Immunisation of pigs by DNA prime and recombinant vaccinia virus boost to identify and rank African swine fever virus immunogenic and protective proteins

Running Title: African swine fever virus immunogenic proteins

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Abstract

African swine fever virus (ASFV) causes an acute haemorrhagic fever in domestic pigs with high socio-economic impact. No vaccine is available limiting options for control. Although live attenuated ASFV can induce up to one hundred percent protection against lethal challenge, little is known of the antigens which induce this protective response. To identify additional ASFV immunogenic and potentially protective antigens we cloned 47 viral genes in individual plasmids for gene vaccination and in recombinant vaccinia viruses. These antigens were selected to include proteins with different functions and timing of expression. Pools of up to 22 antigens were delivered by DNA prime and recombinant vaccinia virus boost to groups of pigs. Responses of immune lymphocytes from pigs to individual recombinant proteins and to ASFV were measured by interferon gamma ELISpot assays to identify a subset of the antigens that consistently induced the highest responses. All 47 antigens were then delivered to pigs by DNA prime and recombinant vaccinia virus boost and pigs were challenged with a lethal dose of Georgia 2007/1 ASFV isolate. Although pigs developed clinical and pathological signs consistent with acute ASFV, viral genome levels were significantly reduced in blood and several lymph tissues in those pigs immunised with vectors expressing ASFV antigens compared with control pigs.
Importance

The lack of a vaccine limits the options to control African swine fever. Advances have been made in the development of genetically modified live attenuated ASFV that can induce protection against challenge. However there may be safety issues relating to the use of these in the field. There is little information about ASFV antigens that can induce a protective immune response against challenge. We carried out a large screen of 30% of ASFV antigens by delivering individual genes in different pools to pigs by DNA immunization prime and recombinant vaccinia virus boost. The response in immunized pigs to these individual antigens was compared to identify the most immunogenic. Lethal challenge of pigs immunised with a pool of antigens resulted in reduced levels of virus in blood and lymph tissues compared to pigs immunised with control vectors. Novel immunogenic ASFV proteins have been identified to test further as vaccine candidates.
African swine fever virus (ASFV) causes an acute haemorrhagic fever, African swine fever (ASF), with high mortality in domestic pigs and has severe socio-economic consequences in affected countries. Currently the disease is endemic or causes sporadic outbreaks in most sub-Saharan African countries. Outside Africa, ASF has been endemic in Sardinia since 1978. Following the introduction of ASF to Georgia in 2007, the disease spread to Eastern Europe including the Russian Federation, Belarus, Ukraine, Lithuania, Poland, Latvia, Estonia, and Moldova (OIE WAHID). The lack of a commercially available vaccine limits disease control options to reliance on rapid detection and implementation of quarantine and slaughter. The complexity of the virus, which encodes 150 to 167 proteins, contributes to the difficulty in obtaining an effective vaccine.

It is well established that pigs that recover from infection with attenuated ASFV isolates can be protected against challenge with related virulent viruses (1-4). Immunisation of pigs with attenuated strain OURT88/3 induces protection against lethal challenge that is dependent on CD8+ cells since protection was abrogated by depletion of this cell subset (5). In pigs several different sub-classes of CD8+ cells have been defined. These include CD8 single positive cells and CD4/CD8 double positive subsets which are perforin positive and have SLA I restricted cytotoxic activity. In addition CD8 is also expressed on additional cell subsets including NK cells (6). Although the depletion studies did not provide information on which CD8+ cell sub-set was important for protection, additional studies have shown a correlation between induction of the CD4+CD8+, perforin+ cytotoxic T lymphocytes (CTL) with protection induced by the attenuated OURT88/3 strain (6). However, protection induced by the attenuated strain NHP/68 has been correlated with high levels of NK cells (4). A role for antibodies is also indicated since passive transfer of antibodies from immune to naïve pigs can induce partial protection (7).
Although live attenuated ASFV strains can induce protection of up to 100% they can cause adverse reactions and may have other safety issues. Vaccines which achieve protection by delivery of one or more virus antigens would have advantages in terms of safety but knowledge of the protective antigens is required. Attempts to induce protection against lethal ASFV challenge by immunisation of pigs with recombinant proteins or by DNA vaccination has had partial success. In one study recombinant proteins p54 and p72 induced partial protection against lethal challenge, although in another study no protection was observed with these proteins despite the induction of neutralising antibodies (8-10). Recombinant ASFV hemagglutinin or CD2v protein (EP402R gene) has also induced partial protection of pigs (11). Partial protection of 30 to 50% of immunised pigs has also been achieved by DNA vaccination with a fusion of the p54 and p30 proteins to the extracellular domain of the CD2v protein, or by immunisation with an expression library (12-14). However, other ASFV proteins are likely to have the potential to be protective, but have not been systematically investigated.

In the current study we carried out a screen of 47 ASFV encoded antigens to determine which induced cellular responses and to assess their potential for inducing protection. These were selected to include proteins with different functions and timing of expression. To achieve this we constructed 3 different ASFV libraries, a gene library for DNA vaccination (15-17), a recombinant vaccinia virus (rVACV) library (18-20) and a library for expression and capture of individual recombinant proteins using an *E. coli in vitro* transcription and translation system. We immunised pigs with pools of these ASFV antigens delivered by a DNA prime and recombinant vaccinia virus boost and ranked the immune responses to the individual captured proteins. Pigs immunised with this pool of antigens had lower levels of virus in blood, tonsil, spleen and submandibular lymph node compared to those immunised with control antigens following challenge with a lethal dose of ASFV.
Materials and Methods

Construction and testing of expression libraries

Sequence data of the Georgia 2007/1 ASFV isolate (Accession number, FR682468) was used to design primers for amplification of ASFV genes shown in Table 1. The PCR fragments were cloned into the plasmid vector pCMVi-LS (15) which includes a cytomegalovirus promoter for expression in mammalian cells upon DNA vaccination and a signal peptide for cell secretion. The same genes, fused to a C-terminal HA epitope, were inserted into the thymidine kinase locus of modified NYVAC replication competent vaccinia virus (18) under control of a vaccinia virus promoter. Expression of a protein of the expected size was confirmed by Western blot analysis of infected cell extracts and probing with anti-HA antibody (not shown). The same ASFV genes were also prepared as PCR-generated template DNAs for thioredoxin-tagged protein expression and capture using an E. coli-based in vitro transcription translation system (see below).

