Available online at www.sciencedirect.com
ScienceDirect
Veterinary Immunology and Immunopathology 124 (2008) 107–119
Veterinary immunology and immunopathology
www.elsevier.com/locate/vetimm

Cytokine mRNA expression and pathological findings in pigs inoculated with African swine fever virus (E-70) deleted on A238L

F.J. Salguero [a,1,*], S. Gil [b,1], Y. Revilla [c], C. Gallardo [a], M. Arias [a], C. Martins [b]

[a] Centro de Investigación en Sanidad Animal, CISA-INIA, Carretera Algete-El Casar s/n, Valdeolmos, 28130 Madrid, Spain
[b] Laboratório de Doenças Infecciosas, CIISA, Faculdade de Medicina Veterinária, Technical University of Lisbon (TULisbon), Av. da Universidade Técnica, 1300-477 Lisbon, Portugal
[c] Centro de Biología Molecular "Severo Ochoa", Universidad Autónoma de Madrid, 28049 Cantoblanco, Madrid, Spain

Received 18 November 2007; received in revised form 24 January 2008; accepted 12 February 2008

Abstract

African swine fever virus (ASFV) induces a variety of immune responses and clinical forms in domestic pigs. As it is the only member of the Asfarviridae family, ASFV encodes many novel genes not encoded by other virus families. Among these genes, A238L may regulate the synthesis of pro-inflammatory cytokines, controlled mainly by NFκB and NFAT pathways. In this study, we inoculated two groups of pigs, one with the ASFV highly virulent E-70 isolate, deleted on A238L gene, and the other group with the parental E-70 isolate. No significant differences were observed in the clinical signs or pathology between both groups. However, the TNF-α mRNA expression was strongly enhanced in the PBMC from pigs inoculated with the virus deleted in A238L, reinforcing the role of the A238L gene in the inhibition of the NFκB pathway of expression of cytokines. No up-regulation of pro-inflammatory cytokines was observed in the PBMC of animals inoculated with the E-70 isolate, even though apoptosis and haemorrhages were evident and might be related to the presence of bystander monocyte-macrophages expressing these cytokines. Other studies using ASFV deleted in other genes inoculated in the natural hosts should be performed to gain further insight into the role of these genes in the pathogenesis of ASF.

Keywords: Virus; African swine fever; Cytokine; Pathology

1. Introduction

African swine fever (ASF) is a porcine infectious disease that affects only porcine species, both domestic and wild pigs of all breeds and ages. It is caused by an icosahedral, double-stranded DNA virus classified as the only member of the family Asfarviridae (Dixon et al., 2005). ASF virus (ASFV) causes unapparent persistent infections, in wild pigs in Africa, warthogs (*Phacochoerus aethiopicus*), bushpigs (*Potamochoerus porcus*) and the soft tick vector, *Ornithodoros* spp. (Plowright et al., 1969; Wardley et al., 1983; Dixon et al., 2004). In domestic pigs and European wild boars, ASFV induces a variety of immune responses and clinical forms of disease ranging from fatal hyper-acute, acute and sub-acute, to chronic or unapparent forms of disease in the

* Corresponding author. Present address: Veterinary Laboratories Agency (VLA-Weybridge), Department of Pathology, Woodham Lane, New Haw, Addlestone, Surrrey KT15 3NB, United Kingdom. Tel.: +44 1932 357299: fax: +44 1932 357217.
E-mail addresses: fjsbodes@hotmail.com, j.Salguero@vla.defra.gsi.gov.uk (F.J. Salguero).
[1] Contributed equally to this work.

0165-2427/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetimm.2008.02.012

host (Martins and Leitão, 1994). The most characteristic lesions in acute ASF infection are haemorrhages in lymphoid organs and severe lymphoid depletion due to apoptosis of T and B lymphocytes (Moulton and Coggins, 1968; Wilkinson and Wardley, 1981; Gomez-Villamandos et al., 1995a,b,c,d; Carrasco et al., 1996a,b, 1997; Oura et al., 1998; Salguero et al., 2002, 2004, 2005). The ASFV *in vivo* infection remains to be one of the most interesting and controversial model for swine diseases in which the virus replicates in reticulo-endothelial cells of lymphoid tissues and organs of domestic swine (Mebus, 1988; Salguero et al., 2002; Dixon et al., 2004), being the monocyte-macrophages the main target cells for viral replication (Mebus, 1988; Martins and Leitão, 1994) which is believed to play a critical role in the pathogenesis of the disease (Colgrove et al., 1969; Gomez-Villamandos et al., 1995c; Gómez del Moral et al., 1999; Salguero et al., 2002, 2005).

ASFV encodes many novel genes not encoded by other virus families. The ability of the virus to persist in the natural hosts and in domestic pigs, which recover from infection with less virulent isolates, shows that the virus has effective mechanisms to evade host defence systems (Dixon et al., 2004).

ASFV isolates show a broad spectrum of virulence degree. However, mechanisms underlying these characteristics are not fully understood. Genome sequence of ASFV isolates has revealed genes responsible for virulence. Among others, different authors have assessed the role of several ASFV genes as virulence factors through the construction of different ASFV deleted virus. This strategy has shown that deletion of NLS gene (Zsak et al., 1998) and of the multigenic families MGF 360 and MGF 530 (Neilan et al., 2002) reduced significantly the ASFV virulence in inoculated pigs in comparison with the effect of infection with the parental isolates. ASFV deleted in the thymidine kinase (TK) gene in the Malawi and Haiti isolates has shown to induce a decrease in the viral growth on macrophage cultures and decreased the mortality rate *in vivo* (Moore et al., 1998).

ASFV encodes also proteins able to modulate the host defence mechanisms namely, two proteins homologous to IAP and Bcl-2, named A224L and A179L, known to inhibit apoptosis in the infected cell and thus promoting survival of infected macrophages and facilitate the production of progeny virions (Yáñez et al., 1995; Brun et al., 1996; Revilla et al., 1997; Oura et al., 1998; Nogal et al., 2001; Rodriguez et al., 2002). Other viral genes modulate signalling pathways in infected macrophages, thus interfering with the expression of a large number of immunomodulatory genes (Dixon et al., 2004). One potent immunomodulatory protein, A238L, inhibits both activation of the host NFκB transcription factor and by means of the inhibitions of calcineurin phosphatase activity and cyclooxygenase-2 expression through a nuclear factor of activated T cell-dependent transactivation pathway (Miskin et al., 1998, 2000; Dixon et al., 2004; Granja et al., 2006a).

Synthesis of pro-inflammatory cytokines is controlled mainly by NFκB and NFAT pathways. NFκB is a dimmer of two heterologous proteins (p65 and p50) held in an inactive complex with an endogenous inhibitor, IκB, in the cytoplasm. After cell activation, IκB is phosphorylated and subsequently degraded by cytoplasmic proteosomes. The released NFκB translocates to the nucleus and activates the synthesis of pro-inflammatory cytokines (Powell et al., 1996). The A238L gene of ASFV has 21% of identity and 40% of homology at the amino acid level to porcine IκBα (Powell et al., 1996) and it can replace and bind to the p65 sub-unit of NFκB blocking the NFκB translocation to the nucleus, which leads to a reduction of pro-inflammatory molecules mRNA levels (Revilla et al., 1998; Tait et al., 2000; Granja et al., 2006b). As the A238L sequence lacks the phosphorylation sites it may indicate that this IκB homologue fails to respond to stimulation of the cells and retains his ability to bind to NFκB. Furthermore, A238L inhibits the activity of the calcium/calmodulin-regenerated phosphatase calcineurin (CaN) (Miskin et al., 2000) that regulates a number of different pathways including the NFAT family of transcription factors. These facts reinforce that A238L protein is a potent immunosuppressor that may contribute for the viral evasion to the host immune response (Miskin et al., 2000; Granja et al., 2006a,b). More recently, biochemical analysis confirms that A238L is a protein with a predicted potent immunosuppressive function (Granja et al., 2006b).

In order to characterize better the role of A238L gene in ASFV infections with virulent isolates, we have extended studies on different aspects of the pathogenesis of the *in vivo* infection using the E70ΔA238L deletion mutant (Granja et al., 2004). We have also performed a further characterization of the role of A238L gene as a modulator of the immune response namely on the apoptosis of host cells and on the cytokine mRNA expression profiles.

