

Title: Identification of Protective Antigens of African Swine Fever Virus – NPB #13-102

Investigator: Daniel L. Rock, Co-PI: Denis Kolbasov

Institution: University of Illinois, Urbana-Champaign and the National Research Institute for Veterinary Virology and Microbiology, Pokrov, Russian Federation

Date Submitted: January 2, 2015

Industry Summary:

African swine fever (ASF) is arguably the most significant emerging disease threat for the swine industry worldwide. Devastating ASF outbreaks and continuing epidemic in the Caucasus region and Russia (2007 – to date) highlight significance of this disease threat. There is no vaccine for ASF available; however, it is clear that vaccination is possible since protection against homologous reinfection has been definitively demonstrated. Vaccine progress is hindered by lack of knowledge concerning the extent of ASFV strain variation and the viral antigens responsible for protective immunity. A safe and efficacious DIVA (Differentiate Infected from Vaccinated Animal) compatible vaccine would be a critical tool for emergency disease response and control and would reduce the risk for U.S. pork producers. Identification of protective antigens of ASFV is the necessary first step toward eventual vaccine development. Here, we demonstrate that two ASFV proteins, CD2v and C-type lectin, are necessary for protection against ASFV. These viral proteins represent significant protective antigens for ASFV that should be targeted in vaccine design and development. For additional information contact

D. L. Rock - dlock@illinois.edu or D. Kolbasov - kolbasovdenis@gmail.com.

Keywords:

African swine fever, ASF, protective antigens, viral CD2v, C-type lectin, protective immunity, ASF vaccine

Scientific Abstract:

African swine fever (ASF) is an emerging disease threat for the swine industry worldwide. No ASF vaccine is available and progress is hindered by lack of knowledge concerning the extent of ASFV strain diversity and the viral antigens responsible for protection in the pig. Available data from vaccination/challenge experiments in pigs indicate ASF protective immunity maybe hemadsorption inhibition (HAI) serotype-specific. Recently, we have shown that two ASFV proteins, CD2v (EP402R) and C-type lectin (EP153R), are necessary and sufficient for mediating HAI serologic specificity (Malogolovkin et al., 2014). Here using ASFV inter-serotypic chimeric and/or gene-deleted viruses and vaccination/challenge experiments in pigs we demonstrate that CD2v and C-type lectin proteins are necessary for homologous protective immunity. These viral proteins represent significant protective antigens for ASFV that should be targeted in vaccine design and development.

These research results were submitted in fulfillment of checkoff-funded research projects. This report is published directly as submitted by the project's principal investigator. This report has not been peer-reviewed.

For more information contact:

National Pork Board • PO Box 9114 • Des Moines, IA 50306 USA • 800-456-7675 • Fax: 515-223-2646 • pork.org

Introduction:

ASF is an acute viral hemorrhagic disease of domestic swine with mortality rates approaching 100% (Coggins, 1974; Mebus, 1988; Montgomery, 1921). Devastating ASF outbreaks and continuing epidemic in the Caucasus region and Russia (2007 – to date) highlight significance of this disease threat (Gogin *et al.*, 2013; Sanchez-Vizcaino *et al.*, 2013). No ASF vaccine is available, though protection against homologous virus challenge has been observed (Detray, 1957; Gomez-Puertas *et al.*, 1998; Leitao *et al.*, 2001; Lewis *et al.*, 2000; Malmquist, 1963; Ruiz *et al.*, 1981; Zsak *et al.*, 1996). Vaccine and disease control progress is hindered by lack of knowledge concerning the ASF virus (ASFV) antigens responsible for inducing protective immunity and their diversity in nature.

