Review

Vectored vaccines to protect against PRRSV

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ABSTRACT

PRRSV is the causative agent of the most important infectious disease affecting swine herds world-wide, producing great economic losses. Commercially available vaccines are only partially effective in protection against PRRSV. Moreover, modified live vaccines may allow virus shedding, and could revert generating virulent phenotypes. Therefore, new efficient vaccines are required. Vaccines based on recombinant virus genomes (virus vectored vaccines) against PRRSV could represent a safe alternative for the generation of modified live vaccines. In this paper, current vectored vaccines to protect against PRRSV are revised, including those based on pseudorabies virus, poxvirus, adenovirus, and virus replicons. Special attention has been provided to the use of transmissible gastroenteritis virus (TGEV) as vector for the expression of PRRSV antigens. This vector has the capability of expressing high levels of heterologous genes, is a potent interferon-α inducer, and presents antigens in mucosal surfaces, eliciting both secretory and systemic immunity. A TGEV derived vector (rTGEV) was generated, expressing PRRSV wild type or modified GP5 and M proteins, described as the main inducers of neutralizing antibodies and cellular immune response, respectively. Protection experiments showed that vaccinated animals developed a faster and stronger humoral immune response than the non-vaccinated ones. Partial protection in challenged animals was observed, as vaccinated pigs showed decreased lung damage when compared with the non-vaccinated ones. Nevertheless, the level of neutralizing antibodies was low, what may explain the limited protection observed. Several strategies are proposed to improve current rTGEV vectors expressing PRRSV antigens.

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1. Introduction

PRRSV is the causative agent of the most important infectious disease affecting the porcine herds worldwide. The immune response to PRRSV is poorly understood but, in spite of this, some vaccines are being commercialized. Commercial vaccines are mostly modified live vaccines based on attenuated European or North American PRRSV strains (i.e., Ingelvac®-PRRS from Boehringer Ingelheim, Amervac®-PRRS from Hipra, or Pyrsvac-183 from Syva labs). Nevertheless, some inactivated vaccines are also available (i.e., Progressis® from Merial, Ingelvac®-PRRS KV from Boehringer Ingelheim, or Suipravac®-PRRS from Hipra). Modified live vaccines have been preferentially used, as they can establish protective immunity, measured by viral load in blood and tissues. Nevertheless, current vaccines against PRRSV have several limitations. In general, modified live vaccines protect against challenge with homologous isolates. They could also protect against heterologous viruses (Diaz et al., 2006; Zuckermann et al., 2007). Furthermore, live vaccines provide partial protection against clinical disease but did not prevent infection (Osorio et al., 1998) and, more importantly, they can revert to virulence (Botner et al., 1997; Nielsen et al., 2001). As the attenuated vaccines induce an immune response resembling that induced by PRRSV natural infection, they do not induce high levels of neutralizing antibodies. Killed PRRSV vaccines, on the other hand, in general, have been less effective in prevention of both infection and disease (Ostrowski et al., 2002).

1.1. Immunity of PRRSV

The innate immune response against PRRSV is very weak, probably contributing to the delay in subsequent humoral and cellular immune responses, and also to virus persistence (Kimman et al., 2009). PRRSV does not induce interferon (IFN)-α production (Albina et al., 1998; Calzada-Nova et al., 2010), a key element in host antiviral response, leading to a minimal production of inflammatory cytokines and activation and recruitment of natural killer (NK) cells (Murtaugh et al., 2002). PRRSV-induced suppression of type I IFN production is due to the interference in the activation of IFN-β promoter stimulator 1 (IPS-1), located downstream of sensor molecule RNA helicase RIG-I. The inactivation of IPS-1 avoids IFN regulatory factor (IRF) 3 activation and, consequently, type I IFN production (Luo et al., 2008). Therefore, to design an effective vaccine against PRRSV, it would be advisable to increase the production of type I IFN. To date, different adjuvants promoting the production of IFN have been tested, in addition to the current vaccines formulations, with limited success (Charerntantanakul, 2009).

