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Virology Report

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Contents:

Introduction

As it is well known, successful vaccination in many viral diseases was reached by classical empirical approaches: attenuated or inactivated virus-based vaccines, viral protein immunization, etc. However, previous attempts to develop vaccines against ASFV have not reached expectations and the reasons of several vaccination failures are unknown yet. Then, a deeper knowledge of basic virology questions in ASFV would be necessary and it would be mandatory that next vaccination approaches should be based in a broader knowledge of virus-host interactions for this agent.

A summary of these research priorities in general would start by standardization of protocols used in the several research groups working in ASFV:

- Standardization of vaccination protocols, define ASFV susceptible cell lines to use, define the route/s for experimental infections, vaccination and challenge.

- Deep sequencing and functional genomics. Standardization of sources of material for this analysis and its preparation.
- Increase Knowledge on early infection stages and virus-host interaction analysis to design and develop new intervention strategies.
- Studies on pathogenesis of natural survivors and to analyze vaccine failures in ASFV from the pathogenesis point of view.

Continue virology developments that lead to novel approaches for ASF disease control:

- Virus deletion mutants as attenuated vaccines
- New molecular targets to be included in vaccine strategies
- New tools for molecular diagnosis of the disease
- Unsolved epidemiology questions related to virus transmission, role of vectors, etc.
- Other preventive strategies in disinfectants and antivirals

Functional Genomics

ASFV is a large DNA virus that infects monocytes/macrophages (Mo/MO) of different species of suids, causing an acute and frequently fatal disease. The viral genome consists of a single molecule of linear, covalently close-ended, dsDNA. The genomes of different isolates vary in length between 165,795 and 191,036 excluding the terminal inverted repeat sequences, and encode between 151 and 167 open reading frames. Furthermore, five different multigene families MGF 110, MGF 360, MGF 530/505, MGF 300 and MGF 100 are found in genome regions close to the termini. Large length differences between genomes of different isolates are due to gain or loss of members of these multigene families. MGF 110 contains 14 members and individual isolates contain between 5 and 11 of these. MGF 360 has 22 members and between 11-18 copies are present in different isolates. MGF 530/505 encompasses 11 members and between 8-10 copies are present. MGF 300 has 4 copies with 3 or 4 present and MGF 100 has 3 copies with 2 or 3 present.

- The complete nucleotide sequence of 11 isolates has been determined. These include the tissue culture adapted Ba71V isolate of African swine fever virus (ASFV-Ba71V) and 10 field isolates from Europe (OUR88/, etc.) and Africa (Malawi, etc.) and very recently, Georgia.

- Virions enclose more than 50 proteins including a number of enzymes and factors needed for early mRNA transcription and processing.

- The expected functions of ASFV gene´s products have been described as reviewed in: H. Takamatsu, C. Martins, J.M. Escribano, C.Alonso, L.K. Dixon, M.L. Salas, Y. Revilla. Asfarviridae Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses. Elsevier (2012) pp. 153–162.

Gaps Functional Genomics:

-Deep sequencing: To obtain complete nucleotide sequence of:

- ✓ isolates from different origins and hosts (pig, wild boar, warthog, tick).
- ✓ natural occurring (or lab) attenuated isolates and comparative analysis with virulent isolates. Comparative pathogenesis with deletion mutants.

- ✓ isolates from natural occurring survivors Crucial gap
- Standardize protocols for sequencing: isolates directly obtained from the animal, virus preparations should be free of cellular DNA.
- Differences in genomic sequences involving gene functions: Focus in crucial genes ("Hot spots", Multigenic families).

-**Function of several genes involved in virus entry, virus traffic, cellular and viral protein synthesis control.**

-Transcriptional map of the virus.

-Complete virus proteome.

Research priorities:

- Functional genomics of isolates from different origins and hosts and sequence comparison in terms of expression and function of the different genes.
- Sequence analysis of natural occurring attenuated isolates.

Determinants of virulence

Virus persists in both ticks and wild pigs following infection. In domestic pigs, ASF occurs in numerous disease forms, ranging from highly lethal to subclinical infections (Mebus, C. A**.** 1988), depending on contributing viral and host factors, some of which remain not fully understood (Chapman, D.A.G., et al. 2008). ASFV infects mainly cells of the mononuclear-phagocytic system. Several ASFV genes have been identified both in European and African pathogenic isolates, with functions involving virulence and host range (Zsak, L., Z. et al 1996, Zsak, L., et al., 1998, Borca, M.V. et al1998, Borca *et al*., 1993). While these genes have been shown to be important for ASFV virulence, they alone are not sufficient, indicating that other viral determinants or combination must play significant roles in viral virulence.