Although ASFV serologic assays used for disease diagnosis have focused on conserved cross-reactive viral proteins (Cubillos *et al.*, 2013; Gallardo *et al.*, 2013), evidence indicates distinct antigenic types of ASFV exist based on hemadsorption inhibition (HAI) serologic typing (Coggins, 1968; Detray, 1957; Malmquist, 1963; Pan *et al.*, 1974; Vigarío *et al.*, 1970; Vigarío *et al.*, 1974). Eight ASFV serogroups (SG) have been identified, although more likely exist (Balyshev *et al.*, 1995; Balyshev *et al.*, 2010; Sereda & Balyshev, 2011; Sereda *et al.*, 1992; Vishnjakov *et al.*, 1995). Notably and of particular significance for vaccine development, ASF protective immunity appears to be serotype-specific, as viruses within a serogroup cross protect against one another (Balyshev *et al.*, 1995; Sereda & Balyshev, 2011; Sereda *et al.*, 1992; Vishnjakov *et al.*, 1991; and Malogolovkin *et al.* unpublished data). Recently, we have shown that two ASFV proteins, CD2v (EP402R) and C-type lectin (EP153R), are necessary and sufficient for mediating HAI serologic specificity (Malogolovkin *et al.*, 2014). **We hypothesized that ASFV CD2v and C-type lectin proteins are responsible in part for the serotype specific cross-protective immunity observed for ASF and that they are significant protective antigens for ASF.**

Stated Objectives from original proposal:

Objective 1: Evaluation of ASFV CD2v and C-type lectin as a protective antigens for ASF

The experimental approach used here to evaluate ASFV CD2v and C-type lectin proteins as protective antigens involves vaccination/challenge experiments using ASFV inter-serotypic recombinant (chimeric) and/or gene-deleted viruses and natural infection in pigs as we have previously described for prior ASF vaccination/challenge experiments (Lewis *et al.*, 2000; Moore *et al.*, 1998; Zsak *et al.*, 1998; Zsak *et al.*, 1996). This experimental approach alleviates many of the potential confounding issues related to evaluating individual ASF viral proteins as protective antigens that have characterized earlier work in this field. Specifically, it ensures proper expression and post-translational modification of the putative ASFV protective antigen, delivery of the appropriate antigen dose and proper antigen presentation to the host in the context of natural infection.

Materials & Methods:

ASF viruses - Attenuated strains Congo (KK-262, a serogroup 2 virus (SG2)) and France (FK-32/135, a serogroup 4 virus (SG4)) were used for immunizations and for construction of ASF recombinant viruses. Congo K49 virus (the virulent parent of KK-262), a highly virulent African ASFV isolate, was used for challenge infections. Immunization with France (FK-32/135) does not protect animals from virulent Congo K49 virus challenge (Malogolovkin *et al.* unpublished data).

Construction of ASF recombinant viruses - To assess the role of CD2v and C-type lectin proteins in ASFV protective immunity, chimeric ASFVs recombinant at the CD2v/C-type lectin gene locus were constructed and evaluated in vaccination/challenge experiments in pigs. Attenuated ASF viruses Congo (KK-262, (SG2)) and

France (FK-32/135, (SG4)) were used to generate chimeras (Congo^{France CD2v/Lectin} and France^{Congo CD2v/Lectin}, respectively) in which native CD2v/C-type lectin genes were deleted and replaced by those from the heterologous virus. Recombinants were constructed essentially as described previously, using recombination vectors with heterologous CD2v/C-type lectin gene sequences and GFP reporter gene flanked by 0.5-0.7 kb homologous sequence to mediate recombination (Diel *et al.*, 2011; Lewis *et al.*, 2000; Zsak *et al.*, 1996). COS-1 cells were infected with parental virus (m.o.i =5) and transfected with recombination vectors. Recombinant viruses were isolated by limiting dilution and plaque assay using fluorescence microscopy. Viral purity and recombinant sequence fidelity was verified by PCR and DNA sequencing. Chimeric viruses exhibited normal growth characteristics and hemadsorption in macrophage cell cultures (Malogolovkin *et al.*, 2014).

