

IMMUNIZATION OF PIGS WITH RECOMBINANT PLASMIDS CONTAINING GENES OF UBIQUITINATED p30, p54 AND CD2v PROTEINS OF AFRICAN SWINE FEVER VIRUS

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Three recombinant plasmid constructs, expressing chimeric proteins containing human ubiquitin fused to an ectodomain of one of the potentially protective proteins (p30, p54 and CD2v) of the attenuated MK-200 strain of African swine fever virus (ASFV), were created as potential inducers of specific antiviral cellular immunity. Three-time immunization of pigs with the mixture of these plasmids led to the formation of virus-specific cytotoxic T-lymphocytes (CTL), but did not induce production of virus-specific antibodies. After challenge with the homologous parental virulent ASFV strain M-78 at a dose of 10^3 HAD₅₀, all five animals (four immunized pigs and one naïve) fell between the 4th and 7th days post infection. The obtained results demonstrated that induction of CTL did not protect pigs against challenge with the virulent ASFV. Balanced activation of CTL and antibody-mediated cellular mechanisms should be investigated.

Key words: African swine fever, recombinant plasmids, immunogenicity, protectivity, ubiquitin

INTRODUCTION

African swine fever virus (ASFV) is a large DNA virus of the *Asfarviridae* family [1,2]. ASFV causes an acute haemorrhagic fever in domestic pigs and wild boars (*Sus scrofa*) with a mortality rate of up to 100% [3]. The disease control is limited due to the absence of vaccines [4]. African and European isolates of ASFV are known. In addition, there are some attenuated laboratory-derived strains, which cause in susceptible animals chronic or inapparent forms of the disease and protect these animals from death or disease after subsequent infection with virulent isolates or strains belonging to the same seroimmunological group [5-8].

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Immune protection against ASFV is realized through a synergistic action of cytotoxic T-lymphocytes (CTL) and antibody-mediated cell cytotoxicity (AMCC), directed against viral proteins expressed on the surface of infected monocytes/macrophages and destroying these cells before maturation of virions [9,10]. Structural proteins p30/p32 (CP204L), p72 (E183L) and transmembrane glycoprotein gp 110-140 (CD2v, haemagglutinin (HA)) of ASFV were considered as potential inducers of virus-specific CTLs [8,10-13]. It is important to note, that gp 110-140 (CD2v, HA) is the only protein of ASFV that has been shown to be associated with serotype specificity and hemadsorbing activity [7,8,14]. Due to their antibody-inducing property, the proteins p30, p54 and p72 of ASFV have been characterized as highly immunogenic [15-17]. The components of recombinant DNA vaccine against ASFV include at least p30, p54 and CD2v [10]. This conclusion is based on: (i) the localization of virus-induced proteins in the envelope of virions and in the plasma membrane of infected cells; (ii) the dynamics of detection of antibodies to specific viral proteins; (iii) the results of immunization of pigs with various recombinant constructs.

Immunization of pigs with recombinant p30 and p54, or with gp 110-140 purified from ASFV infected cells, or with HA from cells infected with recombinant baculovirus, provided full or partial protection from death after challenge with homologous virulent isolates of ASFV [10,14,18]. At the same time, it was shown that all pigs immunized with p30, p54, p72 and p22 proteins, expressed in cells infected with recombinant baculovirus, have died after subsequent challenge with virulent ASFV [19]. Thus, the knowledge about protective proteins of ASFV and immunological protection mechanisms is still limited. Studies on immunization with recombinant DNA, encoding potential protective proteins of ASFV, may be a perspective approach for solving these problems.

It is important that DNA vaccines induce not only humoral but also a cellular mechanisms of specific protection [20-22]. Immunization of pigs with the recombinant plasmid pCMV-PQ, encoding p30(P) and p54(Q) fused together, did not induce detectable humoral immune response (four animals in each group were immunized three times in dose of 600 µg of recombinant DNA in 1.5 ml PBS, administered at 14 days intervals). Adding the extracellular domain of HA (sHA) to this recombinant construct (pCMV-sHAPQ) induced strong humoral immune response but no protection against lethal ASFV-challenge [23]. For the stimulation of predominantly CD8⁺ T-cell response a new plasmid construct (pCMV-UbsHAPQ) encoding sHA, p30 and p54 fused to ubiquitin (Ub) was designed. Immunization with pCMV-UbsHAPQ induced specific T-cell response in the absence of antibodies and provided partial protection (33% (2/6 pigs)) from lethal challenge with homologous virulent ASFV. The protection correlated with proliferation of the antigen specific CD8⁺ T-cells [24].

