transfection System (Invitrogen) were previously setup transfecting the pCMV-GFP plasmid into primary swine fibroblasts using different electroporation settings. The best condition was selected considering the percentage of transfected cells (GFP^+) with respect to live cells. The conditions used gave a 36.14% of GFP^+ cells and a mortality of 4.60%. Although we could not assure that these values were constant in the following assays, it served as a proof of concept for demonstrating that our primary fibroblasts could express proteins under the pCMV promoter.

We started using a collection of 73 recombinant plasmids belonging to an ASFV gene expression library available in the lab (Appendix IV). Although this gene expression library contains almost 100 recombinant plasmids, the 15 tested in Chapter 5 and others encoding already known immunogenic proteins were not included. In a first screening step, mixes of 10 or 11 plasmids were electroporated into fibroblasts to, later on, test the individual plasmids from the mixes capable to specifically induce IFNγ response. Expression of similar ASFV gene expression plasmids was confirmed by adding a FLAG-tag sequence at the C-terminus of the

ASFV gene and detecting it by immunofluorescence (Appendix V), as described in Materials and Methods. However, not all the clones of the gene expression library here employed were tested, thus existing the possibility of some of them not being expressed.

From the ASFV gene expression library, we identified one single clone: pCMV-Ub-MGF505-7R, capable of specifically stimulating IFNγ expression in all the tested animals except for one (Figure 4-3). The non-responder pig (pig 13) did show ASFV-specific IFNγ-SC, thus discarding a possible immunosuppressed state of the PBMCs. Nevertheless, a failure in some specific electroporation events (one transfection per plasmid) could not be excluded.

To the best of the author's knowledge, this is the first ASFV antigen promiscuously inducing $CD8^+$ T-cell response in vaccinated and protected pigs, and might represent a good candidate for future diagnostic and vaccine developments. Remarkably, an individual peptide from MGF505-7R (MGF505-$7R_{334-341}$: NSTLVIRI), was identified in our immunopeptidomics assays using PAMs infected with BA71ΔCD2 (Table 4-6). The fact that this peptide was recognized by a small proportion of ASF-convalescent pigs confirm its SLA I-restricted nature and argue positively in favor of the advantage of using the full-length MGF505-7R protein, containing multiple $CD8^+$ T-cell determinants, in our future developments.

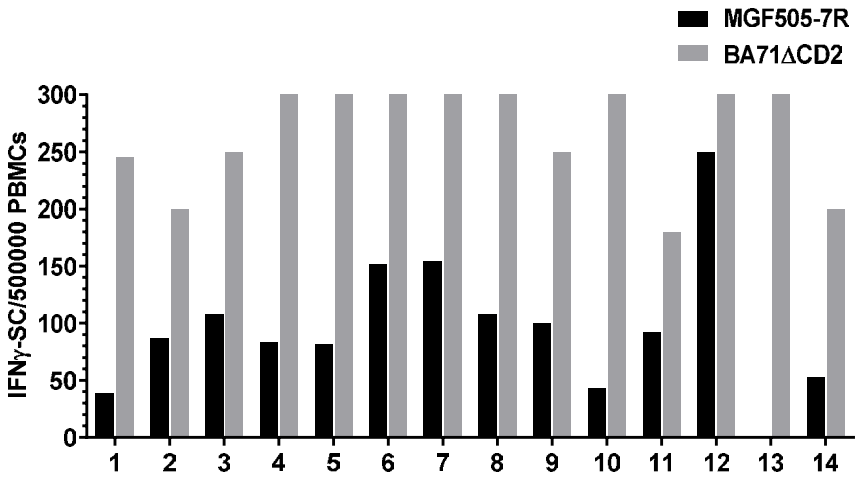


Figure 4-3. In black, IFNγ response assessed by ELISpot assay using fibroblasts transfected with the pCMV-Ub-MGF505-7R plasmid as APCs, and PBMCs from ASF-convalescent animals as effector cells. The number of spots when stimulating with fibroblasts transfected with the pCMV-Ub empty plasmid, which never exceeded ten, were subtracted from the represented values. In grey, the levels of ASFV-specific IFNγ-SC are represented.

Interestingly, two additional antigenic proteins were identified using this methodology: A238L and MGF100-1L. Despite those two proteins showed less promiscuity than MGF505-7R, they were still broadly recognized by ASFV-specific T cells. Thus, fibroblasts transfected with the recombinant plasmids encoding the full-length A238L and MGF100-1L induced an IFNγ response in 60% and 50% of the animals after ASFV *in vitro* stimulation, respectively (Figure 4-4). Interestingly, peptides previously identified from these proteins by immunopeptidomics analysis: A238L$_{81\text{-}91}$ and MGF100-1L$_{68\text{-}86}$, specifically stimulated an IFNγ response in 30% and 50% of the pigs, respectively (Table 4-1, 4-6).

The rest of the proteins from which antigenic peptides were identified by immunopeptidomics were not included in this screening since their immunogenicity and protective potential will be described later in Chapters 5 and 6.

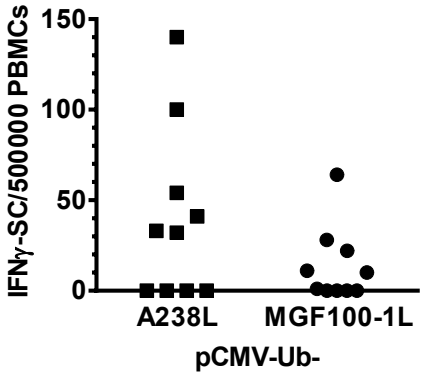


Figure 4-4. IFNγ response to A238L and MGF100-1L full-length Georgia2007/1 proteins assessed by ELISpot assay using fibroblasts transfected with the pCMV-Ub-A238L and pCMV-Ub-MGF100-1L plasmids respectively as APCs and PBMCs from ASF-convalescent animals as effector cells.

4.3. FINAL REMARKS

The results obtained in the present work strongly suggest that immunopeptidomics analysis of PAMs *in vitro* infected with ASFV are a more reliable strategy than *in silico* predictions for the identification of ASFV-specific $CD8^+$ T-cell epitopes. However, peptides are not uniformly recognized by all pigs, probably reflecting their marked restriction for specific SLA alleles. This can be overcome by the use of full-length proteins, which here served to identify MGF505-7R as a novel immunodominant and promiscuous ASFV antigen, and

two additional antigens: A238L and MGF100-1L recognized by at least 50% of the animals tested. Therefore, focusing on full-length proteins instead of epitopes could be a more suitable approach for the identification of ASFV antigens with potential to promiscuously induce specific T-cell responses.

In addition, the observation that ASFV-specific SLA I-restricted epitopes were identified from PAMs *in vitro* infected with BA71 and BA71ΔCD2, while Georgia2007/1 seems to suppress peptide presentation, may open new avenues to understand the evasion strategies used by this specific virus strain and to design more rational vaccine strategies in the future.

CHAPTER 5

M448R and MGF505-7R: two immunodominant ASFV antigens with protective potential

5.1. INTRODUCTION

Although the crucial role of $CD8^+$ T cells in protection against ASFV has been established (Oura et al., 2005), the relevant antigens specifically stimulating this cell subset and inducing protective responses have not been determined so far. Knowledge of these antigens could be key for understanding how $CD8^+$ T cells mediate their protective effect, and further developing rational ASF subunit vaccines.

MS technologies permit the high-throughput identification of peptides associated to MHC molecules from cells or tissues. These so-called immunopeptidome studies provide a unique opportunity to identify pathogen epitopes presented during infection. We previously demonstrated that analysis of the SLA I immunopeptidome of PAMs *in vitro* infected with ASFV can be an effective tool to identify ASFV $CD8^+$ T-cell epitopes (Chapter 4). However, the marked restriction of peptides for specific SLA alleles is one of the major drawbacks of peptide-based vaccination approaches. Therefore, in the present study we focused on full-length proteins. According to the identification of SLA I-restricted peptides by MS-based immunopeptidomics, 15 ASFV-encoded proteins were selected as potential inducers of $CD8^+$ T-cell responses.

In order to assess their immunogenicity and protective potential, pigs were inoculated with the 15 selected recombinant plasmids, each encoding an ASFV antigen with an ubiquitin sequence at the N-terminus, aiming to optimize SLA I presentation and enhance the induction of $CD8^+$ T-cell responses (Argilaguet et al., 2012; Lacasta et al., 2014; Rodriguez and Whitton, 2000). In view of the unsuccessful results previously obtained in the lab when using DNA

immunizations to induce protective immunity against Georgia2007/1 (Rodríguez et al., unpublished results), here we explored a heterologous regimen including DNA prime immunization with the 15 selected antigens followed by inoculation with a low dose of the live attenuated BA71ΔCD2 ASFV, as explained in Materials and Methods. The cross-protective capabilities of BA71ΔCD2, conferring protection not only against the parental BA71 but also against heterologous viruses including the Georgia2007/1 isolate, should allow the enhancement of any cross-protective response induced by the recombinant plasmids, thus increasing the chance of identifying relevant antigen-specific T cells. In addition, this immunization protocol allowed to evaluate the capability of pigs primed with the selected 15 antigens to achieve protection against a Georgia2007/1 lethal challenge, in comparison to a control group primed with a plasmid not encoding any ASFV-specific protein.

In the first experiment here described, partial protection against a Georgia2007/1 challenge in pigs receiving the 15 selected antigens as a DNA prime vaccination was observed. One protein, M448R, showed the most immunodominant nature among the antigens included in the plasmid cocktail. Moreover, M448R exhibited a promiscuous cellular response in ASFV-convalescent pigs not receiving a DNA prime vaccination.

As described in Chapter 4, the use of fibroblasts transiently expressing ASFV antigens as APCs for autologous PBMCs from ASF-convalescent pigs, allowed identifying another ASFV protein: MGF505-7R, with a promiscuous nature similar to that of M448R. Therefore, both M448R and MGF505-7R were promising candidates to be further explored for their importance in ASFV protective immunity. In consequence, the present study describes a second *in vivo*

experiment designed to assess the immunogenicity and protective potential of M448R and MGF505-7R combined, using a prime-boost immunization protocol as above explained.

5.2. RESULTS

5.2.1. DNA immunization with a cocktail of plasmids encoding 15 ASFV pre-selected proteins confers partial protection against Georgia2007/1 challenge infection

Based on previous results of an SLA I-immunopeptidomics study of ASFV-infected PAMs, 15 ASFV antigens were selected as potential candidates to induce $CD8^+$ T-cell responses, and their immunogenicity and protective potential against a Georgia2007/1 lethal challenge was assessed.

The selected set included three ASFV potential enzymes likely involved in nucleic acid metabolism: D339L (RNA polymerase subunit 7), EP424R (putative methyl transferase), and M448R (RNA ligase); as well as I243L, an assumed transcription factor (J. M. Rodríguez et al., 1996). Also two proteins involved in virion morphogenesis were included: the structural protein p37, product of the processing of the polyprotein pp220 (Simón-Mateo et al., 1993), and the chaperon B602L. Multigene family 505 members MGF505-1R and MGF505-3R were also selected, along with seven proteins of unknown function: B475L, DP238L, H339R, I226R, I73R, I9R, and K145R. All the above mentioned proteins represent early, late and intermediate proteins during the ASFV replication cycle (Table 5-1).

Expression of the ASFV proteins here tested was confirmed by anti-FLAG-tag immunofluorescence in transfected RK13 cells as described in Materials and Methods (Appendix V).

Table 5-1. ASFV selected to be tested for their immunogenicity and protective potential against Georgia2007/1 due to the identification of SLA I-restricted peptides by immunopeptidomics.

Protein	Activity/Similarity	Temporal expression
B475L	-	-
B602L	Chaperone	Late
p37 (CP2475L/partial)	Structural polyprotein	Late
D339L	RNA polymerase subunit 7	-
DP238L	-	Early
EP424R	FTS J-like methyl transferase domain	-
H339R	-	Late
I226R	-	Intermediate/Late
I243L	Transcription factor SII	Early/Intermediate/Late
I73R	-	Early
I9R	-	-
K145R	-	Late
M448R	RNA ligase	-
MGF505-1R	-	Early
MGF505-3R	-	Early

Following the immunization protocol shown in Figure 3-1, three out of five (60%) pigs primed with the 15 recombinant plasmids survived the lethal challenge with Georgia2007/1. Conversely, and in line with previous results from the lab using the low dose of BA71ΔCD2, only one out five (20%) of the control pigs immunized once with the low dose of BA71ΔCD2 survived (Figure 5-1).

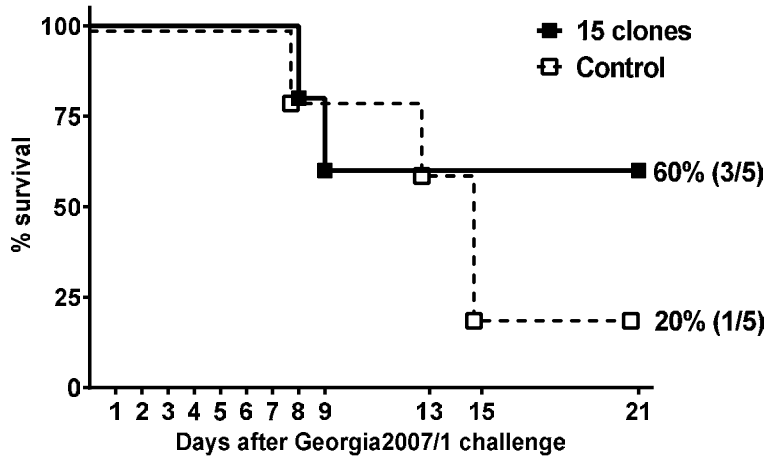


Figure 5-1. Survival percentage after Georgia2007/1 lethal challenge of pigs primed with either the selected 15 recombinant plasmids (15 clones) or the empty pCMV-Ub plasmid (Control). Both groups were immunized with a low dose of the LAV BA71ΔCD2.

During the experiment, animals were monitored daily for ASF typical clinical signs, including fever, lethargy, general body condition, digestive signs, respiratory signs and cyanosis. Even though the four surviving pigs developed transient ASF-compatible symptomatology, the three primed with the 15 recombinant plasmids showed milder clinical signs compared to the survivor from the control group (Figure 5-2, 5-4, pig 185). These results correlated with: i) delayed and shorter viremia in serum in surviving animals in the group primed with the 15 recombinant plasmids, ii) a reduction of 1 to 2 $\log_{10}$ in their maximum titers of the challenge virus (Figure 5-3A, pig 180), and iii) no detectable virus at any time post-challenge (Figure 5-3A, pigs 181 and 184). In addition, a one to two $\log_{10}$ reduction in nasal shedding was observed compared to both the non-surviving animals and the survivor in the control group (Figure 5-3B, D).

The survivor from the control group (pig 185) showed a high and prolonged fever peak (>41°C for 5 days) starting at 4 dpc accompanied with an apathic behavior (Figure 5-2, 5-4B), while the three surviving animals in the 15 recombinant plasmids-primed group had mild fever lasting at most three days (Figure 5-4A). Pig 181 experienced sporadic symptoms coinciding with mild fever peaks, and pig 184 had no apparent symptomatology throughout the study (Figure 5-2). Although survivor pig 180 also showed apathy and evident dyspnea. The onset of clinical signs was delayed compared to both the control that survived and the non-survivors (Figure 5-2). Pigs 182 and 183 of the "15 clones" group succumbed at 9 and 8 dpc, respectively, and their temperature and viral load in sera and in nasal swabs were similar to those in the control group (Figure 5-3, 5-4).

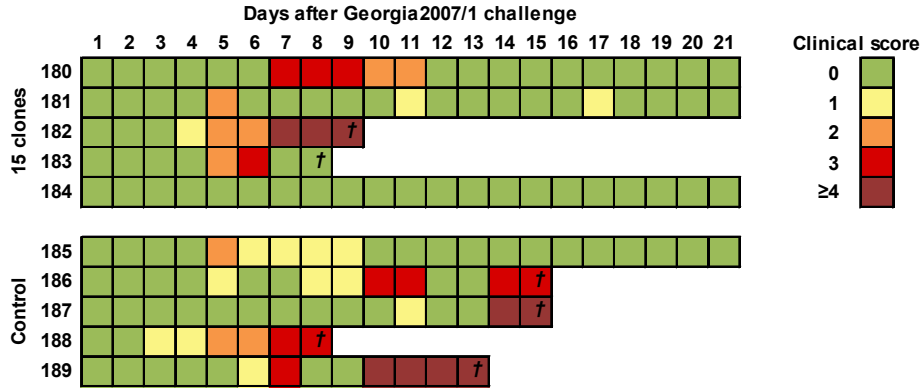


Figure 5-2. Clinical score of the pigs after Georgia2007/1 challenge. Clinical condition included apathy, body condition, digestive signs, respiratory signs and cyanosis, each receiving a score from 0 to 3. The final clinical score in the graph corresponds to the sum of these values. † indicates day of euthanasia.

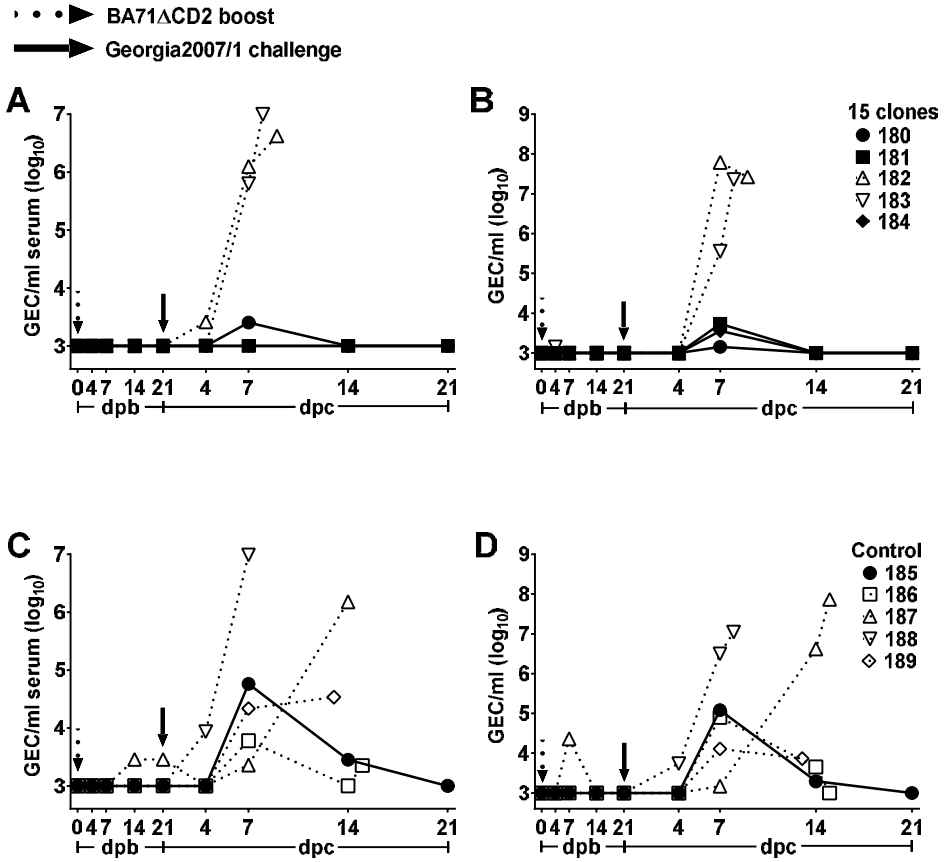


Figure 5-3. Detection of ASFV DNA by qPCR in serum samples and nasal swabs of the pigs after the BA71ΔCD2 vaccination (dpb) and the Georgia2007/1 challenge (dpc). (A, B) Detection of ASFV DNA in serum and nasal swabs, respectively, from the individual animals primed with the 15 recombinant plasmids. (B, C) Detection of ASFV DNA in serum and nasal swabs, respectively, from the individual control animals. Surviving animals are represented in solid lines, while dashed lines indicate that animals had to be euthanized after challenge.