Animal vaccination/challenge experiments - Pigs (30 to 35 kg) were vaccinated and then challenged using protocols detailed below. Survival rate and time-to-death were recorded. Clinical signs of ASF (fever [a rectal temperature greater than or equal to 104°F], anorexia, lethargy, shivering, cyanosis, and recumbency) were monitored daily. Blood samples were collected at regular intervals post vaccination and for 30 days post challenge (DPC). Titration and quantitative PCR of ASFV in blood samples was performed as previously described (Zsak *et al.*, 1996; Zsak *et al.*, 2005). Serologic assays were performed using serum samples collected just prior to challenge infection. HAI assays were performed in swine macrophages as previously described (Ruiz Gonzalvo *et al.*, 1996; Vishnjakov *et al.*, 1995). ASFV ELISA assays were performed using procedures recommended by the manufacturer (Id-vet.com). Challenge data were assessed by Student's t-test or by Mann-Whitney Rank Sum Test if data failed Shapiro-Wilk normality testing.

Experiment 1 Design - Pigs were vaccinated intramuscularly (one site) with 10⁶ HAU of either Congo-attenuated (KK-262) or Congo^{France CD2v/Lectin} viruses and boosted with the same virus and dose at 21 days post-vaccination. One week later, vaccinated and control animals were challenged intramuscularly with 10³ HAU of Congo K49 and monitored for 30 days.

Experiment 2 Design - Pigs were vaccinated intramuscularly (two sites) with 10⁶ HAU of either Congo-attenuated (KK-262) or Congo^{France CD2v/Lectin} viruses and boosted with the same virus and dose at 21 days post-vaccination. At 42 days post-vaccination, vaccinated and control animals were challenged intramuscularly with 10³ HAU of Congo K49 and monitored for 30 days.

Experiment 3 Design - Pigs were vaccinated intramuscularly (two sites) with 10⁶ HAU of either Congo-attenuated (KK-262), France-attenuated (FK-32/135) or France^{Congo CD2v/Lectin} viruses and boosted with the same virus and dose at 21 days post-vaccination. At 42 days post-vaccination, vaccinated and control animals were challenged intramuscularly with 10³ HAU of Congo K49 and monitored for 30 days.

Results:

Homologous CD2v/C-type lectin are necessary for protection against ASF

To assess the role of CD2v and C-type lectin proteins in ASFV protective immunity, chimeric ASFVs recombinant at the CD2v/C-type lectin gene locus were constructed and evaluated in vaccination/challenge experiments in pigs. The chimeric virus used in experiments 1 and 2 was Congo^{France CD2v/Lectin}, a Congo-attenuated (KK-262) based chimeric virus where the native Congo CD2v/C-type lectin genes were deleted and replaced by those from the heterologous virus, virulent France (F-32). Pigs were vaccinated intramuscularly with either Congo-attenuated (KK-262) or Congo^{France CD2v/Lectin} viruses and then challenged intramuscularly with 10³ HAU of virulent Congo K49. We hypothesized that if homologous ASFV CD2v/C-type lectin proteins are important protective antigens, then animals immunized with Congo^{France CD2v/Lectin} should exhibit reduced or no protection when challenged with virulent Congo K49.

Animals immunized with Congo-attenuated (KK-262) virus demonstrated solid levels of protection when challenged with virulent Congo K49 (Table 1). Survival rates of 80% and 100% were observed for immunized animals in experiments 1 and 2, respectively. Apart from a transient fever response observed in approximately 20% of the animals, clinical signs of ASF were not observed. Viremia was not detected in any of the Congo-attenuated (KK-262) immunized animals post challenge infection (PCI). At the time of challenge, 60% to 100% (experiment 1 and 2, respectively) of the Congo-attenuated (KK-262) immunized animals were ASFV serologically positive by ELISA. An HAI serologic response to Congo virus was not detected in experiment 1 animals but was present in all experiment 2 animals with titers ranging from 1:8 to 1:32.

In contrast, animals immunized with Congo^{France CD2v/Lectin} virus were not protected from challenge with virulent Congo K49 (Table 1). Mortality rates of 80% and 100% were observed for animals in experiments 1 and 2, respectively. Disease onset was rapid with fever and other ASF clinical signs evident at approximately 3 to 4 days PCI. High-titered viremic loads were detectable at 3-5 days PCI and persisted until death of the animals. An HAI serologic response to Congo virus was not observed in any Congo^{France CD2v/Lectin} virus vaccinated animals.