Production of DNA templates and antigen capture by in vitro transcription translation

Linear expression elements (LEEs; (21)) were used as template DNA for the in vitro transcription translation reactions. LEEs were prepared as described previously (16) except using either iProof High-Fidelity (Bio-Rad) or Pfu Turbo (Agilent) DNA polymerase plus template purification with the QIAquick PCR Purification Kit (Qiagen). These LEEs allowed expression of each antigen as a fusion with an N-terminal thioredoxin tag and a C-terminal His5 tag.

The in vitro transcription translation reactions were 200 µL total volume. To a Protein LoBind tube (Eppendorf cat. no. 022431081) was added 50 µL of Dynabeads M-280
tosylactivated magnetic beads (Invitrogen cat. no. 142.03). The beads were washed three times in 2.4 M ammonium sulfate, 1 M boric acid, pH 9.5 (Buffer A), and then coated with antibody overnight at 37°C with shaking at 1000 rpm (Eppendorf Thermomixer) in a combined 30 µL of Buffer A and 30 µL of anti-thioredoxin mouse IgG2a monoclonal antibody (GenScript cat. no. A00180). The supernatant was removed and verified to contain <0.05 mg/mL IgG as determined by absorbance at 280 nm. The beads were blocked in 2 mL of 0.5% bovine serum albumin in PBS (10 mM Na$_2$HPO$_4$, 1.76 mM KH$_2$PO$_4$, 136 mM NaCl, 2.7 mM KCl, pH 7.4) for 1 hour at 37°C and 1000 rpm, washed three times with PBS, and suspended in PBS using the above-determined volume. A 50 µL volume of these beads was added per well to a 96-well plate (1.2 mL per square well, U-bottomed, with lid, ABgene cat. no. AB-1127) and the supernatant removed. To the beads was added 100 µL of Starter Reaction as previously described (16) and containing 1 µg of LEE template DNA. The reaction proceeded as described (16), including addition of 100 µL of Feed Reaction (yielding a Total Reaction volume of 200 µL), washes in PBS, and storage at -20°C. Antigen yields were determined by phosphorimager analysis following SDS-PAGE (as described (16), except with sample heating at 85°C) from parallel reactions containing $^{35}$S-methionine (Supplemental Figure 1). Twenty-eight of the 47 antigens were obtained in full-length form. The remaining 19 antigens that could not be efficiently generated as full-length products were expressed as fragments that, when combined, encompassed the full-length forms (Supplemental Figure 1).

**African swine fever viruses and cells**

The Georgia 2007/1 isolate (22) was grown in primary macrophages from pig bone marrow. Virus stocks for pig inoculation were prepared from a spleen suspension from an infected pig and titres were determined by limiting dilution using haemadsorption to detect virus infected cells and titres were calculated by the Spearman-Karber method (23).
**Pig immunisation and challenge**

Pigs were either outbred cross-bred Large White and Landrace from a high health status farm or inbred Babraham pigs (24) from a herd kept at The Pirbright Institute. Pigs were of average size 15 kg at the start of experiments. Plasmid DNA was delivered in DNA gold micronanoplex bullets by gene gun (16) by 5 non-overlapping shots to the ear-pinnae of pigs, at a pressure of 450 psi. Plasmid DNA was delivered by a double prime one day apart to alternate ears, and by a double boost two weeks later. CpG oligonucleotide was also bound to the bullets to act as an adjuvant for porcine TLR3 and 4. The total amount of DNA administered was 10 μg per shot. As controls, pigs were vaccinated in the same way with the same dose of DNA in a pool of 3 negative control plasmids.

Recombinant vaccinia viruses (rVACVs) were delivered by scarification or by using a needleless delivery device at four different sites with 100 μl total volume at each site. rVACVs were delivered to pigs 4 weeks after the last DNA boost in experiment 1. In experiment 2 pigs were boosted at 3 weeks and 5 weeks with rVACV after the last DNA immunisation. Each rVACV was present at 10⁶ pfu for each vaccination. As controls, pigs were vaccinated with a pool of rVACV expressing irrelevant antigens. Pigs were challenged intramuscularly with 10⁴ HAD₅₀ Georgia 2007/1 virus two weeks after the last rVACV boost. Pigs were observed for development of clinical signs and these were scored using the scoring system used previously (25). Blood and tissue samples were collected to measure levels of virus replication.

**Binding of DNA to gold particles**

Target genes from ASFV and negative control genes (influenza hemagglutinin [HA]; human α1-antitrypsin [AAT]; and HIV envelope gp120) were cloned into the immunization vector pCMVi-LS (15). DNA plasmids were loaded on DNA-gold micronanoplexes as previously
described (15, 16) with the following specifications. The amount of DNA per bullet was 5 µg of pooled antigens, divided equally across the antigens, plus 5 µg of CpG adjuvant (Invitrogen oligonucleotide 5'-ggTGCATCGATGCAGgggggG-3' where g indicates phosphorothioated nucleotides). Prior to preparation of DNA-nanogold (16), the combined DNA, without CpG, was precipitated in 0.3 M sodium acetate, pH 5.5, and 70% ethanol. The DNA pellet was washed with 70% ethanol, dried 15 minutes at room temperature, and dissolved in water.