2. Materials and methods

2.1. Animals, virus and experimental design

Large White X Landrace pigs ($n = 8$) of either sex, weighing 30 kg at the beginning of the experiment were

used. The animals, which were clinically healthy and free of antibodies against the viruses of ASF, classical swine fever, Aujeszky's disease and porcine reproductive and respiratory syndrome were housed in isolation (bio-containment level 3) at the Centro de Investigación en Sanidad Animal (CISA-INIA) in Valdeolmos, Spain. Animals were divided in two groups of four pigs. Two animals from group 1 (#1 and #2) received 10^5 haemagglutinating doses $(HAD)_{50}$ of the highly virulent isolate of ASFV E70 by intramuscular inoculation in the shoulder. The other two animals from group 1 (#3 and #4) received 10^6 HAD_{50} of ASFV E70 by the same route. Two animals from group 2 (#5 and #6) received 10^5 HAD_{50} of an ASFV E70 deletion mutant of A238L gene (E70ΔA238L) by intramuscular inoculation in the shoulder. The other two animals from group 2 (#7 and #8) received 10^6 HAD_{50} of ASFV E70ΔA238L by the same route. Clinical signs such as fever, lethargy, anorexia, diarrhoea and any other were checked daily and blood samples were taken from the jugular vein at 3, 5, 6 and 7 days post-inoculation (dpi). Animals were tranquilized with azaperone (Stresnil®; Janssen Animal Health, Beerese. Belgium) and humanely slaughtered with a lethal dose of sodium thiopental (Thiovet®; Vet Limited, Leyland, Lancashire, England). Three animals were unexpectedly found dead during the experiment. This experiment was carried out under the guidelines of the European Union (Directive 86/609/EEC) and was approved by the site ethical review committee.

2.2. E70 ASFV A238L deleted mutant construction

The A238L-defective mutant ΔA238L virus was obtained as previously described (Granja et al., 2004) by insertion of the *Escherichia coli* β-glucuronidase (β-*gus*) gene into the viral A238L open reading frame. The recombinant E70ΔA238L was obtained by infecting COS-7 cell monolayers with the viral strain E70.

The lack of gene in the recombinant E70ΔA238L virus was assessed by Southern blot hybridization. Briefly, DNA samples obtained from purified E70 and ΔA238L viruses were digested with the restriction endonuclease EcoRI, subjected to electrophoresis in agarose gels, and transferred to nylon membranes following standard procedures (Southern, 1975). The DNA probes, specific for the β-*gus* and A238L genes and for the SalI I' fragment of E70 genome, were labelled with a digoxigenin DNA labelling kit (Boehringer Mannheim) using manufacturer's instructions, and hybridizations were done, as described elsewhere (Almendral et al., 1990).

2.3. Virus titration and specific antibodies

ASFV titration in sera of animals was measured by haemadsorption test. Primary leukocyte cultures were used for the titration of infectious virus in blood recovered from infected pigs. Cultures were prepared as previously described (Malmquist and Hay, 1960; Carrascosa et al., 1982). Cells were seeded in 96-well tissue culture grade microtitre plates (200 μl; 1.6×10^6 cells per well) in homologous swine serum, and incubated in a humidified atmosphere containing 5% CO_2 at 37 °C. After 3 days, cultures were infected with 10-fold dilutions of the sample supplemented with 5 μg/ml gentamicine sulphate (BioWhittaker) and incubated for 24 h at 37°. After inoculation, a preparation of 1% homologous red blood cells in buffered saline was then added to each well. The plates were examined for haemadsorption during a 6-day period and the virus titre was estimated.

Specific antibodies against ASF virus were measured as previously described by Pastor et al. (1989, 1990) and Arias et al. (1993).

2.4. Histopathology and immunohistochemistry

Samples from the spleen, liver, lung, gastrohepatic and renal lymph nodes were fixed in 10% buffered formalin and Bouin solution. After fixation, the samples were dehydrated through a graded series of alcohol to xylol and embedded in paraffin wax. For structural and immunohistochemical analysis, sections (3 μm) were cut and stained with haematoxylin and eosin (HE) or processed for immunohistochemical examination.

The avidin–biotin-peroxidase complex (ABC) technique was used to detect monocyte-macrophages, with a monoclonal anti-SWC3 antibody (Biovet-UCO, Córdoba, Spain), as previously described (Salguero et al., 2002) and viral protein 73 (vp73) of ASFV (with a monoclonal antibody raised against vp73 [Ingenasa, Madrid, Spain] diluted 1:10 as described by Perez et al., 1994). After dewaxing and dehydration, endogenous peroxidase activity was quenched by incubation with hydrogen peroxide 3% in methanol for 45 min at room temperature. Enzymatic digestion with 0.1% pronase (Sigma Chemical Company, Poole, Dorset, UK) in phosphate-buffered saline (PBS) for 10 min at room temperature (RT) to unmask vp73 antigen; or with 0.1 M citric acid (pH 6.0) for 5 min in microwave oven to detect SWC3. Tissue sections were then rinsed in 0.01 M PBS, pH 7.4, and incubated with 10% normal goat serum (NGS) (Sigma) for 30 min at room temperature. Primary antibody, diluted in NGS 10%,

was incubated overnight at 4 °C. A secondary goat anti-mouse immunoglobulin G (Dako, Glostrup, Denmark) diluted 1 in 100 in PBS was used. An avidin–peroxidase-complex kit (Vectastain ABC Kit Elite; Vector) was used and 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma) diluted to 0.035% in Tris-buffered saline (pH 7.6) containing hydrogen peroxide 0.01% was applied for 1 min as chromogen.

The slides were then counterstained with Mayer's haematoxylin for 1 min, dehydrated, and mounted. Specific primary antibodies were replaced by PBS, normal mouse serum or normal rabbit serum, in negative control sections.

2.5. In situ detection of apoptosis

Tissue sections were dewaxed, rehydrated, and treated for quenching of endogenous peroxidase activity as described above. Sections were permeabilised by incubation in PBS containing proteinase K (Roche Diagnostics Corporation, Indianapolis, USA) for 20 μg/ml in PBS for 20 min and washed twice for 5 min in PBS. The terminal deoxynucleotidyltransferase-mediated dUTP nick end labelling (TUNEL) method was used for the histological detection of apoptotic cells. The cells were detected with a kit containing horseradish peroxidase (In Situ Cell Death Detection kit, POD®; Roche) according to the manufacturer's directions. The color reaction was developed with DAB and slides were counterstained with Mayer's haematoxylin. Negative controls were always included in each series of sections assayed.

2.6. Transmission electron microscopy

Samples for transmission electron microscopy (TEM) were fixed in 2.5% glutaraldehyde and embedded in Epon 812 (Fluka, Buchs, Switzerland). Sections (50 nm) were stained with uranyl acetate and leas citrate and viewed with a Philips CM-10 electron microscope at the "Servicio Central de Apoyo a la Investigacion", University of Cordoba, Spain.

2.7. mRNA quantification of TNF-α, IL-1, IL-4, IFN-γ and cyclophilin expression in pigs inoculated with E70 and E70ΔA238L

Samples of peripheral blood mononuclear cells (PBMC), obtained as previously reported (Gil et al., 2003), from experimentally inoculated pigs at different days before and after inoculation with E70 and E70ΔA238L virus were used to quantify the mRNA expression of cyclophilin, IL-1, IL-4 and IFN-γ, following previously reported protocols (Gil et al., 2003). Briefly, RNA was extracted and quantified after the addition of Trizol (Gibco-BRL, 15596) to individual PBMC samples. Cells were disrupted by repetitive pipetting, kept at room temperature during 5 min to allow complete dissociation of nucleoprotein complexes and added with chloroform. Tubes were vigorously hand-shaken, incubated (15–30 °C, 2 min) and finally centrifuged (12,000 rpm, 15 min, 4 °C, Haereus Minifuge). Following centrifugation, the upper-phase containing RNA (approximately 600 μl) was transferred to a new tube, added with isopropanol (500 μl) and glycogen (1 μl at 20 mg/ml), incubated (15–30 °C, 10 min) and centrifuged (12,000 rpm, Haereus Minifuge, 4 °C). Supernatant was carefully removed and the precipitated RNA was recovered by centrifugation with 1 ml of 75% ethanol (7500 rpm, 5 min, 4 °C). The RNA was dissolved in diethylpirocarbonate (DPC)-treated water and stored at −70 °C until further use. RNA samples were quantified in spectrophotometer (DU40 Beckman), at 260 and 280 nm (A) and total RNA was calculated as described in the literature.