Although mortality rates were similar, ASF disease course for Congo^{France CD2v/Lectin} virus- vaccinated animals was affected when compared to mock-vaccinated animals. Significant delays in viremia onset and magnitude as well as an increased time-to-death (experiment 1) were observed (Table 1).

Notably, these data indicate that the solid protection afforded by immunization with Congo-attenuated (KK-262) was lost when the CD2v/C-type lectin genes present in Congo-attenuated (KK-262) were replaced with those from a heterologous virus, virulent France (F-32). Thus, homologous ASFV CD2v /C-type lectin proteins are necessary for protection from ASFV infection.

Table 1. Homologous CD2/Lectin is necessary for protection against ASFV

Experiment	Mortality (%)		Fever (%)		Viremic Load (genomes/ml) ⁴		Pre-challenge Serology	
	(%)	TTD ¹	(%) ²	TTF ³	3dpi ⁵	Max Load ⁶	ASFV-ELISA ⁷	Congo-HAI ⁸
Experiment 1								
Mock (n=3)	100	5.7 (0.3)*	100	3.0 (-)	6.7e8 (3.1e8)	2.7e9 (2.4e8)**	0	0
Congo ^{France CD2/Lectin} (n=5)	80	12.0 (3.7)*	80	3.5 (0.3)	-	4.4e8 (6.3e7)**	20	0
Congo-attenuated (n=5)	20	12.0 (-)	20	7.0 (-)	-	-	60	0
Experiment 2								
Mock (n=3)	100	7.4 (0.5)	100	3.0 (-)	5.1e8 (1.2e8)	1.4e10 (3.1e9)***	0	0
Congo ^{France CD2/Lectin} (n=7)	100	7.4 (0.4)	100	3.6 (0.2)	6.1e4 (3.0e4)	2.1e9 (1.2e9)***	100	0
Congo-attenuated (n=6)	0	-	17	16.0 (-)	0	-	100	100

¹TTD, average time-to-death in days post-challenge with standard error of the mean (SEM) in parenthesis. Similar asterisks (*) indicate two values significantly different (P>0.05) by equal variance Test, or by Mann-Whitney Rank Sum Test if data failed Shapiro-Wilk normality testing.

²%, percentage of animals developing rectal temperature greater than or equal to 40°C

³TTF, average time-to-fever in days post-challenge with SEM in parenthesis

⁴Viremic load, average viral load in viral genomic copies per milliliter of blood, with SEM in parenthesis

⁵3dpi, average viremic load in animals with viremic signal at three days post-infection

⁶Max load, average maximum load for all animals demonstrating viral load in blood at any day sampled post-challenge

⁷ELISA, % animals developing positive antibody titers using ID Vet ELISA

⁸HAI, % animals developing HAI antibody titers >1:2 against ASFV strain Congo

Immunization with homologous CD2v/C-type lectin proteins affects ASF disease course

To determine if CD2v and C-type lectin proteins are sufficient for mediating ASFV protective immunity, a chimeric ASFVs recombinant at the CD2v/C-type lectin gene locus were constructed and evaluated in vaccination/challenge experiments in pigs. The chimeric virus used in experiment 3 was France^{Congo CD2v/Lectin}, a France-attenuated (FK-32/135) based chimeric virus where the native France CD2v/C-type lectin genes were deleted and replaced by those from the heterologous virus, virulent Congo (K-49). Pigs were vaccinated intramuscularly with either Congo-attenuated (KK-262), France-attenuated (FK-32/135) or France^{Congo CD2v/Lectin}

and then challenged intramuscularly with 10³ HAU of virulent Congo K49. We hypothesized that if homologous ASFV CD2v/C-type lectin proteins are sufficient for mediating ASFV protective immunity, then animals immunized with France^{Congo CD2v/Lectin} should be partially or fully protected when challenged with virulent Congo K49.