Quantitative PCR

ASFV DNA was detected in blood and tissues using qPCR as described previously (25, 26). The results were determined as genome copies per ml of blood or per mg of tissue.

Analysis of immune responses against ASFV and against individual recombinant proteins

Development of T cell immune responses to ASFV was analysed by interferon gamma (IFNγ) ELISpot and proliferation assays as described previously (27). The ASFV Georgia isolate used in the assay was grown in porcine bone marrow cells and 10^5 HAD_{50} was added per well. Individual thioredoxin-tagged antigens were produced by transcription and translation in vitro in an E. coli system and captured by anti-thioredoxin antibody bound to magnetic beads. The beads with antigen attached were suspended in RPMI 1640 medium and approximately 2.5 µg of in vitro translated protein was added to individual wells in 96 well ELISpot plates. Immune lymphocytes from immunised pigs (6 × 10^5 cells per well) were added and incubation carried out overnight at 37°C before detection of IFNγ producing cells. The development of ASFV specific antibodies was measured using a competition ASFV ELISA (INGENASA PPA3 COMPPAC). ELISAs were performed as described (13) with the following specifications. Per well, 0.1 µg of in vitro translated protein was used; pig serum
was used at 1:500 dilution; well washes were performed in PBS with 0.05% Tween-20 and
with the ELISA plate on top of a magnetic rack; and detection was with TMB (3,3',5,5'-
tetramethylbenzidine) substrate plus HCl and measurement at a wavelength of 450 nm. Total
IgG responses used HRP-conjugated goat anti-swine IgG (H+L) secondary antibody at
1:3000 dilution. Secondary antibodies for IgG1 and IgG2 responses were HRP-conjugated
mouse anti-pig IgG1 (clone K139 3C8) and IgG2 (clone K68 Ig2) monoclonal antibodies at
1:100 dilution.

Results

Delivery of ASFV genes pools by DNA prime and recombinant vaccinia virus boost to
rank immune responses in Babraham pigs [Experiment 1].

ASFV antigens were selected to represent proteins of different functions, timing of
expression and predicted cellular localization. These functional classes included enzymes
involved in virus genome replication and transcription, proteins with roles in immune
evasion, virion structural proteins, membrane proteins and proteins of unknown function. The
larger proteins were cloned in several fragments to facilitate their expression. This approach
was taken to represent the diversity of antigens expressed during an ASFV infection. These
47 genes or gene fragments were cloned in vectors for gene immunization, expression in
recombinant vaccinia virus vectors and for recombinant protein expression by in vitro
transcription and translation (see Materials and Methods). In the first experiment inbred
Babraham pigs were immunized to compare and rank the responses to individual antigens.
Four groups of pigs were immunized by DNA prime and rVACV boost as described in
Methods with 4 weeks between the final DNA vaccination and rVACV boost. Group 1
contained 6 pigs immunized with a set of 20 ASFV antigens comprising different functional
classes (labelled Set 1; Table 1) plus p30 (CP204L) and VP72 (B646L), two well
characterized immunogenic ASFV proteins. Group 2 contained 6 pigs immunized with another set of 20 ASFV antigens of different functional classes (labelled Set 2; Table 1) plus p30 (CP204L) and VP72 (B646L). Group 3 contained 5 pigs immunized with 10 ASFV antigens which are known or predicted to be present on the surface of the intracellular mature or extracellular ASFV virion particles (Table 1), in addition to p30 (CP204L) and VP72 (B646L). These were selected as proteins likely to be important for induction of antibody responses. Group 4 contained 5 pigs and these were immunized with p30 (CP204L) and VP72 (B646L) alone (Table 1). The reason for including the p30 (CP204L) and VP72 (B646L) antigens in each group was to determine if the size and composition of the antigen pool influenced the immune response to these proteins.

Cellular immune responses to the antigens were analyzed by stimulating PBMCs from immunized pigs with recombinant individual ASFV proteins produced by \textit{in vitro} transcription and translation, or with ASFV Georgia 2007/1 isolate. Responses were measured as numbers of IFN$\gamma$ producing cells by ELISpot assays. Figure 1 shows results from IFN$\gamma$ ELISpot assays performed with lymphocytes from the 6 pigs in Group 1 immunized with the 20 antigens of Set 1. The level of response in different pigs after stimulation with whole ASFV Georgia 2007/1 virus varied between 37 and 132 IFN$\gamma$ producing cells per million cells (Figure 1) and the background response to media alone varied between 13 and 35% of this value (Figure 1). A number of individual antigens induced a greater number of IFN$\gamma$ producing cells than whole virus and the antigens which induced a response of at least two-thirds of that induced by Georgia 2007/1 in at least four of the six pigs. Antigen 127 (p30) induced the greatest number of IFN$\gamma$ producing cells from five of the 6 pigs in Group 1, varying from 145 to 422% compared to 100% induced by ASFV Georgia 2007/1 (Figure 1). The response for antigen 070 (F317L) varied from 96 to 334% of the response compared to whole virus. Antigen 052 (MGF505-4R) induced from 89
to 261% of the response observed following stimulation of cells with whole virus. Eight antigens, 004 (MGF360-11L), 052 (MGF505-4R), 070 (F317L), 074b (F1055L), 111 (B602L), 128 (CP530R), 167 (E199L), 127 (CP204L/p30) induced responses greater than 70% of that induced by ASFV Georgia 2007/1 in all 6 pigs. A further 4 antigens 002 (MGF360-11L), 053 (MGF505-4R), 054 (MGF505-5R) and 122 (G1211R) induced a level of response of 70% that of the Georgia 2007/1 ASFV in cells from 3 or more of the pigs.