In the experiment of Lacasta *et al.*, 8 pigs were immunized with ASFVublib (DNA-library encoding short-length restriction fragments from the ASFV genome fused to ubiquitin gene) and 4 pigs were immunized with pCMV-Ub [25]. Animals (seven weeks old) in each group were injected two times a dose of 600 µg of recombinant DNA in

1.5 ml PBS, administered at 14 days intervals. Immunization of pigs with ASFVUblib confirmed the importance of ubiquitination. The partial protection of pigs after lethal ASFV challenge has been achieved in the absence of the vaccine-induced antibodies, supporting the hypothesis that CD8⁺ T-cells play a crucial role in protection against ASFV [25]. Studies of the protective properties of the recombinant plasmids showed the CD2v and ubiquitin as the key components in the composition of the candidate DNA vaccines against ASFV [24].

In order to develop a DNA vaccine against ASFV seroimmunotype III we have constructed a set of hybrid plasmids containing fragments of ASFV genes *CP204L*, *E183L* and *EP402R* from attenuated strain MK-200 (pCI-neo/ASFV/p30, pCI-neo/ASFV/p54 and pCI-neo/ASFV/CD2v). By immunoblotting, the polypeptides of the expressed recombinant proteins were identified in the HEK293T cell lysates and characterized for their molecular weights. We identified a 21.6 kDa polypeptide after pCI-neo/ASFV/p30 transfection, a major (20.9 kDa) and a minor (36.3 kDa) polypeptide after pCI-neo/ASFV/p54 transfection, and, finally, major polypeptides of 39.8 kDa and 63.1 kDa, together with minor polypeptides of 28.8 kDa and 104.7 kDa when pCI-neo/ASFV/CD2v transfected [26]. However, immunization of pigs using cultures of autologous antigenically active leukocytes, transfected by the same plasmids, did not induce any antibody response or protection from the subsequent challenge with the virulent ASFV [27].

In this work, three recombinant plasmids were constructed, each of which containing a gene of the human ubiquitin B fused to a gene encoding an ectodomain of one of the three immunodominant ASFV proteins: p30, p54 and CD2v. Pigs were inoculated three times with a mixture of the obtained recombinant DNA constructs, and the immune response was evaluated by immunological reactions *in vitro* and the protection studies *in vivo*.

MATERIALS AND METHODS

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Computer analysis, PCR and cloning of the genes encoding ubiquitinated p30, p54 and CD2v proteins

Analysis of the nucleotide sequence of the genome of the ASFV isolate Georgia 2007/1 (GenBank accession number (Acc#) FR682468.1) and human ubiquitin B gene (GenBank Acc# BT020104.1) was performed using the Web resources of NCBI (<http://www.ncbi.nlm.nih.gov/>). PCR primers, containing specific restriction sites, for amplification and cloning of partial ORFs of the p30 (nucleotides 142 to 546 of the gene *CP204L*, GenBank Acc# MK211506.1), p54 (nucleotides 160 to 597 of

the gene E183L, GenBank Acc# MK234865.1), and CD2v (nucleotides 49 to 651 bp of the gene *EP402R*, GenBank Acc# KM609347.1) from the attenuated ASFV strain MK-200 into the plasmid pCI-neo (Cat# E1841; Promega, Madison, WI, USA), were designed using the OLIGO software version 7.60 [28], in accordance with the strategy of DNA cloning described by Gibson *et al.* [29,30], and were published earlier [26]. Previously obtained recombinant plasmids pJET1.2/p30-M200/2, pJET1.2/p54-M200/1 and pJET1.2/CD2v-M200/10 were used as the templates [26]. The nucleotide sequences of genes encoding p30, p54 and CD2v of the ASFV strain MK-200 have 100% identity with the same genes of the ASFV strains M-78 (CD2v: GenBank Acc# KM609346.1) and MOZ/1979 (p30: GenBank Acc# EU874310.1; p54: GenBank Acc# EU874372.1).

The full-length ORF of the human ubiquitin B was amplified using the following primers: F-UBB76A (Forward): 5'-ATCG^ACTAGCCGCCACCATGCAGATCTTCGTGAAAACCCTTACC-3' (NheI) and R-UBB76A (Reverse): 5'-TCGAACCC^AGGGCACCTCTCAGACGCAGGACCA-3' (SmaI). The codon of the C-terminal glycine (76th amino acid (a.a.) residue) of the ubiquitin B was mutated into arginine (resulting in a product named UBB76A) to prevent cleavage of the chimeric protein during translation [31, 32]. The mRNA pool, isolated from human peripheral blood mononuclear cells (PBMC) using guanidine thiocyanate-phenol-chloroform extraction method [33], was used as a template. The cDNA was synthesized using M-MLV reverse transcriptase (α Ferment, Moscow, Russia). PCR amplification was performed on the "Tercyc MC2" thermocycler (DNA-technology, Moscow, Russia), using a high-fidelity Pfu DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA).