Reverse transcription of mRNA into cDNA was performed using 2.5 μg of total RNA mixed with oligo $(dT)_{12–18}$ primers (Gibco BRL, 18064-014) and incubated (70 °C, 10 min). The mixture was chilled on ice and incubated (45 °C, 50 min) with five times concentrated reverse transcriptase buffer (50 mM Tris–HCl pH 8.4, 75 mM KCl (Gibco BRL, 18064-014); 3 mM $MgCl_2$ (Gibco BRL, 18064-014); 500 μM of each deoxynucleotide; 10 mM dithiothreitol (Gibco BRL, 18064-014) and 200 U of Superscript II, RT Moloney Murine Leukemia virus reverse transcriptase (Gibco BRL, 18064-014). Reactions were incubated (90 °C, 5 min) and samples of the cDNA obtained were stored at −20 °C until further use.

2.8. Oligonucleotide primer pairs

Primer pairs were selected from different exons, as determined from porcine or consensus DNA sequences from other species, to ensure that amplified cDNA could be distinguished from any amplified genomic DNA contaminants. Primers for TNF-α, IL-1 and IL-4 were designed on the basis of the sequences obtained from the GenBank Data Bank. Primers for cyclophilin and IFN-γ were used as described by Dozois et al. (1997) (Table 1).

Cyclophilin was used as the housekeeping gene in order to normalize cytokine quantification results.

Choice of cyclophilin was based on previous studies in that no differences on the expression of this gene were found on PBMCs from pigs experimentally inoculated with ASFV of different virulence (data not shown).

2.9. PCR amplification of porcine cytokines and housekeeping gene (cyclophilin) cDNA using a fluorimeter based real-time PCR

Quantitative PCR was performed by real-time PCR technology in the ''Ycycler instrument'' (BIO-RAD). For quantification of mRNA expression of TNF-α, IL-1, IL-4, IFN-γ and cyclophilin, cDNA samples were diluted in DPC treated water and used for PCR amplification with the enzymatic system ''IQ SYBR Green Supermix'' (BIO-RAD, 170-8880) using an adapted protocol according to manufacturer's instructions. Briefly, a reaction mix was prepared: 12.5 μl master mix, 8.5 μl of water, 1.5 μl of forward primer and 1.5 μl of reverse primer, 1 μl of cDNA. The optimised runs were as follows: 1 cycle of denaturation (95 °C during 600 s), 50 cycles of amplification: denaturation (95 °C during 15 s), annealing temperature depending on the cytokine (Table 1), during 30 s and extension (72 °C during 30 s) and a final melting program at 75 °C during 5 s at 20 °C/s ramp rate and heating of the samples until 95 °C. The different quantitative PCRs of the several cytokines tested showed the same PCR efficiency (data not shown).

Quantification of the above-mentioned cytokines was assessed using standard curves of amplification obtained with 10^3, 10^4, 10^5 and 10^6 DNA molecules of recombinant plasmids. Plasmids for cyclophilin, IL-1, IL-4, and TNF-α were obtained as previously described (Gil et al., 2003) and used for amplification with corresponding primers (as described above), using a PCR PerkinElmer conventional machine and sequenced to confirm the identity of the amplicon. Dilutions (in number of molecules) of plasmids were quantified by spectrophotometry (Sambrook et al., 1989) and used as standards in real-time PCR for the quantification of mRNA expression of different cytokines.

Quantification was obtained with the fluorescence data in the log-phase portion of the curve and a crossing line was set above the level of the background noise band to give a value for a crossing point. Crossing point values of the standards were expressed by a standard curve obtained as a fractional cycle number determined at the log-phase of the product amplification, plotted against log concentration, which was used to determine the number of molecules of the PCR products. Melting curves obtained after amplification were used to verify the specificity of the PCR products/amplicons by comparison with the melting curves of the correspondent recombinant plasmids.

Results on quantification of mRNA expression of different cytokines are shown as the average ratio (±standard error) between the number of molecules of each cytokine and cyclophilin as housekeeping gene (*N* molecules of mRNA of each cytokine/*N* molecules of mRNA of cyclophilin). Results were statistically analysed using the ANOVA test.

3. Results

3.1. Clinical signs and gross pathology

Details of inocula, doses, type and date of death as well as the date of onset of the clinical signs and gross pathological findings are summarised in Table 2. Mostly all of the animals of both of the groups developed pyrexia from 3 to 4 dpi. Clinical signs were very similar in both of the groups and consisted mainly in anorexia and inapetence. Skin hyperemia and cyanosis was observed frequently form 3–5 dpi in almost all the animals and diarrhoea with presence of blood in faeces was only observed in two animals from group 1 at 6 dpi.

One animal from group 1 died at 3 dpi and two animals from group 2 died at 5 and 6 dpi. The other animals were humanely slaughtered at 5–7 dpi. After necropsy, the most evident gross lesion was the haemorrhagic splenomegaly and the haemorrhagic lymphadenitis mainly observed in the gastrohepatic and renal lymph nodes and with less intensity in the mesenteric and mandibular lymph nodes. Kidney petechiae were observed in two animals from each group and other gross lesions found in some animals consisted in ascitis, hydrothorax and hydropericardium.

3.2. Viremia and specific antibodies

Animals from groups 1 and 2 developed viremia observed from 3 dpi (the first blood sample obtained) onwards (Fig. 1). Baseline pre-inoculation levels of viremia were null in all of the animals. Specific antibodies against ASFV were not detectable in any animal during the experiment.

3.3. Histopathological, immunohistochemical and ultrastructural findings

Results of the histopathological findings, detection of virus by immunohistochemistry and TEM, infiltration of monocyte-macrophages observed by SWC3

Table 1
Primers and optimised conditions used for quantification of mRNA expression of cyclophilin, TNF-α, IL-1, IL-4 and IFN-γ

Cytokine (accession number)	Oligonucleotide sequences (5′–3′)	Annealing temperature (°C)
Cyclophilin (Gil et al., 2003)	(F)-TAA CCC CAC CGT CTT CTT (R)-TGC CAT CCA ACC ACT CAG	55
TNF-α (Gil et al., 2003)	(F)-TGC CTA CTG CAC TTC GAG GTT ATC (R)-TGA GTC GAT CAT CCT TCT CCA GCT	53
IL-1 (Gil et al., 2003)	(F)-ACA GAA GTG AAG ATG GCC AAA GTC (R)-TCA TGT TGC TCT GGA AGC TGT ATG	55
IL-4 (NM 214123)	(F)-CTC ACA TCG TCA GTG CAA ATA GAG (R)-CGT CTT TAG CCT TTC CAA GAA GTC	55
IFN-γ (NM 213948)	(F)-GTT TTT CTG GCT CTT ACT GC (R)-CTT CCG CTT TCT TAG GTT AG	57

immunodetection and apoptosis of lymphocytes studied by TUNEL and TEM are summarised in Table 3.

Animals from both of the groups showed severe haemorrhages in the spleen accompanied by lymphoid depletion affecting to T and B areas. These changes were observed together with virus infection identified by immunodetection of vp73 in a large number of monocyte-macrophages mainly in the red pulp and marginal zone but also in lymphoid follicles. Lymphoid depletion was identified as lymphocyte apoptosis by TUNEL technique and by TEM. Apoptotic lymphocytes displayed condensation and fragmentation of nuclear chromatin with preservation of the cell membrane (Fig. 2). Several monocyte-macrophages were infiltrating the lymphoid follicles and the number of this cell population was increased in the red pulp (Fig. 3).

Renal and gastrohepatic lymph nodes presented also haemorrhages and lymphoid depletion in all of the animals together with viral infection, that was very evident mainly in the lymph node medulla as observed by immunohistochemical detection of vp73. No differences were found in virus replication in cells for E70 and E70ΔA238L; replication sites were visible as organelle-free areas containing membranous structures and virions (hexagonal particles 175–190 nm in diameter, some with an electron dense nucleoid) at

Table 2
Inoculation, clinical signs and macropathology of pigs from group 1 (#1, #2, #3 and #4) and group 2 (#5, #6, #7 and #8)

	Pig number							
	#1	#2	#3	#4	#5	#6	#7	#8
Inoculum	E-70	E-70	E-70	E-70	ΔA238L	ΔA238L	ΔA238L	ΔA238L
Dose $(HAD)_{50}$	10^5	10^5	10^6	10^6	10^5	10^5	10^6	10^6
Death (dpi)	6	6	5	3	6	7	5	5
Post mortem[a]	Eutha	Eutha	Eutha	F.D.	F.D.	Eutha	Eutha	F.D.
Clinical signs (date of onset)								
Pyrexia (dpi)	4	3	3	3	3	3	3	3
Anorexia (dpi)	5	3	3	3	3	3	3	3
Diarrhoea (dpi)	6	6	–	–	–	–	–	–
Skin lesions (cyanosis, hyperemia) (dpi)	5	5	5	–	5	4	3	3
Macropathology[b]								
Hemorrhagic splenomegaly	+++	+++	+++	+	+++	+++	+++	–
Lymphadenitis gastrohepatic L.N.	+++	+++	+++	–	+++	+++	+++	–
Lymphadenitis renal L.N.	+++	+++	+++	–	+++	+++	+++	–
Lymphadenitis mesentheric L.N.	++	++	–	–	++	–	–	–
Lymphadenitis mandibular L.N.	–	–	++	–	–	–	–	–
Kidney petechiae	++	+++	–	–	–	+	+++	–
Ascitis	–	++	–	–	–	–	–	++
Hydrothorax/hydropericardium	++	++	++	–	–	++	–	+++

[a] Euthanasia (Eutha) or found dead (F.D.).
[b] –, none; +, light; ++, moderate; +++, severe.