For Group 2 immunized with the 20 antigens of Set 2 (Table 1), the IFNγ response of cells from 3 pigs to the individual antigens were analyzed (Figure 2) and antigens which induced a response at least two-thirds of that induced by whole virus in all three pigs are shown in Table 3. Antigen 127 (p30) induced the highest response in all 3 pigs ranging from 201 to 249% of that induced by ASFV Georgia 2007/1. Antigen 084 (EP364R) also induced high responses in all pigs ranging from 108 to 174% that of ASFV Georgia 2007/1 (Table 3).

Seven antigens, 084 (EP364R), 123 (G1211R), 124 (G1211R), 126a (CP2475L), 133a (NP1450L partial), 163 (E183L), 127 (CP204L/p30) induced responses greater than 70% that of ASFV Georgia 2007/1 strain in all three pigs (Figure 2).

Cellular immune responses were compared, using IFNγ ELISpot assays, in pigs from groups 1, 2 and 4 to the antigens 127 (CP204L/p30) and 113 (B646L/p72) to evaluate if the responses varied when antigen sets varied in content and size. As expected, cells from pigs in Group 4 immunized with antigens 127 (CP204L/p30) and 113 (B646L/p72) alone responded less well to whole virus compared to cells from pigs in Groups 1 and 2 that were immunized with antigen sets of 20 plus 127 and 113. After the response to media alone was subtracted, the responses to antigens 113 and 127 in Group 4 varied between 37 and 147 or 34 and 334 spots per million cells, respectively. The responses to the two antigens in the three different groups were not significantly different (One-way ANOVA; Figure 3). This showed that
differences in the composition or the size of the pool did not affect the cellular immune response to two well-characterised ASFV antigens CP204L/p30 and B646L/p72.

The antibody response to antigen 127 (CP204L/p30) was analyzed by ELISA using sera from the immunized pigs in Groups 1 to 4 (Figure 4). The results showed that responses to p30 were consistently high in all pigs from all groups and therefore that the size of the antigen pool had not affected antibody responses to this individual protein. Analysis of the isotype of antibodies induced identified both IgG1 and IgG2 antibodies indicating a broad isotype spectrum (Figure 5). Antibody responses to individual antigens in Group 3 were compared by ELISA for one of the pigs (pig 395, Figure 6). This showed that following immunization, antibody response to individual antigens varied. Responses to antigen 127 (CP204L/p30) were highest and approximately 3 to 4 fold above background levels. Other antigens showing a greater than two-fold increase in response included antigen 194 (L10L) 145 (D117L) and 205 (EP153R). Neutralizing antibodies were not detected in sera from any of the pigs (data not shown).

Immunisation of pigs with a pool of 47 ASFV genes and effect on ASFV challenge (Experiment 2)

The experiment described above indicated that good cellular and antibody responses had been induced in pigs following immunisation by a DNA prime/rVACV boost and that delivery of the antigens in larger pools had not caused a great reduction in response to individual antigens. We therefore carried out a second experiment in which pigs where challenged with a lethal dose of ASFV after the DNA prime and rVACV boost immunisation. The previous experiments had shown that the p30 protein was very immunogenic and induced strong antibody and cellular responses. We considered it possible that including this antigen may direct the immune responses to p30 rather than more broadly to other potentially protective
antigens. Therefore we immunised pigs with pools of DNA and rVACV expressing ASFV
310 antigens containing or lacking the p30 protein.

Three groups of 6 Babraham pigs were immunised. Group A pigs were immunised with DNA
312 and rVACV expressing all of the previously analysed 47 ASFV antigens (Table 1), Group B
313 with all ASFV antigens except for that encoding CP204L/p30 (Table 1) and Group C only
314 with control antigens gp160, AAT and HA (see Methods). The regime for immunisation was
315 a double prime with DNA plasmids (10 µg total) and CpG adjuvant and 2 weeks later a boost
316 with DNA (10 µg total) and CpG adjuvant. Pigs were boosted with rVACVs 3 weeks later
317 with a second boost 2 weeks after that. Three weeks after the final rVACV boost, pigs were
318 challenged intramuscularly with 10⁴ HAD₅₀ ASFV Georgia 2007/1 and the development of
319 clinical signs was recorded. Blood samples were collected at days 0, 3 days post-challenge
320 and at termination for measurement of virus load. Tissue samples were collected at post-
321 mortem. Pigs in all 3 groups developed clinical signs on day 3 post-challenge and were
322 euthanized on days 5 or 6 when they reached the moderate severity humane end-point for the
323 experiment (see Figure 7). Statistical analysis by 2 way Anova of mean clinical scores per
324 group showed scores were significantly higher in groups A and B compared to control group
325 C (P= 0.0001) on days 2, 3 and 4 post-challenge. This suggests a possible immune
326 enhancement of disease in those pigs immunized with vectors expressing ASFV antigens
327 although the mechanism is unknown.

Post-mortem examination showed that all pigs had signs typical of the early stages of acute
329 ASF disease including signs of haemorrhage and enlargement in spleen and lymph nodes.

The virus genome load in tissues and blood was measured using qPCR (Figure 8). This
331 showed a significant reduction (One-way ANOVA) in virus load in blood at day 3 post-
332 challenge, and in the spleen, tonsil and sub-mandibular lymph nodes in pigs in Groups A and
B which were immunized with pools of DNA and rVACVs expressing ASFV antigens compared to pigs immunized with the control antigens in Group C. In fact, viral load was 10 to 100 fold lower in the submandibular lymph node, tonsils and blood of the pigs in Groups A and B when compared to the controls, and viral load was reduced to undetectable levels in the spleens of Group A and B. However in the gastro-hepatic and mesenteric lymph nodes similar levels of virus genome were detected in samples from all three groups of pigs (Figure 7). These results indicate that clearing of virus had occurred in some tissues and blood by the day the pigs were killed.