As expected, animals immunized with Congo-attenuated (KK-262) virus demonstrated solid levels of protection as observed in experiments 1 and 2 (Table 1) while animals immunized with France-attenuated (FK-32/135) virus were not protected from challenge with virulent Congo K49 (Table 2). A 80% mortality rate was observed for France-attenuated (FK-32/135) immunized animals. Disease onset was rapid with fever and other ASF clinical signs evident at 3 days PCI. High-titered viremic loads were detectable in animals at 3 days PCI and persisted until death of the animals. Pre-challenge HAI titers to Congo virus ranging from 1:4 to 1:16 were observed in all Congo-attenuated (KK-262) immunized animals.

Table 2: Immunization with homologous CD2/Lectin affect ASF disease course

Experiment	Mortality (%)		Fever (%)		Viremic load (genomes/ml) ⁴		Pre-challenge serology	
	(%)	TTD ¹	(%) ²	TTF ³	3dpi ⁵	Max load ⁶	ASFV-ELISA ⁷	Congo-HAI ⁸
Mock (n=3)	100	7.0 (0.0)	100	3.0 (-0.0)	8.7e7 (4.7e7)	6.9e8 (3.3e8)	0	-
France-attenuated (n=5)	80	6.3 (0.5)*	100	3.0 (-0.0)	6.7e7 (6.2e7)	5.4e7 (5.0e7)	100	-
France ^{Congo CD2/Lectin} (n=4)	100	8.0 (0.4)*	100	3.8 (-0.3)	8.5e6 (4.3e6)	8.6e6 (4.3e6)	100	0
Congo-attenuated (n=3)	0	-	0	-	-	-	100	100

¹TTD, average time-to-death in days post-challenge with standard error of the mean (SEM) in parenthesis. Similar asterisks (*) indicate two values that are significantly different (P>0.05) by equal variance T-Test, or by Mann-Whitney Rank Sum Test if data failed Shapiro-Wilk normality testing.

²%, percentage of animals developing rectal temperature greater than or equal to 40.0°C

³TTF, average time-to-fever in days post-challenge with SEM in parenthesis

⁴Viremic load, average viral load in viral genomic copies per milliliter of blood, with SEM in parenthesis

⁵3dpi, average viremic load of animals with viremic signal at three days post-infection

⁶Max load, average maximum load for all animals demonstrating viral load in blood at any day sampled post-challenge

⁷ELISA, % of animals developing positive antibody titers using DVet ELISA

⁸HAI, % of animals developing HAI antibody titers 1:2 against ASFV strain Congo

Immunization with France^{Congo CD2v/Lectin} failed to protect animals from challenge with virulent Congo K49. Rapid disease onset (~ 4 DPC), high titered viremias (detectable at 3 DPC) and a 100% mortality rate were observed (Table 2). An HAI serologic response to Congo virus were not detected in France^{Congo CD2v/Lectin} virus-vaccinated animals prior to challenge. However, the ASF disease course observed for France^{Congo CD2v/Lectin} virus-vaccinated animals differed from that seen in France-attenuated (FK-32/135) virus-vaccinated animals. With France^{Congo CD2v/Lectin} virus-vaccinated animals disease onset was delayed by approximately 1 day, viremia titers were reduced approximately 90% (at 3 DPC) and time to death was significantly increased by approximately 1.5 days when compared to France-attenuated (FK-32/135) vaccinated animals. Since France-attenuated (FK-32/135) and France^{Congo CD2v/Lectin} viruses differ only at the CD2v/C-type lectin gene locus, the partial protective effect observed here against virulent Congo challenge may be due to the homologous Congo CD2v/C-type lectin genes present in France^{Congo CD2v/Lectin}.