ASFV genes associated to *pathogenesis and virulence* in domestic swine that do not affect viral replication in macrophages:

-UK (DP96R) and 23-NL (DP71L or I14L) Right variable region (Zsak, L., Z. et al 1996)

-MGF360 and MGF530 affect virulence in swine and *growth in MO*, and complement NL virulence function (Afonso C.L., et al 2004, Borca M.V., et al 1994).

-MGF360 reduces *replication in ticks* (Burrage T.G. et al, 2004)

Immune evasion genes:

-Genes affecting pathogenesis and virus replication EP402R (8DR; CD2). Mediates haemadsorption and affect immune response (Borca M.V., et al 1994, Borca M.V.et al, 1998, Rodriguez J.M. et al 1993)

-Deletion does not affect virulence in swine or viral growth in macrophages: A238L (IkB) (Granja A.G. et al 1006), EP153R (8CR; lectin-like) (Neilan J.G. et al, 1999)

- MGF360 and MGF530 mutants increased interferon I early response genes (Afonso C.L. et al 2004)

In conclusion, several genes have been identified both in European and African pathogenic isolates, with functions involving virulence and host range. These genes are important for ASFV virulence, but not sufficient, indicating that other viral determinants or combination of these should play significant roles in viral evasion.

Gaps Virulence determinants:

- To identify new virulence determinants.
- Differences in virulent and attenuated virus in specific genes.

Research Priorities

-Generation of deletion mutants of individual viral genes and deletion mutants of more than one gene.
-Generation of recombinants mutants over expressing genes that modulate immune response.
-In vivo experiences with recombinant viruses.

Genetics

ASFV transcripts are 3'-polyadenylated and 5'-capped. Both early and late gene transcripts are of defined length; sequences of seven or more consecutive thymidylate residues in the coding strand are signals for mRNA 3'-end formation.

Viral genes are expressed in an ordered cascade. Early genes are expressed prior to DNA replication; expression of late genes is dependent on the onset of DNA replication. Synthesis of some early genes continues throughout infection. Intermediate genes are expressed late but their expression does not depend on the onset of DNA replication.

Immunogenicity of these proteins was confirmed by demonstrating the corresponding antibodies in sera from pigs infected either with the Malta isolate or with the OURT88/3‑OURT88/1 isolate combination. Furthermore, the majority of these ORFs were also recognized by immune antiserum from the natural host, the bush pig, following secondary challenge with the virulent Malawi (SINT90/1) isolate of ASFV. Thus, it is possible that some of these determinants may be important in protection against virus infection. (Kollnberger S.D. et al, 2002).

A porcine microarray containing 2,880 cDNAs was used to investigate the response of macrophages to infection by a virulent African swine fever virus (ASFV) isolate, Malawi LIL20/1, showing about one hundred twenty-five targets to be altered at either early and late times postinfection compared with targets after mock infection, including several proinflammatory cytokines and chemokines, surface proteins, and proteins involved in cell signaling and trafficking pathways.

The modifications in the translational machinery induced by ASFV has been recently analyzed, (Castello A et al, 2009), showing that during ASFV infection, eIF4G and eIF4E are phosphorylated (Ser1108 and Ser209, respectively), whereas 4E-BP1 is hyperphosphorylated at early times post infection and hypophosphorylated after 18 h.

EIF4F components are indispensable for ASFV protein synthesis and virus spread, since eIF4E or eIF4G depletion in COS-7 or Vero cells strongly prevents accumulation of viral proteins and decreases virus titers.

Degradation of cellular polyadenylated mRNAs and recruitment of the translation machinery to viral factories may contribute to the inhibition of host protein synthesis, facilitating ASFV protein production in infected cells (Castello, A et al. 2009).

Gaps:

- What´s the mechanism to control the ordered cascade expression of the viral proteins? Is it cellular, viral or both?
- What are the gene (s) involved? Is g5R, the ASFV decapping enzyme, one of them?
- What´s the molecular mechanism for the virus-induced cellular shutoff and genes involved. Is it related to attenuation?

Research priorities:

-To analyze the processing of viral and cellular mRNAs during the infection in swine macrophages. Comparison of virulent and attenuated ASFV strains in terms of modulation of eIF4F, ribosomes and mitochondria localization.

Host-Pathogen interactions

Early virus-host cell interactions are key determinants of viral tropism and pathogenesis. For a better understanding of the biology of the virus the identification of targets at early stages of infection could reveal important clues to the design of possible intervention strategies. Being an area under current active research, many questions remain unresolved.