Discussion

In this study we aimed to identify immunogenic and potentially protective antigens encoded by ASFV for future investigation as targets for vaccine development. In addition, the identification of immunogenic proteins would provide additional biomarkers for serological diagnosis. The development of a safe and efficacious vaccine for ASFV would have a high economic impact since currently no vaccine is available and disease control relies on implementation of quarantine and mass slaughter of exposed pigs.

Previously, expression library immunisation has been used to identify immunogenic and candidate protective antigens encoded by genomes of complex pathogens. This approach has several advantages since it does not require prior knowledge of the protective antigens and it may identify potentially protective antigens that are either not exposed or are expressed at low levels during infection with the pathogen. Here we examined an expression library comprised of approximately 30% of the full ASFV genome. These antigens were selected to include virion or cell surface proteins based on established or predicted cellular locations, or known immunogenicity. In addition, virus proteins of other functional classes or expression times were selected. This approach mimicked the profile of proteins expressed during ASFV
infection and included a number of highly conserved enzymes. The cloning strategy for expression library construction has varied between random cloning of different sized fragments to the cloning of intact ORFs if sufficient sequence data is available (28).

Our approach was to use DNA prime and recombinant vaccinia virus boost for delivery of complete or large fragments of ASFV genes to pigs. This dual modality of delivery has been demonstrated to enhance the immune response to the expressed transgenes and to be effective in inducing memory cytotoxic T cell responses as well as inducing antibody responses. The replication competent modified NYVAC vaccinia virus strain was selected for this purpose since it has been shown to have a good immunogenicity and safety profile (18). To provide flexibility in screening of different antigens, the ASFV genes were individually cloned in both a DNA vector and rVACV and were then delivered in pools to pigs. The antigens selected included the previously described immunogenic proteins discussed in the introduction in addition to a further 6 antigens which were known to be expressed on the surface of the intracellular mature or extracellular enveloped ASFV particles or on the surface of infected cells. These included KP177R, an early membrane protein, a KP177R related protein L10L, and structural proteins B438L, D117L, O61R, and E120R (32). These antigens were selected as potentially important for induction of a protective antibody response or for use as serological markers of infection. An additional 30 ASFV antigens were selected semi-randomly to represent genes of different functional classes, including enzymes involved in replication, immune evasion proteins, multigene family members, or proteins of unknown function. In total 47 whole or partial ASFV genes were screened.

In a first experiment we screened the immune responses to individual antigens delivered in pools of 22 (Groups 1 and 2, Table 1) by DNA prime and rVACV boost. The antigens p30 and p72 were included in each pool but otherwise the pools were not overlapping. Cellular
immune responses to individual proteins were assayed by stimulation of lymphocytes from immunised pigs with recombinant proteins produced by *in vitro* translation in an *E. coli* system and detection of the numbers of responding cells by IFN-γ ELISpot assay. By this approach we ranked the antigens according to the relative levels of response induced and consistency of response between different pigs. In both groups of pigs the strongest response in all but one of the pigs was to antigen 127 (CP204L/p30). Other antigens in Set 1 which consistently induced high responses included antigens 070 (F317L) 052 (MGF50-4R), 004 (MGF360-11L), 074b (F1055L), 111 (B602L), 128 (CP530R), and 167 (E199L). In Set 2, antigens 084 (EP364R), 123 (G1211R), 124 (G1211R), 126a (CP2475L), 133a (NP1450L partial), and 163 (E183L) consistently induced strong responses.

The phenotype of the IFNγ producing cells stimulated by antigens was not further characterised. However it is expected that they would include predominantly CD4+ T cells but could also include CD8+ T cells if the recombinant antigen was taken up in antigen presenting cells and processed for presentation of peptides with SLA I. It is known that CP204L/p30 is a highly immunogenic protein and stimulates a strong antibody response during ASFV infection. CP204L/P30 has also been shown to contain epitopes that stimulate CTL responses. The other top-ranked antigens have not previously been identified as proteins against which antibody or cellular immune responses are directed. Antibody responses induced against the individual proteins were tested by ELISA for a subset of antigens selected as known virus or cell surface proteins. The antibody response to p30 protein was highest. Proteins 145 (D117L) and 205 (EP153R) also induced antibody responses more than two fold above that seen at day 0. Further investigation is needed to establish if these proteins could be used as novel targets for serological diagnosis. However, ASFV-specific neutralizing antibodies were not induced.
The immunisation and challenge experiment conducted demonstrated that pigs immunised with pools of 46 or 47 ASFV antigens by DNA prime and rVACV boost had decreased levels of ASFV genome in blood and some tissues after challenge compared to control pigs. We cannot exclude that virus is not cleared earlier, but that its distribution to specific tissues is inhibited or altered. Our results suggest that some of the antigens have protective potential. Future experiments will be directed at determining if specific pools of antigens can induce a protective immune response to ASFV challenge. The goal of this work is to develop a safe vaccine strategy for prevention of ASF in pigs.

Acknowledgements

We acknowledge the Animal Services Staff at The Pirbright Institute for help with animal experiments and Greg Golden Biodesign Institute for Project Management. Financial support was provided by DEFRA project SE1514, SE1515, BBSRC projects BB/G530468/1, BB/H008969/1 and Arizona State University.