Discussion:

Results presented here provide compelling evidence that ASFV CD2v (EP402R) and C-type lectin (EP153R) proteins are necessary for homologous protective immunity (Table 1) and thus significant ASFV protective antigens. Further, the fact that the solid protection afforded by immunization with Congo-attenuated (KK-262), a serogroup 2 virus, was lost when the Congo CD2v/C-type lectin genes were replaced with those from a virulent France strain (F32), a serogroup 4 virus, directly supports the emerging concept that ASF protective immunity is HAI serotype-specific (Balyshev *et al.*, 1995; Sereda & Balyshev, 2011; Sereda *et al.*, 1992; Vishnjakov *et al.*, 1991; and Malogolovkin *et al.*, unpublished data).

Results from experiments 1 and 2 also suggest a lesser role for other, yet to be identified, ASF viral proteins in inducing protective host responses. ASF disease course for Congo^{France CD2v/Lectin} virus-vaccinated animals was affected when compared to mock-vaccinated animals. Delays in viremia onset and magnitude, as well as an increased time to death (experiment 1), were observed in the Congo^{France CD2v/Lectin} virus-vaccinated group (Table 1).

While we have shown that ASFV CD2v and C-type lectin proteins are necessary for homologous protective immunity, our efforts to determine if these proteins alone are sufficient for mediating protection were inconclusive (Table 2). We hypothesized if homologous ASFV CD2v/C-type lectin proteins are sufficient for mediating ASFV protective immunity, then animals immunized with France^{Congo CD2v/Lectin} should be protected when challenged with virulent Congo K49. Immunization with France^{Congo CD2v/Lectin}, using the protocol described for Experiment 3, failed to protect animals from lethal infection when challenged with virulent Congo K49. However, there were indications of a partial protective effect (increased time to disease onset, decreased viremic loads and increased time to death) for France^{Congo CD2v/Lectin} virus-immunized animals when compared to animals immunized with France-attenuated (FK-32/135). As France-attenuated (FK-32/135) and France^{Congo CD2v/Lectin} differ only at the CD2v/C-type lectin gene locus, Congo CD2v/C-type lectin proteins are directly implicated in mediating this altered disease course.

The presence of a ASFV HAI serologic response in animals appears to correlate with protection from severe/lethal infection (Ruiz-Gonzalvo et al., 1993; Zsak and Rock, 2010 and experiments described here). Notably, the lack of a pre-challenge HAI serological response to Congo virus in France^{Congo CD2v/Lectin} virus-immunized animals may be highly significant for the lack of complete protection observed here. It is known that ASFV HAI antibodies appear late and at relatively low titer during infection and that virulence of the infecting viral strain may influence HAI antibody production (Malmquist, 1963; Ruiz Gonzalvo *et al.*, 1986; Ruiz Gonzalvo & Coll, 1993; Vigario *et al.*, 1970). Conceivably, the immunization schedule used here and/or the France-attenuated (FK-32/135) strain used to express the Congo CD2v/C-type lectin genes were not adequate to obtain HAI responses in France^{Congo CD2v/Lectin} immunized pigs. Further research is needed to optimize the immunizing potential of CD2v/C-type lectin proteins to obtain HAI titers at level comparable to those observed in immune protected animals.

In summary, we demonstrate that two ASFV proteins, CD2v and C-type lectin, are necessary for homologous protection against ASFV. These viral proteins represent significant protective antigens for ASFV that should be further evaluated and targeted for vaccine design and development.

References

- Balyshev, V. M., Fedorishhev, I. V. & Salina, M. V. (1995). Study of serotype interactions of ASF virus strains both in vitro and in vivo. In *Virusniye bolezni zhivotnikh*, p. 230. Vladimir.
- Balyshev, V. M., Kalantaenko, J. F. & Zhoukov, A. N. (2010). Immunobiological and molecular-genetic characteristics of ASF virus isolates detected in the RF. In *Orientirovannyye i fundamentalniye issledovaniya i ih realizatsiya v agropromishlennom komplekse Rossii: materialy Vserossiyskoy nauchnoy konferentsii*, pp. 94-98.
- Coggins, L. (1968). A modified hemadsorption-inhibition test for African swine fever virus. *Bull Epizoot Dis Afr* 16, 61-64.
- Coggins, L. (1974). African swine fever virus. Pathogenesis. *Prog Med Virol* 18, 48-63.
- Cubillos, C., Gomez-Sebastian, S., Moreno, N., Nunez, M. C., Mulumba-Mfumu, L. K., Quembo, C. J., Heath, L., Etter, E. M., Jori, F., Escribano, J. M. & Blanco, E. (2013). African swine fever virus serodiagnosis: a general review with a focus on the analyses of African serum samples. *Virus Res* 173, 159-167.