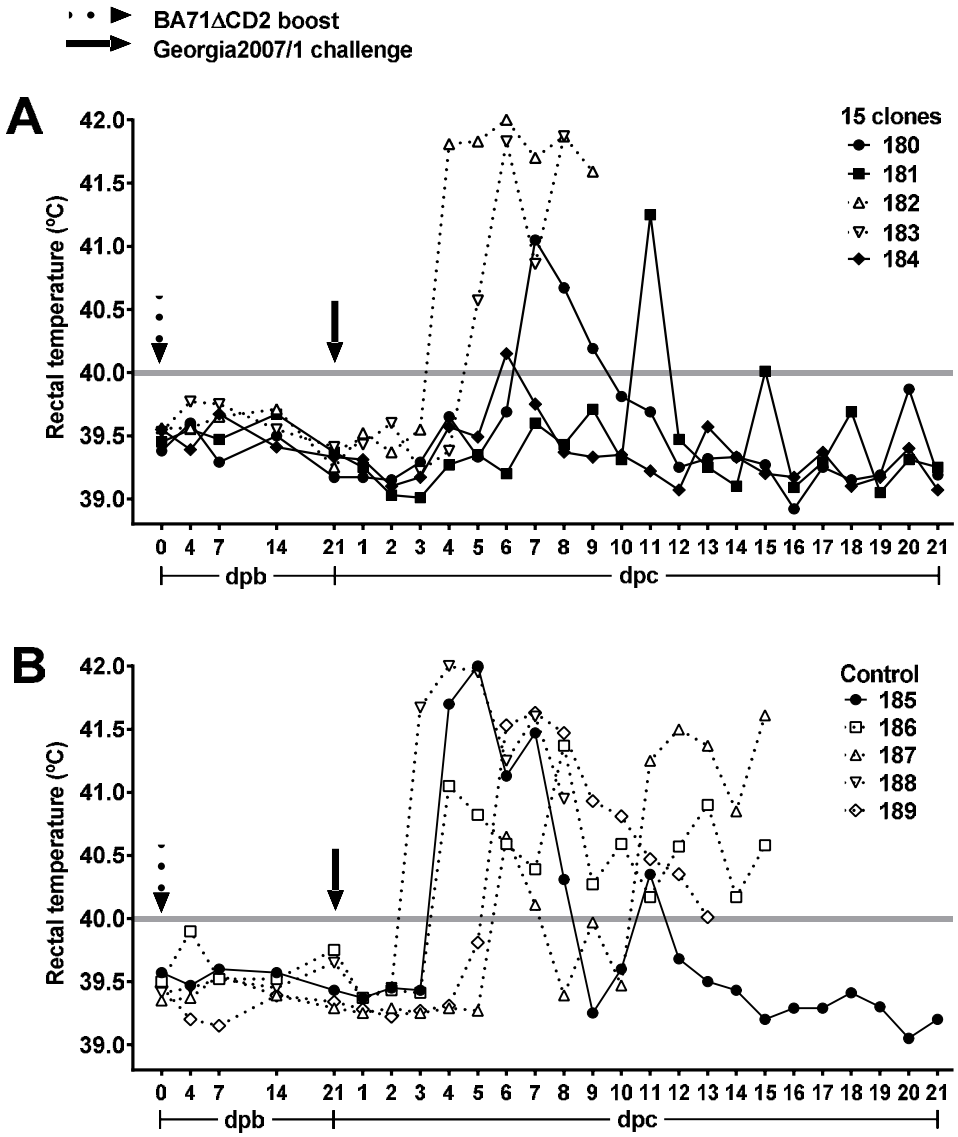


Figure 5-4. Rectal temperature values of the pigs after the BA71ΔCD2 vaccination (dpb) and the Georgia2007/1 challenge (dpc). (A) Evolution of the rectal temperature of pigs primed with the 15 clones. (B) Individual temperature values of control animals. Surviving animals are represented in solid lines, while dashed lines indicate that animals had to be euthanized after challenge.

5.2.2. Immunization with the 15 recombinant plasmids induces ASFV-specific T cells, but no antibody response is detected

Administration of the 15 recombinant plasmids did not induce any specific antibody response but it did induce detectable ASFV-specific IFNγ response at 14 dpp (Figure 5-5), thus indicating the immunogenicity of at least one of the 15 included antigens. The results here obtained confirmed previous results from the lab with ubiquitinated constructs designed to induce specific CTL responses while abolishing humoral responses (Argilaguet et al., 2012; Lacasta et al., 2014).

After the BA71ΔCD2 vaccination, all the animals seroconverted and developed ASFV-specific T response (Figure 5-5). No clear correlation of protection was observed considering the level of antibodies or T cells induced after immunization, since control animals vaccinated only once with BA71ΔCD2 did also show a notable ASFV-specific immune response. Notwithstanding, the two animals from the "15 clones" group that did not survive the Georgia2007/1 challenge showed the lowest level of antibodies and ASFV-specific T response at the time of challenge infection. The T-cell response induced directly after DNA immunization (Figure 5-5A) very likely contributed to a better control of ASFV infection and virus clearance in the "15 clones"-primed group.

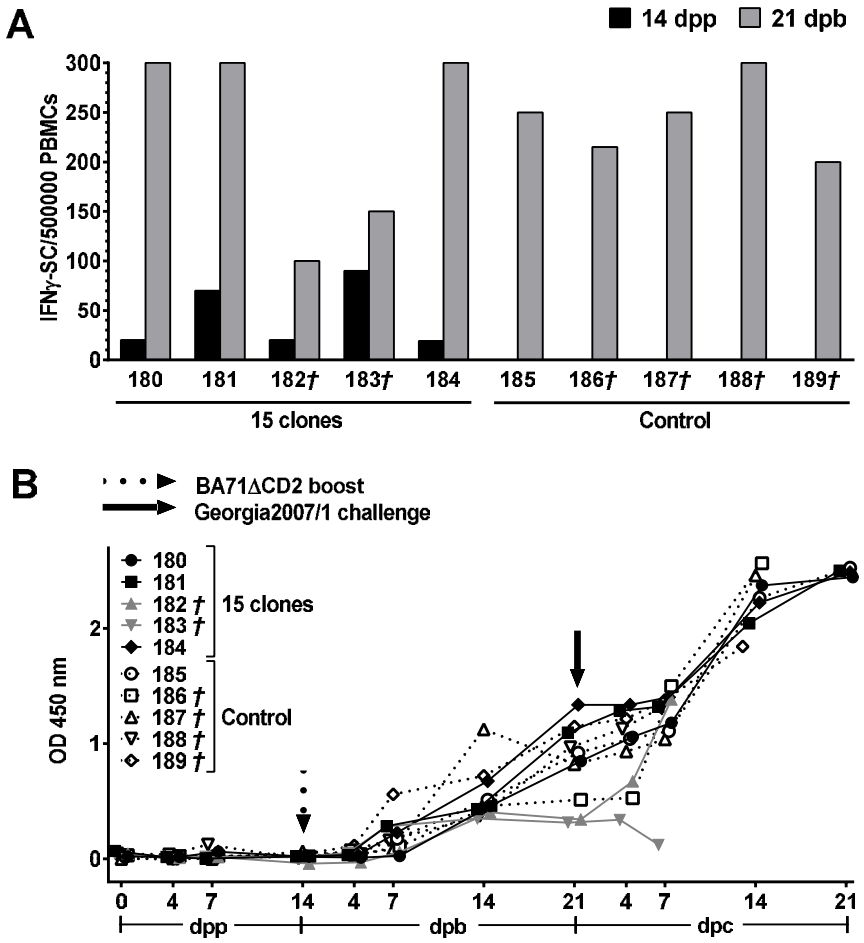


Figure 5-5. (A) ASFV-specific IFNγ response assessed by ELISpot using BA71ΔCD2 as stimulus at 14 days after the 2nd DNA immunization (14 dpp) and at 21 days after BA71ΔCD2 boost (21 dpb). 300 spots is considered the limit of detection of the assay. (B) ASFV-specific antibodies in sera. Results are expressed as OD values at a wavelength of 450 nm. † indicates animals that had to be euthanized after challenge.

5.2.3. M448R shows an immunodominant nature in ASFV-convalescent animals previously primed with the 15 recombinant plasmids

Once confirmed the immunogenicity of the administered plasmid cocktail, it was aimed to determine the immunogenic profile of each of the 15 ASFV antigens used. To this end, swine fibroblasts were electroporated with each individual recombinant plasmid contained in the immunization mix and used as APCs in an ELISpot assay with autologous PBMCs obtained at 14 dpc as effector cells.

Interestingly, high levels of IFNγ-SC (>50 spots) were exclusively detected in all the animals when PBMCs were incubated with fibroblasts transfected with pCMV-Ub-M448R. The number of M448R-specific IFNγ-SC was comparable to that obtained when transfecting the mix of the 15 recombinant plasmids (Figure 5-6). This result suggests that T-cell immunity towards M448R was largely responsible for the immunogenicity observed after immunization with the 15 recombinant plasmids. Furthermore, M448R-specific T cells primed could have contributed to the milder course of Georgia2007/1 infection and increased survival in the "15 clones" group.

The number of spots when using ASFV as stimulus after the boost was much higher than when using swine fibroblasts transfected with the pCMV-Ub-M448R plasmid, most probably explained by different reasons. Firstly, ASFV infects APCs much better than plasmids transfect them, therefore being more efficiently processed. Secondly, PBMCs from ASFV recovered pigs might also recognize other antigens present in ASFV than those contained in the plasmid mix.

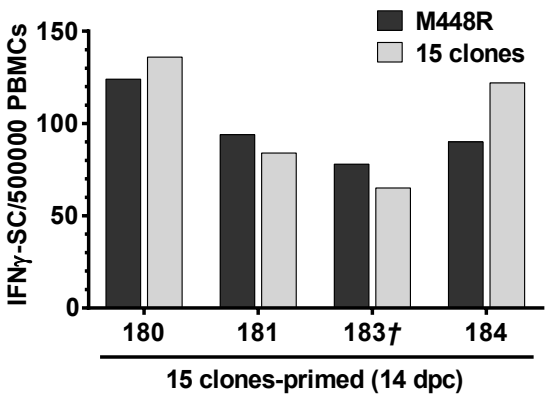


Figure 5-6. IFNγ response to swine fibroblasts transfected with pCMV-Ub-M448R or the 15 recombinant plasmid cocktail at 14 dpc. Animal 183 died at 8 dpc, so PBMCs from 14 dpb were tested. Animal 182 was not analyzed due to a contamination in the swine fibroblast cell culture.

5.2.4. M448R induces a specific T-cell response during ASFV infection without a prior DNA prime

In a next step, it was attempted to determine if M448R induced ASFV-specific $CD8^+$ T cells not only when pigs were primed with pCMV-Ub-M448R, but also after ASFV infection. For this, PBMCs from BA71ΔCD2-immunized animals and challenged with a lethal dose of Georgia2007/1 (not previously primed with the pCMV-Ub-M448R plasmid) were tested in an ELISpot assay with autologous swine fibroblasts transfected with the pCMV-Ub-M448R plasmid. Strikingly, an IFNγ response against M448R when expressed in the pCMV-Ub plasmid was induced in 7 out of 9 animals (Figure 5-7), thus confirming the promiscuous nature of M448R and the presence of immunodominant T-cell epitopes within it.

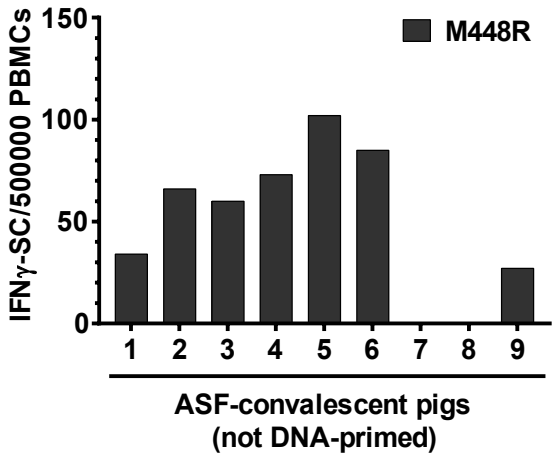


Figure 5-7. IFNγ response assessed by ELISpot assay using swine fibroblasts transfected with the pCMV-Ub-M448R plasmid as APCs and PBMCs from ASF-convalescent animals as effector cells (21 days post Georgia2007/1 challenge).

5.2.5. Immunization with pCMV-Ub-M448R and pCMV-Ub-MGF505-7R confers partial protection against Georgia2007/1 lethal challenge

Given the immunodominant feature of M448R and its protective potential, we decided to include it in future experimental vaccine formulations, together with MGF505-7R, a second antigen promiscuously recognized by ASF-convalescent pigs (Chapter 4). Therefore, the immunogenicity and protective potential of these two antigens was assessed by priming a group of pigs with two DNA plasmids encoding M448R and MGF505-7R proteins, with an ubiquitin sequence at the N-terminus, and boosting with a low dose of BA71ΔCD2 (Figure 3-1).

In line with previous results using a low dose of BA71ΔCD2, only one animal in the control group out of five (20%) survived the Georgia2007/1 challenge infection. In contrast, in the pCMV-Ub-M448R+pCMV-Ub-MGF505-7R-

immunized group three out of five pigs survived the lethal challenge infection (Figure 5-8).

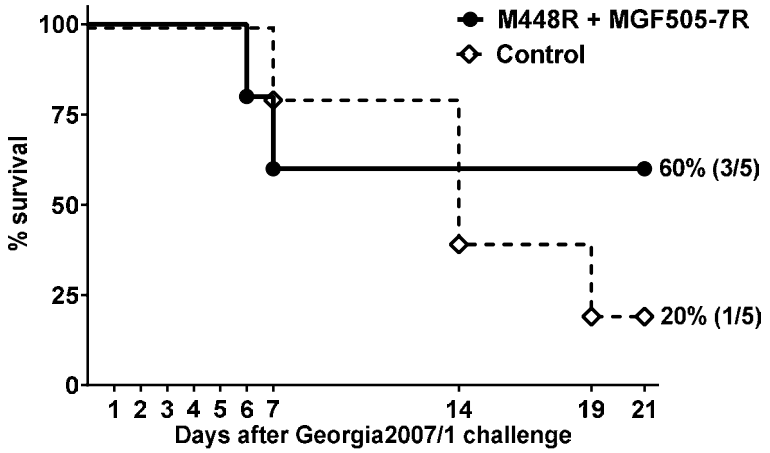


Figure 5-8. Percentage of surviving pigs within the M448R+MGF505-7R-primed group (solid line) and the control group (dashed line) after a Georgia2007/1 lethal challenge infection.

Surviving animals from the DNA-primed group showed lower and shorter fever peaks than the control pigs (Figure 5-10). Thus, pig number 89 showed no fever (Figure 5-10A) and no other clinical signs at any time after the Georgia2007/1 challenge (Figure 5-9), and pig number 90 showed a brief peak of fever at 20-21 dpc (Figure 5-10). The third survivor in this group (pig 88) showed mild apathy and the body condition was slightly affected (clinical score never exceeded 2), but was completely recovered by day 14 post-challenge (Figure 5-9). Pigs number 86 and 87 from the DNA-primed group succumbed the infection showing ASF clinical signs indistinguishable from that found in control pigs (vaccinated with BA71ΔCD2 only). These included severe ASF symptoms, such as lethargy, depression, visible vertebrae and/or ribs, dyspnea and cyanosis (scoring at least 4 in the clinical signs scale) (Figure 5-9).

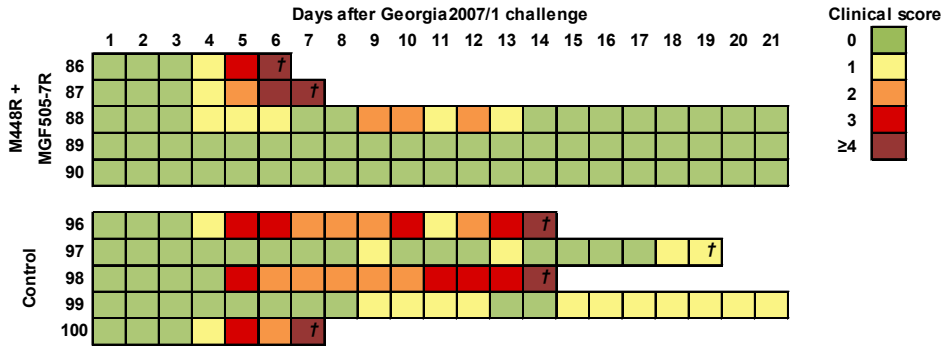


Figure 5-9. Individual clinical scores after the Georgia2007/1 challenge. Clinical condition included apathy, body condition, digestive signs, respiratory signs and cyanosis, each receiving a score from 0 to 3. The final clinical score in the graph corresponds to the sum of these values. † indicates day of sacrifice.

ASF-clinical signs in the control group were more evident with two exceptions. Pig number 97 was found dead at 19 dpc, after suffering a mild ASF symptomatology (Figure 5-9) and high fever (>41°C) for two consecutive days before dying (Figure 5-10B). Pig number 99 suffered prolonged lethargy starting at 9 dpc and lasting until the end of the trial and also developed cyanosis in ears and tail (Figure 5-9), but ultimately survived the Georgia2007/1 challenge.

Despite the survival percentage in the M448R+MGF505-7R-primed group was the same than when priming with the 15 clones (60%), the animals seemed to cope much better with the Georgia2007/1 infection, at least according to the clinical signs observed.