Each of the amplified DNA fragments of genes CP204L, E183L and EP402R was fused downstream to the ubiquitin B gene, and ligated into the plasmid pJET1.2 (Thermo Fisher Scientific). The obtained recombinant plasmids and the acceptor plasmid pCI-neo were cleaved by NheI and XhoI restriction enzymes (Thermo Fisher Scientific) and ligated using T4 DNA Ligase (Thermo Fisher Scientific). The obtained recombinant plasmids were used to transform the *E. coli* RosettaTM 2(DE3) pLysS Competent Cells (Cat# 71403; Novagen, MilliporeSigma, MA, USA) using heat shock method in the presence of Ca²⁺ ions [34]. Extraction and purification of the plasmid DNAs from the selected ampicillin-resistant transformants were performed using GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific). The presence of the specific insertions in the recombinant plasmids was checked by PCR using previously described specific primers [26] and by restriction analysis.

Preparative amounts of the obtained plasmids (pUBB76A_p30, pUBB76A_p54 and pUBB76A_CD2v) were purified using the Plasmid Mega Kit (Cat# 12183, Qiagen, Venlo, Netherlands).

The nucleotide sequence of the chimeric insert of the recombinant plasmid was verified by Sanger DNA sequencing using Applied Biosystems 3130 genetic analyzer (Life Technologies, Carlsbad, CA, USA) [35]. The alignment and analysis of DNA

sequences were performed using BioEdit software version 7.2.5 (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) [36]. For virtual cloning and assembling of chimeric constructs the SnapGene® Viewer software version 3.2.1 (GSL Biotech; http://www.snapgene.com/products/snapgene_viewer/) was used.

Cell culture and viruses

Primary swine blood leucocyte (PSBL) cell culture was prepared by the following procedure. 30-40 ml of the pig's whole blood was collected from the cranial vena cava into a glass tube containing heparin (20 IU/ml). The tube was placed vertically in an incubator or water bath at 37°C to allow the cells to settle. The upper fraction consisting of plasma and leukocytes was collected and centrifugated at 2000 rpm (~1300 g) for 15 min. Supernatant liquid was removed and the pellet was suspended in the Eagle's minimal essential medium (EMEM) (final concentration of 4×10^6 leucocytes per ml) containing 10% of autologous serum (inactivated by heating at 56 °C for 30 min), penicillin (100-200 IU/ml) and streptomycin (100-200 mg/ml). The cells were incubated in 24 or 96 well cell culture plates at 37 °C in an atmosphere containing 5% CO₂.

Virulent ASFV strain Mozambique-78 (M-78) and the derived from M-78 attenuated ASFV strain MK-200 (genotype V, seroimmunotype III) were used in this study [7].

The infectivity titer of the ASFV was determined in a cell culture of PSBL using eight wells for each 10-fold dilution. The results were recorded 5-7 days post inoculation (d.p.i.) by identification of infected cells by hemadsorption (HAD) [37]. The titer of the virus was calculated by the Kerber method in modification of Ashmarin and expressed in 50% Haemadsorbing Units (HAU₅₀) per ml [38].

The attenuated strain MK-200 of ASFV was selected at Federal Research Center for Virology and Microbiology (FRCVM, formerly VNIIVViM) by serial passage of the virulent strain M-78 in a culture of pig bone marrow cells. The average yield of MK-200 *in vitro* is $10^{7.5}$ – $10^{8.0}$ HAU₅₀/ml. Intramuscular inoculation of the MK-200 in a dose of $10^{3.0}$ – $10^{4.0}$ HAU₅₀ renders protection against the virulent strain M-78 to about 50% of pigs on day 7, and 81.2% of pigs on day 14 after inoculation. Viremia after infection of pigs with MK-200 virus strain remains on the level of $10^{3.5}$ – $10^{4.0}$ HAU₅₀/ml for up to 45 days. The virus remained attenuated during five consecutive passages in pigs (duration of observation), caused a mild reaction in 67% of inoculated pigs, and was not harmful for piglets 2-4 months of age.

Immunization and challenge experiment in pigs

Five pigs with a weight of 20 kg were used in the experiment: four pigs (No.1-4) were immunized three times with an interval of 14 days (on day 0, 14 and 28) with a mixture of the recombinant plasmids pUBB76A_p30, pUBB76A_p54 and pUBB76A_CD2v (1.0 mg of each plasmid in 1.5 ml volume for one immunization for each pig), and one

pig was used as a control (No.5). The plasmid mixture was divided into three equal parts (3 x 0.5 ml) and injected parenterally into three points: the trapezius muscle of the neck, into the quadriceps muscle of the thigh, and subcutaneously into the ear. All manipulations with animals were carried out in accordance with the ethical norms and rules for the care and use of laboratory animals [39].

On days 0, 14, 28 and 42 from the start of the experiment, blood samples were collected from each animal (from the cranial vena cava): 8 ml into a test tube with a coagulant (3.2% buffered sodium citrate solution) to obtain sera, and 3 ml into a test tube with an anticoagulant (BD Vacutainer® Heparin blood collection tubes containing lithium heparin (BD Biosciences, San Jose, CA, USA)) for the determination of the number of the interferon-*gamma* (*IFN-γ*) secreting cells.