References

1. DeTray DE. 1957. African swine fever in warthogs (Phocohaerus aethiopicus). J Amer Vet Med Ass 130:537-540.
2. Manso-Ribeiro J, Nunes-Petisca, J.L., Lopez-Frazao, F., and Sobral, M. 1963. vaccination against ASF. Bulletin Office International Epizootic 60:921-937.
3. Boinas FS, Hutchings GH, Dixon LK, Wilkinson PJ. 2004. Characterization of pathogenic and non-pathogenic African swine fever virus isolates from Ornithodoros erraticus inhabiting pig premises in Portugal. Journal of General Virology 85:2177-2187.
4. Leitao A, Cartaxeiro C, Coelho R, Cruz B, Parkhouse RME, Portugal FC, Vigario JD, Martins CLV. 2001. The non-haemadsorbing African swine fever virus isolate ASFV/NH/P68 provides
a model for defining the protective anti-virus immune response. Journal of General Virology 82:513-523.

5. Oura CAL, Denyer MS, Takamatsu H, Parkhouse RME. 2005. In vivo depletion of CD8(+) T lymphocytes abrogates protective immunity to African swine fever virus. Journal of General Virology 86:2445-2450.

6. Denyer MS, Wileman TE, Stirling CMA, Zuber B, Takamatsu HH. 2006. Perforin expression can define CD8 positive lymphocyte subsets in pigs allowing phenotypic and functional analysis of Natural Killer, Cytotoxic T, Natural Killer T and MHC un-restricted cytotoxic T-cells. Veterinary Immunology and Immunopathology 110:279-292.

7. Wardley RC, Norley SG, Wilkinson PJ, Williams S. 1985. The Role of Antibody in Protection against African Swine Fever Virus. Veterinary Immunology and Immunopathology 9:201-212.

8. Barderas MG, Rodriguez F, Gomez-Puertas P, Aviles M, Beitia F, Alonso C, Escribano JM. 2001. Antigenic and immunogenic properties of a chimera of two immunodominant African swine fever virus proteins. Archives of Virology 146:1681-1691.

9. Gomez-Puertas P, Rodriguez F, Oviedo JM, Brun A, Alonso C, Escribano JM. 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.

10. Neilan JG, Zsak L, Lu Z, Burrage TG, Kutish GF, Rock DL. 2004. Neutralizing antibodies to African swine fever virus proteins p30, p54, and p72 are not sufficient for antibody-mediated protection. Virology 319:337-342.

11. RuizGonzalvo F, Rodriguez F, Escribano JM. 1996. Functional and immunological properties of the baculovirus-expressed hemagglutinin of African swine fever virus. Virology 218:285-289.

12. Argilaguet JM, Perez-Martín E, Gallardo C, Salguero FJ, Borrego B, Lacasta A, Accensi F, Diaz I, Nofrarias M, Pujols J, Blanco E, Perez-Filgueira M, Escribano JM, Rodriguez F. 2011.
Enhancing DNA immunization by targeting ASFV antigens to SLA-II bearing cells. Vaccine 455:5379-5385.

13. Argilaguet JM, Perez-Martin E, Nofrarias M, Gallardo C, Accensi F, Lacasta A, Mora M, Ballester M, Galindo-Cardiel I, Lopez-Soria S, Escribano JM, Reche PA, Rodriguez F. 2012.

DNA Vaccination Partially Protects against African Swine Fever Virus Lethal Challenge in the Absence of Antibodies. Plos One 7.

14. Lacasta A, Ballester M, Monteagudo PL, Rodriguez JM, Salas ML, Accensi F, Pina-Pedrero S, Bensaid A, Argilaguet J, Lopez-Soria S, Hutet E, Frederique Le Potier M, Rodriguez F. 2014.

Expression Library Immunization Can Confer Protection against Lethal Challenge with African Swine Fever Virus. Journal of Virology 88:13322-13332.

15. Borovkov A, Magee DM, Loskutov A, Cano JA, Selinsky C, Zsemlye J, Lyons CR, Sykes K. 2009.

New classes of orthopoxvirus vaccine candidates by functionally screening a synthetic library for protective antigens. Virology 395:97-113.

16. Hansen DT, Robida MD, Craciunescu FM, Loskutov AV, Doerner K, Rodenberry J-C, Wang X, Olson TL, Patel H, Fromme P, Sykes KF. 2016. Polyclonal Antibody Production for Membrane Proteins via Genetic Immunization. Scientific Reports 6.

17. Stemke-Hale K, Kaltenboeck B, DeGraves FJ, Sykes KF, Huang J, Bu CH, Johnston SA. 2005.

Screening the whole genome of a pathogen in vivo for individual protective antigens. Vaccine 23:3016-3025.

18. Kibler KV, Gomez CE, Perdiguero B, Wong S, Trung H, Holechek S, Arndt W, Jimenez V, Gonzalez-Sanz R, Denzler K, Haddad EK, Wagner R, Sekaly RP, Tartaglia J, Pantaleo G, Jacobs BL, Esteban M. 2011. Improved NVVAC-Based Vaccine Vectors. Plos One 6.

19. Vijaysri S, Jentarra G, Heck MC, Mercer AA, McInnes CJ, Jacobs BL. 2008. Vaccinia viruses with mutations in the E3L gene as potential replication-competent, attenuated vaccines: Intra-nasal vaccination. Vaccine 26:664-676.
20. White SD, Conwell K, Langland JO, Jacobs BL. 2011. Use of a negative selectable marker for rapid selection of recombinant vaccinia virus. Biotechniques 50:303-4.

21. Sykes KF, Johnston SA. 1999. Linear expression elements: a rapid, in vivo, method to screen for gene functions. Nature Biotechnology 17:355-359.

22. Rowlands RJ, Michaud V, Heath L, Hutchings G, Ora C, Vosloo W, Dwarka R, Onashvili T, Albina E, Dixon LK. 2008. African swine fever virus isolate, Georgia, 2007. Emerging Infectious Diseases 14:1870-1874.

