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Short Communication

BacMam immunization partially protects pigs against sublethal challenge with African swine fever virus

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ABSTRACT

Lack of vaccines and efficient control measures complicate the control and eradication of African swine fever (ASF). Limitations of conventional inactivated and attenuated virus-based vaccines against African swine fever virus (ASFV) highlight the need to use new technologies to develop efficient and safe vaccines against this virus. With this aim in mind, in this study we have constructed BacMam-sHAPQ, a baculovirus based vector for gene transfer into mammalian cells, expressing a fusion protein comprising three in tandem ASFV antigens: p54, p30 and the extracellular domain of the viral hemagglutinin (secretory hemagglutinin, sHA), under the control of the human cytomegalovirus immediate early promoter (CMVie). Confirming its correct *in vitro* expression, BacMam-sHAPQ induced specific T-cell responses directly after *in vivo* immunization. Conversely, no specific antibody responses were detectable prior to ASFV challenge. The protective potential of this recombinant vaccine candidate was tested by a homologous sublethal challenge with ASFV following immunization. Four out of six immunized pigs remained viremia-free after ASFV infection, while the other two pigs showed similar viremic titres to control animals. The protection afforded correlated with the presence of a large number of virus-specific IFNγ-secreting T-cells in blood at 17 days post-infection. In contrast, the specific antibody levels observed after ASFV challenge in sera from BacMam-sHAPQ immunized pigs were indistinguishable from those found in control pigs. These results highlight the importance of the cellular responses in protection against ASFV and point towards BacMam vectors as potential tools for future vaccine development.

African swine fever (ASF), a fatal hemorrhagic disease in domestic pigs and European wild boar (Dixon et al., 2005) is caused by African swine fever virus (ASFV), the sole member of the family *Asfarviridae*. In spite of being a permanent problem in sub-Saharan African countries for over a century, efforts on ASFV-vaccine development declined after the disease was eradicated from continental Europe in the mid nineties. The recent appearance of the disease in the Caucasus area and its rapid spread to neighboring countries has highlighted again the relevance of developing an effective vaccine against ASF (www.promedmail.org- OIE, WAHID; Rowlands et al., 2008). Early attempts to use conventional vaccines against ASFV, such as inactivated or attenuated viruses, were unsuccessful or not practicable in the field due to safety concerns (Kihm et al., 1987; Kovalenko et al., 1965; Mebus, 1988; Sanchez Botija, 1963). Immunization with subunit vaccines based on specific ASFV antigens resulted in lack of protection (Neilan et al., 2004) or partial protection using protocols that were not practical for use in the field (Barderas et al., 2001; Gomez-Puertas et al., 1998; Ruiz-Gonzalvo and Coll, 1993; Ruiz-Gonzalvo et al., 1996). Recent results from our laboratory demonstrate (i) that both humoral and cellular responses induced in pigs by DNA vaccines could be exponentially improved by targeting the encoded antigens to antigen presenting cells (Argilaguet et al., 2011) and (ii) that protection against a lethal challenge could be afforded in the absence of detectable antibodies (Argilaguet et al., 2012), demonstrating the important role that cellular responses play in protection and confirming previous results using attenuated ASFV (Canals et al., 1992; King et al., 2011; Oura et al., 2005).

To optimize the immune responses induced and thus the protection afforded against ASFV, we tested the potential of a new vaccination strategy based on the use of so-called BacMam

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viruses, which are baculovirus vectors encoding antigens under the control of vertebrate cell-active promoters that allow high-level transgene expression in mammalian cells (Keil et al., 2009; Kost and Condreay, 2002; O'Grady et al., 2011). Despite the fact that the natural host cells for baculoviruses are derived from arthropods, the virus is also able to efficiently transduce vertebrate cells in which baculoviruses are unable to replicate. Thus, BacMam viruses are safe and efficient and therefore highly suited for use as vaccine vectors (Brun et al., 2008; Condreay and Kost, 2007; Fang et al., 2010; Hitchman et al., 2009; Huser and Hofmann, 2003; Koroleva et al., 2010; Kost et al., 2007; M. Li et al., 2009; Paul et al., 2010). So far, most BacMam immunization experiments have been performed in small laboratory animal models, being proved to efficiently induce humoral and cellular responses (Bai et al., 2008; Brun et al., 2008; Facciabene et al., 2004; Hu, 2008; Y. Li et al., 2009; Tang et al., 2011; Wang et al., 2007; Wu et al., 2009). In addition, baculoviruses induce strong innate immune responses after inoculation of mammals with a bias towards Th1-like responses (Abe and Matsuura, 2010; Suzuki et al., 2010).