Target cell: The virus replicates in macrophages with markers characteristic of late stages of differentiation (McCullough K.C. et al, 1999; Sanchez-Torres C. et al, 2003). Mature macrophages CD163 positive.

ASFV attachment proteins characterized so far are p12, p54, p30. Some of these proteins have been involved in the induction of neutralizing or infection inhibitory antibodies in infected or vaccinated pigs.

Viral receptor/s: The cellular receptor for ASFV is still unknown. Porcine CD163 scavenger receptor participates in the natural host cell infection and it has been proposed as a putative ASFV receptor (Sanchez-Torres C. et al, 2003). Expressed on most tissue macrophages but not on other myeloid cells is one of the most reliable markers of the monocyte/macrophage lineage (Perez C. et al, 2008). Future studies should characterize virus receptor/s and this would be crucial to develop vaccines that elicit antibodies preventing virus binding to receptors.

ASF Virus entry: The entry of a virus in a host cell determines whether a successful productive infection would occur or not. ASFV entry route is a matter of current debate. Early studies characterized ASFV entry as saturable and specific receptor-mediated (Alcami A,. et al.,

1989a,b; Alcami A., et al., 1990; Valdeira ML and Geraldes A, 1985). Electron microscopy observations found ASFV particles frequently adsorbed to invaginations similar to clathrin coated pits (Alcami A. et al, 1989a). Recent studies showed that ASFV entry involves dynamin-dependent and clathrin-mediated endocytosis. In addition, this entry mechanism requires cholesterol. Also, macropinocytosis has been described as a major entry pathway (Sanchez EG et al, 2012), and even phagocytosis (Basta S et al, 2010). Nevertheless, these routes might not be equally effective to initiate infection. These observations should be further extended in order to determine whether these could be cooperative (Alonso C., et al, 2012) or alternative pathways of entry. Also, it is not known whether both mechanisms would be consistent with saturable and specific receptor-mediated endocytosis. Moreover, the entry mechanism/s used in macrophages remains unresolved.

Endocytic pathway progression and uncoating: In contrast, there is a consensus about the virus requirements immediately after entry (Valdeira ML and Geraldes A, 1985; Valdeira ML et al., 1998; Cuesta-Geijo M.A. et al., 2012). Endocytosis and its intraluminal acidic pH are both required for a productive infection. Which is the crucial endocytosis stage/s to be reached for a successful infection? ASFV desencapsidation occurs at late endosomes (Cuesta Geijo M.A. et al, 2012).

Viral transport: ASFV p54 interacts with microtubule motor dynein for intracellular transport (Alonso C. et al., 2001). Cytoplasmic dynein is a minus-end-directed microtubule motor protein that mediates a range of functions, including the transport of viruses to their replication site close to the microtubule organizing center (MTOC; Vallee RB et al, 2012). Dynein-p54 is a high affinity chemical interaction that forms a stable molecular weight complex in vitro. A short peptide sequence mimicking the p54-dynein binding domain (DBD) binds and competes this interaction *in vitro*. In infected cells, treatment with this peptide decreases infectivity, virus replication and production (Hernaez B et al, 2010).

ASFV nuclear phase: ASFV DNA is detected in the nucleus and cytoplasmic replication sites by in situ hybridization and radioactive labeling (Ballester M et al., 2010; Garcia-Beato R et al., 1992; Rojo G et al., 1999). ASFV DNA found in mature viral particles is derived from both nuclear and cytoplasmic fragments (Ortin J et al., 1979; Rojo G et al., 1999). Proteins p37 and 17, products of polyprotein pp220, a component of the ASFV core shell, are targeted to the nucleus (Eulalio A et al, 2004, 2006). Recent studies reported that ASFV infection disrupts nuclear organization at an early stage of infection. Increased lamin A/C phosphorylation is found at 4 hpi, followed by lamina network disassembly in the proximity of the replication site. Other nuclear elements redistributed are RNA polymerase II, the splicing speckle SC35 marker, and the B23 nucleolar marker (Ballester M et al., 2011; Basta S et al, 2010).