23. D.J. F. 1978. Statistical Methods in Biological Assay. Charles Griffin & Company Ltd.:391-401.

24. Signer EN, Jeffreys AJ, Licence S, Miller R, Byrd P, Binns R. 1999. DNA profiling reveals remarkably low genetic variability in a herd of SLA homozygous pigs. Research in Veterinary Science 67:207-211.

25. King K, Chapman D, Argilaguet JM, Fishbourne E, Hutet E, Cariolet R, Hutchings G, Ora CAL, Netherton CL, Moffat K, Taylor G, Le Potier M-F, Dixon LK, Takamatsu H-H. 2011. Protection of European domestic pigs from virulent African isolates of African swine fever virus by experimental immunisation. Vaccine 29:4593-4600.

26. King DP, Reid SM, Hutchings GH, Grierson SS, Wilkinson PJ, Dixon LK, Bastos ADS, Drew TW. 2003. Development of a TaqMan (R) PCR assay with internal amplification control for the detection of African swine fever virus. Journal of Virological Methods 107:53-61.

27. Gerner W, Denyer MS, Takamatsu HH, Wileman TE, Wiesmuller KH, Pfaff E, Saalmuller A. 2006. Identification of novel foot-and-mouth disease virus specific T-cell epitopes in c/c and d/d haplotype miniature swine. Virus Research 121:223-228.

28. Sykes K. 2008. Progress in the development of genetic immunization. Expert Review of Vaccines 7:1395-1404.

29. Borca MV, Irusta P, Carrillo C, Afonso CL, Burrage T, Rock DL. 1994. African Swine Fever Virus Structural Protein P72 Contains a Conformational Neutralizing Epitope. Virology 201:413-418.
30. GomezPuertas P, Rodriguez F, Oviedo JM, RamiroIbanez F, RuizGonzalvo F, Alonso C, Escribano JM. 1996. Neutralizing antibodies to different proteins of African swine fever virus inhibit both virus attachment and internalization. Journal of Virology 70:5689-5694.

31. Malogolovkin A, Burmakina G, Tulman ER, Delhon G, Diel DG, Salnikov N, Kutish GF, Kolbasov D, Rock DL. 2015. African swine fever virus CD2v and C-type lectin gene loci mediate serological specificity. Journal of General Virology 96:866-873.

32. Salas ML, Andres G. 2013. African swine fever virus morphogenesis. Virus Research 173:29-41.

Figure Legends

Table 1 Antigens tested by DNA prime and recombinant vaccinia virus boost in pigs

The antigens selected for testing by immunisation of pigs are indicated with the relevant antigen set in the first column, an antigen number in the second column and the gene name and predicted function in the third column. The remaining columns show the antigens that were present (“+”) in the different groups in the immunisation Experiment 1 (Groups 1-4) and in the immunisation and challenge Experiment 2 (Groups A and B).

Figure 1 Interferon gamma ELISpot results for Group 1 antigens

Six Babraham pigs were immunised with a pool of 22 DNA plasmids and boosted 4 weeks later with rVACV each expressing individual recombinant antigens as shown in Table 1, Group 1. PBMCs purified 4 weeks post-boost were stimulated overnight with the indicated antigens, media alone (BG), or the Georgia 2007/1 ASFV isolate (ASFV-G). The IFNγ response was determined by ELISpot and each graph shows the results for one pig. Error bars indicate the standard deviation from the mean of duplicate wells. The black, green, blue and red bars highlight the response to media alone, antigen 113 (B646L/p72), antigen 127 (CP204L/p30) and Georgia 2007/1, respectively.
Figure 2. Interferon gamma ELISpot results for Group 2 and Group 4 antigens

Babraham pigs were immunised with pools of DNA plasmids and boosted 4 weeks later with rVACV each expressing an individual recombinant antigen as shown in Table 1. Numbers of IFN gamma producing cells were measured by ELISpot in 3 (407, 409 and 412) of the 5 pigs immunised with 22 antigens as shown in Table 1, Group 2. These included antigens 113 and 127 which were included in all groups ELISpot results are shown from 3 pigs (403, 418 and 423) of the 5 pigs immunised with antigens 113 and 127 alone (see Table 1 Group 4). PBMCs purified 4 weeks post-boost with rVACV were stimulated overnight with the indicated antigens, media alone (BG), or the Georgia 2007/1 ASFV isolate (ASFV-G). The IFNγ response was determined by ELISpot and error bars indicate the standard deviation from the mean of duplicate wells. The black, green, blue and red bars highlight the response to media alone, antigen 113 (B646L/p72), antigen 127 (CP204L/p30) and Georgia 2007/1, respectively. PBMCs from some pigs (397, 411, 424 in Group 2 and 417, 421 from Group 4) failed to respond to stimulation by antigens or virus and results from these pigs are not shown. This may be due to experimental error in storage or recovery of cells after freezing.

Figure 3. Inter-group comparison of response to antigen 113 and 127.

Graph shows the mean and standard deviation of the IFNγ response from Groups 1, 2 and 4 to antigen 113 (B646L/p72) or antigen 127 (CP204L/p30).

Figure 4. Antibody response to p30 in pigs from Groups 1, 2, 3, 4

Serum antibody responses to recombinant protein p30 were measured by ELISA in pigs (x-axis shows the pig number) immunised with antigens shown in Table 1 Group 1 (Panel A), Group 2 (Panel B), Group 3 (Panel C) and Group 4 (Panel D). Open bars indicate results from pre-bleeds collected prior to immunisation and closed bars indicate final bleeds collected at termination of the experiment. Error bars are the standard deviation from duplicate measurements. Results are shown for all of the immunised pigs (Group 1 n=6, Group 2 n=6. Group 3 n=5 and Group 4 n=5).