In addition, on day 42, 3 ml of whole blood were collected from each pig to prepare the PSBL cell culture (using a media, supplemented with 10% of the simultaneously obtained autologous serum) to study their susceptibility and ability to support of ASFV infection. PSBL cells from each pig were transferred into 24-well plates (4x10⁶ cells/ml/well) and cultured at 37 °C in an atmosphere containing 5% CO₂ for 48 hours. Then cultures of PSBL were infected with the virulent ASFV strain M-78, at a dose of 10² HAU₅₀ per well. On the 4th day post infection (when dense hemadsorption was observed in all wells) the infected PSBL cultures were frozen at minus 70 °C. The accumulation of the virus in each well was determined by titration in a culture of PSBL cells from a healthy control pig (non-immunized) in 96-well plates.

Detection of virus-specific antibodies

The indirect ELISA for the detection of ASFV-specific antibodies (Abs) in the blood serum of pigs was performed using a validated 'Diagnostic kit for indirect ELISA for African Swine Fever (VNIIVViM ASF-ELISA Ab/Ag)' (VNIIVViM, Volginsky, Russia) [40]. The ELISA antigen was prepared from infected cells grown in the presence of pig serum [41]. The results were recorded at a wavelength of 405 nm on a Sunrise™ microplate reader (Tecan, Männedorf, Switzerland). Antibodies to p30 were also determined using an IDvet multi-antigen indirect ELISA kit for the detection of antibodies against P32, P62, and P72 of the ASF virus in porcine serum, plasma or blood filter paper samples (ID Screen® African Swine Fever Indirect; Grabels, France).

Detection of ASFV-induced production of *IFN-γ* in cultures of PBMC using Enzyme-Linked ImmunoSpot (ELISPOT)

Whole blood samples were collected in BD Vacutainer® Heparin blood collection tubes containing lithium heparin (BD Biosciences). Mononuclear cells were isolated from the blood by the gradient centrifugation method using Histopaque-1077 (Sigma diagnostics Inc., Livonia, MI, USA), they were washed twice, resuspended to a concentration of 5x10⁵ cells per ml in serum-free CTL-Test medium (Cellular

Technology Limited, Cleveland, OH, USA) containing 2 mM L-glutamine and gentamicin (80 mg/l).

ELISPOT analysis for the detection of ASFV-induced production of IFN- γ in cultures of PBMC was performed using the 'Pig IFN- γ Single-Color ELISPOT' (ImmunoSpot[®]) kit (Cellular Technology Limited, USA) [42]. Monoclonal anti-IFN- γ antibodies with a concentration of 5 μ g/ml in 100 μ l of phosphate-saline buffer (PBS) with a pH of 7.2 were adsorbed in strip wells overnight at 4 °C. The strips were washed with PBS, then 5×10^5 PBMC and the virulent ASFV strain M-78 at a dose of 10^5 HAU₅₀ were added into the wells. The background secretion of IFN- γ by mononuclear cells in the presence of culture medium was used as a negative control. After 24 hours of incubation at 37 °C in an atmosphere with 5% CO₂, the cells were removed, biotinylated secondary anti-porcine IFN- γ antibodies were added into the strip wells and were incubated for 2 hours at room temperature. Then, the strips were incubated at room temperature with streptavidin-peroxidase (30 min). The water-insoluble 3,3',5,5'-tetramethylbenzidine (TMB) (15 min) was used for staining. The reaction was stopped by gently washing the strips with distilled water.

For calculation of the number of ASF-specific IFN- γ secreting cells, the number of spots in unstimulated wells was subtracted from the number of spots in virus-stimulated wells. The amount of the cytokine-producing cells was expressed as the number of responding cells per 10^6 PBMC.

RESULTS

Construction of recombinant plasmids

For efficient presentation of antigenic epitopes through protein degradation in the proteasomes, the ectodomains of the p30 (135 aa), p54 (146 aa) and CD2v (201 aa) proteins of the attenuated ASFV strain MK-200 were fused with the leading sequence of the ubiquitin B gene (76 aa; UBB76A), the C-terminal glycine residue of which was replaced with arginine to prevent ubiquitin cleavage during translation [31,32]. The schematic representation of the inserts of the obtained recombinant pCI-neo-based plasmids (pUBB76A_p30, pUBB76A_p54 and pUBB76A_CD2v) are shown on Fig. 1.

Determination of the plasmids' protective efficiency in pigs

Pigs were immunized by mixture of the recombinant plasmids pUBB76A_p30, pUBB76A_p54 and pUBB76A_CD2v as written above ('Material and Methods' section 'Immunization and challenge experiment in pigs').

14 days after the 3rd immunization (on day 42 after the start of the experiment), the immunized and control animals were challenged by intramuscular inoculation of the virulent ASFV strain M-78, at a dose of 10^5 HAU₅₀.