- Detray, D. E. (1957). Persistence of viremia and immunity in African swine fever. *Am J Vet Res* 18, 811-816.
- Finney, D. J. 1978. In *Statistical methods in biological assays*, 2nd ed., p. 394–398. Macmillan Publishing Co., New York, N.Y.
- Gallardo, C., Soler, A., Nieto, R., Carrascosa, A. L., De Mia, G. M., Bishop, R. P., Martins, C., Fasina, F. O., Couacy-Hymman, E., Heath, L., Pelayo, V., Martin, E., Simon, A., Martin, R., Okurut, A. R., Lekolol, I., Okoth, E. & Arias, M. (2013). Comparative evaluation of novel African swine fever virus (ASF) antibody detection techniques derived from specific ASF viral genotypes with the OIE internationally prescribed serological tests. *Vet Microbiol* 162, 32-43.
- Gogin, A., Gerasimov, V., Malogolovkin, A. & Kolbasov, D. (2013). African swine fever in the North Caucasus region and the Russian Federation in years 2007-2012. *Virus Res* 173, 198-203.
- Gomez-Puertas, P., Rodriguez, F., Oviedo, J. M., Brun, A., Alonso, C. & Escribano, J. M. (1998). The African swine fever virus proteins p54 and p30 are involved in two distinct steps of virus attachment and both contribute to the antibody-mediated protective immune response. *Virology* 243, 461-471.
- King, K. et al. 2011. Protection of european domestic pigs from virulent African isolates of African swine fever virus by experimental immunization. *Vaccine* 29: 4593-4600.
- Leitao, A., Cartaxeiro, C., Coelho, R., Cruz, B., Parkhouse, R. M., Portugal, F., Vigario, J. D. & Martins, C. L. (2001). The non-haemadsorbing African swine fever virus isolate ASFV/NH/P68 provides a model for defining the protective anti-virus immune response. *J Gen Virol* 82, 513-523.
- Lewis, T., Zsak, L., Burrage, T. G., Lu, Z., Kutish, G. F., Neilan, J. G. & Rock, D. L. (2000). An African swine fever virus *ERV1-ALR* homologue, *9GL*, affects virion maturation and viral growth in macrophages and viral virulence in swine. *J Virol* 74, 1275-1285.
- Malmquist, W. A. (1963). Serologic and immunologic studies with African swine fever virus. *Am J Vet Res* 24, 450-459.
- Malogolovkin, A., Burmakina, G., Tulman E.R., Delhon, G., Diel, D.G., Salnikov, N., Kutish, G. F., Kolbasov, D. and Rock, D. L. 2014. African swine fever virus CD2v and C-type lectin gene loci mediate serologic specificity. *J. Gen. Virol.* Dec 18. pii: jgv.0.000024. doi: 10.1099/jgv.0.000024.
- Mebus, C. A. (1988). African swine fever. *Adv Virus Res* 35, 251-269.
- Montgomery, R. E. (1921). On a form of swine fever occurring in British East Africa (Kenya Colony). *J Comp Pathol* 34, 159-191; 243-262.
- Moore, D.M., Zsak, L., Neilan, J.G., Lu, Z. and Rock, D.L. 1998. The African swine fever virus thymidine kinase gene is required for efficient replication in swine macrophages and for virulence in swine. *J Virol* 72: 10310-10315.
- Oura, C.A.L., Denyer, M.S., Takamatsu, H. and Parkhouse, R.M.E. 2005. In vivo depletion of CD8+ T lymphocytes abrogates protective immunity to African swine fever virus. *J Gen Virol* 86: 244.