Viral factory: The viral assembly site or viral factory (VF) comprise a robust collection of newly synthesized viral proteins and viral DNA and are located at the perinuclear area corresponding to the MTOC. VFs are characteristically devoid of organelle markers (Alonso C. et al, 2012). Its formation starts as multiple smaller spots or early factories that are motile around the nucleus and coalesce in a single location coincident with the MTOC at subsequent time points (Hernaez B et al, 2006). Mitochondria organize around the VFs that contain high number of ribosomes (Rojo G et al., 1998) and the ER and Golgi complex are disassembled as infection progresses

(Netherton CL et al., 2006). ASFV also induces profound changes in the cytoskeleton. Intermediate filaments proliferated in the cytoplasm forming a robust vimentin cage around the factories (Stefanovic S et al., 2005). Microtubules depolimerize and actin cytoskeleton progressively disassembled. Other host factors necessary for VF formation are prenylated proteins, especially Rho GTPases (Quetglas J.I. et al, 2012) and cholesterol biosynthesis inhibitors as statins alter its formation. Precursor viral membranes derived from the ER at the VF are associated to ASFV transprenyltransferase (ORF B318L), which is an essential and late gene (Alejo A. et al., 1997; Andres G. et al., 1997). Farnesyl pyrophosphate (PP) and geranyl geranylPP synthesized by B318L product serve as substrates for protein prenylation, required during virus replication and morphogenesis (Alejo A. et al, 1999).

Gaps and Research priorities ASFV-host interaction:

- Target cell: to clarify which stage of differentiation is crucial to select the source of primary macrophages.
- Identification of viral receptor/s and interaction with viral attachment proteins – vaccines that elicit antibodies preventing virus binding to receptors.
- Viral entry mechanisms and cellular proteins and lipids involved.
- Clarify the alternative/cooperative pathways to enter the plasma membrane.
- Virus traffic: viral and cellular factors involved in traffic at viral entry and exit.
- Characterize early steps: endosomal passage and further steps to finish uncoating and start replication
- Further characterization of the nuclear phase and viral factory formation
- Characterize molecular targets at early infection steps to develop intervention strategies.
- Investigate pathogenesis and the effect of virus entry inhibitors effect in ticks.

ASFV control of cell stress, survival and death

ASFV induce apoptosis in infected cells at relatively late times after infection (24-48 hpi) (Ramiro-Ibañez F. et al, 1996). However, the signal triggering the apoptotic process, occur at an early post-binding stage, as it occurs with vaccinia virus (Ramsey-Ewing A and Moss B, 1998). ASFV uncoating is required and apoptosis is blocked with lysosomal acidification inhibitors (Carrascosa A. el al, 2002). The dynamics of caspase expression starts with ER stress-caspase 12 and mitochondrial-caspase 9 activation at 16 hpi, followed by executor caspase 3 activation at 48 hpi (Galindo I. et al, 2012). This activation was found to be critical at early infection.

Membrane blebbing with formation of vesicles filled with virus, at the end of apoptotic process is an efficient system for ASFV spread. Blebbing suppression (Blebbistatin, Rock-I inhibitor) reduces extracellular virus fraction but does not modify total virus production (Galindo I. et al, 2012).

Proteomic analysis of cellular proteins after infection led to the identification of several chaperones overexpression 10-24 hpi (Alfonso P et al, 2004). The high level of viral protein production saturates the ER chaperones protein-folding capacity and disturbs ER homeostasis inducing the Unfolded Protein Response (UPR). ER stress after infection is reflected by caspase 12 activation, chaperones calnexin and calreticulin but not ERp57 or BiP overexpression (Galindo

I et al, 2012; Netherton CL et al, 2004). ATF6 is a transmembrane protein which functions as UPR sensor, and is activated and translocated to the nucleus upon ASFV infection. This activation is essential for a successful infection (Galindo I. et al 2012).

Induction of apoptosis in infected cells limit virus replication by reducing progeny virion production. Therefore many viruses elicit mechanisms to inhibit apoptosis in infected cells (Hay S and Kannourakis G, 2002). ASFV encodes two proteins similar to host antiapoptotic proteins. One of these is similar to cellular IAP proteins and acts by binding to caspase 3 inhibiting its activity (Nogal et al., 2001). The second of these is similar to the Bcl2 protein family (Afonso C.L. et al., 1996) and has been shown to inhibit apoptosis (Brun et al., 1996; Revilla et al., 1997) by binding proapoptotic members of the Bcl2 family (Galindo, I. et al 2008). The IAP protein is expressed late in infection (Chacon et al., 1995), and is not essential for virus replication, whereas the A179L-Bcl2 protein is an early and essential protein in the viral cycle (Revilla Y. et al. 1997). Although the A224L-mediated inhibition of caspase-3 in mammalian cells has been shown (Nogal ML et al., 2001), together with the demonstration of a role for A224L in NFkB activation (Rodríguez, C. et al. 2002), an intriguing possibility is that IAP may have a more critical role during virus infections of the tick vector.