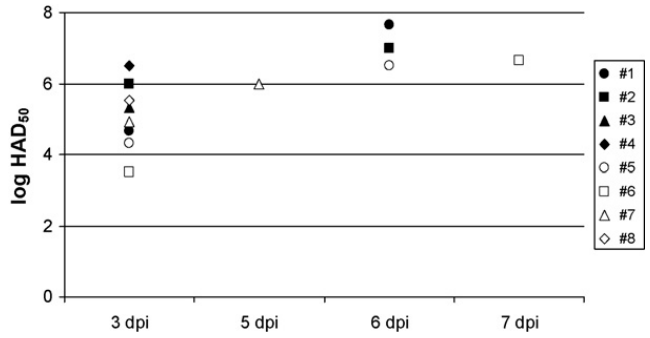

Fig. 1. Viremia in animals from group 1 (#1, #2, #3 and #4) and group 2 (#5, #6, #7 and #8) at 3, 5, 6 and 7 dpi, expressed in log HAD_{50} in pulmonary alveolar macrophages incubated with blood from inoculated animals.

varying stages of maturation. The cytopathic effect caused by viral replication was observed as a peripheral margination of the chromatin, rounding of nuclei and presence of cytoplasmic vacuoles. Some virus budding from the infected cells was also observed and free virions were observed enveloped in a single membrane in the external layer gained from the host cell (Fig. 3).

Other histopathological finding was the presence of periportal mononuclear infiltration in the liver of some of the animals from both of the groups. The liver suffered for viral infection, affecting to Kupffer cells and circulating monocytes in all of the animals. Hepatocyte infection was only observed in two animals from group 1 (Fig. 4). Viral infection was also observed in alveolar and pulmonary intravascular macrophages of the lungs.

3.4. Quantification by real-time PCR of mRNA expression of TNF-α, IL-1, IL-4 and IFN-γ in pigs inoculated with E70 and E70ΔA238L virus

Quantification by real-time PCR of mRNA expression of TNF-α, IL-1, IL-4, IFNγ and cyclophilin in pigs inoculated with E70 and E70ΔA238L was carried out at different days of the experiment as shown in the following figures. Four pigs were used for the quantification of the cytokines at day 0, 3 and 5 dpi. On 6 and 7 dpi, two and one pigs were used, respectively.

Table 3
Histopathology, infiltration of monocyte-macrophages (M-mΦ), viral infection and lymphocyte apoptosis in pigs from group 1 (#1, #2, #3 and #4) and group 2 (#5, #6, #7 and #8)

	Pig number							
	#1	#2	#3	#4	#5	#6	#7	#8
Spleen								
Haemorrhages	+++	+++	+++	+++	+++	+++	+++	+++
Lymphoid depletion	+++	+++	+++	+	++	+++	+++	+++
Infiltration of M-mΦ	+++		++	++		++	+++	
Viral infection	+++	+++	++	+++	+++	++	+++	+++
Lymphocyte apoptosis	+++	+++	++	+	++	+++	++	+++
Gastrohepatic L.N.								
Haemorrhages	+++	+	++	+	+	+++	++	+
Lymphoid depletion	++	+++	+	+	++	+++	++	++
Infiltration of M-mΦ	+++		+++	+		+	++	+++
Viral infection	+++	+++	++	++	+++	++	++	++
Lymphocyte apoptosis	++	++	+	+	++	++	++	+
Renal L.N.								
Haemorrhages	+++	+	++	−	−	+++	++	+
Lymphoid depletion	++	+++	++	+	−	+++	++	+
Infiltration of M-mΦ	+++		+++	+		+	++	++
Viral infection	+++	+++	+++	++	++	++	++	++
Lymphocyte apoptosis	++	++	+	+	+	++	++	+
Liver								
Mononuclear infiltrates	+++	++	+	−	−	+	−	−
Viral infection	+++[a]	+++[a]	+	++	++	+	++	++
Lung								
Viral infection	+++	+++	++	++	++	+	+	+

−, none; +, light; ++, moderate; +++, severe.
[a] Presence of viral antigen in hepatocytes.

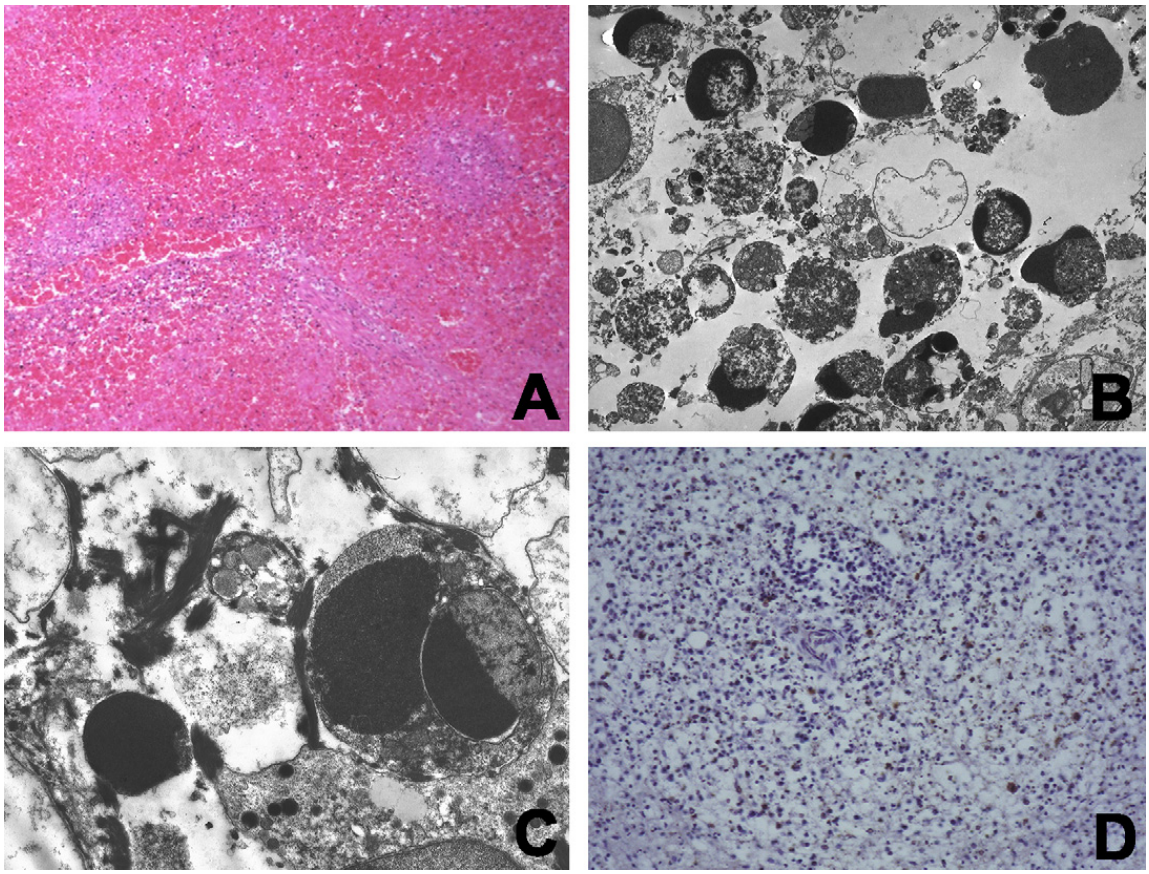

Fig. 2. Spleen. Severe haemorrhages and lymphoid depletion (HE staining; 100×) in animal #7 (A). Apoptosis of lymphocytes observed as condensation and fragmentation of the chromatin with preservation of the cell membrane in a lymphoid follicle from animal #7 (B, 5400×) and from animal #2 (C, 9200×), with presence of fibrin strands. Lymphocyte apoptosis detected by TUNEL in animal #7 (D, 200×).