In the present study, a recombinant BacMam encoding three ASFV antigens from the E75 virus isolate under the control of the vertebrate-specific CMV promoter was developed for vaccine purposes. The new recombinant baculovirus (BacMam-sHAPQ) encodes two immunodominant ASFV proteins: full length p54 and p30, fused in tandem to the carboxyl-terminus end of the extracellular domain of the viral hemagglutinin (secretory hemagglutinin, sHA; residues 21 to 204 of the HA). We have recently described the capability of this chimeric protein (sHAPQ) to induce potent antibody and cellular responses in pigs when administered as a DNA vaccine (Argilaguet et al., 2012). Briefly, the ORF encoding sHAPQ was excised from the pCMV-sHAPQ plasmid together with the CMV promoter sequence using the restriction enzymes *Eco*RI and *Sal*I. The fragment was then cloned into the recombination vector pBacPH-GFPpolyA cleaved with *Eco*RI and *Sma*I (Keil et al., 2009). The recombinant Bacmam-sHAPQ was obtained by transposition in *Escherichia coli* as described (Keil et al., 2009). The correct *in vitro* expression of the chimeric sHAPQ protein in mammalian cells was assessed by transduction of KOP/R cells with BacMam-sHAPQ followed by indirect immunofluorescence with specific antibodies (Fig. 1A). Once *in vitro* expression was confirmed, the immunogenicity of BacMam-sHAPQ was tested in swine, the natural domestic host of ASFV (animal care and procedures were performed under the supervision of the Ethical and Animal Welfare Committee of the UAB, permit number DMAH-5796). The *in vivo* experimental approach followed is shown in Fig. 1B. Briefly, six Landrace × Large White pigs were immunized three times at 15 day intervals with 10^7 plaque forming units (pfu) of BacMam-sHAPQ (in a volume of 1.5 ml saline). As a control group, six additional animals received three doses of the wild type baculovirus (BacMam control). In order to minimize the probability of inoculation failures, one third of each vaccine dose was injected in the rectus femoris quadriceps muscle, one third in the trapezius muscle of the neck and the last third was subcutaneously injected in the ear; a protocol previously optimized in the laboratory for DNA vaccination of pigs (Argilaguet et al., 2011). Two weeks after the last boost, all animals were inoculated twice with an intramuscular sub-lethal dose of 10^2 HAU_{50} (hemadsorbing units) of the homologous E75 ASFV with a two-day-interval to test the protective capacity of BacMam-sHAPQ (Fig. 1B). This infection protocol is alternative to the lethal challenge with 10^4 HAU_{50} of the E75 strain (equivalent to 20 LD50) normally used in the laboratory (Argilaguet et al., 2011, 2012). This strategy, widely used in HIV research (Hessell et al., 2009; Moldt et al., 2012; Tenbusch et al., 2012), avoids the overestimation of vaccine induced immunological requirements to confer protection against a pathogen and might better reflect natural field conditions, including ASFV-tick bite transmission (Endris et al., 1991; Groocock et al., 1980; Mellor and Wilkinson, 1985).

Pigs were bled 15 days after each immunization and at different times post-infection (p.i.) to monitor both the immune responses induced and the virus titres in blood. All animals were sacrificed at day 17 p.i. (Fig. 1B).

As expected from previous experiments carried out in our laboratory using this protocol of infection, none of the control pigs developed any ASF-specific clinical signs, with the exception of a short peak of moderate fever (<41.5 °C) in some animals (data not shown). In contrast to the mild clinical signs, all six control pigs showed high levels of virus in their blood. The titres peaked at day 10 p.i. and then declined slowly from day 11 p.i. until the end of the experiment at day 17 p.i. (Fig. 2). Conversely, four out of six pigs (66.6%) immunized with the BacMam-sHAPQ showed total protection, with no virus detected in their blood at any time tested (Fig. 2).