A179L-Bcl2 protein inhibits the action of several pro-apoptotic BH3- only proteins, known to be rapid inducers of apoptosis, such as activated Bid, BimL, BimS, BimEL, Bad, Bmf, Bik, Puma, and DP5 (Galindo I. et al., 2008). It also interacts at the mitochondrial membrane, A179L action is exerted on key pro-apoptotic Bcl2 family members, such as Bax and Bak. This viral Bcl2 has a dual role as regulator of apoptosis and autophagy. This protein interacts with Beclin1 and negatively regulates autophagy (Hernaez B et al, 2012). Autophagy regulation in ASFV infection is, however not fully understood and is a key determinant in many virus infections.

Moreover, ASFV encodes a homolog of the neurovirulence factor ICP34.5 of HSV-1 and the cellular gene GADD34. This homolog is the DP71L (23NL/MyD 88) gene (Zsak L. et al., 1996). The cytoprotective effect of DP71L is exerted by binding the catalytic subunit of protein phosphatase 1 (PP1). This binding causes the dephosphorylation of eukaryotic translation initiation factor 2 α (eIF2α), thereby preventing the inhibition of protein synthesis produced by ER stress and the UPR (Rivera J. et al., 2007). The prevention of the protein synthesis inhibition caused by eIF2α phosphorylation is an important virus-host interaction that ensures viral protein synthesis and cell survival in several virus models. HSV-1 ICP34.5 (He et al., 1997), papilloma virus (Kazemi et al., 2004), and coronavirus (Cruz JL et al., 2011) follow a similar strategy to that used by ASFV to overcome protein synthesis inhibition during its adaptation to the host. Moreover, a number of viruses have evolved mechanisms to inhibit viral nucleic acid sensing by interferon-inducible protein kinase (PKR) and activation of eIF2α, the latter promoting cell death (Domingo-Gil E et al., 2011; Ramelot TA et al., 2002).

Interestingly, deletion of DP71L from a virulent ASFV (isolate E70) reduces the virulence of the virus in pigs (Zsak L et al., 1996); however, this effect was not reproducible for the highly pathogenic Malawi isolate. Moreover, deletion of this gene does not modify eIF2α phosphorylation. This observation thus suggests the presence of alternative mechanisms to prevent eIF2α phosphorylation (Zhang F et al., 2010), as described for other DNA viruses (e.g. HSV-1). Also, DP71L inhibits the early induction of ATF4 and its downstream target CHOP (Zhang

F et al., 2010), a transcription factor that is commonly up-regulated as a result of the UPR, but not in ASFV infection (Galindo I et al., 2012; Netherton CL et al., 2004). Other functions undertaken by the HSV-1-homologous gene, such as the inhibition of autophagy by means of Beclin-1 inhibition (Orvedahl A et al., 2007); do not occur in ASFV DP71L.

Gaps:

- Investigate the relevant function of apoptosis induction at early infection and characterize the viral and cellular genes involved.
- Investigate the functional role of apoptosis for virus spread in porcine macrophages and in the *in vivo* infection
- The different ASFV genes and mechanisms controlling apoptosis acting at different times post-infection.
- Characterize the relevance of autophagy and ER stress as key elements for ASFV infection.
- ASFV gene(s) involved in cellular and viral protein synthesis, e.g. G5R, a potential ASFV mRNA decapping enzyme?

Research priorities:

-Generation of deletion mutants in virulent strains (E70, Lisbon60) and attenuated (NHV) lacking of genes found to be important in the regulation of apoptosis *in vitro* by Ba71V infection of Vero cells, e.g. A224L, EP153R.
- Use of these deletion mutants to infect animals for further attenuation.
- Further characterize molecular targets essential for ASFV infection in apoptosis-, ER stress- and autophagy- related signaling.
- Targeting the cellular machinery of protein synthesis, apoptotic and ER stress pathways as a vaccination strategy.

Pathogenesis

ASFV is very well adapted to its natural hosts (Wilkinson PJ, et al 1984) warthogs and soft ticks. In these hosts the virus causes unapparent infections which can persist for months or years. Pigs which recover from less virulent infections remain persistently infected for long periods (Leitao A et al., 2001; Villeda CJ et al., 1993; Wilkinson PJ, 1984; Wilkinson PJ et al., 1981). The soft tick vector also remains persistently infected for long time periods transmitting the virus to both domestic pigs and wildlife hosts.

Acute form of ASF is caused by virulent strains in domestic pigs present mortality rates over 99% in 5-12 days of infection. Chronic or subacute form is produced by less virulent isolates but the role of host factors could play a role in the natural infection. These animals survive infection and develop immunity against related virulent viruses (King K et al, 2011; Zsak L et al, 1996). It remains unexplored if this is due to host factors or to less virulent strains in natural occurring survivors.