A significant enhancement in the expression of TNF-α was observed in PBMC from inoculated pigs with E70ΔA238L from 5 to 7 dpi when compared with day 0 (Fig. 5A). In opposition expression of TNF-α in pigs inoculated with E70 decreased after inoculation along the different days of the experiment (Fig. 5B).

Quantification on mRNA expression of IL-1 did not show significant differences along the different days of the experiment in PBMC from inoculated pigs with E70ΔA238L when compared with day 0 (Fig. 6A), while expression of IL-1 in pigs inoculated with E70 showed a significant decrease of expression from 3 dpi when compared to day 0 (Fig. 6B).

Quantification on mRNA expression of IL-4 (Fig. 7A and B) and IFN-γ (Fig. 8A and B) in PBMC of pigs inoculated either with E70 or E70ΔA238L did not show significant differences along the different days of the experiment.

Amplicons of the different studied cytokines and cyclophilin were runned on an 1.5% agarose gel (Fig. 9).

4. Discussion

ASF remains to be a controversial disease and the role of several ASFV genes has been widely studied in *in vitro* but not *in vivo* infections. Acute ASF is characterised by lymphopenia and a state of immunodeficiency (Sanchez-Vizcaino et al., 1981), but the factors contributing to the development of these phenomena are unknown. The main target cell of ASF is the monocyte-macrophage (Mebus, 1988) and this is believed to play a critical role in the pathogenesis of the disease (Colgrove et al., 1969; Gomez-Villamandos et al., 1995c; Gómez del Moral et al., 1999). To the other hand, monocyte-macrophages secrete a large range of soluble mediators in pigs, including IL-1, IL-6 and TNF-α (Murtaugh et al., 1996).

Expression of pro-inflammatory and immunoregulatory cytokines at mRNA level was previously studied in porcine macrophages infected with ASFV isolates of different virulence (Gil et al., 2003). Studies using cultures of non-stimulated porcine blood derived

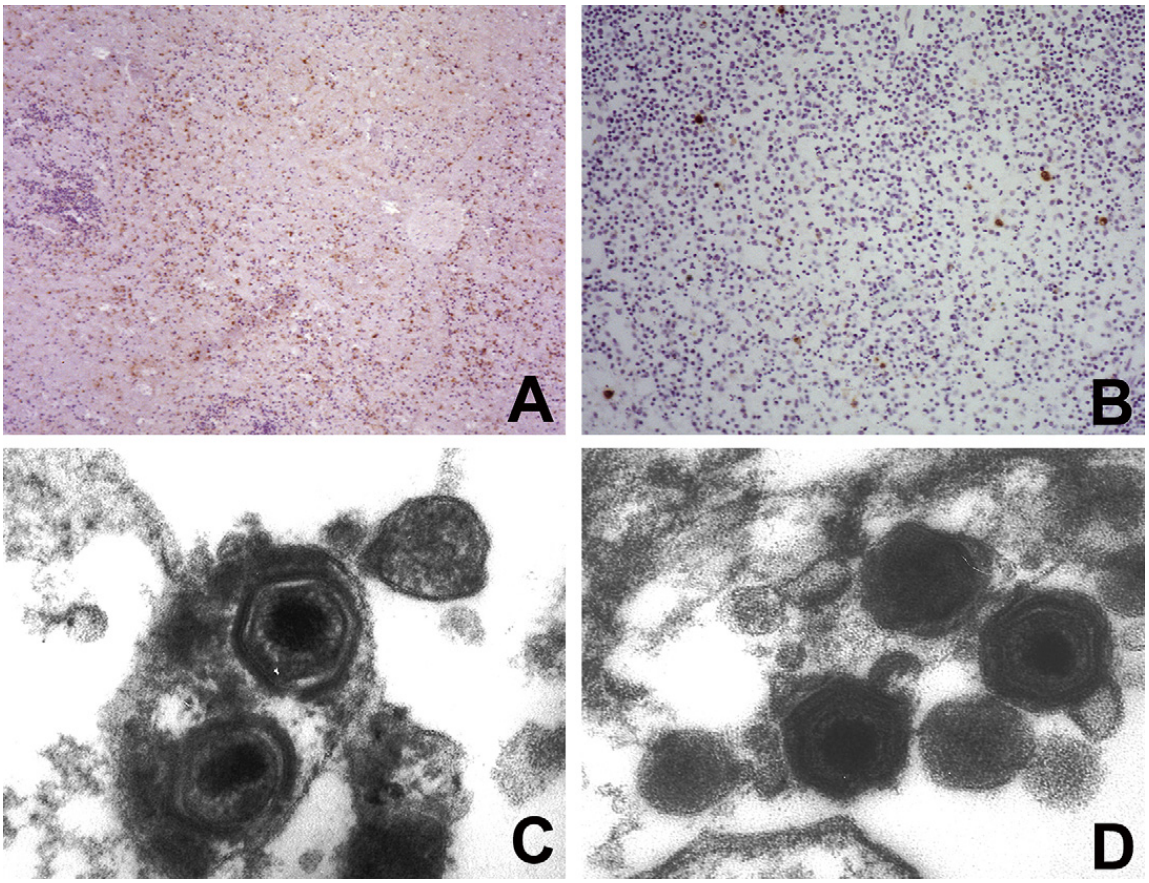

Fig. 3. Infiltration of M-mΦ (immunodetection of SWC3, ABC) in the areas of lymphoid depletion and red pulp of spleen from animal #7 (A, 100×). Immunodetection of vp73 in gastrohepatic lymph node of animal #6 (B, ABC, 200×). ASFV virions observed by TEM in the gastrohepatic lymph nodes of animal #1 (C, 150,000×) and animal #6 (D, 150,000×).

macrophages infected *in vitro* with the highly virulent ASFV/L60 and the low virulent NH/P68 at 6 h post-infection show a different effect of the infection by both isolates as an increase of mRNA expression of TNF-α, IL-6, IL-12 and IL-15 was identified in macrophages infected with the low virulent NH/P68 in comparison with results obtained with the highly virulent L60 (Gil et al., 2003).

Expression of pro-inflammatory cytokines in ASFV-infected cells, is in part regulated by the transcription factors NFκB (Granja et al., 2004, 2006a,b). An ASFV gene, A238L, with similarity to the inhibitor of NFκB

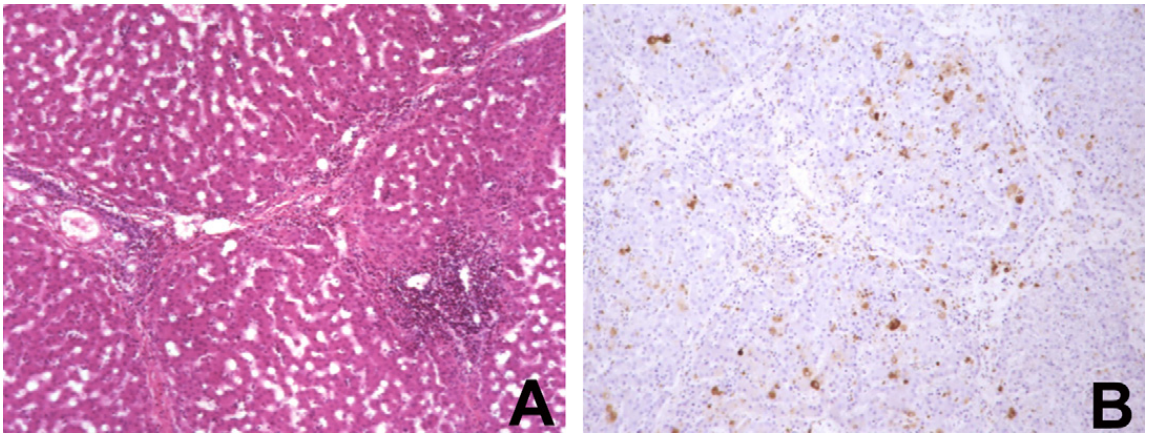

Fig. 4. Liver. Periportal infiltration of mononuclear cells in animal #2 (A, HE, 200×). Vp73 immunodetection in Kupffer cells, circulant monocytes and hepatocytes in animal #2 (B, ABC, 200×).