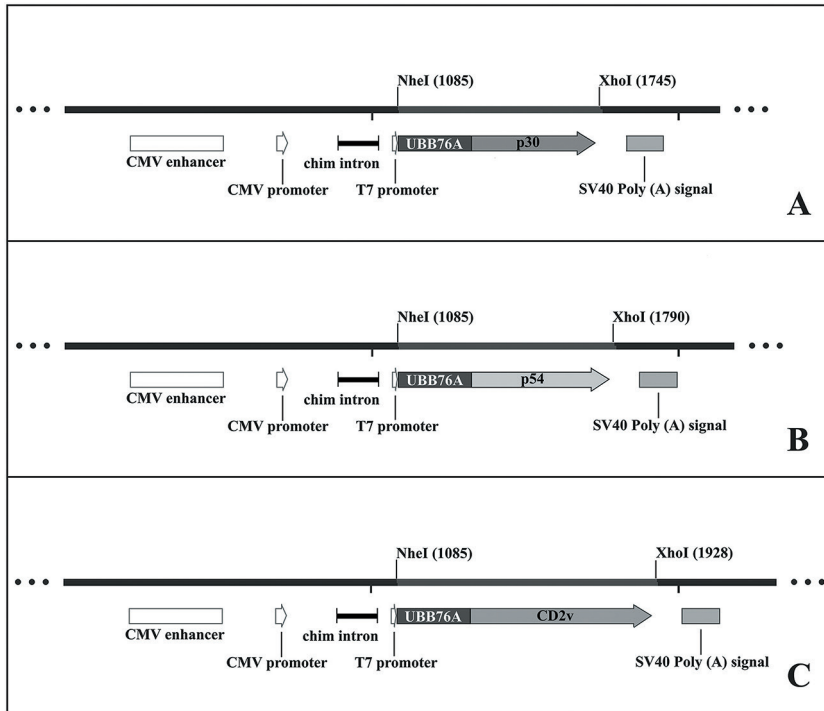


Figure 1. Schematic diagram of the chimeric ORFs of the plasmids: A – pUBB76A_p30; B – pUBB76A_p54; C – pUBB76A_CD2v.

CMV enhancer – human cytomegalovirus immediate early enhancer; CMV promoter – human cytomegalovirus promoter; chim intron – chimeric intron; T7 promoter – T7 bacteriophage RNA polymerase promoter; UBB76A – sequence encoding the human ubiquitin B (with c-terminal glycine mutated to arginine); p30 – sequence encoding an ectodomain of the p30 protein of ASFV strain MK-200; p54 – sequence encoding an ectodomain of the p54 protein of ASFV strain MK-200; CD2v – sequence encoding an ectodomain of the CD2v protein of ASFV strain MK-200; SV40 Poly (A) signal – SV40 virus polyadenylation signal; NheI и XhoI – restriction sites.

On the 3rd d.p.i. with the virulent ASFV, all animals had fever (body temperature increased up to 40.3°C - 41.4°C). Two of the animals (the immunized animal No.3 and the control animal No.5) had a frothy discharge from the mouth, bloody diarrhea, and body temperature 41.1°C and 41.4°C, respectively. On the 4th and 5th d.p.i. these animals died. On the 3rd day the body temperature of the immunized pig No.2 was 41.1°C, on the 4th d.p.i., it decreased to 39.0°C, but on the 5th d.p.i. the body temperature rose again to 40.9°C. On the 6th d.p.i. the animal No.2 died. The immunized pigs No.1 and No.4, had a stably increased body temperature (between 40.3-40.4°C) over the course of infection, and they died on 6th and 7th d.p.i., respectively (Fig. 2). Thus, the immunization of pigs with the plasmids did not protect them from death or illness.

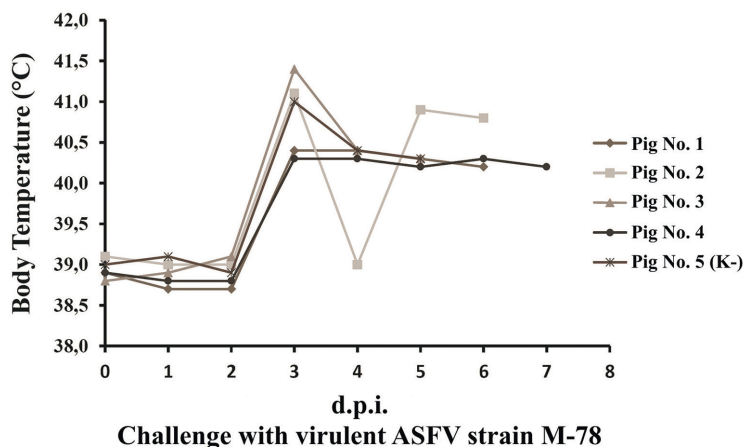


Figure 2. Body temperature of the immunized (pigs No.1-4) and control (pig No.5) animals after challenge with the virulent ASFV strain M-78 in the dose of 10^3 HAU₅₀.