- Pan, I. C., Trautman, R., Hess, W. R., DeBoer, C. J., Tessler, J., Ordas, A., Botija, C. S., Ovejero, J. & Sanchez, M. C. (1974). African swine fever: comparison of four serotests on porcine serums in Spain. *Am J Vet Res* 35, 787-790.
- Ruiz, G. F., Carnero, M. E. & Bruyel, V. (1981). Immunological responses of pigs to partially attenuated ASF and their resistance to virulent homologous and heterologous viruses. In *FAO/CEC Expert Consultation in ASF Research*, pp. 206-216. Edited by P. J. Wilkinson. Rome.
- Ruiz Gonzalvo, F., Carnero, M. E., Caballero, C. & Martinez, J. (1986). Inhibition of African swine fever infection in the presence of immune sera in vivo and in vitro. *Am J Vet Res* 47, 1249-1252.
- Ruiz-Gonzalvo, F., et al. 1993. Characterization of a soluble hemagglutinin induced in African swine fever virus-infected cells. *Virology* 196: 769-777.
- Ruiz Gonzalvo, F., Rodriguez, F. & Escribano, J. M. (1996). Functional and immunological properties of the baculovirus-expressed hemagglutinin of African swine fever virus. *Virology* 218, 285-289.
- Sanchez-Vizcaino, J. M., Mur, L. & Martinez-Lopez, B. (2013). African swine fever (ASF): five years around Europe. *Vet Microbiol* 165, 45-50.
- Sereda, A. D., Solovkin, S. L., Fugina, L. G. & Makarov, V. V. (1992). [Immune reactions to the African swine fever virus]. *Vopr Virusol* 37, 168-170.
- Sereda, A. D. & Balyshev, V. M. (2011). [Antigenic diversity of African swine fever viruses]. *Vopr Virusol* 56, 38-42.
- Vigario, J. D., Terrinha, A. M., Bastos, A. L., Moura Nunes, J. F., Marques, D. & Silva, J. F. (1970). Serological behaviour of isolated African swine fever virus. Brief report. *Arch Gesamte Virusforsch* 31, 387-389.
- Vigario, J. D., Terrinha, A. M. & Moura Nunes, J. F. (1974). Antigenic relationships among strains of African swine fever virus. *Arch Gesamte Virusforsch* 45, 272-277.
- Vishnjakov, I., Mitin, N., Karpov, G., Kurinnov, V. & Jashin, A. (1991). Differentiation African and classical swine fever viruses. *Veterinariya* 4, 28-31.
- Vishnjakov, I. F., Mitin, N. I. & Petrov, J. I. (1995). Seroimmunological classification of African swine fever virus natural isolates. In *Actualniye voprosi veterinarnoy virusologii Materiali naucho-practicheskoy konferentsii VNIIVViM "Klassicheskaya chuma sviney neotlozhniye problemi nauki a praktiki"*, pp. 141-143. Pokrov.
- Zsak, L., Lu, Z., Kutish, G. F., Neilan, J. G. & Rock, D. L. (1996). An African swine fever virus virulence-associated gene NL-S with similarity to the herpes simplex virus ICP34.5 gene. *J Virol* 70, 8865-8871.
- Zsak, L., Caler, E., Lu, Z., Kutish, G.F., Neilan, J.G. and 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.
- Zsak, L., Borca, M. V., Risatti, G. R., French, R. A., Lu, Z., Kutish, G. F., Neilan, J. G., Callahan, W. M. and Rock, D. L. 2005. Preclinical Diagnosis of Africa Swine Fever in Contact Exposed Swine by a Portable Real-Time PCR Assay. *J. Clin. Micro.* 43(1) 112-119.

Zsak, L., Burrage, T.G., Beilan, J.G., Lu, Z., Kutish, G.F., and Rock, D.L. 2010. African swine fever virus cd2-like protein mediates monocyte infection-inhibition. XVII International Poxvirus, Asfivirus, and Iridovirus Symposium. Sedona, Arizona. June 5-10: P7.19.