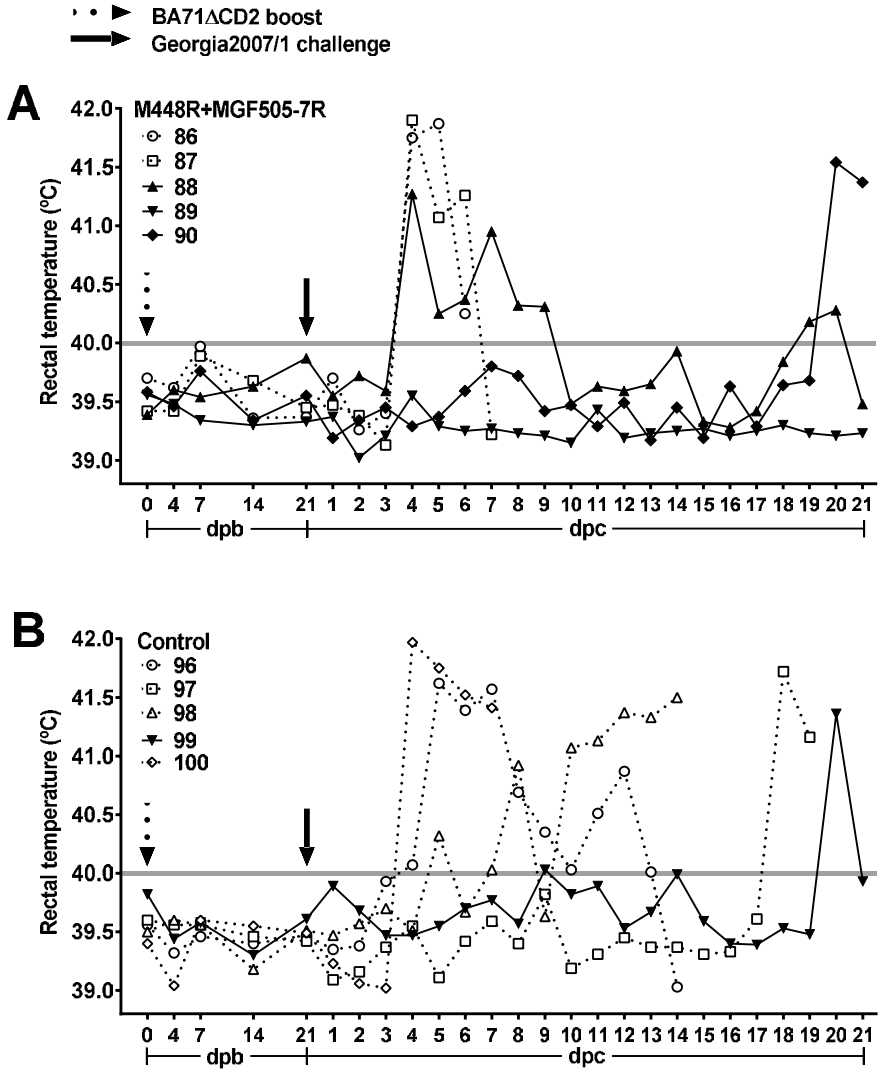


Figure 5-10. Evolution of the rectal temperature of pigs after BA71ΔCD2 boost and Georgia2007/1 inoculation. (A) Individual rectal temperature values of pCMV-Ub-M448R + pCMV-Ub-MGF505-7R-immunized pigs. (B) Individual rectal temperature values of control animals. Surviving animals are represented in solid lines, while dashed lines indicate that animals had to be euthanized after Georgia2007/1 challenge.

5.2.6. Priming with pCMV-Ub-M448R and pCMV-Ub-MGF505-7R contributes to reduced virus titers in serum and reduced nasal shedding after Georgia2007/1 challenge infection

Serum and nasal swabs were collected at the indicated sampling days and then tested for the presence of ASFV DNA by qPCR. After the BA71ΔCD2 vaccination, no viral DNA was found in either serum or nasal swabs from the DNA-primed animals. Conversely, and evidencing the replication of our LAV, a peak of viral DNA in the serum was detected in one control animal after administration of BA71ΔCD2 (Figure 5-11, pig 98).

After Georgia2007/1 inoculation, the level of viral DNA in the serum of the animals that survived always remained below 10^6 GEC/ml, in contrast with the pigs that had to be sacrificed, all reaching at least 10^7 GEC/ml at some time point after the infection (Figure 5-11). Focusing on the surviving animals, no ASFV DNA was detected in serum from animals 89 and 90, and low levels were found in nasal swabs, except for the virus peak found at 21 dpc in animal 90 (Figure 5-11A, B). These results concurred with the absence of clinical signs reported in these animals. The detection of ASFV DNA in both serum and nasal swab from animal 88 at 7 dpc is consistent with the mild ASF symptomatology observed in this surviving animal. Despite the prolonged lethargy and cyanosis reported, the surviving animal in the control group (pig 99), was capable of controlling virus replication, showing low virus DNA levels in serum and reduced nasal shedding (Figure 5-11C, D). The severe ASF clinical signs observed in the animals that had to be sacrificed from the DNA-primed group coincided with both high virus titers in serum and nasal swabs, and no difference was found between the succumbing animals in the immunized and the control group (Figure 5-11).

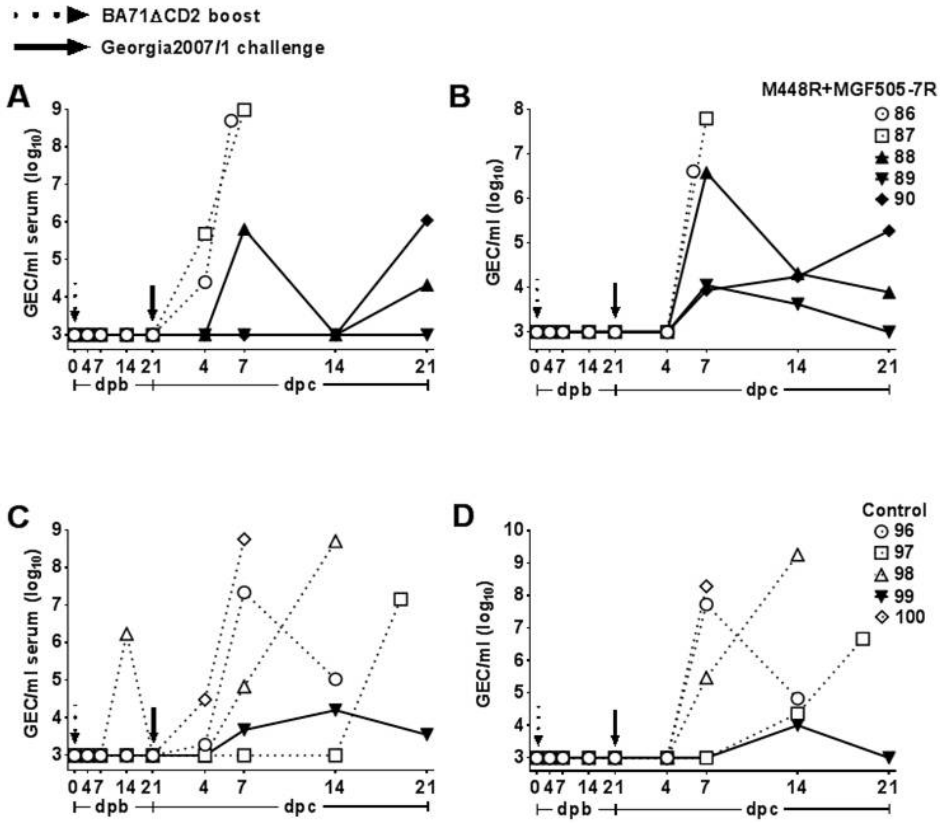


Figure 5-11. Detection of ASFV DNA by qPCR in serum and nasal swabs of the pigs before and after Georgia2007/1 inoculation. (A, B) Detection of ASFV DNA in serum and nasal swabs, respectively, from the individual animals primed with pCMV-Ub-M448R+pCMV-Ub-MGF505-7R. (B, C) Detection of ASFV DNA in serum and nasal swabs, respectively, from the individual control animals. Surviving animals are represented in solid lines, while dashed lines indicate that animals had to be euthanized after Georgia2007/1 challenge infection.

5.2.7. DNA immunization with pCMV-Ub-M448R and pCMV-Ub-MGF505-7R induces ASFV-specific T-cell response capable of recognizing both M448R and MGF505-7R antigens *in vitro*

As expected, administration of the pCMV-Ub-M448R and pCMV-Ub-MGF505-7R plasmids did not induce any detectable ASFV-specific antibody response (Figure

5-12). No difference was observed regarding antibody response among surviving and succumbing animals before Georgia2007/1 inoculation, all showing elevated levels at the day of challenge (except for pig number 100, which also showed weak T-cell response).

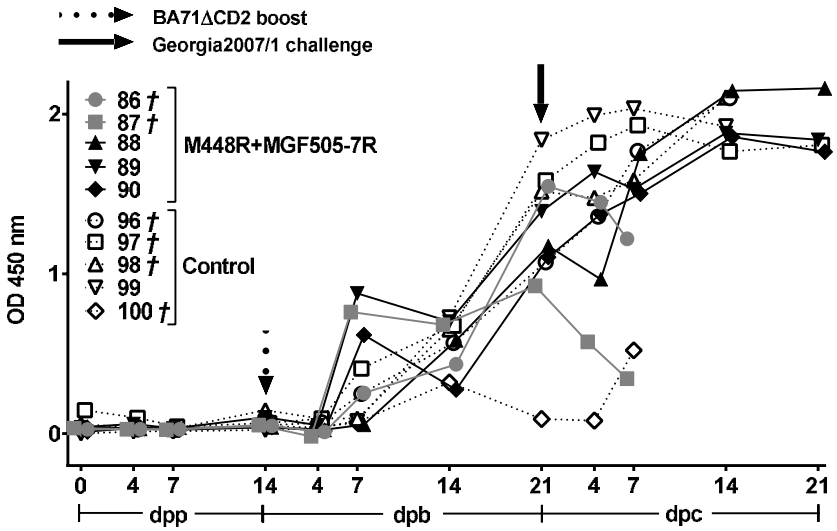


Figure 5-12. ASFV-specific antibody detection in serum by ELISA. Results are expressed as OD values at a wavelength of 450 nm. † indicates animals that had to be euthanized after Georgia2007/1 challenge.

As expected, IFNγ response against swine fibroblasts transfected with pCMV-Ub-M448R and pCMV-Ub-MGF505-7R were detected seven days after the second DNA immunization in DNA-primed animals, but not in the control group (Figure 5-13). Although low levels of IFNγ-SC were detected (likely because of low immunogenicity of DNA vaccines in large animals), this confirmed the immunogenicity of the tested antigens when administered in a DNA-based

formulation. Notably, at this early time point, the two animals not showing M448R- and MGF505-7R-specific T-cell response (pigs 86 and 87) were the ones that later succumbed the Georgia2007/1 challenge.

In line with previous results showing the promiscuous and immunodominant nature of both M448R and MGF505-7R during ASFV infection, after the BA71ΔCD2 vaccination all the animals except one control (pig 100) were capable of recognizing their autologous swine fibroblasts transfected with the recombinant plasmid cocktail containing pCMV-Ub-M448R and pCMV-Ub-MGF505-7R (Figure 5-13). The control that did not respond showed low cellular and humoral responses throughout the whole experiment, reflecting perhaps an immunosuppressed state.

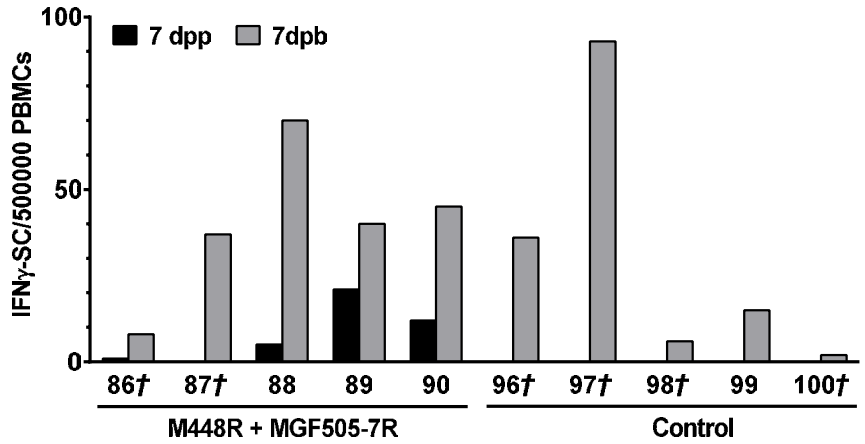


Figure 5-13. IFNγ response assessed by ELISpot in PBMCs isolated 7 days after the DNA prime (7 dpp) and 7 days after the BA71ΔCD2 vaccination (7 dpb), using swine fibroblasts transfected with both pCMV-Ub-M448R and pCMV-Ub-MGF505-7R as APCs. *†* indicates animals that had to be euthanized after Georgia2007/1 challenge infection.

In order to characterize the response induced by each one of the antigens here tested, ELISpot assays were performed with swine fibroblasts transfected with either pCMV-Ub-M448R or pCMV-Ub-MGF505-7R. Simultaneous IFNγ response to M448R and MGF505-7R were detected in all the animals (except pig 86, which did not recognize MGF505-7R), thus discarding a possible immunodominance effect between M448R and MGF505-7R when administered in a DNA-based formulation. At a group level, the IFNγ response to both M448R and MGF505-7R of the three surviving animals (pigs 88, 89 and 90) was higher than the two animals that died (pigs 86 and 87) at all the analyzed time points (Figure 5-14). Again confirming the presence of M448R- and MGF505-7R-specific T cells in Georgia2007/1-convalescent animals without a prior DNA prime immunization, the control animal that survived (pig 99) showed a notable IFNγ response to both antigens at the end of the trial (Figure 5-14, 21 dpc).

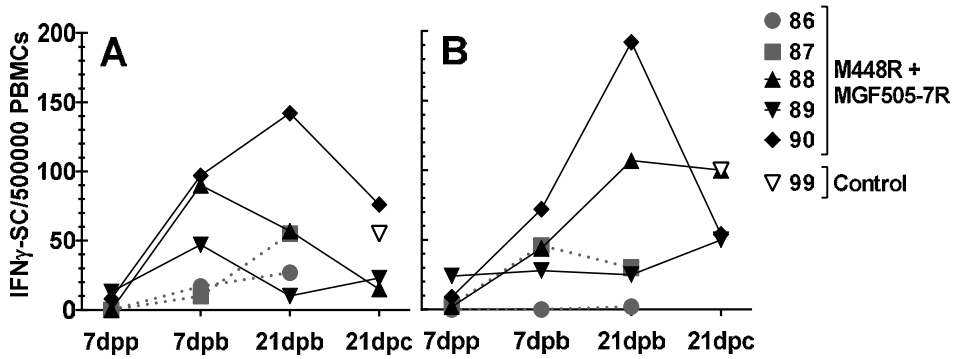


Figure 5-14. Kinetics of the IFNγ response throughout the study in PBMCs co-cultured with autologous swine fibroblasts transfected with either (A) pCMV-Ub-M448R or (B) pCMV-Ub-MGF505-7R. Surviving animals are represented in solid lines, while dashed lines indicate animals that had to be euthanized after challenge.

Aiming to determine if the DNA prime immunization had an effect on the magnitude of ASFV-specific T-cell response, the number of IFNγ-secreting cells responding to BA71ΔCD2 after the vaccination with BA71ΔCD2 was assessed both early and late after the boost (at days 7 and 21 dpb, respectively).

At 21 dpb, all pigs showed indistinguishable ASFV-specific T-cell response, with the exception of the low responder pig 100. IFNγ response was also detected in all the animals at 7 dpb (Figure 5-15), despite no significant differences were found between the DNA-primed animals and the control group. It is worth mentioning that the best IFNγ-responder within the control group, pig 99, was the only survivor (Figure 5-15).

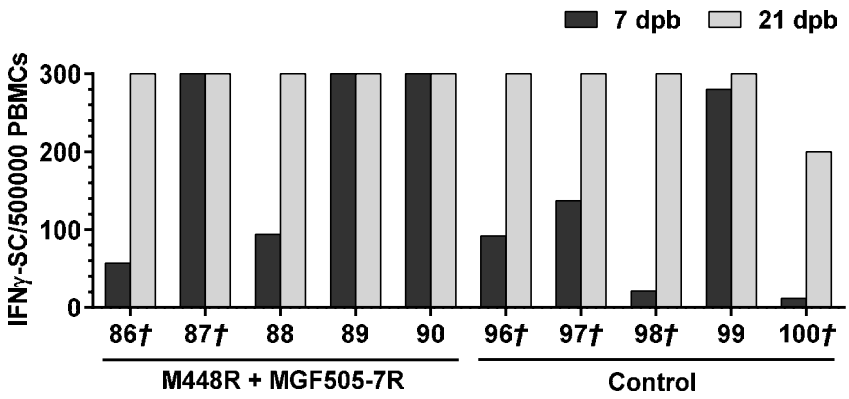


Figure 5-15. IFNγ-secreting cells in response to BA71ΔCD2 assessed by ELISpot assay 7 days (7 dpb) and 21 days (21 dpb) after the BA71ΔCD2 vaccination. *†* indicates animals that had to be euthanized after Georgia2007/1 challenge.

5.3. FINAL REMARKS

In the present study, we confirmed the feasibility of inducing ASFV-specific cellular response in pigs by administering a cocktail of 15 plasmids encoding full-length ASFV proteins in frame with ubiquitin. We demonstrate here that a heterologous immunization regimen including a DNA prime with the 15 recombinant plasmids followed by a low dose of the LAV BA71ΔCD2 confers partial protection against a Georgia2007/1 challenge. Protein M448R was the main responsible for the immunogenicity of the plasmid cocktail, thus suggesting its protective potential. Moreover, ASF-convalescent animals promiscuously recognized M448R, without receiving a prior DNA prime.

Following the same experimental design, DNA priming with M448R in combination with MGF505-7R, which was also shown to have an immunodominant and promiscuous nature (Chapter 4), did also result in a 60% survival percentage. Further studies should be done to individually define the protective role for M448R and MGF505-7R.

CHAPTER 6

Design of multiepitope-based DNA constructs and assessment of their immunogenicity and protective potential against ASFV

6.1. INTRODUCTION

DNA-based immunization has proved to be sufficient for the pigs to overcome a lethal challenge of ASFV using the E75 isolate, thereby allowing the characterization of epitopes with protective potential (Argilaguet et al., 2012; Lacasta et al., 2014). However, when similar experiments were performed with the Georgia2007/1 ASFV, the results previously obtained with the E75 isolate were not reproducible, thus hampering the identification of relevant antigens and evidencing the need for novel immunization strategies.

In a first attempt to enhance the immunogenicity of our DNA constructs based on the Georgia2007/1 sequence, ASFV proteins in which the presence of $CD8^+$ T-cell determinants was previously described by our group: EP402R (Argilaguet et al., 2012), CP312R and A240L (Lacasta, 2012; Rodríguez et al., unpublished results), were analyzed for the presence of regions containing multiple theoretical CTL epitopes. These protein regions or "SLA I-hot spots" were selected to be included in the vaccine formulation, aiming to induce a wide repertoire of SLA I-restricted immune responses. Optimal proteasomal cleavage sites were added spacing the different protein domains, and the ubiquitin gene was used as a leader sequence. With this design, it was aimed to enhance the SLA I processing and presentation of the epitopes as previously reported (Argilaguet et al., 2012; Lacasta et al., 2014), thus inducing specific $CD8^+$ T-cell responses while abolishing humoral responses. The immunogenicity of this multiepitope-encoding plasmid, referred to herein as multiepitope-I (ME-I), was confirmed *in vivo*. Confirming the efficacy of the strategy, pigs immunized with ME-I induced ASFV-specific T-cell response that specifically recognized peptides from EP402R, CP312R and A240L (Bosch-Camós et al., 2017).

Extending this outcome for the identification of novel Georgia2007/1 antigens, the results described in Chapter 4 were taken into account to select proteins with potential to induce $CD8^+$ T-cell responses. Therefore, a selection was done based on analyses of the immunopeptidome profile of macrophages *in vitro* infected with ASFV, followed by *in silico* CTL epitope predictions of each one of them. Protein regions containing a high density of predicted epitopes and at least one SLA I-restricted peptide identified in the immunopeptidomics assays were selected as "SLA I-hot spots" for the design of a second multiepitope DNA construct (ME-II).