To establish a correlation between protection and the immune response induced by the experimental vaccine, both the humoral and cellular responses were analyzed. The presence of specific antibodies in sera was determined by ELISA (Fig. 3), using either ASFV or the recombinant p30 protein as specific antigens as previously described (Argilaguet et al., 2011). To confirm results, sera were tested by western blot assay using recombinant p54 protein as antigen (Argilaguet et al., 2011). Protection observed did not correlate with the humoral response induced, since none of the BacMam-sHAPQ vaccinated animals showed detectable specific antibody responses before challenge (Fig. 3; day 0 p.i.). Moreover, the kinetics of the specific humoral responses induced after infection were identical in both immunized and control groups (Fig. 3). Lack of induction of specific-antibodies cannot be attributed to a defect on the expression of the protein (Fig 1A), neither to specifics of the antigenic fusion protein, as the same antigen (sHAPQ) induced good humoral responses when encoded by a DNA vaccine (Argilaguet et al., 2012). The failure of BacMam-sHAPQ to induce specific antibodies neither could be ascribed to an inherent defect of the BacMam strategy since this delivery vector has been successfully used for this purpose with many other antigens (Bai et al., 2008; Facciabene et al., 2004; Y. Li et al., 2009). One potential explanation might come from some defect on its *in vivo* presentation to the B cells as it has been described for other antigens (Borrego et al., 2006: Ganges et al., 2005). The failure of BacMam-sHAPQ to induce a humoral response highlights the difficulty of predicting immunological outcomes when developing experimental vaccines and warns against extracting "universal" conclusions. The possibility that specific antibody responses in pigs would have been induced by an increased dose of the BacMam vaccine cannot be ruled out.

The cellular responses induced were measured by an IFNγ-ELISPOT as described (Argilaguet et al., 2011). Peripheral blood mononuclear cells (PBMC) isolated 15 days after each immunization and 17 days after the ASFV challenge (at the time of the sacrifice), were stimulated for 16 h with ASFV E75 isolate (10^6 HAU_{50}/ml) to analyze the presence of virus-specific IFNγ-secreting cells by ELISPOT (Fig. 4). As expected for control animals, no specific responses were detected before challenge (Fig. 4A). Conversely, four out of six pigs immunized with BacMam-sHAPQ, showed specific T-cell responses prior to challenge (Fig. 4A), confirming their correct immunization. The variability of the frequency of specific T-cells found in blood after vaccination with BacMam-sHAPQ could be partially explained by the continuous circulation of T-cells between the periphery and the lymphoid organs (Li et al., 2006). Independently of this, a statistically significant correlation was observed between the magnitude of the immune responses found at 17 dpi in pigs vaccinated with BacMam-sHAPQ and the protection afforded (Figs. 2 and 4B). Thus, total protection correlated (correlation coef-