ASFV infects and replicate in warthogs, bushpigs and soft ticks, but these species develop long term subclinical persistent infections (Wilkinson PJ, 1984).

ASFV targets cells of the monocyte/macrophage lineage (M*0*) producing massive destruction of these important immune defense cells. Apoptosis (Oura CA et al 1998; Ramiro-Ibanez F et al 1996; Gomez-Villamandos JC et al 1995)

Specifically, ASFV target cells are **mature macrophages CD163, CD172**+ (Sanchez-Torres C et al., 2003). Bone marrow derived and fresh blood monocytes are less susceptible to *in vitro* infection than alveolar M*0* (Carrasco L et al., 1992;Rodriguez F et al 1996). M0 cell lines available so far lack most markers of mature macrophage lineage.

Without tick involvement, ASFV enters the body via the tonsils or pharyngeal mucosa (Greig A, 1972; Plowright W et al., 1994) After 24 hpi, the virus is detectable in all tissues. **Virus detected in all secretions and excretions** (Blome S et al., 2013).

Viremia (8h- 24hpi) associated to erythrocytes. Small % infected monocytes in peripheral blood (8609199) and few granulocytes (Carrasco L et al. 1995)

Most virus induced pathogenesis depend on cytokine-mediated damage which causes a severe lymphopenia and immunosuppression on the one hand and activation of endothelial cells and the coagulation system resulting in disseminated intravascular coagulation (DIC).

Increased TNF-α (Gomez del Moral MG et al., 1999), IL-1α, IL-1β and IL-6 coincides with onset of fever, vascular damage and changes in lymphoid tissues (Salguero FJ et al., 2002) and detection of cytokines in tissues correlates with virus detection with antibodies. TNF-α can induce vascular changes and activation of endothelium. Infected pulmonary intravascular M*0* (not alveolar) correlate with lung associated lesions (Carrasco L et al., 2002; Oura CA et al 1998). Fibrin networks and microthrombi/tissue destruction by apoptosis in spleen, lymph nodes, liver, kidneys (Gomez-Villamandos JC et al., 1995; Rodriguez F et al., 1996).

Activation of endothelial cells and the coagulation system and disseminated intravascular coagulation (DIC). Vascular changes are not due to direct infection of endothelial cells but by proinflammatory cytokines release and procoagulant state of the endothelium (Gomez-Villamandos JC et al, 1997). This leads to peripheral consumption of platelets and coagulation factors (Edwards JF et al, 1984; Villeda et al., 1993b) and activation of fibrinolytic system (Villeda et al., 1993b). Acute phase protein increased in serum such as haptoglobin, serum amyloid A (Sanchez-Cordon PJ et al., 2007; Carpintero R et al., 2007). Also, bone marrow destruction of platelet precursors is possible (Blome S et al., 2013).

Opposite findings characterize the chronic form of the disease, namely lymphoid organ hyperplasia (Ramiro-Ibanez F et al, 1997) and immune complex deposition (Hervas J , et al, 1996 a,b; Martin- Fernandez J et al 1991) in kidneys, joints, skin, etc.

Immunology:

Main features from *in vivo* acute infection are immunosuppression, and impairment innate immunity. The IFN response and its control by viral gene products is a major virulence determinant of isolates (Afonso CL et al, 2004)**.** ASFV induces apoptosis of infected macrophages

(Ramiro-Ibanez F et al, 1996), which results in decreased M0 numbers, Lymphopenia and low numbers of all lymphoid subpopulations B, T CD4 and CD8. ASFV virus induces specifically IL-2 downregulation (Canals A et al, 1995). There is a characteristic non-specific activation and apoptosis of B cell populations in vitro and in vivo (Takamatsu H et al 1999; Ramiro-Ibanez F et al, 1997). In the subacute form there is increased B lymphocytes and M*0* numbers (week 1), which peak at the time of viraemia. CD8 and CD4, SLA I, II become elevated (week 2) to return to normal values at week 3 (Ramiro-Ibanez F et al, 1997).