Seeking to increase the chances of success of our experimental vaccine prototype, the heterologous prime-boost immunization regimen described in the Materials and methods section was used. Thus, animals were primed with the DNA plasmids encoding the multiepitope constructs, followed by an intramuscular inoculation with a low dose of the live attenuated BA71ΔCD2 virus. This model was useful not only to confirm the capability of the selected antigens to induce ASFV-specific $CD8^+$ T cells, but also to evaluate their protective potential against a Georgia2007/1 lethal challenge.

6.2. RESULTS

6.2.1. Selection of ASFV proteins with potential to trigger immunodominant $CD8^+$ T-cell responses and design of a multiepitope DNA construct

Results from SLA I-restricted immunopeptidomics assays were used to select ASFV proteins with potential to induce $CD8^+$ T-cell responses. The best protein candidates were selected according to three main criteria: (i) proteins from which

5 or more peptides were identified in SLA I-restricted immunopeptidomics assays, (ii) proteins from which peptides were identified using PAMs from different animals, and (iii) proteins from which a peptide had been recognized by specific T cells obtained from pigs inoculated with the LAV BA71ΔCD2 or with a peptide cocktail including that specific peptide (i.e. antigenic peptides).

With this data in mind, 13 proteins were finally selected for further analysis (Table 6-1). Interestingly, four of these proteins corresponded to ASFV enzymes involved in nucleic acid metabolism: G1211R (DNA polymerase beta), D1133L (helicase), P1192R (DNA topoisomerase II), and EP424R (putative methyl transferase); while another two corresponded to the p150 and p37 structural proteins, encoded by the CP2475L ORF as a pp220 polyprotein precursor (Simón-Mateo et al., 1993). Additionally, two multigene family 505 members resulted selected: MGF505-1R, probably involved in IFN I inhibition (Golding et al., 2016) and absent in the non-pathogenic OURT88/3 and BA71V ASFV (Chapman et al., 2008), and MGF505-9R. Finally, the K145R ORF, previously identified as an immunodominant antigen using sera from convalescent pigs (Kollnberger et al., 2002), was selected together with four additional ORFs with unknown functions: B475L, M1249L, H339R and I226R.

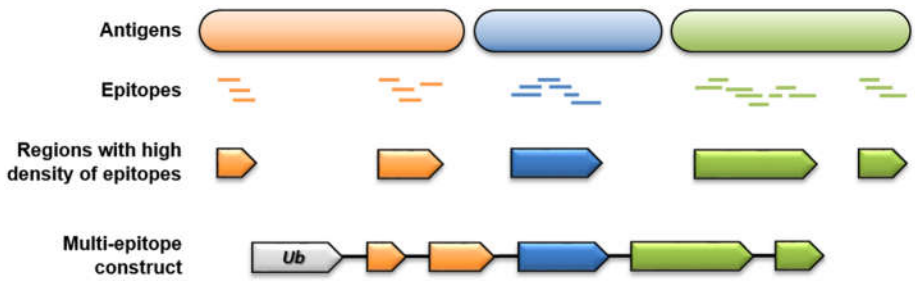


Figure 6-1. Representation of the design process of a multiepitope construct.

In order to encode the 13 selected proteins in a unique ORF, each one was *in silico* analyzed to identify regions with a high density of epitopes using the NetMHCpan 3.0 software as described in Materials and Methods. Finally, a single DNA construct was designed with the selected protein regions (Appendix VI), linked by an optimal proteasomal cleavage site (AAY) (Velders et al., 2001) and with ubiquitin as a leader sequence (Figure 6-1) aiming to enhance their SLA I processing and presentation (Argilaguet et al., 2012; Lacasta et al., 2014; Rodriguez and Whitton, 2000). The final plasmid encodes, including the ubiquitin gene, a protein of 1,884 amino acids in length, hereinafter referred to as multiepitope-II (ME-II).

Table 6-1. ASFV proteins selected to be included in the multiepitope-based DNA construct. From each protein, the table shows the total number of peptides identified by SLA I immunopeptidomics analysis, the number of PAMs in which peptides were found, and the sequence of the antigenic peptides identified.

Protein	Number of peptides	Number of PAMs	Antigenic peptide
B475L	**9**	1	
D1133L	**7**	**2**	YKDETLPYL
p37 (CP2475L/partial)	**5**	**2**	
K145R	**5**	**2**	
M1249L	**5**	**2**	
G1211R	**5**	1	ENIAYERLETL
P1192R	4	1	
p150 (CP2475L/partial)	3	**2**	
EP424R	3	**2**	
H339R	3	1	NPTIIMEQY
I226R	2	**2**	KNILNTLMF
MGF505-1R	2	**2**	
MGF505-9R	2	**2**	

6.2.2. DNA immunization with ASFV multiepitope-based plasmids partially protects against Georgia2007/1 lethal challenge

To test the protective efficacy of the selected ASFV candidates, the prime-boost heterologous vaccination protocol previously described (Figure 3-1) was evaluated. Both ME-I and ME-II were administered to the pigs. As represented in Figure 6-2, three out of five pigs (60%) primed with the multiepitopes survived the Georgia2007/1 challenge, while only one out of the five controls (20%) did, coinciding with the expected results for the low dose of BA71ΔCD2 used.

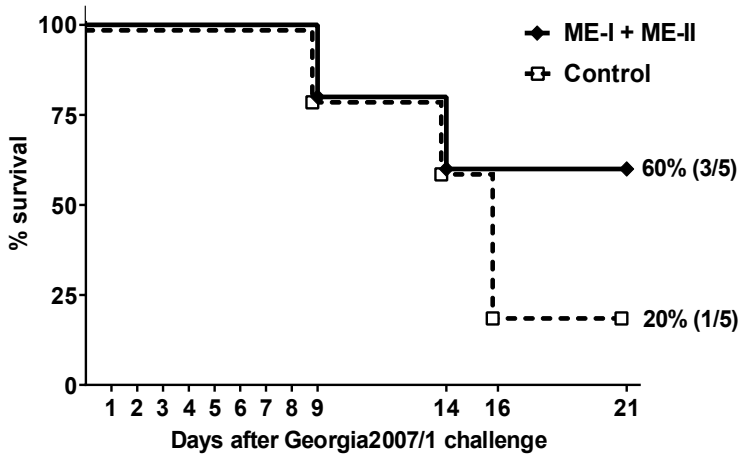


Figure 6-2. Percentage of surviving pigs after the Georgia2007/1 lethal challenge within the group primed with the multiepitopes (solid line) and the control group (dashed line).

In the group immunized with the multiepitopes, two of the three surviving animals (pigs 175 and 176) had not even two consecutive days of fever (Figure 6-5), and ASFV positive samples of serum and nasal swabs of these animals showed low virus titers (Figure 6-4), confirming the success of the DNA priming with our plasmids. Moreover, pig 175 remained free of ASF-compatible clinical

signs throughout the experiment (Figure 6-3). Animal 176 showed slight apathy and evident dyspnea starting at 14 dpc until the end of the trial (Figure 6-3), but not correlating with fever nor ASFV positive serum samples or nasal shedding (Figure 6-4). The third survivor (pig 178) showed continued but minor ASF symptomatology (Figure 6-3), and transient episodes of fever starting at 5 dpc (Figure 6-5), correlating with a prolonged detection of ASFV in both serum and nasal swabs (Figure 6-4). However, at the end of the experiment this animal was almost recovered, showing no fever and only a slight dyspnea, and undetectable levels of ASFV in serum and nasal swabs. Rectal temperature (Figure 6-5) and virus titers (Figure 6-4) of the pigs that had to be sacrificed from the ME-I+ME-II-primed group (177 and 179) were not different from those found in most of the animals in the control group (Figure 6-4, 6-5).

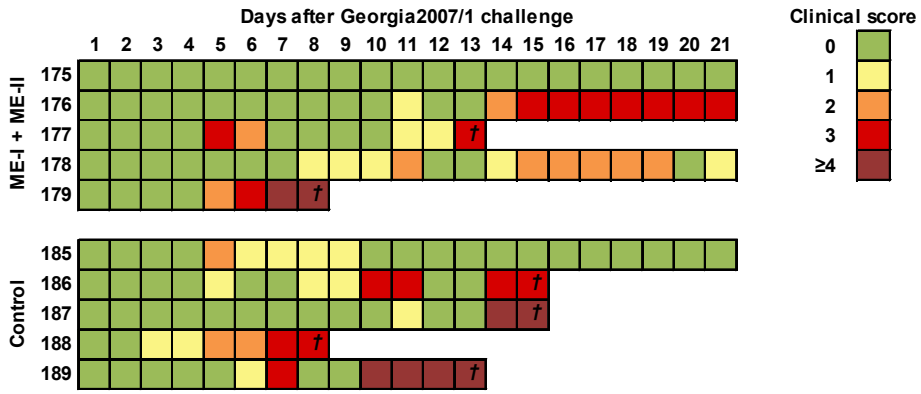


Figure 6-3. Individual clinical scores after the Georgia2007/1 challenge. Clinical condition included apathy, body condition, digestive signs, respiratory signs and cyanosis, each receiving a score from 0 to 3. The final clinical score in the graph corresponds to the sum of these values. † symbolizes the day of death or euthanasia.

The course of infection in the control group was in line with previous results using a low dose of BA71ΔCD2 (Monteagudo et al., 2017). In this group, ASF-compatible clinical signs were apparent from day 3 after the Georgia2007/1

challenge (Figure 6-3), coinciding with the onset of prolonged fever (all the animals had at least five consecutive days of fever) (Figure 6-5B), and in agreement with the virus titers in serum and nasal swabs (Figure 6-4C, D). The surviving control animal (pig 185) had prolonged fever compared to the survivors in the ME-I+ME-II-primed group (Figure 6-5), which also showed a delay in the appearance of symptoms of ASF (Figure 6-3) that could be reflecting a better initial control of the infection.

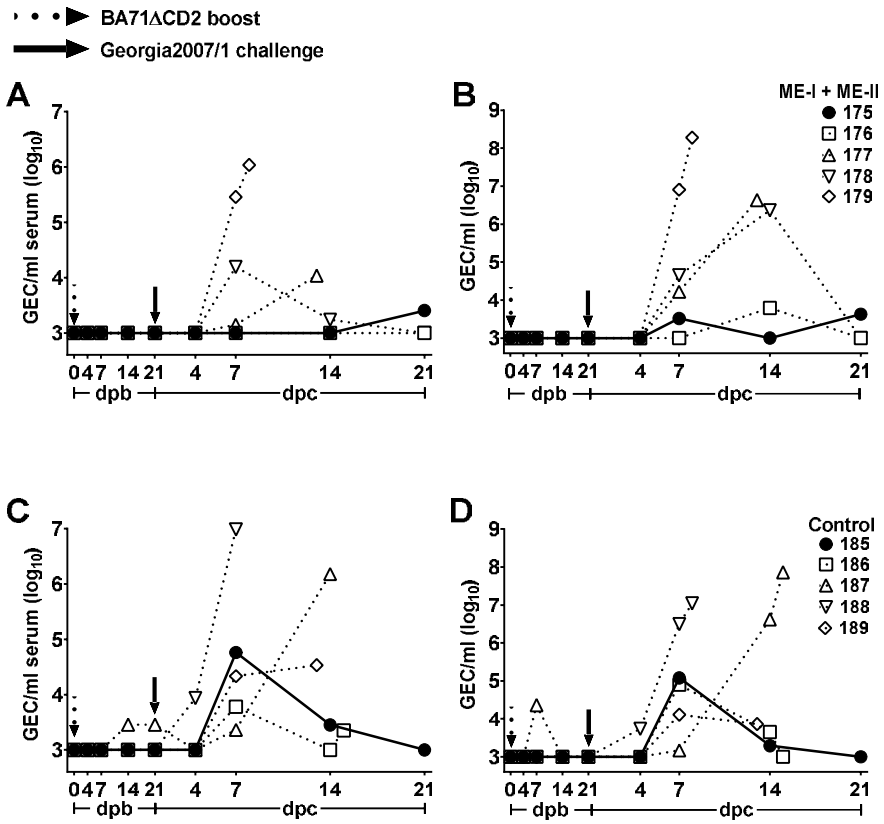


Figure 6-4. Detection of ASFV DNA by qPCR in serum and nasal swabs of the pigs before and after Georgia2007/1 inoculation. (A, B) Detection of ASFV DNA in serum and nasal swabs, respectively, from the individual animals primed with the multiepitopes. (B, C) Detection of ASFV DNA in serum and nasal swabs, respectively, from the individual control animals.

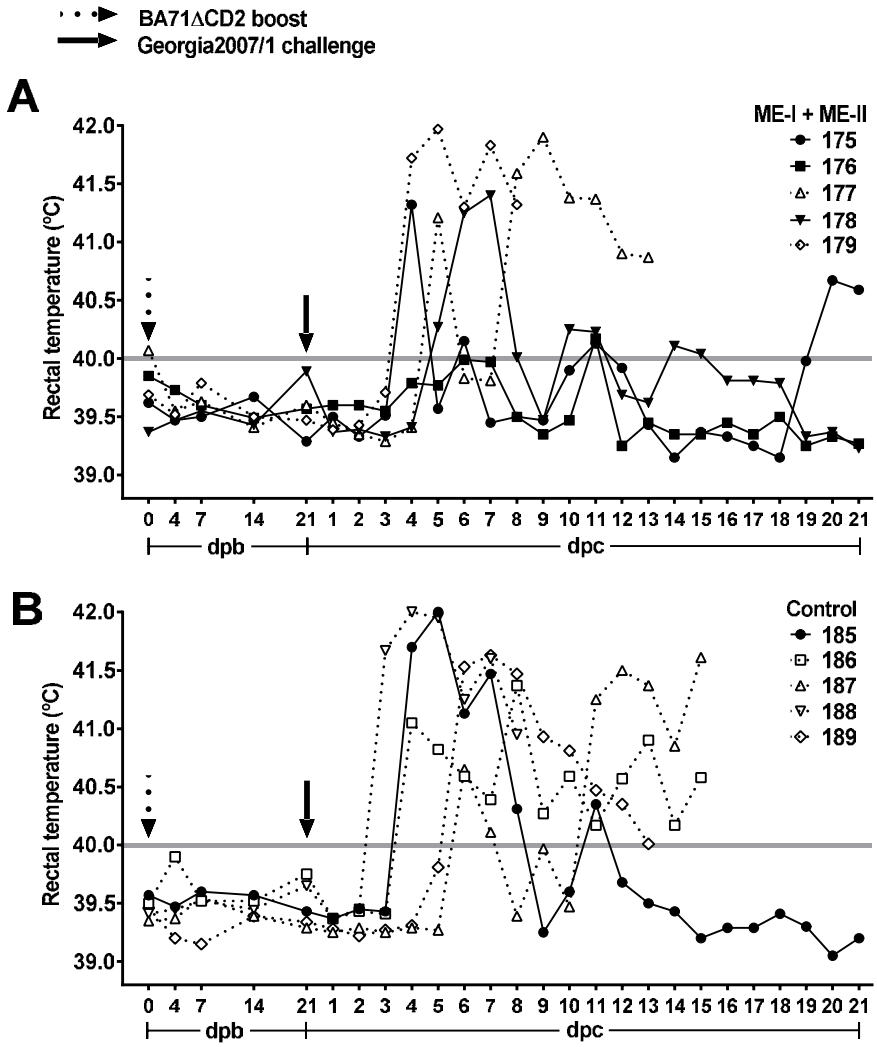


Figure 6-5. Evolution of the rectal temperature of pigs after BA71ΔCD2 boost and Georgia2007/1 inoculation. (A) Individual temperature values of pigs primed with the multiepitopes. (B) Individual temperature values of control animals. Surviving animals are represented in solid lines, while a dashed line symbolizes that the animal succumbed to the challenge.

6.2.3. Multiepitope-based DNA constructs encoding multiple epitopes from ASFV induce ASFV-specific T cells *in vivo*

As expected, inoculation of the multiepitopes did not induce any ASFV-specific antibody response (Figure 6-7B), but it did induce detectable ASFV-specific IFNγ response (Figure 6-7A), confirming the successful DNA priming with the chosen antigens. After the BA71ΔCD2 boost, all the pigs seroconverted and the number of ASFV-specific T cells increased notably (Figure 6-7). As described before, no correlation seems to exist between the level of antibodies or specific T cells at the challenge time point and the protection afforded, at least when measured by the techniques employed here.

Confirming the antigenicity of our multiepitope DNA constructs, IFNγ response was detected by ELISpot when they were transfected into fibroblasts and these used as APCs for autologous PBMCs obtained at 21 dpb (Figure 6-6).

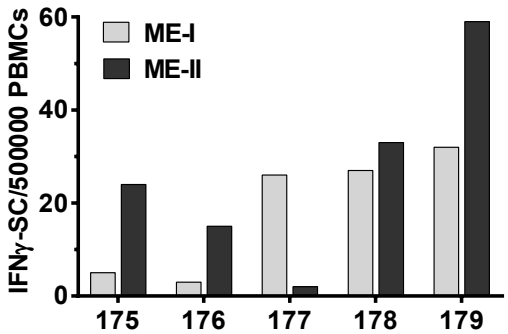


Figure 6-6. Number of IFNγ-SC in PBMCs from multiepitopes-primed animals at 21 dpb when stimulated with autologous fibroblasts transfected with ME-I or ME-II.

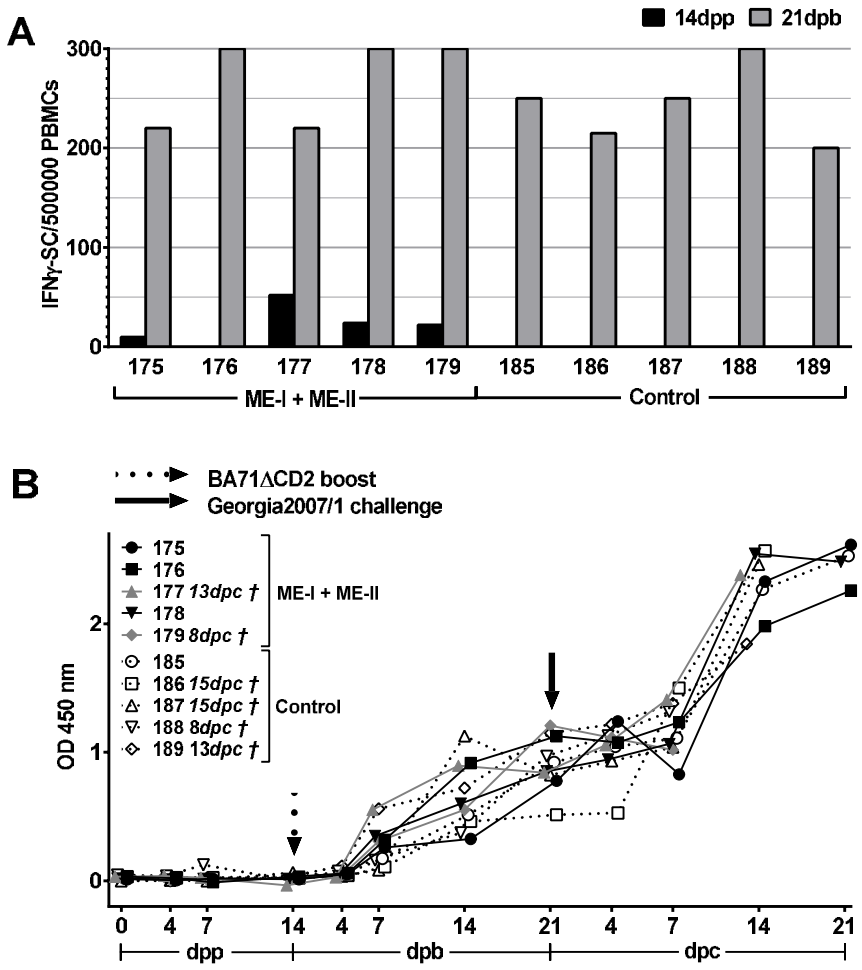


Figure 6-7. ASFV-specific cellular response assessed by ELISpot using BA71ΔCD2 as stimulus at 14 days after the 2nd DNA immunization (14 dpp) and at 21 days after BA71ΔCD2 boost (21 dpb). 300 spots is considered the limit of detection of the assay. (B) ASFV-specific antibodies in sera. Results are expressed as OD values at a wavelength of 450 nm.