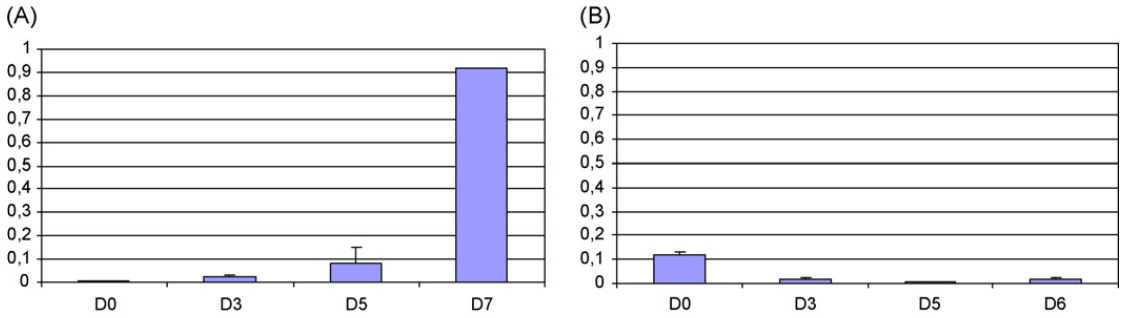

Fig. 5. Quantification of mRNA expression of TNF-α in PBMC of pigs inoculated with E70ΔA238L (A) and E70 (B) at different days of the experiment (D0, D3, D5, D6/D7). Results are shown as average ± standard error (AVG ± S.E.), between the number of molecules of each cytokine and the number of molecules of the housekeeping gene cyclophilin.

(IκB) has been identified in different isolates (Yáñez et al., 1995; Neilan et al., 1997). The similarity between A238L and IκB suggested that A238L may act as a homologue of IκB and inhibit activation of NFκB following virus infection (Revilla et al., 1998). There are several mechanisms by which A238L may act to inhibit NFκB, but the similarity between ankyrin repeats in A238L and IκB suggest that, like IκB, A238L may bind directly to NFκB (Revilla et al., 1998), causing an inhibition of pro-inflammatory cytokine expression which is in accordance with previous studies where a negative correlation was found between the expression of A238L gene using the virulent isolate ASFV/L60 (L60) and several cytokines (Gil et al., 2003). Moreover, A238L binds to the catalytic sub-unit of the serine threonine protein phosphatase calcineurin (Miskin et al., 1998). Thus, one consequence of calcineurin may be inhibition of NFAT-dependent gene transcription in ASFV-infected macrophages (Dixon et al., 2004), including cytokine genes such as COX-2, TNF-α and iNOS (Granja et al., 2004, 2006a, 2006b).

As reported by others for different ASFV isolates (Neilan et al., 1997; Revilla et al., 1998), we found in previous studies (Gil et al., 2003) 100% homology in both strands of the gene A238L in two viral isolates of different virulence (ASFV/NH/P68 and ASFV/L60). The expression of this gene was then quantified in macrophages infected with both isolates and we have further attempted to correlate its expression with the expression of macrophage-derived cytokines at mRNA level in both ASFV infections. As reported (Gil et al., 2003) significantly higher mRNA expression of A238L was observed in macrophages infected with NHV when compared to the mRNA expression of this gene in L60 infected macrophages. Results on the statistical correlation between A238L and cytokine gene expression suggested a negative correlation between the expressions of A238L mRNA and pro-inflammatory TNF-α, IL-1 and IL-6 in L60 infected macrophages. This may relate to the inactivation of NFκB by the A238L gene in accordance with studies by others using highly virulent ASFV (Powell et al., 1996). Similar results observed in relation to IL-12 and IL-15 pointed to a role of the A238L on the regulation of these cytokines. Studies by others using a virulent ASFV isolate (Malawi Lil-20/1) deleted in A238L gene did not show differences in virus replication in macrophages and on viremia levels and mortality of pigs inoculated with the deleted or with parental virus (Neilan et al., 1997).

In our studies pigs were inoculated with E70 (Group 1) and E70ΔA238L (Group 2). This *in vivo* experiment did

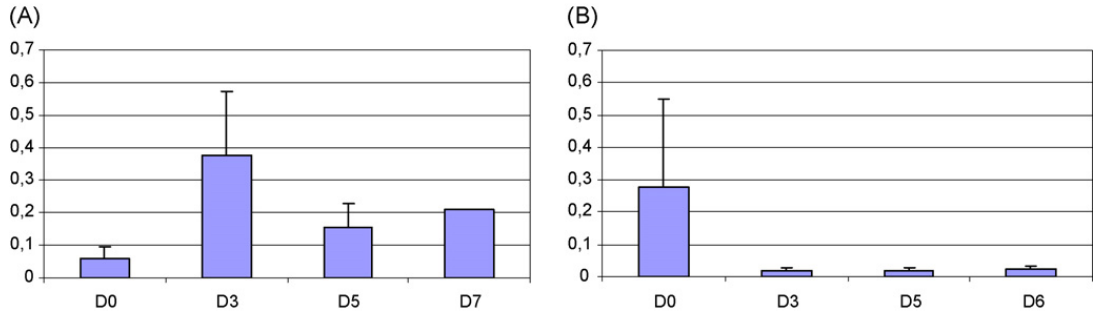

Fig. 6. Quantification of mRNA expression of IL-1 in PBMC of pigs inoculated with E70ΔA238L (A) and E70 (B) at different days of the experiment (D0, D3, D5, D6/D7). Results are shown as average ± standard error (AVG ± S.E.), between the number of molecules of each cytokine and the number of molecules of the housekeeping gene cyclophilin.

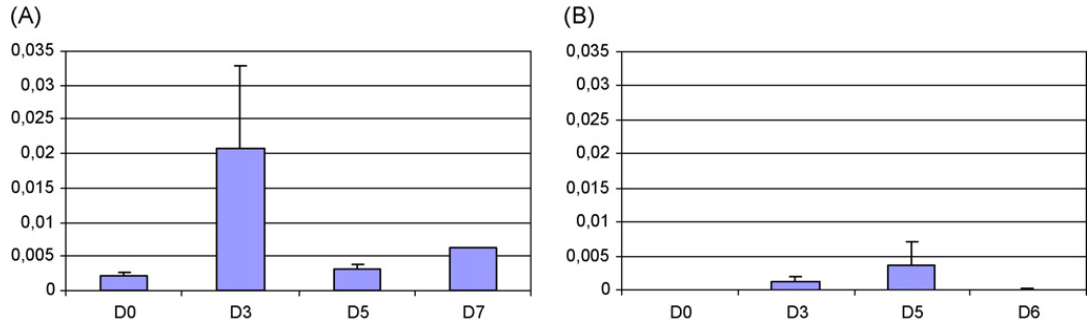

Fig. 7. Quantification of mRNA expression of IFN-γ in PBMC of pigs inoculated with E70ΔA238L (A) and E70 (B) at different days of the experiment (D0, D3, D5, D6/D7). Results are shown as average ± standard error (AVG ± S.E.), between the number of molecules of each cytokine and the number of molecules of the housekeeping gene cyclophilin.

not show significant differences in terms of pathology between groups 1 and 2. Animals from both groups developed clinical signs, gross lesions and histopathological findings similar to those observed in animals inoculated with highly virulent isolates of ASFV (Gomez-Villamandos et al., 1995c,d; Carrasco et al., 1996a,b, 1997; Salguero et al., 2002, 2005). Haemorrhages and lymphocyte apoptosis were observed in lymphoid organs from all of the animals. ASFV encodes two proteins that are similar to host anti-apoptotic proteins (Yáñez et al., 1995; Brun et al., 1996; Nogal et al., 2001) what might be related to the survival of the infected cell resulting in a more efficient productive infection (Oura et al., 1998). Moreover, the number of monocyte-macrophages expressing IL-1, IL-6 and TNF-α in the lymphoid organs during acute ASFV infection is increased along with an increase of the serum level of IL-1β and TNF-α (Gómez del Moral et al., 1999; Salguero et al., 2002).

RT-PCR analysis of the expression of pro-inflammatory cytokines in ASFV infected macrophages showed an initial up-regulation of TNF-α gene expression at between 2 and 4 h post-infection and down-regulation at later times post-infection (Gómez del Moral et al., 1999). This enhancement in the mRNA expression of TNF-α was also observed at 6 h post-infection in macrophage cultures infected with the low virulent NH/P68 isolate in opposition with results observed with the highly virulent L60 isolate (Gil et al., 2003).