Dynamics of ASFV accumulation in the blood of infected pigs

The results of determination of the ASFV infectivity titer in the blood of immunized and control pigs on the 2nd, 4th and 6th d.p.i. have shown that the dynamics of viremia in all animals was similar: from 4.25 to 4.75 lg HAU₅₀/ml on day two, from 6.50 to 7.25 lg HAU₅₀/ml on day four, and from 6.75 to 7.25 lg HAU₅₀/ml on day six (Fig. 3). On the 4th and 6th d.p.i., the maximal viremia was observed in pigs No.1, No.3 and No.5 (from 7.00 to 7.25 lg HAU₅₀/ml, 7.00 lg HAU₅₀/ml, and 7.25 lg HAU₅₀/ml, respectively). In pigs No.2 and No.4 the level of viremia was lower: from 6.75 to 7.00 lg HAU₅₀/ml and from 6.50 to 6.75 lg HAU₅₀/ml, respectively (Fig. 3).

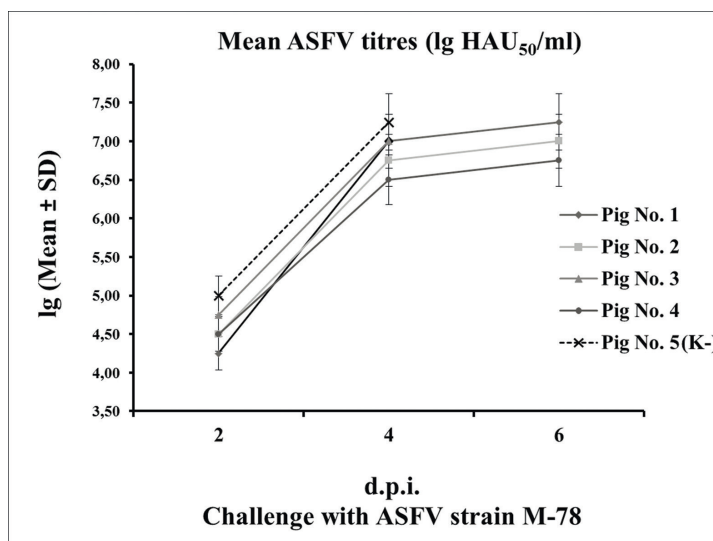


Figure 3. Titers of ASFV in blood samples collected on day 2, 4, and 6 after challenge

Immunological analysis of ASF-induced production of *IFN-γ* in PBMC using ELISPOT (T-cell response)

Immunization with recombinant plasmids induced a virus-specific T-cell response in each of the four pigs (No.1-4). It was determined using the *IFN-γ* ELISPOT after stimulation *in vitro* of PBMC with the virulent ASFV strain M-78. Studies were performed on day 0 and day 14 after each of the three immunizations. In all animals a booster effect of consecutive immunizations was observed, i.e. a consistent increase of the T-cell response (number of *IFN-γ*-producing cells) after each next immunization, with the maximum detected after the third immunization (Fig. 4). The highest absolute values of the T-cell response were in pigs No. 2 and No. 4: the number of *IFN-γ* secreting cells per million of PBMC was in the range from 105 to 178, respectively.

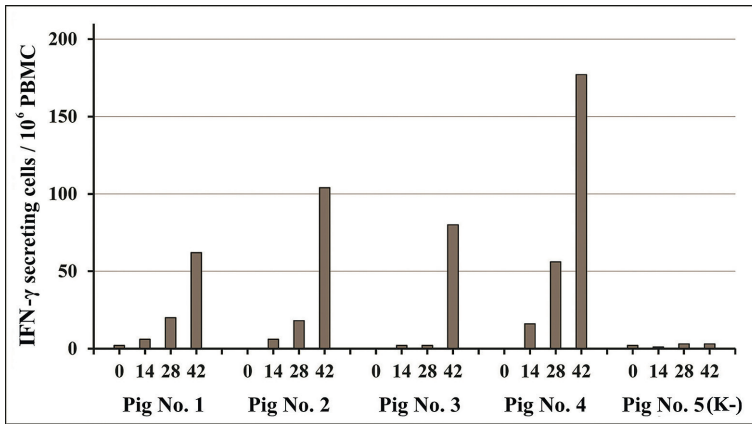


Figure 4. Numbers of ASFV-induced *IFN-γ* secreting cells per million PBMC collected from pigs on day 0 and day 14 after each of three consecutive immunizations. The ASFV-induced *IFN-γ* secreting cells were detected by ELISPOT, using the virulent ASFV strain M-78 for induction. 0, 14, 28, 42 – days of blood collection and testing.

In our studies, T-cell response levels ranged from 62 to 178 *IFN-γ* secreting cells per million PBMC. Similar values of T-cell response were obtained after immunization of pigs with pools of 47 genes of the ASFV (these genes were cloned into the recombinant plasmids (used for DNA prime immunization) and the recombinant vaccinia viruses (used for boost immunization) [43]. The similar level of T-cell response in various pigs were registered by Lacasta and co-workers after stimulation with virulent ASFV isolate Georgia 2007/1 (between 32 and 137 *IFN-γ* secreting cells per million PBMC) [25]. After immunization with ubiquitinated 4029 clones representing 130 kbp of ASFV genome, from 18 to 47 *IFN-γ* secreting cells per million PBMC were recorded after their stimulation by the virulent ASFV isolate E75 [25].