6.2.4. Identification of ASFV antigens: DNA-primed versus not-primed animals

With the aim of determining and rank the immunogenicity of the individual ASFV antigens encoded in the multiepitope-based DNA constructs, pCMV-Ub plasmids encoding each of the full-length proteins included in both ME-I and ME-II were transfected into fibroblasts, and these used as APCs in the ELISpot assay. Expression of the full-length ASFV antigens here employed was confirmed by immunofluorescence as described in Materials and Methods (Appendix V). In order to determine the role that DNA priming can have on induction and modulation of T-cell responses, PBMCs from BA71ΔCD2-inoculated animals both primed with the multiepitopes and without a prior DNA prime were used as effector cells in the ELISpot assay. While almost all the clones stimulated a specific IFNγ response, in both cases proteins CP312R and D1133L showed the most promiscuous nature (Table 6-2, 6-3). Strikingly, while three out of the five ASFV-infected animals not previously primed responded to CP312R and D1133L, IFNγ production was observed in all the DNA-primed animals, suggesting an effect of the DNA prime on the induction of specific T-cell response against these two antigens. An increase on the number of animals responding to A240L was also seen in DNA-primed animals, in line with previous results of the lab (Bosch-Camós et al., 2017). Although once again protection could not be correlated with any of the responses or its magnitude, these results demonstrate the successful DNA priming using designed multiepitopes to characterize two not previously described immunodominant ASFV antigens: CP312R and D1133L, with potential to induce protective T-cell responses.

Table 6-2. IFNγ-secreting cells in PBMCs from BA71ΔCD2-vaccinated animals (14 days post-immunization) assessed by ELISpot using autologous fibroblasts transfected with pCMV-Ub plasmids encoding full-length ASFV proteins in frame with ubiquitin as APCs. +: more than 5 spots, ++: more than 30 spots, +++: more than 50 spots.

		62	63	64	65	99 (control)
ME-I	**EP402R**	+	-	-	-	-
	CP312R	-	+++	++	++	-
	A240L	+++	-	-	-	+
ME-II	**D1133L**	+++	+++	-	+	-
	G1211R	-	+++	-	-	-
	M1249L	-	++	-	-	-
	MGF505-9R	+	-	-	-	-
	P1192R	-	-	-	-	+
	P150 (CP2475L/partial)	-	-	-	+	-
	B475L	+	-	-	-	-
	EP424R	-	-	-	-	-
	H339R	-	+	-	-	-
	I226R	-	-	-	-	-
	K145R	-	-	-	-	-
	MGF505-1R	-	-	-	-	-
	P37 (CP2475L/partial)	+	-	-	-	-

Table 6-3. IFNγ response at 14 dpb assessed by ELISpot using as APCs, fibroblasts transfected with plasmids encoding full-length ORFs and as effector cells, PBMCs from pigs pre-immunized with the multiepitopes. +: more than 5 spots, ++: more than 30 spots, +++: more than 50 spots.

		175	176	177	178	179
ME-I	EP402R	+	-	-	-	-
	CP312R	+	+	++	+	+
	A240L	+	+	+	-	+++
ME-II	D1133L	++	+++	+	+++	++
	G1211R	+++	-	-	-	-
	M1249L	++	-	-	+	-
	MGF505-9R	+	-	-	-	-
	P1192R	-	-	-	-	-
	P150 (CP2475L/partial)	++	-	-	-	+
	B475L	-	-	-	-	-
	EP424R	-	-	-	-	-
	H339R	-	-	-	-	-
	I226R	-	-	-	-	-
	K145R	-	-	-	-	-
	MGF505-1R	-	-	-	-	-
	P37 (CP2475L/partial)	-	-	-	-	-

6.3. FINAL REMARKS

The present study has proven the feasibility of multiepitope DNA constructs to *in vivo* induce ASFV-specific T-cell response and increase survival after a Georgia2007/1 lethal challenge when included in a heterologous prime-boost regimen using the LAV BA71ΔCD2 as a boost. The protective potential of the epitopes encoded in the DNA plasmids has therefore been confirmed, validating antigen selection based on immunopeptidomics studies of ASFV-infected macrophages. The use of allogeneic fibroblasts as APCs in our ELISpot assays allowed to narrow down the potential candidates and to identify CP312R and D1133L as highly promiscuous antigens, which could be good candidates to be included in future vaccine formulations. Further studies using the immunization approach here tested could allow the identification of ASFV-relevant antigens contributing to immunity, which would pave the way for future efforts in designing rational ASF vaccines.

CHAPTER 7

Discussion

After a single introduction in Georgia in 2007, the continuous spread of ASF through Continental Europe, and more recently to Asia, pointed out important gaps in ASF disease control. Development of an effective vaccine would greatly improve management of the disease and eradication actions, thus reducing the high socio-economic losses currently associated to an ASFV outbreak.

As of today, live attenuated viruses seem the most plausible ASF vaccine option in the short-term, but the inherent biosafety issues of this strategy hampers their implementation in ASF-free areas. Therefore, alternative methodologies such as subunit vaccines arise as an option. Lack of knowledge on the molecular mechanisms underlying immunity to ASFV is a major drawback for the design of rational subunit vaccine formulations. Thus, although the crucial contribution of $CD8^+$ T cells has been reported (Oura et al., 2005), the specific $CD8^+$ T-cell antigens eliciting protective immune response are mostly unknown. Therefore, the present thesis was focused on the determination of ASFV antigens inducing protective $CD8^+$ T-cell responses against ASFV, specifically against the Georgia2007/1 isolate.

To this aim, different methodologies have been used throughout this thesis, including: *in silico* theoretical predictions of $CD8^+$ T-cell epitopes, immunopeptidomics studies based on SLA I immunoprecipitation assays using extracts from PAMs infected with ASFV followed by HPLC-MS/MS determinations, *in vitro* functional assays using ASFV-specific PBMCs stimulated with autologous fibroblasts transfected with ASFV-gene expression plasmids, and *in vivo* DNA immunization experiments.

In our initial experiments, Georgia2007/1 challenge infection of pigs resulted in the death of all the animals in less than two weeks after infection. In this fast

course of infection, animals succumbed before being capable of mounting protective responses, therefore depriving us of Georgia2007/1-specific effectors for further functional assays (Rodríguez et al., unpublished results). Furthermore, we could not detect any Georgia2007/1-specific peptide associated with SLA I molecules in our immunopeptidomics assays. These results forced us to focus on alternative strategies to identify SLA I-restricted ASFV epitopes with potential to induce protective responses against the Georgia2007/1 ASFV. The best alternative in our hands was found in using the cross-protective BA71ΔCD2 LAV.

The majority of T-cell epitopes described so far have high (<50 nM) or intermediate (<500 nM) IC50 binding affinity to its corresponding SLA I allele and, therefore, the first cutoff of the multiparametric bioinformatic analysis performed for the identification of Georgia2007/1 epitopes was a theoretical IC50 below 500 nM. As expected for MHC I ligands, 8 to 11 amino acid long peptides were analyzed, but longer peptides were also synthesized if sequences with ten or more overlapping predictions were found. Short peptides can directly bind to MHC molecules on the cell surface, competing with and displacing the host peptides presented. At high concentrations such as the ones used in our *in vitro* stimulation assay, longer peptides should also be able to be presented by MHC I molecules, directly fitting into the binding groove with a bulged conformation (Probst-Kepper et al., 2004; Tynan et al., 2005) or being internalized and processed by APCs, thus finally presenting sequences of more conventional length.

Due to the large size and complexity of ASFV, covering its entire proteome with overlapping 9-mer peptides was out of the scope of this study. Only the best 330 theoretically predicted peptides where selected for its synthesis, aiming to

characterize the potential of our *in silico* prediction method. Only one out of the 330 tested peptides from the *in silico* analysis here performed was *in vitro* recognized by ASFV-specific PBMCs. Several reasons could explain this outcome. Firstly, peptide processing and presentation by the SLA I pathway have remained elusive so far. As a result, the vast majority of prediction programs are set up with experimental data obtained from human and mouse studies. This is the case, for example, of the proteasomal cleavage and TAP transport predictions analyzed. The same happens with SLA binding motifs, which are mainly established based on sequence and structure similarities with HLA molecules. Secondly, the MHCpan3.0 software offers predictions for a limited set of 42 SLA I alleles, whereas more than 190 alleles have been distinguished by the SLA Nomenclature Committee of the International Society for Animal Genetics (Maccari et al., 2017), although not many have been described in detail (Fan et al., 2016; Gao et al., 2017; Zhang et al., 2011). Finally, the impossibility to handle the totality of predicted epitopes (originally almost 9000) forced us to focus our work on a limited collection of peptides. Even though the ranking criteria established was based on scientific grounds, limiting the selection to 330 peptides was not absent of risks, particularly when the specific virus encodes more than 180 ORFs.

This outcome is in line with previous results from the lab using a peptide library based on *in silico* predictions of the E75 ASFV strain (Correa-Fiz, 2014), from which one single peptide out of almost 400 selected was recognized by PBMCs of ASF-convalescent pigs (Rodríguez et al., unpublished results). Conversely, *in silico* predictions of SLA I-restricted epitopes have rendered successful outcomes when used for epitope discovery once the protection-inducing antigens had already been identified or there is a narrowly defined set of

proteins to be analyzed (Argilaguet et al., 2012; Baratelli et al., 2017; Burgara-Estrella et al., 2013).

Immunopeptidomics studies provide a unique opportunity to identify SLA I-associated peptides naturally generated during infection. In our hands, this approach has proven more reliable than bioinformatic analysis for the determination of ASFV $CD8^+$ T-cell epitopes, since five out of 111 identified sequences induced an IFNγ response in ASFV-specific PBMCs. Despite the fact that the peptides characterized by the immunopeptidomics approach are actual SLA I ligands, it must be borne in mind that this does not necessarily imply that these peptides will trigger an effective T-cell response, as can be seen from the results here obtained. In contrast with the poor efficacy of our *in silico* predictions, five from de 111 peptides identified in the immunopeptidomics assays were recognized by ASV-specific T cells. As expected, none of the peptides were recognized by all the PBMCs tested, which could be attributed to SLA I restriction. Haplotyping of the PAMs used in the immunopeptidomics assays revealed differences in the constitutively expressed classical SLA I genes: SLA 1, SLA 2 and SLA 3. Consequently, we could discard the possibility of having used PAMs with a similar haplotype in our assays, which could have been a factor limiting the characterization of promiscuously recognized epitopes.

Macrophages are the main target of ASFV, but other APCs can also be infected. A few studies explored the interaction of DCs with ASFV (Franzoni et al., 2018a, 2018b; Golding et al., 2016; Gregg et al., 1995a, 1995b). DCs are professional APCs and play a central role in initiating the adaptive immune response. Different antigen processing pathways have been described in DCs and macrophages (Dinter et al., 2014), and therefore sequence analysis of the ASFV

peptides presented by DCs could provide novel information on the antigens eliciting $CD8^+$ T cell-mediated protective responses.

Immunopeptidomics studies of other non-conventional APCs should also be considered. Epithelial cells can support ASFV replication at late stages of the infection (Gómez-Villamandos et al., 1995), and their function as APCs has been described. Swine kidney epithelial cells can be easily collected and maintain their viability for several passages, and thus they could be a source of ASFV SLA I-restricted peptides. Porcine γδ T cells have been demonstrated to exhibit APC features, and they have proven able to present soluble ASFV antigens to ASFV-specific $CD4^+$ T cells (Takamatsu et al., 2006). Exogenous soluble antigen taken up by γδ T cells can also be loaded on MHC I molecules by means of antigen cross-presentation, thus inducing $CD8^+$ T-cell response (Brandes et al., 2009; Meuter et al., 2010; Zhu et al., 2016). However, the role that γδ T cells play during ASFV infection should be further addressed, especially considering their large proportion in pigs (15-30% of the total peripheral blood lymphocyte population) (Takamatsu et al., 2006; Yang and Parkhouse, 1996). Another interesting cell subset to be examined are fibrocytes. They are circulating leukocytes that can enter injured tissue and differentiate into fibroblasts, contributing to wound healing. Moreover, they can act as APCs, and their capacity to efficiently stimulate virus-specific CTL response in a MHC I-dependent manner has been reported (Balmelli et al., 2005).

Taking into account that the LAV BA71ΔCD2 is capable to confer cross-protection against Georgia2007/1, it was plausible to assume that some of the peptides identified from BA71ΔCD2-infected PAMs would be common to Georgia2007/1 and BA71 isolates, and also that some of them would be responsible for the protective immune responses described before. Reinforcing

this statement, 86% of the sequences identified from BA71- and BA71ΔCD2-infected PAMs were identical to Georgia2007/1. Despite both BA71 and BA71ΔCD2 are efficiently presented by PAMs, the SLA I-peptides identified are quantitatively and qualitatively different. Thus, the proportion of peptides specifically recognized by PBMCs from ASFV-recovered pigs was significantly higher for BA71ΔCD2 than for BA71, most probably reflecting, at least partially, the successful protection afforded by BA71ΔCD2.

Differences in the peptide repertoire were likewise observed in the sense that out of frame peptides were only identified from BA71ΔCD2-infected samples. Presentation by MHC molecules of peptides encoded in alternate translation reading frames has been described in tumors (Chalick et al., 2016; Probst-Kepper et al., 2001; Robbins et al., 1997) and virus-infected cells (Cardinaud et al., 2004; Mayrand et al., 1998; Zhu et al., 2015), including ASFV-infected cells (Jenson et al., 2000). Although none of the out of frame peptides were efficiently recognized by PBMCs from recovered pigs, their protective relevance should not be discarded.

The molecular mechanisms leading to the attenuation and protective capabilities of BA71ΔCD2 remain to be determined. Nonetheless, the involvement of CD2v in host immune response modulation has long been discussed (Borca et al., 1994, 1998; Kay-Jackson et al., 2004; Pérez-Núñez et al., 2015). Apart from being responsible for the hemadsorption phenomenon observed in ASFV-infected cells (Rodríguez et al., 1993), *in vitro* results showed that CD2v suppresses mitogen-dependent lymphocyte proliferation (Borca et al., 1998), which can contribute to virus expansion and persistence. The immune suppressive effect of CD2v has been attributed to its C-terminal end, located in the cytoplasm of the infected cell, where it interacts with multiple immune

mediators. Thus, CD2v interacts with the SH3P7 actin-binding adaptor protein (Kay-Jackson et al., 2004), a relevant protein in T-cell proliferation and cytokine production (Han et al., 2005), also related to cell trafficking, endocytosis and signal transduction (Fucini et al., 2002; Nakatsu and Ohno, 2003; Warren et al., 2002). Similarly CD2v can interact with the adaptor protein 1 (AP-1) (Pérez-Núñez et al., 2015), in a similar fashion to that described for other intracellular pathogens as a mechanism to restructure cell traffic to benefit viral morphogenesis and egress (Agrawal et al., 2013; Madrid et al., 2005; Tong et al., 1998). In fact, HIV Nef protein induces AP-1 binding to MHC I molecules, which leads to MHC I down-regulation and contributes to HIV immune escape (Kulpa et al., 2013; Wonderlich et al., 2008), in a similar manner than BA71ΔCD2 might do. Future work from the laboratory will attempt to understand the intrinsic mechanisms by which depletion of CD2v from BA71 yields an attenuated ASFV capable to induce efficient and cross-protective immune responses.

Although a deeper analysis of the characteristics and differences of the SLA I peptides from host proteins in PAMs infected with the LAV BA71ΔCD2 and the parental virus BA71 was beyond the scope of this study, the data here obtained can form the basis for future examinations that could provide novel insights into the understanding of the protective immunity induced by BA71ΔCD2.

The IFNγ ELISpot here used as our gold-standard technique for the *in vitro* screening of $CD8^+$ T cells stimulation is possibly limiting the detection of peptides and full-length proteins that have a protective role without IFNγ mediation. Knowing that other arms of the immune response are also crucial in protection, we focused this thesis to unmask $CD8^+$ T-cell determinants with protective potential. Nonetheless, the specific phenotype of the responding cells has not been confirmed in every single experiment. In this respect, lymphocyte

proliferation assay by carboxyfluorescein succinimidyl ester (CFSE) dilution along with assessment of specific expression markers has been occasionally assayed in our laboratory, confirming the identity of the responding cells. However, the procedure is time-consuming, not easy to reproduce and need large amounts of PBMCs, which are major drawbacks for using this technique.

Immunopeptidomics studies can be a powerful tool for understanding T-cell response against pathogens as well as viral immune escape mechanisms (Pymm et al., 2017; Ramarathinam et al., 2018; Schellens et al., 2015; Trujillo et al., 2014). Difficulties in determining ASFV peptides from PAMs infected with the Georgia2007/1 isolate suggests that this strain is possibly more proficient at interfering with the SLA I presentation pathway than other virulent strains, including BA71. Furthermore, Georgia2007/1 strain inhibits IFNγ secretion *in vitro* when added to PBMCs from ASFV-recovered pigs (Rodríguez et al., unpublished results). These results may indicate that the immunosuppressive mechanisms of Georgia2007/1 are stronger than the ones provoked by many other virulent ASFV strains including BA71, E75, and Kenya06, that specifically promote the specific secretion of IFNγ (Rodríguez et al., unpublished results), which might in part account for the higher virulence observed.

I*n vitro* stimulation of ASFV-specific PBMCs with autologous fibroblasts transfected with plasmids encoding full-length ASFV proteins led to the identification of promiscuously recognized antigens, while peptides identified from those antigens induced IFNγ response in a lower percentage of animals. An antigenic molecule usually has many epitopes (Borbulevych et al., 2007; Connelley et al., 2016), which might be presented by various MHC alleles and thus induce immunogenic responses in a wider range of individuals. This effect was observed for proteins MGF505-7R and A238L, promiscuously recognized

ASFV proteins from which SLA I-restricted peptides were characterized by immunopeptidomics. In the case of MGF100-1L, there was no increase in responding animals when using the full-length protein compared to the specific antigenic peptide, most probably due to the inherent nature of the peptide used: a 19-mer selected for the presence of multiple epitopes within its sequence.

To our knowledge, this is the first time that ASFV antigens were promiscuously recognized by T cells from ASF-convalescent animals. In that respect, the ubiquitination strategy might have had an enhancing effect on antigen presentation by SLA I molecules. While virus infections usually lead to the development of a host response against a narrow range of dominant peptides, expression of antigens in fusion with ubiquitin has been previously described to promote $CD8^+$ T-cell responses (Argilaguet et al., 2012; Lacasta et al., 2014), and as a screening tool to identify dominant and subdominant epitopes (Rodriguez et al., 2001, 1998).