In an infected pig, a number of monocyte-macrophage is newly infected every second, so there are always monocyte-macrophages in the first stages of the infection with an up-regulation of pro-inflammatory cytokines. Moreover, A238L protein is predicted to have both pro- and anti-apoptotic functions. Possibly, these are effective at different times post-infection (Dixon et al., 2004).

Overall our results on the cytokine mRNA expression strongly suggest a clear enhancement on the expression of TNF-α in PBMC from pigs inoculated with the virus deleted in the A238L gene. In opposition, we found a decrease on the mRNA expression of this cytokine in the PBMC of pigs inoculated with the parental E70 in accordance to the *in vitro* previously reported studies using the highly virulent isolate Lisbon 60 (Gil et al., 2003). Results on the other pro-inflammatory cytokines using the deleted virus are not so obvious but they suggest the same trend found for TNF-α. IL-1 expression in the PBMCs inoculated with the complete E70 was inhibited but the other cytokines tested did not show any differences in comparison with the control values.

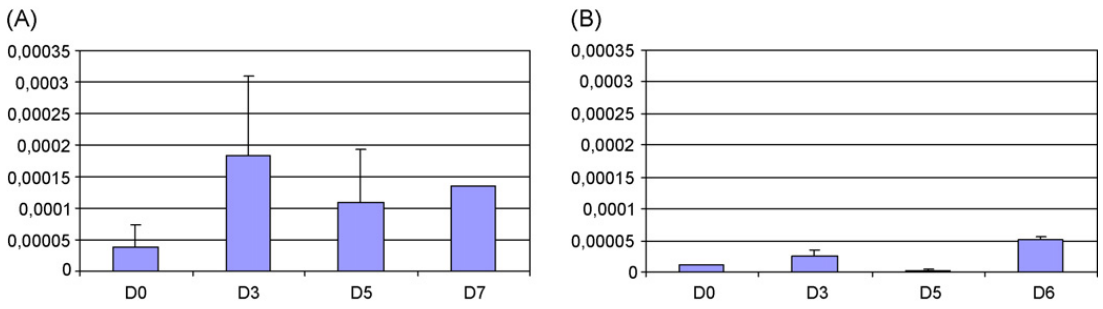

Fig. 8. Quantification of mRNA expression of IL-4 in PBMC of pigs inoculated with E70ΔA238L (A) and E70 (B) at different days of the experiment (D0, D3, D5, D6/D7). Results are shown as average ± standard error (AVG ± S.E.), between the number of molecules of each cytokine and the number of molecules of the housekeeping gene cyclophilin.

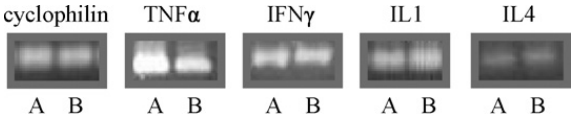

Fig. 9. Agarose gel image showing amplicons of cyclophilin, TNF-α, IFNγ, IL-1 and IL-4 from PBMCs of pig #6 (A) at DPI 7 (pig inoculated with E70ΔA238L) and pig #1 (B) at DPI 6 (pig inoculated with E70).

Our results reinforce the role of the A238L gene in the inhibition of the NFκB pathway of expression of the pro-inflammatory TNF-α cytokine even though no significant differences have been observed in the clinical signs and pathology of animals inoculated with the deletion mutant. An aberrant cytokine profile in situ is directly related to the appearance of lymphocyte apoptosis in the lymphoid organs in acute ASFV infection (Salguero et al., 2002, 2005). In this work, we observed no up-regulation of pro-inflammatory cytokines in the PBMC of animals inoculated with E-70 isolate, but the lymphocyte apoptosis and haemorrhages observed might be related to the presence of bystander monocyte-macrophages expressing pro-inflammatory cytokines (Gomez-Villamandos et al., 1995c; Salguero et al., 2005).

The inoculation of ASFV deletion mutants of other genes or deletion mutants of more than one gene could give us more information about the role of these genes in the pathogenesis of ASF, and, moreover, the inoculation of deletion mutants in the natural hosts, warthogs or bushpigs, in which the clinical signs and the lymphocyte apoptosis are not severe after inoculation with virulent isolates of ASFV (Oura et al., 1998). Moreover, some of these deletion mutants could be valuable tools for future vaccination trials.

Evasion of pig defence mechanisms orchestrated by different ASFV genes remain to be elucidated. The role of such genes should also be studied in infection of the soft tick vector *Ornithodoros* spp. in order to improve the knowledge on the interaction of ASFV isolates with their natural hosts.

Acknowledgements

This work was supported by the project QLK2-CT-2001-02216 and by the post-doctoral fellowship FCT/BPD/14676/2003.

References

Almendral, J.M., Almazan, F., Blasco, R., Vinuela, E., 1990. Multigene families in African swine fever virus: family 110. J. Virol. 64, 2064–2072.

Arias, M., Escribano, J.M., Sanchez-Vizcaino, J.M., 1993. Persistence of African swine fever antibody reactivity on ELISA and immunoblotting assays. Vet. Rec. 133, 189–190.

Brun, A., Rivas, C., Esteban, M., Escribano, J.M., Alonso, C., 1996. African swine fever virus gene A179L, a viral homologue of bcl-2, protects cells from programmed cell death. Virology 225, 227–230.

Carrasco, L., Chacon de Lara, F.M., Martin de las Mulas, J., Gomez-Villamandos, J.C., Hervás, J., Wilkinson, P.J., Sierra, M.A., 1996a. Virus association to lymphocytes in acute African swine fever. Vet. Res. 27, 305–312.

Carrasco, L., Gomez-Villamandos, J.C., Bautista, M.J., Martin de las Mulas, Villeda, C.J., Wilkinson, P.J., Sierra, M.A., 1996b. *In vivo* replication of the African swine fever virus (Malawi 83) in neutrophils. Vet. Res. 27, 55–62.

Carrasco, L., Chacon de Lara, F.M., Martin de Las Mulas, J., Gomez-Villamandos, J.C., Sierra, M.A., Villeda, C.J., Wilkinson, P.J., 1997. Ultrastructural changes related to the lymph node haemorrhages in acute African swine fever. Res. Vet. Sci. 62, 199–204.

Carrascosa, A.L., Santarén, J.F., Viñuela, E., 1982. Production and titration of African swine fever virus in porcine alveolar macrophages. J. Virol. Methods 3, 303–310.

Colgrove, G.S., Haelterman, O.E., Coggins, L., 1969. Pathogenesis of African swine fever virus in young pigs. Am. J. Vet. Res. 30, 1343–1359.

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

Dixon, L.K., Escribano, J.M., Martins, C., Rock, D.L., Salas, M.L., Wilkinson, P.J., 2005. In: Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A. (Eds.), Vírus Taxonomy. VIII Report of the ICTV. Elsevier/Academic Press, London, pp. 135–143.

Dozois, M.D., Oswald, E., Gautier, N., Serthelon, J., Fairbrother, J., Oswald, 1997. A reverse transcription-polymerase chain reaction method to analyse porcine cytokine gene expression. Vet. Immunol. Immunopathol. 58, 287–300.

Gil, S., Spagnuolo-Weaver, M., Canals, A., Sepúlveda, N., Oliveira, J., Aleixo, A., Allan, G., Leitão, A.C., Martins, C.L.V., 2003. Expression at mRNA level of cytokines and A238L gene in porcine blood-derived macrophages infected *in vitro* with African swine fever virus (ASFV) isolates of different virulence. Arch. Virol. 184, 2077–2097.

Gómez del Moral, M., Ortuño, E., Fernández-Zapatero, P., Alonso, F., Alonso, C., Ezquerra, A., Domínguez, 1999. African swine fever virus infection induces tumor necrosis factor alpha production: implications in pathogenesis. J. Virol. 73, 2173–2180.

Gomez-Villamandos, J.C., Hervas, J., Mendez, A., Carrasco, L., Villeda, C., Willkinson, P.J., Sierra, M.A., 1995a. Ultrastructural study of the renal tubular system in acute experimental African swine fever: virus replication in glomerular mesangial cells and in collecting ducts. Arch. Virol. 140, 581–589.

Gomez-Villamandos, J.C., Hervas, J., Mendez, A., Carrasco, L., Villeda, C., Willkinson, P.J., Sierra, M.A., 1995b. Pathological changes in the renal interstitial capillaries of pigs inoculated with two different strains of African swine fever virus. J. Comp. Pathol. 112, 283–289.