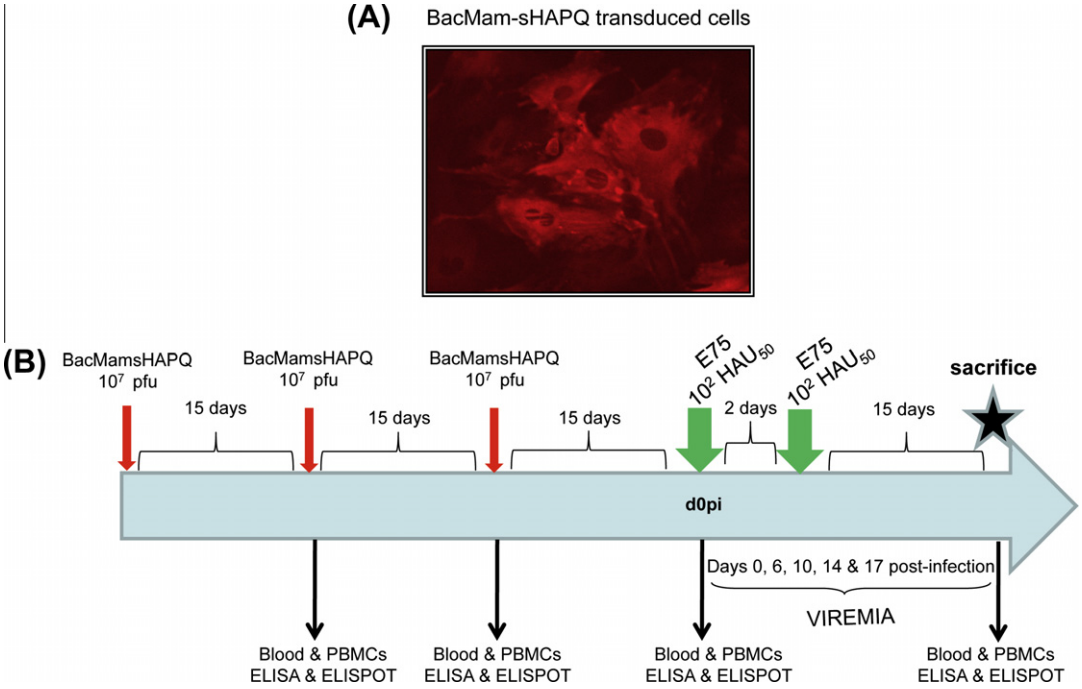

Fig. 1. (A) Transient *in vitro* expression of the BacMam-encoded ASFV-antigens. KOP/R cells were transduced with 10 TCID50/cell BacMam-sHAPQ and 24 h later cells were fixed and stained with a monoclonal antibody against p30, followed by an Alexa 568 conjugated anti-mouse antibody. No specific signal was detectable in cells transfected with the wild type baculovirus (not shown). (B) Schematic representation of the *in vivo* experimental approach. Pigs received three immunizations at 15 day intervals. Two weeks after the last vaccine boost all animals were infected twice with a sub-lethal dose of ASFV with a two-day interval. Pigs were sacrificed at day 17 post-infection.

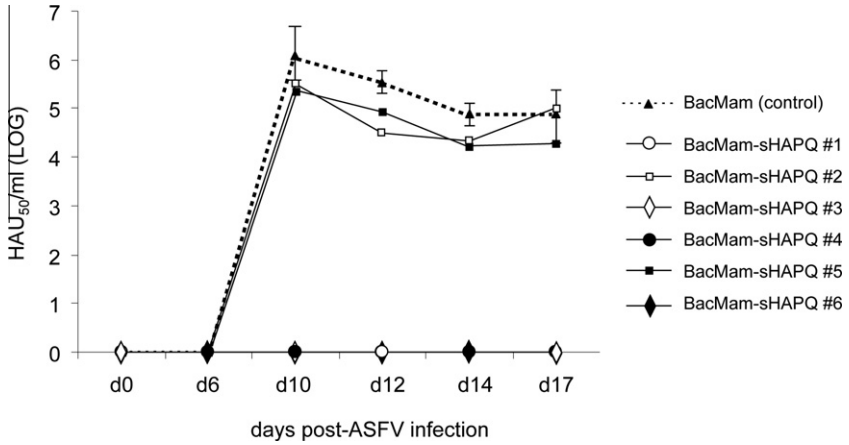

Fig. 2. BacMam-sHAPQ protects 66.6% of pigs against sub-lethal ASFV challenge. Viremia kinetics of BacMam-sHAPQ immunized pigs after ASFV challenge. Results are represented as the logarithm of HAU50/ml serum (mean and standard deviation from control group is shown).

ficient calculated using Monte Carlo simulations, $\rho > 0.42$ with 95% confidence) with the presence of >150 IFNγ-secreting cells per million of PBMCs in their blood by day 17 p.i. (pigs 1, 3, 4, and 6).

Our results are in full agreement with previous observations made using attenuated ASFV in which a direct correlation between protection and the induction of IFNγ-secreting T-cells was postulated (King et al., 2011; Revilla et al., 1992). The lack of protection observed for pigs 2 and 5 seems to corroborate this correlation since these two animals showed significantly lower specific T-cell numbers in their blood at day 17 p.i., perhaps reflecting limitations of certain swine leucocyte antigen (SLA) haplotypes to efficiently present the vaccine-encoded antigens. Finally, the specificity of the detected T-cells was further analyzed by ELISPOT after PBMC stimulation with either the recombinant p30, p54 or HA ASFV proteins. Similar to results obtained after DNA immunization with the same antigens (Argilaguet et al., 2012), specific responses against both the p30 and the p54 ASFV antigens were found, while no specific stimulation was detectable against the full-length HA antigen (data not shown), perhaps reflecting its capacity to inhibit lymphocytic activation *in vitro* (Borca et al., 1998).