The use of transfected fibroblasts as APCs in the screening methodology of the present work has proven a valuable strategy to monitor the immune response of the pigs *ex vivo*. Similar screening of transiently transfected APCs with immune cells from convalescent animals as effector cells was previously described for the identification of $CD8^+$ T-cell antigens in ASF, using both targeted gene approaches and random cDNA clones (Goh et al., 2016; Graham et al., 2006; Jenson et al., 2000), albeit no data about their protective efficacy have been shown. Apart from the antigens identified as promiscuously recognized, few other antigens were recognized individually by some of the tested animals. Further work is needed to unmask their protective potential.

The results above discussed made us focus our *in vivo* work on proteins or polypeptides encoding multiple epitopes. Moreover, previous results from our laboratory using DNA immunization with ubiquitinated plasmids (Argilaguet et al., 2012; Lacasta et al., 2014) encouraged us to continue with this strategy for the identification of ASFV $CD8^+$ T-cell epitopes. The successfulness of the ubiquitination approach was again *in vivo* confirmed by the detection of ASFV-specific cellular responses directly after DNA immunization with recombinant plasmids encoding ASFV proteins with an ubiquitin gene at the N-terminus while as expected, no specific antibody response was observed. Unexpectedly, no significant differences were observed in the kinetics nor in the levels of ASFV-specific IFNγ-secreting cells after administration of BA71ΔCD2 between DNA-primed and control animals, at least at the times tested and by using the IFNγ ELISpot as a readout. The consistent improvement in the survival rate of vaccinated animals versus control pigs, clearly suggests that the T-cell repertoire induced by the DNA priming contributed to a better control of the infection. It is interesting to observe that DNA-based formulations successfully tested so far against ASFV never exceeded the 60% protection here afforded (Argilaguet et al., 2012; Lacasta et al., 2014; Mwangi et al., 2018).

Proteins M448R and MGF505-7R were identified not only as immunodominant $CD8^+$ T-cell determinants described in ASFV, but also as good candidates to be included in future and complex vaccine formulations. M448R orthologues have been described in other dsDNA viruses (Iyer et al., 2006; Yutin et al., 2009). Its RNA ligase activity is involved in tRNA repair (Silber et al., 1972), and hence can facilitate host evasion by bypassing immune response to damaged RNA associated with virus infection (Amitsur et al., 1987; Martins and Shuman, 2004). M448R sequence is conserved among ASFV isolates (Chapman et al.,

2011), a common feature of metabolic enzymes (Peregrín-Alvarez et al., 2009). MGF505-7R ASFV gene, also conserved among ASFV strains (Chapman et al., 2011, 2008), has been described to encode an IFN inhibitor protein (Correia et al., 2013). Curiously enough, three ASFV antigens so far described as potential vaccine targets: M448R, MGF505-7R and CD2v (Argilaguet et al., 2012), are masters of the immune evasion.

Space restriction is one of the main limitations of expression vectors. In this regard, an alternative approach was designed to include as many protective determinants as possible within a single ORF, aiming to induce a broad repertoire of TCR specificities and simplify the vaccine formulation. Instead of designing a typical epitope-based vaccine, in which single epitopes are adjacently encoded with or without spacers, protein domains theoretically encoding multiple overlapping $CD8^+$ T-cell epitopes were used aiming to increase the vaccine coverage.

In vitro recognition of ASFV after only DNA administration confirmed the immunogenicity of the multiepitope constructs. DNA priming resulted in all the animals recognizing both CP312R and D1133L after the BA71ΔCD2 boost. CP312R has been reported as a relevant serological determinant during ASFV infection (Kollnberger et al., 2002; Reis et al., 2007), and was also identified in previous studies of our laboratory as a potential T-cell antigen using an expression library immunization approach (Lacasta, 2012). The D1133L ORF encodes a putative helicase (Yáñez et al., 1993) and, to our knowledge, this is the first report showing its immunogenicity. The increased survival observed after Georgia2007/1 in the DNA-primed group, clearly indicates the presence of at least one protective antigen in the multiepitope DNA constructs, with CP312R and D1133L becoming promising candidates to be further investigated.

DNA vaccination has proven successful before in our laboratory to characterize antigens or epitopes with protective potential against the virulent E75 strain of ASFV (Argilaguet et al., 2012; Lacasta, 2012; Lacasta et al., 2014). However, these results could not be reproduced when working with the highly virulent Georgian isolate, even when using the protective antigens identified for the E75 strain (Rodríguez et al., unpublished results). Similar results have been found in other laboratories by using DNA-based vaccine formulations and even heterologous prime-boosting strategies; capable to render ASFV-specific response, but failing to confer protection against the Georgia2007/1 strain (Jancovich et al., 2018; Lopera-Madrid et al., 2017). The reasons behind this failure could be associated to the higher virulence of Georgia2007/1 compared to E75, perhaps related with the *in vitro* capabilities of the former to inhibit SLA I antigen presentation and IFNγ stimulation. This outcome forced us to look for alternative strategies aiming to strengthen the immune response induced by our DNA constructs. Therefore, a heterologous immunization regimen based on DNA priming followed by a boost with a low dose of BA71ΔCD2 (1000 PFU), previously described as suboptimal in terms of the protection afforded against Georgia2007/1 (Monteagudo et al., 2017), was explored as a part of this thesis. This experimental design should boost the T-cell responses primed by the DNA plasmids, thus improving the chances of the animals to survive the Georgia2007/1 challenge. Furthermore, it should allow the expansion of specific T-cell populations against both immunodominant and subdominant epitopes encoded in the plasmids (Takamatsu et al., 2013), thus inducing a larger repertoire of T cells.

Despite the fact that the prime-boost regimen here explored is not optimal for vaccination in the field, it served as a proof of concept that demonstrates its

feasibility to identify $CD8^+$ T-cell antigens which play a protective role against Georgia2007/1 challenge. A similar immunization strategy using vector-expressed ASFV antigens as a prime, followed by a boost with the naturally attenuated OURT88/3 ASFV isolate was done recently (Murgia et al., 2019). Although that study was focused on antibody response, and neither cell-mediated response nor protective potential were evaluated, their results pointed out that such prime-boost approach can increase recognition of epitopes that are not dominantly presented during infection, thus facilitating their identification.

Determination of correlates of protective immunity to ASF after vaccination remains an important gap that needs to be addressed. A deeper knowledge concerning ASFV immunity will be required for the identification of novel immune correlates of protection, including the exact mechanisms mediated by $CD8^+$ T cells, the role of other cell subsets and the implication of the innate immune system in protective immune response. In this regard, pigs surviving ASFV infection show ASFV-specific CTL responses (Alonso et al., 1997), and higher levels of CD4/CD8$\alpha\beta$ double positive CTLs and increased NK cell cytotoxic activity correlated with ASFV protection (Denyer et al., 2006; Leitão et al., 2001). Nonetheless, there is no other publicly available data to support these observations.

IFNγ response was the immune parameter assessed to determine the antigenicity of the peptides or proteins tested throughout the present work, since it is a marker for $CD8^+$ CTL activation. Notwithstanding, recognition of specific ASFV epitopes might be evoking the release of other cytokines that could as well play relevant roles in the development of protective responses. Functional assays analyzing the induction of pro-inflammatory cytokines, e.g.

IFNα, TNFα, IL-6, IL-12, IL-15 (Gil et al., 2008, 2003), IL-18, IL-1β and IL-1α (Franzoni et al., 2017), increased during infection with low virulent or attenuated ASFV in comparison to highly virulent isolates should be considered. Independently of the nature of the *in vitro* correlate with ASF protection, identifying them is essential, not only for future rational vaccine designs but also to guarantee the animal welfare, avoiding the unnecessary suffering associated to lethal challenging with virulent ASFV strains.

Six of the ASFV proteins included in the DNA priming formulations tested in the present thesis: B602L, D339L, EP402R, G1211R, M1249L and CP2475L, coincided with those recently described by Jancovich and colleagues, who aimed to characterize ASFV antigens with potential to induce protective cellular response. In this case, immunization consisted on a DNA prime followed by a boost with recombinant vaccinia viruses encoding the chosen ASFV antigens. Coinciding with our results, IFNγ response was observed against the DNA polymerase beta protein encoded by the G1211R ORF; the polyprotein pp220, encoded by the CP2475L ORF; and the B602L chaperone involved in virus assembly. Conversely, neither D339L, EP402R nor M1249L induced a notable response (Jancovich et al., 2018). It must be taken into account that inbred swine were used in this study, limiting the conclusion to a single pig haplotype, which does not reflect the real situation found in the field. In contrast, immunization studies using HEK-purified and MVA-vectored antigens, animals did show EP402R-specific IFNγ response (Lopera-Madrid et al., 2017).

Future prospects

Selection of the optimal protective antigens is crucial for the success of ASFV subunit-based immunization approaches. Here we demonstrated that immunopeptidomics studies are a reliable strategy for the identification of ASFV-specific T-cell epitopes, as well as a valuable antigen selection methodology for further determination of antigens with protective potential. The relevance of the ASFV antigens here identified and the specific immune response elicited by them should be further examined in detail in the future. Alternative functional assays should be established to better characterize the specific $CD8^+$ T-cell populations specifically induced, knowledge that could provide novel insights into ASF protective response.

Given the importance of cellular immunity in protection against ASF, future experiments should further validate and confirm the protective potential of the antigens here described: M448R, MGF505-7R, CP312R and D1133L. The four antigens are conserved among ASFV isolates, thus is likely that they will induce specific immune response against several ASFV strains, arising the possibility of being included in future universal ASFV vaccine formulations. Screening of the rest of the Georgia2007/1 proteome should be considered in order to identify other immunogenic proteins with protective potential.

The complex nature of ASFV will most probably need a combination of several antigens to elicit a fully protective response. A better understanding of protective innate and adaptive immunity is fundamental to optimize the vaccine composition, not only to include optimal antigen formulations but also ideal adjuvant and expression platforms.

CHAPTER 8

Conclusions

1. SLA I immunoprecipitation using extracts from PAMs *in vitro* infected with ASFV followed by mass spectrometry-based sequencing, is a more effective approach for the identification of ASFV $CD8^+$ T-cell epitopes than *in silico* predictions, at least with the methodologies here employed.

2. *In vitro* infection of PAMs with BA71 and BA71ΔCD2 allows the identification of a significant collection of ASFV-specific SLA I-restricted peptides, while *in vitro* infection with Georgia2007/1 does not.

3. The use of PBMCs from pigs vaccinated with the cross-protective BA71ΔCD2 as effector cells in functional assays allows confirming the identity of ASFV $CD8^+$ T-cell epitopes, highly conserved between BA71 and Georgia2007/1.

4. ASFV proteins M448R and MGF505-7R are immunodominant antigens promiscuously recognized by PBMCs from BA71ΔCD2-vaccinated pigs.

5. DNA priming with plasmids encoding the full-length M448R and MGF505-7R fused to ubiquitin, to enhance their SLA I antigen presentation and the induction of CTL responses, promotes partially protective responses against lethal Georgia2007/1 challenge.

6. Administration of multiepitope-encoding DNA plasmids designed based on our *in vitro* assays and *in silico* predictions, promotes ASFV-specific T-cell responses, and provides partial protection against lethal challenge with Georgia2007/1. Pigs primed with the multiepitopes plasmids promiscuously recognize ASFV proteins CP312R and D1133L, making them promising candidates as inductors of protective $CD8^+$ T-cell responses against the Georgia2007/1 ASFV.

Appendix

Appendix I

In silico predictions of ASFV CD8+ T-cell epitopes from the Georgia2007/1 proteome.

Protein	Peptide	Length	Start	End	Proteasome	Promiscuity	Overlap	Immunogenicity	TAPREG
A118R	KRKKRIQFLTF	11	33	43	8	2	5	6	10
A118R	LTFLTNLFLY	10	41	50	8	4	3	7	9
A151R	ISYIGNTYKY	10	43	52	8	5	5	7	9
A151R	NTYKYFTF	8	48	55	9	1	6	6	10
A859L	QLARHAIRY	9	572	580	8	4	2	8	9
A859L	ATLRFDTWYKW	11	626	636	8	1	4	8	10
B119L	FQYWTFAF	8	68	75	7	5	3	9	10
B169L	FQNPFIVAL	9	26	34	9	5	2	8	0
B175L	KTVPKFVPTY	10	69	78	8	5	2	6	10
B318L	AQMSALAL	8	155	162	8	5	4	5	10
B385R	TIFDETHFY	9	180	188	8	5	1	8	10
B475L	SIFETLGAY	9	10	18	7	5	4	8	10
B475L	TLGAYFINIFY	11	14	24	8	3	6	9	5
B475L	YFINIFYNFLY	11	18	28	6	4	6	8	4
B475L	KMLDSFYKY	9	62	70	8	5	3	5	10
B602L	LIMTSFIGY	9	61	69	7	4	6	7	9
B602L	LIMTSFIGYVF	11	61	71	8	1	6	7	9
B602L	ALQEWMPSY	9	73	81	6	7	2	6	10
B646L	LMSALKWPIEY	11	455	465	8	5	4	7	7
B646L	SALKWPIEY	9	457	465	8	5	4	7	10
B962L	FQHAIVYF	8	599	606	7	5	2	8	9
C147L	IMDDLVEEY	9	10	18	7	5	2	7	10
C315R	TMYNVLRRAYY	11	257	267	7	5	3	7	10
C315R	KMYEDYYSHF	10	290	299	7	4	4	6	10
C475L	FVWVADLSY	9	115	123	7	5	2	7	10
C475L	NIFNTIPTLTY	11	127	137	8	2	3	8	10
C475L	NTIPTLTY	8	130	137	8	5	3	7	9
C475L	KQVAIHGFAAY	11	207	217	8	1	5	9	10
C475L	AIHGFAAY	8	210	217	8	5	5	8	10
C475L	GFAAYALLY	9	213	221	7	4	5	7	9
C475L	STLPYNYY	8	438	445	6	5	4	6	10
C62L	SVFWRDRRIRF	11	41	51	7	1	6	10	10
C717R	AIMITTEY	8	356	363	7	5	4	8	10

Protein	Peptide	Length	Start	End	Proteasome	Promiscuity	Overlap	Immunogenicity	TAPREG
C717R	AIMITTEYVGY	11	356	366	8	3	4	8	10
C717R	SFMSLLFDLCY	11	394	404	8	4	6	5	10
C717R	MTYYHFNPTSF	11	425	435	7	5	3	7	10
C717R	RQYGLAFVNTF	11	495	505	8	2	4	8	10
C717R	FFNSITAIDFY	11	543	553	9	2	6	8	0
C717R	SITAIDFY	8	546	553	9	3	4	8	8
C84L	MMMFITVY	8	42	49	8	5	3	8	8
C962R	ALANTSANY	9	327	335	7	6	3	6	10
C962R	YFSILAEYVY	10	398	407	9	1	6	8	3
C962R	SILAEYVYSY	10	400	409	7	5	6	7	9
C962R	YSYNGMLEHY	10	407	416	9	8	6	6	9
C962R	RQRSFIQTL	9	536	544	9	6	3	6	9
C962R	ATMVAASNY	9	730	738	7	8	5	6	10
CP2475L	VTMIEAVY	8	214	221	7	5	4	8	9
CP2475L	NQAINTFMY	9	543	551	7	1	7	7	10
CP2475L	QAINTFMYYYY	11	544	554	6	4	7	6	8
CP2475L	FMYYYYVAQIY	11	549	559	8	4	8	7	6
CP2475L	VIYQHFNLEY	10	976	985	7	5	2	7	10
CP2475L	MMVFNQLIASY	11	1547	1557	6	4	7	7	10
CP2475L	YTHAIQALRF	10	1889	1898	8	5	4	7	8
CP2475L	YSFEEIACLMY	11	2019	2029	8	5	4	8	5
CP2475L	AIQNWVQQY	9	2226	2234	8	4	2	7	10
CP530R	LIDFDPLVTFY	11	225	235	7	5	4	8	8
CP530R	VTFYLLLEPY	10	232	241	6	5	4	7	10
CP530R	STINALRF	8	358	365	6	5	3	7	10
CP80R	RTHLITTLDY	10	67	76	6	5	3	8	10
D1133L	RQFMNHYMNFI	11	114	124	7	5	5	5	9
D1133L	TVLNTFEAYGY	11	756	766	7	5	3	8	9
D1133L	FMARPVWTY	9	782	790	8	5	4	8	10
D1133L	ALALISLCY	9	819	827	8	5	3	6	10
D1133L	KTWHEIPLY	9	1000	1008	8	4	2	8	10
D1133L	GMQDGIRWFY	10	1111	1120	7	3	4	9	8
D205R	AMQKLFTYIY	10	2	11	7	3	6	6	10
D205R	KLFTYIYEF	9	5	13	7	3	6	8	8
D205R	FTYIYEFIEYR	11	7	17	5	1	6	10	9
D205R	YIYEFIEY	8	9	16	7	5	6	9	9
D250R	AFSEFIHCHY	10	37	46	9	1	5	8	10
D250R	SMSYFDGKTEY	11	168	178	8	5	4	6	9

Protein	Peptide	Length	Start	End	Proteasome	Promiscuity	Overlap	Immunogenicity	TAPREG
D250R	TEYKHIYFL	9	176	184	9	4	4	6	10
D345L	KTIFNNVRY	9	125	133	8	5	2	8	10
DP96R	MTSSEWIAEY	10	46	55	6	6	4	8	8
E146L	MRFRNCVFTF	10	55	64	8	5	4	7	0
E301R	LMLNTITL	8	290	297	10	1	4	7	10
E423R	LVDHIFNY	8	57	64	8	5	2	8	10
E66L	SIITRYTLKY	10	4	13	6	5	4	6	10
E66L	LTHNHILFTY	10	18	27	7	4	3	8	10
EP1242L	QMFNVDITY	9	61	69	9	2	2	8	10
EP1242L	RVDMNRFFQFY	11	818	828	7	6	3	7	9
EP153R	KTYNHESNY	9	116	124	7	5	3	6	10
EP296R	SLFEGMIWKSY	11	235	245	8	3	4	7	10
EP402R	YTNESILEY	9	150	158	7	8	2	7	0
EP402R	TYLTLSSNYFY	11	190	200	7	1	10	5	10
EP402R	LTLSSNYFYTF	11	192	202	7	1	10	5	9
EP402R	SNYFYTFFKLY	11	196	206	8	2	10	7	10
EP402R	YQYNTPIYY	9	279	287	7	5	2	7	10
EP424R	FISAINHFNY	10	137	146	7	5	4	8	10
EP424R	YTMMHYPTF	9	146	154	8	10	5	5	0
EP424R	ATQRLTPIHLY	11	215	225	7	7	1	7	9
EP424R	YTLNHAFTL	9	270	278	9	5	3	7	0
F1055L	VIMDVFYETY	10	112	121	8	4	5	8	9
F1055L	VFYETYSLPY	10	116	125	7	3	5	6	10
F1055L	RTYNILQRF	9	527	535	8	4	2	7	10
F165R	NFFFFYIMEY	10	23	32	7	1	10	8	9
F317L	TQMDKLGFLL	10	4	13	8	5	3	6	9
F317L	SRRSLVNPWTL	11	246	256	10	1	4	7	9
F317L	RSLVNPWTL	9	248	256	10	2	4	8	7
F334L	FQYIRYFTDNL	11	264	274	7	5	4	8	0
F778R	AMDEAIHAALY	11	520	530	7	7	3	8	7
F778R	VSQSMSLNY	9	712	720	7	5	7	3	10
F778R	SQSMSLNYYFY	11	713	723	7	3	7	4	10
F778R	SMSLNYYFY	9	715	723	7	7	7	6	9
G1211R	AADDTTCYY	9	147	155	8	4	4	7	8
G1211R	IVMAYDIETY	10	206	215	8	5	3	8	9
G1211R	ASLYPSLIMAY	11	519	529	7	3	6	5	10
G1211R	SLYTEVTDAY	10	698	707	5	5	3	8	10
G1211R	STQFMIQY	8	1043	1050	9	5	2	6	9