Gomez-Villamandos, J.C., Hervas, J., Mendez, A., Carrasco, L., Villeda, C., Willkinson, P.J., Sierra, M.A., 1995c. Experimental African swine fever: apoptosis of lymphocytes and virus replication in other cells. J. Gen. Virol. 76, 2399–2405.

Gomez-Villamandos, J.C., Hervas, J., Mendez, A., Carrasco, L., Villeda, C., Willkinson, P.J., Sierra, M.A., 1995d. A pathological study of the perisinusoidal unit of the liver in acute ASF. Res. Vet. Sci. 59, 146–151.

Granja, A.G., Nogal, M.L., Hurtado, C., Salas, J., Salas, M.L., Carrascosa, A.L., Revilla, Y., 2004. Modulation of p53 cellular function and cell death by African swine fever virus. J. Virol. 78, 7165–7174.

Granja, A.G., Nogal, M.L., Hurtado, C., Del Aguila, C., Carrascosa, A.L., Salas, M.L., Fresno, M., Revilla, Y., 2006a. The viral protein A238L inhibits TNF-alpha expression through a CBP/p300 transcriptional coactivators pathway. J. Immunol. 176, 451–462.

Granja, A.G., Sabina, P., Salas, M.L., Fresno, M., Revilla, Y., 2006b. Regulation of inducible nitric oxide synthase expression by viral A238L-mediated inhibition of p65/RelA acetylation and p300 transactivation. J. Virol. 80, 10487–10496.

Malmquist, W.A., Hay, D., 1960. Hemadsorption and cytopathic effect produced by African swine fever virus in swine bone marrow and buffy coat cultures. Am. J. Vet. Res. 21, 104–108.

Martins, C., Leitão, A., 1994. Porcine immune responses to African swine fever virus (ASFV) infection. Vet. Immunol. Immunopathol. 43, 99–106.

Mebus, C.A., 1988. African swine fever. Adv. Virus Res. 35, 251–269.

Miskin, J.E., Abrams, C.C., Goatley, L.C., Dixon, L.K., 1998. A viral mechanism for inhibition of the cellular phosphatase calcineurin. Science 281, 562–565.

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

Moore, D.M., Zsak, L., Neilan, J.G., Lu, Z., Rock, D.L., 1998. The African swine fever vírus thymidine kinase gene is required for efficient replication in swine macrophages and for virulence in swine. J. Virol. 72, 10310–10315.

Moulton, J., Coggins, L., 1968. Comparison of lesions in acute and chronic African swine fever. Cornell Vet. 58, 364–388.

Murtaugh, M.P., Baarsch, M.J., Zhou, Y., Scamurra, R.W., Lin, G., 1996. Inflammatory cytokines in animal health and disease. Vet. Immunol. Immunopathol. 54, 45–55.

Neilan, J.G., Lu, Z., Kutish, G.F., Zsack, L., Lewis, T.L., Rock, D.L., 1997. A conserved African swine fever virus IκB homologue, 5EL, is nonessential for growth in vitro and virulence in domestic swine. Virology 235, 377–385.

Neilan, J.G., Zsak, L., Lu, Z., Kutish, G.F., Afonso, C.L., Rock, D.L., 2002. Novel swine virulence determinant in the left variable region of the African swine fever virus genome. J. Virol. 76, 3095–3104.

Nogal, M.L., Gonzalez de Buitrago, G., Rodriguez, C., Cubelos, B., Carrascosa, A.L., Salas, M.L., Revilla, Y., 2001. African swine fever virus IAP homologue inhibits caspase activation and promotes cell survival in mammalian cells. J. Virol. 75, 2535–2543.

Oura, C.A., Powell, P.P., Parkhouse, R.M., 1998. African swine fever: a disease characterised by apoptosis. J. Gen. Virol. 79, 1427–1438.

Pastor, M.J., Laviada, M.D., Sanchez Vizcaino, J.M., Escribano, J.M., 1989. Detection of African swine fever virus antibodies by immunoblotting assay. Can. J. Vet. Res. 53, 105–107.

Pastor, M.J., Arias, M., Escribano, J.M., 1990. Comparison of two antigens for use in an enzyme-linked immunosorbent assay to detect African swine fever antibody. Am. J. Vet. Res. 51, 1540–1543.

Perez, J., Rodriguez, F., Fernandez, A., Martin de las Mulas, J., Gomez-Villamandos, J.C., Sierra, M.A., 1994. Detection of African swine fever virus protein VP73 in tissues of experimentally and naturally infected pigs. J. Vet. Diagn. Invest. 6, 360–365.

Plowright, W., Parker, J., Peirce, M.A., 1969. The epizootology of African swine fever in Africa. Vet. Rec. 85, 668–674.

Powell, P.P., Dixon, L.K., Parkhouse, R.M., 1996. An IkappaB homologue encoded by African swine fever virus provides a novel mechanism for downregulation of proinflamatory cytokine responses in host macrophages. J. Virol. 70, 8527–8533.

Revilla, Y., Cebrian, A., Baixeras, E., Martinez, C., Vinuela, E., Salas, M.L., 1997. Inhibition of apoptosis by the African swine fever virus Bcl-2 homologue: role of the BH1 domain. Virology 228, 400–404.

Revilla, Y., Callejo, M., Rodriguez, J.M., Culebras, E., Nogal, M.L., Salas, M.L., Viñuela, E., Fresno, M., 1998. Inhibition of nuclear factor κB activation by a virus-encoded IκB-like protein. J. Biol. Chem. 273, 5405–5411.

Rodriguez, C.I., Nogal, M.L., Carrascosa, A.L., Salas, M.L., Fresno, M., Revilla, Y., 2002. African swine fever virus IAP-like protein induces the activation of nuclear factor kappa B. J. Virol. 76, 3936–3942.

Salguero, F.J., Ruiz-Villamor, E., Bautista, M.J., Sánchez-Cordón, P.J., Carrasco, L., Gómez-Villamandos, J.C., 2002. Changes in macrophages in spleen and lymph nodes during acute African swine fever: expression of cytokines. Vet. Immunol. Immunopathol. 90, 11–22.

Salguero, F.J., Sanchez-Cordon, P.J., Sierra, M.A., Jover, A., Nunez, A., Gomez-Villamandos, J.C., 2004. Apoptosis of thymocytes in experimental African swine fever vírus infection. Histol. Histopathol. 19, 77–84.

Salguero, F.J., Sanchez-Cordon, P.J., Nunez, A., Fernandez de Marco, M., Gomez-Villamandos, J.C., 2005. Proinflammatory cytokines induce lymphocyte apoptosis in acute African swine fever infection. J. Comp. Pathol. 132, 289–302.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning, a Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, New York.

Sanchez-Vizcaino, J.M., Slauson, D.O., Ruiz-Gonzalvo, F., Valero, F., 1981. Lymphocyte function and cell-mediated immunity in pigs with experimentally induced African swine fever. Am. J. Vet. Res. 42, 1335–1341.

Southern, E.M., 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98, 503–517.

Tait, S.W.G., Reid, E.B., Greaves, D.R., Wileman, T.E., Powell, P.P., 2000. Mechanism of inactivation of NF-κB by a viral homologue of IκBα (signal-induced release of IκBα results in binding of the viral homologue to NF-κB). J. Biol. Chem. 275, 34656–34664.

Wardley, R.C., de, M., Andrade, C., Black, D.N., de Castro Portugal, F.L., Enjuanes, L., Hess, W.R., Mebus, C., Ordas, A., Rutili, D., Sanchez Vizcaino, J., Vigario, J.D., Wilkinson, P.J., Moura Nunes, J.F., Thomson, G., 1983. African swine fever virus. Brief review. Arch. Virol. 76, 73–90.

Wilkinson, P.J., Wardley, R.C., 1981. African swine fever. In: Gibbs, E.P.J. (Ed.), Virus Diseases of Food Animals. A World Geography of Epidemiology and Control. Academic Press, New York, pp. 767–786.

Yáñez, R.J., Rodriguez, J.M., Nogal, M.L., Yuste, L., Enriquez, C., Rodriguez, J.F., Viñuela, E., 1995. Analysis of the complete nucleotide sequence of African swine fever virus. Virology 208, 249–278.

Zsak, L., Caler, E., Lu, Z., Kutish, G.F., Neilan, J.G., Rock, D.L., 1998. A nonessential African swine fever virus gene UK is a significant virulence determinant in domestic swine. J. Virol. 72, 1028–1035.