ASFV infection induces cytokine secretion TNFα (Del Moral M.G et al 1999), TGF-beta or inhibits others (Salguero FJ et al 2002). The fact that ASFV has adapted to infect both mammalian macrophages and an invertebrate tick host suggests that this virus may have evolved immune evasion genes focused on adaptative and innate immunity (de Oliveira VL et al. 2011). Macrophages infected by ASFV synthesize cytokines that have an impact on the development of inflammatory responses (pro-inflammatory cytokines such as IFN type I, IL1, IL6 and TNF-α) and cytokines that participate in the development of specific immune mechanisms (immunoregulatory cytokines such as IL12, IL15, IL18). Studies using porcine blood-derived macrophages infected in vitro with two ASFV isolates of different virulence, the highly virulent L60 and the low virulent NHV, demonstrated a particular effect of infection by the latter isolate in that significantly increased levels of transcripts for TNFα, IL6, IL12 and IL15, (Gil, S., et al, 2008), in contrast to the effect of infection with ASFV/L60 (Gil S, et al. 2003). These results suggest a role of ASFV/NHV in inducing inflammatory and specific cellular-based responses in the natural host, which has been largely described to be important in protection (Martins C, et al, 1993; Leitao A, et al 2001). Levels of protein expression and secretion were measured for two proinflammatory cytokines, interleukin 1 beta and tumor necrosis factor alpha, during a time course of infection with either the virulent Malawi LIL20/1 isolate or the OUR T88/3 nonpathogenic isolate. The results revealed differences between these two ASFV isolates in the amounts of these cytokines secreted from infected cells (Zhang, F. et al. 2006).

- Expression of these cytokines is partially regulated by the transcription factor nuclear factor kappa beta (NFκB) and NFAT transcription factors.

- An African swine fever virus gene with similarity to the inhibitor of NFκB (IκB), has been identified in the pathogenic African isolate Malawi Lil-20/1 (5EL) (Neilan J.G. et al 1997) and a cell-culture-adapted European virus BA71V (A238L) (Revilla, Y et al 1998).

- Data showed that A238L interacts with the RelA subunit of NFκB, where it is able to prevent binding of NFκB to target sequences and inhibit NFκB-dependent gene expression (Revilla Y. et al 1998). The 5EL gene from Malawi isolate also provides a mechanism that enables ASFV to evade host defense systems by preventing the transcription of cellular NFAT transcription factor (Miskin, J.E.,et al 1998). ASFV is thus able to control the transcriptional activation of immunomodulatory genes dependent on NFkB and NFAT pathways, such as cyclooxygenase- 2 (COX-2) (Granja A. et al, 2004) and TNF-alpha, through a mechanism involving the control of the transcriptional coactivator p300. (Granja A et al 2006; Sánchez EG et al 2012). Given the central role of NFκB and NFAT in regulating the expression of many proinflammatory genes, it is amazing that deletion of 5EL from the Malawi Lil-20/1 genome had no effect on disease onset, disease course, or viral virulence (Neilan, J.G. et al., 1997). Importantly, preliminary results obtained by

immunizing pigs with a recombinant NHV, lacking of A238L gene, have shown lower protection against the challenge with Armenia virulent strain than the parental virus (Y. Revilla, C. Gallardo, personal communication).

- A second ASFV protein probably involved in evading host defense systems is the protein encoded by the Ba71V-EP402R gene (CD2v). The CD2v extracellular domain has 15 potential sites for N-linked glycosylation. The cytoplasmic domain varies in length between different virus isolates due to the presence of variable numbers of repeats of sequence KPCPPP (Borca MV et al., 1994b; Rodriguez et al., 1993). ASFV CD2v protein is required for the characteristic rosetting of red blood cells around ASFV-infected cells (Borca MV et al., 1994b; Rodriguez JM et al., 1993). The binding of ASFV particles to red blood cells probably provides a mechanism for virus dissemination, as, in pigs infected with virulent ASFV from which the EP402R gene encoding CD2v was deleted, dissemination of virus to lymph nodes was delayed compared to wild type virus and onset of disease was delayed although mortality rates were not reduced (Borca MV et al., 1998).

Gaps in ASF pathogenesis:

-Natural route of infection (oro-nasal) should direct the route of vaccination and challenge. Standardization of route and dose is required.
- Survivors (natural and experimental) and subacute disease pathogenesis characterization.
- Clarify other gaps in the epidemiology of the disease.
- Natural appearance of survivors: are those due to changes in the virus isolate?, to modifications in cytokine profile?, to the genetic background of animals?
- Analysis of vaccine failure from the pathogenesis point of view.
- Identification of the relevant immune response to elicit protection: cellular vs. humoral.
- Predictive profile of immune response elements (innate and adaptive imm.) and cytokines for the disease outcome.

Research priorities:

- The study of virus evasion strategies: viral genes and cellular pathways regulated by the virus *in vivo* through the construction of specific deletion mutants; relevant to understanding virus pathogenesis and further attenuation of non-virulent ASFV strains.