Protein	Peptide	Length	Start	End	Proteasome	Promiscuity	Overlap	Immunogenicity	TAPREG
G1340L	VMMPEIETMY	10	97	106	7	6	4	7	8
G1340L	YQFDLLYY	8	229	236	7	5	4	7	10
G1340L	MSVSTFWPYTL	11	463	473	8	1	7	8	1
G1340L	STFWPYTL	8	466	473	8	1	7	8	9
G1340L	NVALLQNTY	9	538	546	9	2	5	6	10
G1340L	ALLQNTYAY	9	540	548	6	5	5	6	10
H124R	MSMIGPYLNVY	11	51	61	8	6	4	7	4
H124R	SMIGPYLNVY	10	52	61	8	5	4	7	10
H240R	VVQYLTPIFY	10	139	148	7	5	4	7	9
H339R	KLWAAYEGY	9	84	92	6	3	4	8	10
H339R	AAYEGYFKY	9	87	95	6	5	3	7	10
H339R	KLYTAALGVY	10	200	209	8	3	3	7	10
I10L	VSLGGTGECYY	11	136	146	8	5	3	7	9
I215L	MVSRFLIAEY	10	1	10	8	5	1	8	10
I226R	RQLSFVLL	8	51	58	9	3	4	6	9
I226R	TLMFAVRY	8	150	157	7	4	4	7	10
I243L	IQMMFFRTL	9	81	89	8	7	4	7	1
I267L	FQSRHIHHFTL	11	66	76	7	7	3	8	0
I267L	LTMASLARF	9	252	260	7	5	6	6	6
I329L	RSNTPTYLYY	10	136	145	8	6	3	7	8
I329L	FSNNNTFLY	9	182	190	6	8	3	7	0
I7L	MLYNEPLGTY	10	28	37	7	5	4	7	10
I8L	RLYPHIFY	8	88	95	8	4	4	8	10
I9R	STFSSQDFDEY	11	63	73	7	5	4	5	10
K145R	SIQQLFLTMY	10	48	57	8	5	4	6	9
K205R	MVSFENFIERY	11	38	48	7	3	3	9	10
K421R	LLYKLDPEY	9	78	86	6	4	6	5	10
K78R	SSMHSGMLY	9	36	44	7	10	4	4	9
M1249L	STMYSVSPVF	10	266	275	8	6	6	4	9
M1249L	SVSPVFTSGY	10	270	279	7	6	7	6	7
M1249L	TSGYMPLLY	9	276	284	7	4	7	5	8
M1249L	LLYDLYRAGY	10	282	291	6	3	6	7	10
M1249L	AVNVCLPLVY	10	555	564	9	5	2	6	10
M1249L	AVIYIYAY	8	583	590	7	5	3	7	10
M1249L	KTSTLIYLRAY	11	772	782	7	4	4	7	9
M1249L	RAYELFLKY	9	780	788	6	5	5	7	10
M1249L	ASLEFNTFYAF	11	922	932	7	3	5	9	9
M1249L	SLEFNTFYAFY	11	923	933	6	5	5	9	7

Protein	Peptide	Length	Start	End	Proteasome	Promiscuity	Overlap	Immunogenicity	TAPREG
M1249L	AVDSAVRIFLY	11	1068	1078	7	5	4	7	9
M1249L	RVTDPASALLY	11	1130	1140	8	5	5	6	6
M448R	LTIYHWDDPEY	11	146	156	7	5	2	9	10
M448R	KTLKTVYPEY	10	248	257	8	5	3	6	9
MGF100-1R	YLMRYTQIYKY	11	103	113	7	7	5	6	4
MGF110-11L	SIARYFDRCMY	11	84	94	8	5	5	7	9
MGF110-12L	YQSPTTPWCFY	11	19	29	8	5	4	8	5
MGF110-14L	IVNRNSWGCFY	11	72	82	8	6	3	7	3
MGF110-1L	LSIPTLLYTY	10	10	19	8	5	4	7	8
MGF110-1L	SVFRHNEFCTY	11	77	87	8	6	3	8	10
MGF110-8L	TVSRWNGICSY	11	81	91	8	5	1	8	9
MGF110-9L	HIMSCTNPTY	10	101	110	9	5	5	5	10
MGF110-9L	MIATIALISY	10	138	147	6	4	4	8	10
MGF110-9L	ATIALISY	8	140	147	6	7	4	7	10
MGF110-9L	MQAATRLLIFL	11	157	167	9	1	6	8	9
MGF110-9L	CMYEAHFRIHY	11	252	262	8	5	1	9	10
MGF300-1L	LLSWGASPEY	10	78	87	8	5	3	7	9
MGF300-1L	STLYCIFLAIY	11	179	189	7	3	5	8	9
MGF300-4L	YVYSKTFY	8	165	172	7	5	6	5	10
MGF360-10L	KLFNDNPFPAY	11	136	146	8	4	3	7	10
MGF360-10L	AMLACVRFY	9	266	274	8	3	3	7	10
MGF360-11L	NVFDLHELY	9	221	229	8	4	2	7	10
MGF360-11L	IQDYSYSAIYY	11	247	257	7	3	7	5	9
MGF360-12L	SLSTLLLKY	9	174	182	7	5	6	5	10
MGF360-12L	SLSTLLLKYWY	11	174	184	8	5	6	6	10
MGF360-12L	AMLSSIQYY	9	266	274	8	5	4	4	10
MGF360-13L	VQYYNIGNIFF	11	271	281	8	1	4	8	10
MGF360-15R	LIEFLTGFFY	10	16	25	7	3	5	9	9
MGF360-15R	VMDMICLDYY	10	37	46	7	5	5	6	9
MGF360-15R	HEWEISIDYAL	11	71	81	7	4	2	8	10
MGF360-18R	RLYDANIY	8	93	100	9	4	3	7	9
MGF360-18R	QVMYLLYKY	9	159	167	8	4	4	5	10
MGF360-1L	YSMAILYKL	9	184	192	8	5	6	6	0
MGF360-1L	ILYKLTEAIQY	11	188	198	8	3	7	6	7
MGF360-1L	LTEAIQYFY	9	192	200	6	4	6	7	8
MGF360-1L	AIQYFYQRY	9	195	203	8	3	6	6	10
MGF360-1L	CMYDCNYTTIY	11	244	254	7	5	3	7	9
MGF360-21R	FSEMLTRYWY	10	176	185	7	3	6	7	8

Protein	Peptide	Length	Start	End	Proteasome	Promiscuity	Overlap	Immunogenicity	TAPREG
MGF360-21R	SEMLTRYWYSM	11	177	187	7	4	6	7	0
MGF360-21R	YSMAILYNL	9	185	193	8	5	5	7	0
MGF360-21R	ILYNLTEAIQY	11	189	199	8	3	7	7	10
MGF360-21R	IQYFYQRYRHF	11	197	207	8	1	5	7	10
MGF360-21R	STYDGNYSTIY	11	246	256	7	6	3	6	10
MGF360-2L	KLTEAIQY	8	191	198	8	1	7	7	9
MGF360-2L	IQYFYQRY	8	196	203	8	1	5	7	10
MGF360-4L	FVYNRFILY	9	162	170	7	4	3	8	10
MGF360-4L	STYKNTESFY	10	363	372	6	8	3	5	10
MGF360-6L	STYEYTETF	9	356	364	7	4	4	8	9
MGF360-8L	TEWGANIYYGL	11	80	90	8	4	2	8	10
MGF360-8L	RAISFFYQTY	10	189	198	8	4	4	6	10
MGF360-8L	QTYGHLSMWRL	11	196	206	9	1	5	6	10
MGF360-9L	QRYAHLHRWRL	11	193	203	9	2	3	8	10
MGF360-9L	NQAMFHSIQFY	11	257	267	9	2	5	6	10
MGF360-9L	AMFHSIQFY	9	259	267	9	5	5	6	10
MGF360-9L	IQFYNIGNIFF	11	264	274	8	1	4	8	10
MGF360-9L	SMLSLNCY	8	300	307	9	5	2	5	10
MGF505-10R	IVDDYIRFLFY	11	499	509	8	5	2	9	2
MGF505-11L	LLYAATLY	8	142	149	7	5	4	7	9
MGF505-11L	SMMQTAIQKNY	11	231	241	7	5	5	5	10
MGF505-11L	SQMTVIDSVYY	11	312	322	8	1	7	7	10
MGF505-11L	TVIDSVYY	8	315	322	8	5	7	6	10
MGF505-11L	SVYYSIIKY	9	319	327	8	4	6	6	10
MGF505-11L	LIDSLIERFRY	11	528	538	7	5	4	8	8
MGF505-1R	LLAWEGNLYY	10	76	85	8	5	4	8	10
MGF505-1R	ATYNHRKILIY	11	270	280	8	5	1	7	10
MGF505-1R	RLMNFIYDRCY	11	427	437	8	3	3	8	9
MGF505-2R	YLHETLFEL	9	160	168	8	4	4	8	0
MGF505-2R	AISKRDLTMY	10	197	206	8	2	6	5	10
MGF505-2R	KLPRYVIEY	9	311	319	7	3	4	8	9
MGF505-3R	LLLSWEADPRY	11	75	85	8	4	2	8	10
MGF505-3R	AVVGALESKY	10	86	95	8	3	4	6	10
MGF505-4R	LTMGYSLLF	9	204	212	6	9	4	5	7
MGF505-4R	LTGFIDYYYSY	11	352	362	8	2	4	7	10
MGF505-5R	NVSLLSIGY	9	200	208	8	3	4	6	10
MGF505-5R	LSIGYTLLF	9	204	212	8	3	4	7	10
MGF505-5R	ILADFIGYHSY	11	346	356	7	6	4	8	8

Protein	Peptide	Length	Start	End	Proteasome	Promiscuity	Overlap	Immunogenicity	TAPREG
MGF505-6R	SQIQDWHILL	10	102	111	8	5	2	8	8
MGF505-6R	LVDIIHSIY	9	427	435	6	4	4	8	10
MGF505-7R	SQIQDWHVLL	10	102	111	8	5	2	8	8
MGF505-9R	STYTEIVKY	9	355	363	9	5	2	7	10
NP1450L	RRWLRVICL	9	102	110	10	4	2	8	10
NP1450L	ATQQLALNY	9	645	653	7	6	3	5	10
NP1450L	FSFGRTLVY	9	776	784	8	6	4	7	10
NP1450L	MRMLIRIEL	9	1008	1016	10	2	2	8	10
NP1450L	ILDLIRLQY	9	1032	1040	7	5	4	7	8
NP1450L	ILQWHLLYETY	11	1136	1146	8	3	3	8	9
NP1450L	SSIGDTMELY	10	1327	1336	8	6	3	7	9
NP419L	VVTPKHLTY	9	355	363	10	4	4	5	10
NP868R	TLINPDDEYLY	11	176	186	8	4	4	7	8
NP868R	YLYEIEIEY	9	184	192	7	4	4	9	9
NP868R	YMDPFSFEEL	10	570	579	8	4	5	7	0
O174L	YQLDLFTAL	9	97	105	9	7	2	7	0
P1192R	REMFTRIEL	9	189	197	8	3	2	8	10
P1192R	SIAENVFKTHY	11	372	382	8	5	3	7	9
P1192R	KTMPVEFYY	9	584	592	6	8	3	7	10
P1192R	SLANHTVKYY	10	605	614	8	5	2	6	10
P1192R	YVADHMFY	8	744	751	8	8	2	6	4
P1192R	NTYYEILY	8	1026	1033	8	4	6	8	10
P1192R	ILYAWLPY	8	1031	1038	5	6	5	8	7
P1192R	REHAVLKL	8	1049	1056	10	1	4	6	10
P1192R	RIIMETAIVRY	11	1057	1067	8	2	3	8	10
Q706L	LTARFARALKY	11	36	46	7	4	4	7	10
Q706L	YQFLIYTAF	9	47	55	8	5	3	8	2
Q706L	RRFRFVSL	8	137	144	8	5	1	7	10
QP509L	MVYTTRPVSL	10	76	85	10	1	4	7	9
QP509L	SIILLTYGY	9	392	400	6	5	6	7	10
R298L	AMYHILIIEY	10	99	108	7	2	4	9	10
R298L	GTWEYVCPEFY	11	189	199	8	2	6	8	10
R298L	YVCPEFYYY	9	193	201	7	5	6	7	4
R298L	YVCPEFYYYGY	11	193	203	7	4	6	7	1
R298L	FYYYGYYYQL	10	198	207	8	3	7	6	7
R298L	YQLPLTVWTI	10	205	214	7	5	5	8	0
R298L	NLFRFRAENFY	11	220	230	7	2	4	9	9
S183L	YSLNNWARY	9	10	18	7	7	3	8	0

Protein	Peptide	Length	Start	End	Proteasome	Promiscuity	Overlap	Immunogenicity	TAPREG
S183L	QAFEHLYAF	9	128	136	7	4	3	7	10
S273R	TLQRWAIKY	9	96	104	7	5	1	8	10
S273R	SMMDFERVHY	10	113	122	8	6	3	8	10
X69R	NEYWAIHLFFI	11	19	29	5	4	2	10	10
X69R	SQFWNYTM	8	47	54	6	7	3	8	9

Appendix II

ASFV peptides selected for the presence of multiple predicted $CD8^+$ T-cell epitopes.

Protein	Peptide sequence	Length	Start	End	# Overlapping predictions
A118R	MAMSIPRMINKRKKRIQFLTF	21	23	43	9
A151R	VSEISYIGNTYKYFTF	16	40	55	12
B475L	TLGAYFINIFYNFLY	15	14	28	3
B602L	REQYIRRLIMTSFIGYVFKALQEW	24	54	77	14
C475L	KQVAIHGFAAYALLY	15	207	221	11
C62L	KVISNTAVSVFWRDRRIRF	19	33	51	11
C717R	AFDILKYGYPMQQSGY	16	47	62	2
C717R	GEYRYRFVWYQPF	13	104	116	2
C717R	ILMDEDSFMSLLFDLCYGAY	20	388	407	10
C717R	FFNSITAIDFYAIARNLRSML	21	543	563	3
C962R	YFSILAEYVYSYNGMLEHYM	20	398	417	11
C962R	FQMTATMVAASNYNFII	17	726	742	12
CP204L	SQVVFHAGSLY	11	23	33	5
CP2475L	SQVDLNQAINTFMYYYYVAQIY	22	538	559	17
CP2475L	IQNNRSMMMVFNQLIASYITRFY	23	1540	1562	13
CP2475L	YMSRYNKEPLMPF	13	1795	1807	3
D205R	MAMQKLFTYIYEFIEYRKM	19	1	19	13
D250R	KRYSLAFSEFIHCHYSI	17	32	48	7
E146L	IAYMRFRNCVFTF	13	52	64	3
E423R	RSRTASSAELYRKMLYAY	18	152	169	4
EP152R	FLKQSGLQSFYLYIQPDHTCF	21	40	60	10
EP402R	CTYLTLSSNYFYTFFKLYYIPL	22	189	210	23
EP424R	AINHFNYTMMHYPTFNW	17	140	156	3
F1055L	NVIMDVFYETYSLPYNI	17	111	127	8
F1055L	LQFNYSFQSHMYAIMLLTK	19	905	923	12
F165R	KQASISSILNFFFFYIMEYFVAV	23	14	36	24
F778R	FVSQSMSLNYYFY	13	711	723	15
G1211R	TAADDTTCYYRM	12	146	157	2
G1340L	IQISISSMSVSTFWPYTLSSK	21	456	476	17
G1340L	KMDINVALLQNTYAYLF	17	534	550	7
I267L	SNMGYMLTMASLARFIINTASFNK	24	246	269	13
K145R	TQLYRSIQQLFLTMYK	16	43	58	7
K421R	AEHSKALATLLYKLDPEY	18	69	86	11
M1249L	STMYSVSPVFTSGYMPLLYDLYRAGYL	27	266	292	17

Protein	Peptide sequence	Length	Start	End	# Overlapping predictions
M1249L	YLRAYELFLKYL	12	778	789	4
M1249L	QTALKASLEFNTFYAFY	17	917	933	11
M1249L	RVTDPASALLYSIEF	15	1130	1144	3
MGF100-1L	LQMAPGGSYFITDNMTEEF	19	68	86	3
MGF100-1R	YINIYMYLMRYTQIYKYPL	19	97	115	10
MGF110-9L	HIMSCTNPTYYDWFDELM	18	101	118	10
MGF110-9L	KQYSRMRMQAATRLLIFL	18	150	167	13
MGF300-1L	YAKNYSLSTLYCIFLAIYY	19	172	190	8
MGF300-4L	RLYVYSKTFYRK	12	163	174	6
MGF360-10L	AISYVYQHFKYL	12	195	206	6
MGF360-11L	EMMHIACIQDYSYSAIYYCFI	21	240	260	15
MGF360-12L	KTDLLNNEFSLSTLLLKYWYAI	22	165	186	13
MGF360-14L	YFYKRHKNHLYW	12	195	206	3
MGF360-2L	KLTEAIQYFYQRYSHFK	17	191	207	11
MGF360-3L	TTNSMLNEISFSEMLTKYWY	20	167	186	11
MGF360-6L	SFDSHDFYVLIDF	13	150	162	2
MGF360-8L	YQTYGHLSMWRL	12	195	206	3
MGF360-9L	NQAMFHSIQFYNI	13	257	269	7
MGF505-11L	HIMHLTSSQELFEFFHLFI	19	111	129	12
MGF505-11L	ISMMQTAIQKNYFRFFKK	18	230	247	9
MGF505-11L	SQLDSQMTVIDSVYYSIIKY	20	308	327	15
MGF505-5R	HSYTYMVDFMREF	13	354	366	3
NP1450L	INYQRAPTWYSEV	13	575	587	2
NP419L	YADRYALFQKLTPALF	16	363	378	3
NP868R	QFQPSDFPLAFLYYHPDTSSF	21	495	515	9
O61R	VVIMAIMLYYFWWMPR	16	23	38	13
P1192R	IEFNTYYEILYAWLPYRR	18	1023	1040	10
QP509L	ETAFTLQGEYIYF	13	51	63	8
QP509L	ALSQAHSASIILLTYGYGR	19	384	402	12
R298L	GTWEYVCPEFYYYGYYYQLPLTVWTI	26	189	214	13

Appendix III

SLA I-restricted ASFV peptides identified by MS-based immunopeptidomics.