Humoral immunity

The presence of antiviral Abs in the blood serum of immunized pigs was investigated by indirect ELISA. The presence of recombinant proteins p30, p54 and CD2v in the

lysates of HEK293T cells transfected with plasmids pCI-neo/ASFV/p30, pCI-neo/ASFV/p54 and pCI-neo/ASFV/CD2v without ubiquitination [27] was confirmed by immunoblotting [37] using positive serum from VNIIVVIM ASF-ELISA Ab/Ag diagnostic kit [40]. No Abs to ASFV proteins were detected in the blood sera samples from pigs No.1-4, obtained on days 14, 28, 42 after the start of immunization.

Susceptibility of the PSBL of immunized animals to ASFV infection

We have reported earlier, that cultures of PSBL, obtained from pigs inoculated with the attenuated ASFV strain, can be considered as a surrogate *in vitro* model, representing protective immunological reactions occurring *in vivo* [44]. In this study we investigated whether the immunization of pigs with recombinant plasmids had any impact on the susceptibility of PSBL to ASFV infection.

It was determined that the maximum of ASFV ($\geq 10^7$ HAU₅₀) were accumulated in cultures of PSBL cells from pigs No.3 and No.5, whereas virus yield in the culture of PSBL cells from pigs No.1, No.2 and No.4 was 0.75-1.25 lg HAU₅₀/ml lower than that in PSBL from the naïve animal (pig No.5) (Fig. 5).

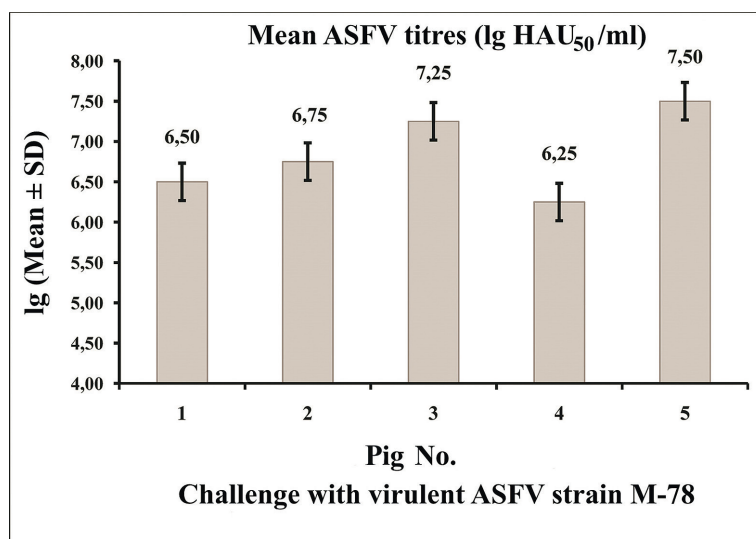


Figure 5. Level of virus accumulation in the cultures of PSBL cells infected *in vitro* by ASFV strain M-78.

On day 42 after the start of immunization (14 days after the third immunization) the samples of the whole blood were collected from each pig for preparation of the PSBL culture (using a media, supplemented by 10% of the simultaneously obtained autologous serum). After 48 hours of incubation at 37°C in an atmosphere containing 5% CO₂ the cultures of PSBL were infected with the virulent ASFV strain M-78, at a dose of 10² HAU₅₀ per 4x10⁶ cells, and incubated for another 4 days. The level of virus accumulation in the infected PSBL was determined by infectivity titration using a culture of PSBL cells from a healthy control pig (non-immunized).

DISCUSSION

Predominance of the cellular immune response and limited protective role of the virus-induced antibodies is one of the problems in the development of the effective ASF vaccines [45].

For this reason, no protection against ASFV has been achieved after immunization by the inactivated or subunit experimental vaccines [46-48], which, as a rule, are capable of inducing predominantly humoral immunity. Possible perspectives of using attenuated or genetically modified strains to protect domestic pigs from ASFV are geographically limited to the central and western regions of sub-Saharan Africa, where the seroimmunological diversity of ASFV is insignificant [49,50].

Investigation of the protective effectiveness of recombinant plasmids containing genes of the potentially protective ASFV proteins is important in both, theoretical and practical aspects.

Immunization with recombinant DNA constructs containing genes of the protective proteins can induce antibodies and CTL [21]. But it is important to find an optimal balance in the stimulation of effector mechanisms of the immune response. It was reported earlier, that excessive induction of the virus specific Abs leads to accelerated death of the challenged pigs [23]. In order to diminish induction of Abs and to increase the specific CD8⁺ T-cell responses the chimeric DNA construct, encoding antigenic determinants of p30, p54 and sHA proteins fused to ubiquitin, were designed. As expected, immunization of pigs with recombinant plasmid pCMV-UbsHAPQ did not induce humoral response in pigs, but induced virus specific CTL and provided partial protection of immunized pigs from lethal challenge with homologous virulent ASFV strain [24].