Figure 5. IgG1 and IgG2 responses to p30 in immunised pigs from Group 3
Serum IgG1 (A) and IgG2 (B) responses to recombinant protein p30 were measured by ELISA in all 555 pigs (n=5) from Group 3. The x-axis shows the pig number. Gray bars indicate no added serum, open bars indicate results from pre-bleeds prior to immunization and closed bars indicate final bleeds collected at termination. Error bars are the standard deviation from duplicate measurements.

Figure 6. Antibody response to individual antigens from Group 3

Antibody responses to each of the 12 individual antigens (see Table 1 Group 3) are shown on the x-axis as measured by ELISA in sera from pig 395 from Group 3. Open bars indicate pre-bleeds and closed bars indicate final bleeds. Error bars are the standard deviation from duplicate measurements. Serum from the other 4 pigs immunised in Group 3 were not analysed.

Figure 7. Clinical scores from pigs immunised with different pools of antigens following lethal challenge with virulent ASFV Georgia 2007/1

Three groups of 6 Babraham pigs were immunised with pools of DNA plasmids twice two weeks apart. Each immunisation contained 10 µg DNA total and CpG adjuvant. Animals were boosted after a further 3 weeks and 5 weeks with rVACVs expressing antigens as shown in Table 1 Experiment 2 (Groups A and B). Group A consisted of all 47 antigens and Group B all except CP204L/p30. Group C contained irrelevant control antigens gp160, AAT and HA. Three weeks after the final rVACV boost pigs were challenged with 10^4 HAD_{50} virulent Georgia 2007/1 isolate by the intramuscular route. Clinical scores (y-axis) were recorded at different days post-challenge (x-axis). The results are shown for individual pigs in each group and for the average for each group.

Figure 8. ASFV genome in blood and tissue samples collected from different pigs

Levels of ASFV DNA detected in samples collected from pigs immunised with different groups of ASFV antigens (Table 1, Groups A and B) or with unrelated Group C antigens are shown as log 10 genome copies per ml of blood or g of tissue (y-axis). Each panel shows results for viremia at different days post-challenge or different tissues collected at termination as indicated. Results for individual pigs are shown as blue circles (Group A), red squares (Group B) or green triangles (control
Group C). Black lines indicate the mean of each group and asterisks indicate significant differences between Group C and either Group A or Group B (ANOVA: * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001).
| Set | Gene Number | Gene Name and Predicted Function | Experiment (1), Group 1 | Experiment (1), Group 2 | Experiment (1), Group 3 | Experiment (1), Group 4 | Experiment (2), Group A | Experiment (2), Group B |
|-----|-------------|---------------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|
| Set 1 | 139 | IF44L p72 – major capsid protein | + | + | + | | | |
| | 167 | CP204L – polyprotein precursor | + | + | | + | | |
| | 122 | NS3/6-1L (VP30/L) | + | + | | | | |
| | 196 | NS2/3-1L (VP16/17) | + | + | | | | |
| | 154 | NS1/10-2L | + | + | | | | |
| | 153 | NS1/10-4L | + | + | | | | |
| | 197 | NS3/30-2R | + | | | | | |
| | 152 | NS2/50-1R – partial | + | | | | | |
| | 150 | NS2/50-4R – partial | + | | | | | |
| | 194 | L10L-KP177R related gene | + | + | | | | |
| | 205 | EP153R – C-type lectin | + | + | | | | |
| | 019 | F313L – carboxenon | + | + | | | | |
| | 076a | F1055L – helicase | + | + | | | | |
| | 096 | F1055L – helicase | + | + | | | | |
| | 083b | EP432R – CO2 like protein | + | + | + | | | |
| | 101 | B941L – structural protein | + | + | | | | |
| | 111 | B601L – chaperone for virus assembly | + | + | + | | | |
| | 122 | G1211R – DNA polymerase – partial | + | + | | | | |
| | 118 | G1340L – polyprotein precursor p60, p62 | + | + | | | | |
| | 167 | E139L – VWS like | + | + | | | | |
| | 121c | T36L – TWS (TIS11b) | + | + | | | | |
| Set 2 | 165 | NS3/30-1R | + | + | | | | |
| | 079 | K265R – unknown | + | | | | | |
| | 076b | K265R – unknown | + | | | | | |
| | 198 | EP384R – unknown | + | + | | | | |
| | 086a | M1248L – partial | + | + | | | | |
| | 086b | M1248L – partial | + | + | | | | |
| | 117 | B407L | + | + | | | | |
| | 131d | G1390L – partial | + | + | | | | |
| | 123b | G1215R – DNA polymerase – partial | + | + | | | | |
| | 124 | G1211R – DNA polymerase – partial | + | + | | | | |
| | 130a | CP247R – polyprotein precursor of structural proteins p150, 27, 18, 14 – partial | + | + | | | | |
| | 126a | CP247R – polyprotein precursor of structural proteins p150, 27, 18, 14 – partial | + | + | | | | |
| | 133a | NP1450L – RNA polymerase subunit 1 – partial | + | + | | | | |
| | 135 | NP1450L – RNA polymerase I | + | + | | | | |
| | 142 | D035L | + | + | | | | |
| | 145 | D115L – structural protein | + | + | | | | |
| | 163 | E186L – polyprotein I | + | + | | | | |
| | 195 | NS2/3-1R | + | + | | | | |
| | 196 | NS2/50-1R | + | + | | | | |
| | 107 | complement (381375, 381378) – unknown | + | + | | | | |
| | 132 | M65R – p23 structural protein | + | + | | | | |
| | 154 | H108R | + | + | | | | |
| | 155 | E120R | + | + | | | | |
| | 159 | G104R – polyprotein precursor | + | + | | | | |
| | 205 | EP153R – C-type lectin | + | + | | | | |