					BA71		Georgia2007/1	
Protein	**Peptide sequence**	**Length**	**BA71**	**BA71ΔCD2**	**Start**	**End**	**Identity (%)**	**Sequence**
A137R	NSYTKKMEL	9			47	55	100	
A151R	ENDLYHTNY	9			97	105	100	
A179L	VIFNIKYFL	9			149	157	100	
A238L	DKDGNSALHYL	11			84	94	100	
B117L	LTRHIYNTV	9			95	103	100	
B125R	AKDLDNNKEL	10			5	14	100	
B125R	NKKEEYLRM	9			99	107	100	
B475L	NKKLYEKML	9			56	64	100	
B475L	DSFIPKEYSQSI	12			93	104	100	
B475L	LPFIIKNRKENY	12			134	145	100	
B475L	LPSEHFSNEEY	11			260	270	100	
B475L	RKQELLTSQEL	11			288	298	100	
B475L	SQELTSKSPNN	11			295	305	100	
B602L	SKPTHTTKTLL	11			82	92	100	
B602L	KVDEFYYKY	9			406	414	100	
B602L	VDEFYYKY	8			407	414	100	
B646L	KPYVPVGFEY	10			65	74	100	
B646L	NVRFDVNGNSL	11			190	200	100	
B646L	LLQNGSAVLRYST	13			634	646	100	
B646L	LQNGSAVLRYST	12			635	646	100	
C129R	VPYDNISKLY	10			87	96	100	
C129R	YAKPDAIML	9			96	104	100	
C257L	NKYLERQDL	9			115	123	100	
C315R	KKISNYQLL	9			32	40	100	
C475L	SPNHVEDAY	9			76	84	100	
C475L	HKNDKNITL	9			248	256	100	
C475L	TPADKSLFLFY	11			343	353	100	
C475L	LPDVSTLPYNY	11			434	444	100	
CP123L	WANEAEHLI	9			74	82	100	
CP123L	AKSLFKEFL	9			112	120	100	
CP2475L	NKALQKVGL	9			298	306	100	
CP2475L	HNKQEFQSY	9			564	572	100	

					BA71		Georgia2007/1	
Protein	**Peptide sequence**	**Length**	**BA71**	**BA71ΔCD2**	**Start**	**End**	**Identity (%)**	**Sequence**
CP2475L	ITKTFVNNI	9			654	662	100	
CP2475L	DNAPAGHYY	9			710	718	100	
CP2475L	TPEEAAQRVY	10			824	833	90	TAEEAAQRVY
CP2475L	VNDALSTRW	9			839	847	100	
CP2475L	EFYQKLFSF	9			917	925	100	
CP2475L	IPIYLKENY	9			1645	1653	100	
CP2475L	YINQALHEL	9			2157	2165	88.9	YINQALHEI
CP312R	MKTKHVIKL	9			166	174	100	
D1133L	HNAQLFVQNF	10			76	85	100	
D1133L	YKDETLPYL	9			402	410	100	
D1133L	GPDPKISSNAY	11			429	439	100	
D1133L	TPDGGPELAKY	11			693	703	100	
D1133L	EPMEQVLIHY	10			914	923	100	
D205R	EFIEYRKMVL	10			12	21	100	
D205R	EFIEYRKMVLL	11			12	22	100	
D205R	VPYDKFVQM	9			26	34	100	
D250R	TAMQLKTSI	9			3	11	100	
D339L	RSKKDFKQI	9			192	200	100	
DP238L	YSEKEKETI	9			212	220	88.9	YSEEEKETI
E111R	YNIKTKEY	8			99	106	100	
E120R	LTHNMEKI	8			60	67	100	
E183L	YTHKDLENSL	10			174	183	100	
E248R	TNTVNQYSHY	10			171	180	100	
E301R	NTITLNNTI	9			293	301	88.9	NTITLNTTI
EP1242L	RVHGSGVSL	9			417	425	100	
EP1242L	TNAFELAQRY	10			626	635	100	
EP364R	PAFPNPQKKI	10			100	109	100	
EP424R	NKIKLLNEYL	10			379	388	90	NKIKLLNDYL
EP424R	YVLEKKHKL	9			406	414	100	
F317L	DFFIYEDERL	10			277	286	100	
F317L	DFFIYEDERLLMF	13			277	289	100	
F334L	EFISRDEGM	9			199	207	100	
G1211R	MPIDIHEVRY	10			35	44	100	
G1211R	ENIAYERLETL	11			93	103	90.9	ENIVYERLETL
G1211R	EPEPGERFSY	10			891	900	100	

					BA71		Georgia2007/1	
Protein	**Peptide sequence**	**Length**	**BA71**	**BA71ΔCD2**	**Start**	**End**	**Identity (%)**	**Sequence**
G1340L	DFVDLNDIHTI	11			1190	1200	100	
H233R	SPFSLAHLEY	10			6	15	100	
H233R	NAYAHKLNI	9			212	220	100	
H339R	NKIESSVHLL	10			245	254	100	
H339R	NPTIIMEQY	9			271	279	100	
H339R	DSKNISPRI	9			286	294	100	
H359L	FNLAVKTHF	9			176	184	100	
I226R	TNLYLKQEL	9			35	43	100	
I226R	KNILNTLMF	9			145	153	100	
I243L	NTILTNKI	8			20	27	100	
I73R	TAKNIKVVI	9			22	30	100	
I9R	YKIYIHSDL	9			75	83	100	
K145R	KLDPIGFINY	10			58	67	100	
K145R	YIKTSKQEYL	10			67	76	100	
K145R	AKIVEEGGEES	11			135	145	100	
M1249L	KNIRLIDF	8			43	50	100	
M1249L	YAATVARI	8			689	696	100	
M1249L	TPAELLSQKEFY	12			722	733	100	
M448R	RAKIPAQEI	9			58	66	88.9	RAKIPAQE**T**
MGF110-6L	RKEWKKDEL	9			110	118	77.8	RKEWKK**N**E**H**
MGF360-10L	NKLFDLHNL	9			218	226	100	
MGF360-8L	DVIRLFTEW	9			74	82	100	
MGF360-8L	VPMNIFVKY	9			169	177	88.9	VPMN**V**FVKY
MGF505-1R	YAIHHAPKL	9			452	460	100	
MGF505-1R	TNINIVNKY	9			494	502	100	
MGF505-2R	RKHIEKLLL	9			275	283	100	
MGF505-3R	KKYQHKHIL	9			264	272	100	
MGF505-5R	DWLSEHVIQRL	11			17	27	90.9	DWL**G**EHVIQRL
MGF505-7R	NSTLVIRI	8			334	341	87.5	NSTLVIR**L**
MGF505-9R	YNIHNITGY	9			423	431	100	
NP1450L	RAKHPNTYI	9			1200	1208	100	
NP419L	TPALFKKHLY	10			374	383	100	
P1192R	AAYVGKGTTI	10			230	239	100	
P1192R	NSTIHTATI	9			265	273	100	
P1192R	VIKKFQQTI	9			326	334	88.9	V**V**KKFQQTI

					BA71		Georgia2007/1	
Protein	**Peptide sequence**	**Length**	**BA71**	**BA71ΔCD2**	**Start**	**End**	**Identity (%)**	**Sequence**
Q706L	AKNIRILFL	9			197	205	100	
QP383R	NKLQDISKVL	10			147	156	90	NKLQDIS**N**VL
QP383R	PSMKKINTI	9			238	246	-	
QP383R	KKINTIHKL	9			241	249	-	

Appendix IV

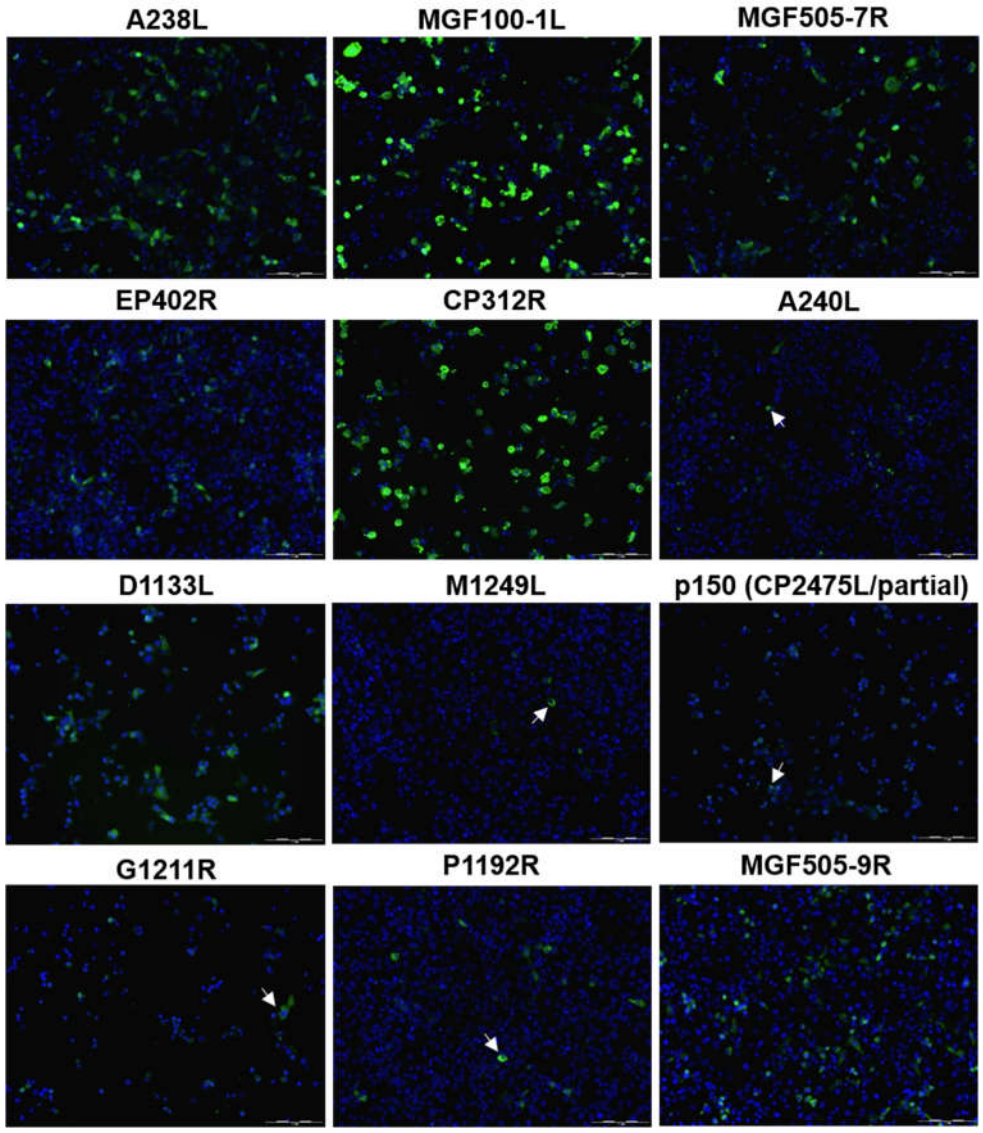
ASFV E75 gene expression library encoding each ORF with the ubiquitin gene at the N-terminus and under the control of the CMV promoter.

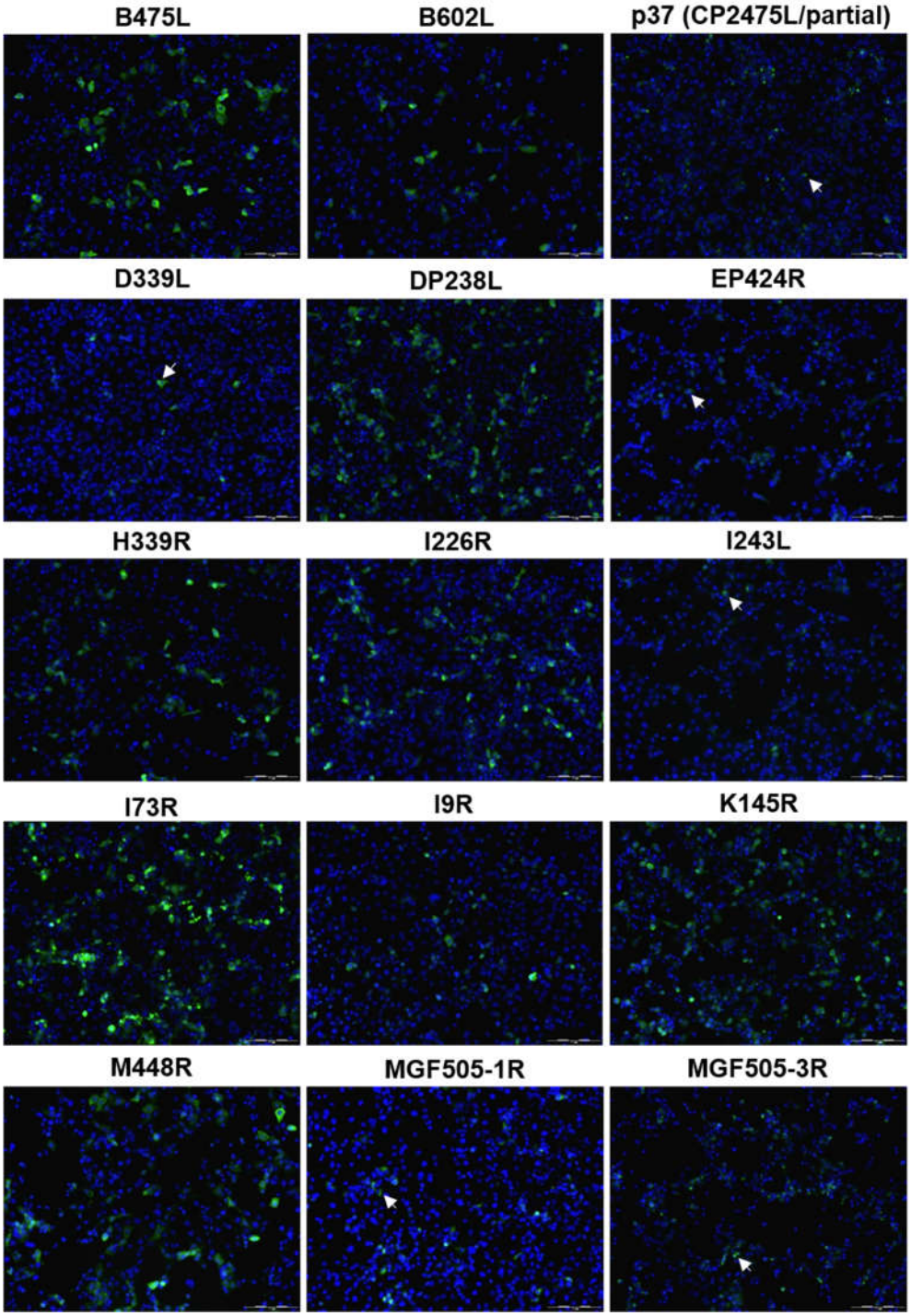
Plasmid mix	E75 locus	Protein name
Mix 1	2	DP93R
	3	MGF360-2L
	4	KP177R
	6	L60L
	7	MGF360-3L
	8	MGF110-1L
	10	MGF110-13L
	13	MGF110-12L
	14	MGF110-14L
	15	MGF360-4L
Mix 2	16	MGF360-6L
	17	X69R
	18	MGF300-1L
	20	MGF300-2R
	22	MGF300-4L
	23	MGF360-8L
	25	MGF360-10L
	26	MGF360-11L
	28	MGF360-12L
	30	MGF360-14L
Mix 3	31	MGF505-2R
	33	MGF505-4R
	35	MGF505-6R
	36	MGF505-7R
	39	A224L
	40	A104R
	41	A118R
	45	MGF360-15R
	46	A238L
	47	A859L
Mix 4	48	A179L
	49	A137R
	50	F317L
	53	F165R
	55	K205R
	56	K78R
	63	EP152R
	66	EP364R
	77	C62L
	83	B438L

Plasmid mix	E75 locus	Protein name
Mix 5	92	B407L
	93	B175L
	94	B263R
	104	O174L
	109	D250R
	110	D129L
	111	D79L
	116	D345L
	117	S183
	118	S273R
	120	H359L
Mix 6	121	H171R
	122	H124R
	132	E184L
	133	E183L
	135	E301R
	137	E199L
	138	E165R
	139	E248R
	140	E120R
	141	E296R
	142	E111R
Mix 7	143	E66L
	144	I267L
	151	I215L
	152	I177L
	153	I196L
	158	MGF100-2L
	161	I8L
	163	I10L
	164	L11L
	167	DP96R
	168	MGF360-19R

Appendix V

Immunofluorescence assay of RK13 cells transfected with each pCMV-Ub-ASFV ORF-FLAG recombinant plasmid.




B475L
B602L
p37 (CP2475L/partial)
D339L
DP238L
EP424R
H339R
I226R
I243L
I73R
I9R
K145R
M448R
MGF505-1R
MGF505-3R

Appendix VI

Protein domains included in the multiepitopes-encoding DNA plasmids.

Multiepitope-I

Protein	Start	End	Length	Relevant peptides previously identified	#Overlapping predictions
EP402R	66	91	26		4
	94	105	12		2
	139	160	22		4
	278	295	18		5
	183	206	24		10
CP312R	7	151	145	SFPLCLEMGVVKVFE*, KNNGIDVNSIYGSDD*	8
	260	300	41		10
A240L	64	133	70	SEFIAEIAVLL*	17
	175	206	32		6

Multiepitope-II

Protein	Start	End	Length	Peptide identified by MS-based immunopeptidomics	#Overlapping predictions
B475L	36	71	36	NKKLYEKML	14
	84	171	88	DSFIPKEYSQSI, LPFIIKNRKENY	23
	226	305	80	LPSEHFSNEEY, RKQELLTSQEL, SQELTSKSPNN	9
P37 (CP2475L/partial)	538	591	54	HNKQEFQSY	23
	625	662	38	ITKTFVNNI	6
	708	760	53	DNAPAGHYY, NIRDLIGRSL	9
	824	877	54	TAEEAAQRVY, VNDALSTRW	11
P150 (CP2475L/partial)	900	938	39	EFYQKLFSF	8
	1634	1663	30	IPIYLKENY	8
	2142	2179	38	YINQALHEI	6
D1133L	56	133	78	HNAQLFVQNF	19
	393	463	71	YKDETLPYL, GPDPKISSNAY	22
	867	950	84	EPMEQVLIHY	21
G1211R	3	60	58	MPIDIHEVRY	14
	88	218	131	ENIVYERLETL	43
H339R	245	324	80	NKIESSVHLL, NPTIIMEQY, DSKNISPRI	11
I226R	19	72	54	TNLYLKQEL	19
	118	165	48	KNILNTLM	14
M1249L	27	79	53	KNIRLIDF	7
	677	755	79	YAATVARI, TPAELLSQKEFY	15
MGF505-1R	427	460	34	YAIHHAPKL	9
	493	530	38	TNINIVNKY	7
MGF505-9R	74	101	28	RQYDLIQKY*	7
	128	151	24	NMLPIFQKY*	6
	294	316	23	SSINYCVNPF*	8
	408	473	66	YNIHNITGY	11
P1192R	189	241	53	AAYVGKGTTI	15
	485	531	47	TIMVRNEQL	6
K145R	30	116	87	KLDPIGFINY, YIKTSKQEYL	25
EP424R	341	413	73	NKIKLLNDYL, YVLEKKHKL	14

*Antigenic peptides from a peptide library already present in the lab (unpublished)

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