- Generation of virus recombinants by manipulation of A238L in virulent and attenuated ASFV strains. Infection with these recombinants to assess their impact in protection and pathogenesis in the pig and protection assays.

- Develop and analyze iInhibitors and immunosupressors to change the acute disease course.

Disinfectants and Antivirals

African swine fever is a threat to pig production and has a major impact on national economies. The minimum measures of rapid detection and eradication are essential but not always sufficient to contain outbreaks in time. Disinfectants are an important help to other

epidemiological countermeasures. Searching for an antiviral product with intracellular action, high inhibitory efficiency and low cellular toxicity, is a critical challenge raised to the scientific community at present. The possibility to obtain an antiviral with low specificity should help to control a wide range of possible virus infections in animals. Antiviral drugs can be employed in an economically useful manner to minimize the outbreak and the associated damage. An study from the Central Veterinary Institute (LEI, CVI) from Wageningen UR for Classical swine fever estimated that comparing all scenarios reveals that the economic damage is smallest if all the pigs in a radius of 2 kilometers around the infected farm are treated with antiviral drugs, at a price level of less than 10 euros for a daily dose of the antiviral drug (Bergevoet et al, 2012). This encourages research in antiviral drugs against ASFV and to pursue a similar study of economic impact in disease control, especially in areas with high livestock density. Such a survey might conclude that stockpiling of antiviral drugs by the authorities is helpful for epidemic outbreaks.

Disinfectants against ASFV

Fomites: (3)4-log reduction standard

-Porous surfaces (wood): 2% Citric acid, 2000 ppm sodium hypochlorite or chlorine (Krug PW et al 2012).

-Hard non-porous surfaces (plastic, steel): Sodium hypochlorite 1000 ppm, Sodium carbonate 4% (Krug PW et al 2011) (US Environmental Protection Agency). Others to be tested: iodine (potassium tetraglicine triiodide), and quaternary ammonium compounds.

Food products:

Sausages: salt (NaCl) and phosphate supplemented salt were capable to inactivate ASFV within 48 h. In contrast to the other viruses (FMDV, CSFV and SVDV), ASFV was the most stable virus even at higher temperatures (Wieringa-Jelsma T et al 2011).

Survival in pork products (Farez S et al 1997; Morgan DO et al 1978)

30 days in peperoni or salami sausage/104 days frozen meat or chilled meat. 112 days in Iberian loins-140 days other Iberian hams-399 in Parma hams

Antivirals in ASFV

Antivirals would reduce the susceptible population around the outbreak focus, provide instantaneous protection without culling the susceptible animal, and do not induce an anti-virus antibody response, facilitating serological testing to regain the virus-free status.

Antivirals from natural sources: plant phytoalexins: resveratrol, oxyresveratrol (Galindo I et al, 2011), microalgae (Fabregas J et al, 1999);

Drugs approved for human use: Cholesterol lowering statins (Quetglas JI et al, 2012); antitumoral lauryl-gallate (Hurtado C et al 2008); anticonvulsivant valproic acid (Vazquez-Calvo A et al, 2011).

Research stage inhibitors: dynamin inhibitors (Cuesta-Geijo MA et al, 2012), topoisomerase inhibitors (fluoroquinolones; Mottola, C. et al; 2013, 23415476), serine protease inhibitors (GalindoI et al, 2012), specific peptides (Hernaez B et al, 2010) and miscelanea (Basta S et al, 2010).

Gaps and research priorities Disinfectants and Antivirals:

- Develop Indications/Applications protocols
- Consider regional regulatory issues for application
- To develop new countermeasures against the disease include the development of antivirals based on virus-host interaction knowledge.
- Use antivirals as tools to analyze variations in the experimental disease course.
- Sudies on indications, price-tolerance, applications, e.g.:
  - ✓ Outbreaks in large farms to lower viral load until slaughter
  - ✓ Create a "safety belt" in neighboring farms from areas of dense pig population
  - ✓ To reduce the negative impact of an ASFV outbreak

Resources:

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-Bergevoet, R.H.M., M.A.P.M. van Asseldonk and J. Backer. Economic aspects of antiviral agents to control Classical Swine Fever epidemics LEI report 2012-066 ISBN/EAN: 978-90-8615-602-3 43.
-Dixon, L.K., C.Alonso, J.M. Escribano, C. Martins, Y. Revilla, M.L. Salas, and H. Takamasu. Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of viruses. Chapter: Asfarviridae A.M.Q. King, M.J. Adams, E.B. Carstens and E.J. Lefkowitz Eds. Elsevier Amsterdam, New York, 2012. ISBN: 978-0-12-384684-6

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