In our study we used an immunization approach similar to that reported earlier by Argilaguet *et co-workers* [24]. However, in our experiments we used three separate recombinant plasmids, expressing chimeric proteins consisting of human ubiquitin B fused with an ectodomain of one of the potentially protective proteins (p30, p54, CD2v) of the attenuated ASFV strain MK-200. This strategy provides an expression of each antigenic component independently of each other. Three-time immunization of pigs with the mixture of these DNA constructs resulted in the formation of the virus specific CTL without induction of virus specific Abs. All five pigs (four immunized pigs and one control animal) died on 4th to 7th day after challenge with the virulent ASFV strain M-78. Nevertheless, it is interesting to note the following facts: (i) three out of five pigs (pigs No.1, 2 and 4) had lived longer after challenge with the virulent virus, than pig No.3 or naïve animal (pig No.5); (ii) had higher numbers of IFN- γ secreting cells after 1st and 2nd immunizations; (iii) had lower level of accumulation of ASFV in the PSBL cell culture prepared on the 14th day after 3rd immunization; (iv) pigs No.2 and No.4 had lower level of viremia on 4th and 6th day after challenge. It is possible to suppose that pigs No.1, 2 and 4 had developed some antiviral immune

mechanisms, though insufficient for protection against ASFV infection and death. Since no virus-specific Abs were detected in immunized animals, the CTL could be the possible restricting mechanism.

We have reported that in pigs inoculated with the high dose (10^8 HAU₅₀) of the attenuated ASFV strain FK-135, AMCC was registered from the 3rd d.p.i., and the primary CTL – from the 6th d.p.i. [10]. Studies *in vitro* using reconstructed syngenic PSBL cell culture demonstrated that on the 6th day after inoculation with the attenuated ASFV strain FK-135, the role of Abs in the inhibition of accumulation of the homologous virulent strain F-32 was more significant, than that of CTL [44]. Thus, induction of only the CTL is not enough for the efficient protection against ASF. It is necessary to induce an antibody-dependent cellular mechanisms, balanced by CTL.

Taken together, we constructed three recombinant plasmids encoding chimeric proteins, consisting of human ubiquitin fused to one of the potentially protective ASFV proteins. Three-time immunization of four pigs with the mixture of these plasmids induced the formation of the virus-specific cytotoxic T-lymphocytes, but not virus-specific Abs, and did not protect animals against challenge with the virulent ASFV. It is possible, that the complete absence of the virus-specific Abs is counterproductive. Therefore, other schemes of immunization combining plasmids with potentially protective ASFV proteins alone or fused to ubiquitin should be investigated. Another interpretation of our data could be that the failure of these constructs to protect against lethal challenge is not necessarily the result of the lack of humoral immunity. It possibly could be because of the absence of other ASFV genes in the vaccine or even the method of immunization.

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Authors' contributions

IA, KA, and MD carried out the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. LV, ST carried out the immunoassays. TI participated in the sequence alignment. ŠM participated in the design of the study and performed the statistical analysis. SA conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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IMUNIZACIJA SVINJA SA REKOMBINANTNIM PLAZMIDIMA KOJI SADRŽE GENE UBIKVITARNIH p30, p54 i CD2v PROTEINA VIRUSA AFRIČKE KUGE SVINJA

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Kao potencijalni induktori specifičnog antivirusnog celularnog imuniteta, kreirane su 3 rekombinantne plazmidne konstrukcije, koje predstavljaju himerne proteine, koje sadrže humani ubikvitin, na čijem ektodomenu je inkorporiran po jedan od sledećih potencijalno zaštićenih proteina (p30, p54 i CD2v), a koji potiču od atenuiranog soja afričke kuge svinja MK - 200. Navedenim plazmidima je izvršena trostruka imunizacija svinja, koja je kod njih dovela do posledičnog formiranja virus specifičnih citotoksičnih T limfocita (CTL), ali bez uticaja na indukciju sinteze virus specifičnih antitela. Nakon

veštačke infekcije ovih imunizovanih svinja i to homolognim virulentnim sojem virusa afričke kuge svinja M - 78, u dozi od 10^3 HAD₅₀, svih 5 svinja (4 imunizovane svinje i 1 neimunizovana - kontrolna) su uginule između 4 i 7 dana nakon inficiranja. Dobi-jeni rezultati su pokazali da indukcija samo celularnog imunog odgovora, odnosno citotoksičnih T limfocita, ne štiti svinje od infekcije virulentnim sojem virusa afričke kuge svinja. Izbalansiranu aktivaciju citotoksičnih T limfocita i antitelima posredovane ćelijske imunske mehanizme trebalo bi dalje istražiti.