In summary, the results presented here clearly demonstrate the capability of BacMam-sHAPQ immunization to protect pigs against a sub-lethal homologous challenge with ASFV in the absence of antibodies. Furthermore, a direct association was found between the protection afforded and the magnitude of the T cell responses induced. Further work should be done to analyze in more detail the phenotype of the specific cells induced after BacMam-sHAPQ immunization. Further work is also required to test the degree of protection achieved with this strategy when animals are infected with a higher dose of the virus, in an attempt

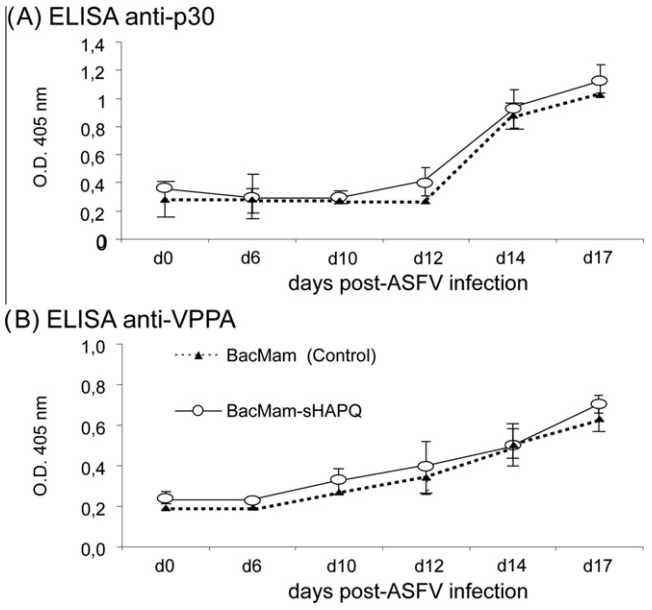

Fig. 3. BacMam-sHAPQ immunization does not induce ASFV-specific antibodies. Sera collected at different times after immunization and infection were used to monitor by ELISA the kinetics of the specific anti-p30 (A) and anti-VPPA (B) antibodies. Data shown correspond to average O.D. values and standard deviations obtained per each immunization group.

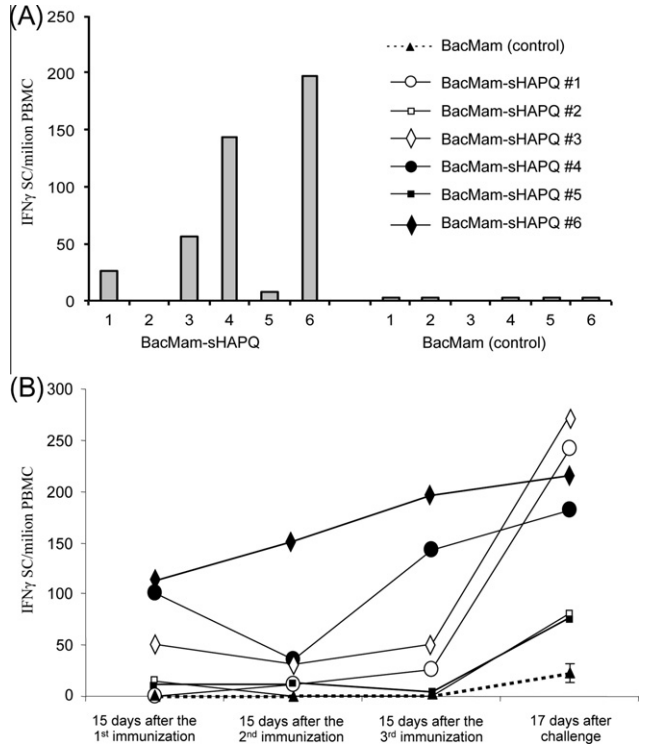

Fig. 4. Protection associates with presence of IFNγ-secreting cells. PBMCs obtained at different time points were *in vitro* stimulated with ASFV to analyze the presence of virus-specific IFNγ-secreting T-cells by ELISPOT. (A) Presence of ASFV-specific IFNγ-secreting T-cells before virus challenge. (B) Presence of high levels of ASFV-specific IFNγ-secreting T-cells at day 17 after challenge.

to reflect the current scenario in the Caucasian regions where disease might be transmitted by pig to pig contact and/or through ingestion/exposure to blood or tissues from infected pigs (Rahimi et al., 2010).

We are currently focussing our efforts on two complementary strategies in order to improve the vaccine immunogenicity: (1) Identifying new ASFV antigenic proteins and/or peptides which could be added to vaccine formulations thus enhancing the chances of inducing broader immune responses in SLA-heterogenous pig populations and (2) testing BacMam-sHAPQ in different prime-boost regimes to try to induce concurrent cellular and humoral immune responses.

Claim disclosure

Part of this work has been subjected to patent by CReSA (Ref. PCT/ES2008/000264 and P26002ES00).

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