A hallmark of the swine humoral response against PRRSV is the production of non-neutralizing antibodies detected early in the infection, followed by a low neutralizing antibody (NAb) titer that is detected more than 3 weeks after infection (Kimman et al., 2009; Murtaugh et al., 2002). One possible explanation for the late detection of NAbs is the difference on technique sensitivity, as ELISA has higher sensitivity than neutralization assays. Therefore, the presence of very low titers of NAbs early in the infection cannot be completely discarded. Early non-neutralizing antibodies are mainly induced by nucleocapsid (N), M and GP5 proteins, and have been involved in antibody-dependent enhancement of PRRSV infection (Mateu and Diaz, 2008; Murtaugh et al., 2002). NAbs are induced by GP3, GP4, GP5 and M proteins, although the ones recognizing GP5 are the most relevant for protection (Kim and Yoon, 2008; Ostrowski et al., 2002). Two B cell epitopes were identified in GP5 protein ectodomain: an immunodominant epitope (IDE), that has been proposed to act as a decoy epitope, and an epitope critical for neutralization (ECN), that is recognized by NAbs (Ostrowski et al., 2002). Several hypothesis have been proposed to explain the delay in NAbs induction by GP5 protein, such as the presence of the IDE, and glican-shielding of the ECN (Lopez and Osorio, 2004). The role of NAbs in protection was demonstrated by passive transfer of these antibodies (Osorio et al., 2002). Protection of swine against PRRSV infection correlated with the level of NAbs and it was proposed that an efficient vaccine must induce NAb titers of 1:32 to prevent PRRSV infection (Lopez et al., 2007).

PRRSV infection results in a weak and delayed T cell mediated immune response that should be necessary for the elimination of the virus (Mateu and Diaz, 2008; Murtaugh et al., 2002). It has been shown that the induction of IFN-γ secreting cells, complementing neutralizing antibodies, provides partial protection against PRRSV (Zuckermann et al., 2007). As interleukin (IL)-10 levels inversely correlate with IFN-γ response, it has been proposed that the expression of IL-10 may be responsible for the suppression of T cell responses (Charerntantanakul et al., 2006; Kimman et al., 2009). M protein is the most potent inducer of T cell proliferation, followed by GP5, GP3 and GP2 (Bautista et al., 1999), and may play a role in protection. Different vaccine adjuvants have been tested to improve T cell responses to PRRSV. Nevertheless, in addition to the adjuvants included in vaccine formulation, only IL-2 and CpG oligodeoxynucleotides enhanced protection conferred by current vaccines (Charerntantanakul, 2009).

1.2. Factors affecting PRRSV vaccine development

There are three main problems for the development of more efficient vaccines against PRRSV: the correlates of protection are not well known, PRRSV may induce negative regulatory signals for the immune system, and there is a extremely large antigenic variability in PRRSV structural proteins. As indicated above, the PRRSV heterodimer GP5-M must be the main inducer of protective humoral and cellular responses. Nevertheless, minor structural proteins are also required for PRRSV virion infectivity (Wissink et al., 2005) and may play a role in protection. Also, there is limited information about the T cell epitopes implicated in the induction of a protective T cell response (Mateu and Diaz, 2008).

One of the mechanisms used by viruses to suppress or evade the host immune response is the induction of regulatory T cells (Treg). Porcine Treg phenotype is CD4$^+$ CD25$^+$ Foxp3$^+$ (Kaser et al., 2008), as that described for human and mice (Belkaid, 2007). Tregs have been classified in natural and induced. The latter ones can be subdivided in three subtypes: Treg1 (TR1) secreting IL-10, T helper 3 (Th3) secreting transforming growth factor (TGF)-β, and converted Tregs (Belkaid, 2007). It has been recently described that American

type PRRSV-infected dendritic cells induced Tregs, an effect that was reverted by the addition of IFN-α. The induced Treg population is a Th3 type, as it promotes TGF-β but not IL-10 expression (Silva-Campa et al., 2009). In contrast, dendritic cells infected with EU type PRRSV viruses did not induce Treg cells, although they exhibited an unbalanced ability to stimulate T cell immune responses (Silva-Campa et al., 2010). The impact of Treg induction on delayed immune responses after PRRSV infection remains to be established, as well as the viral proteins involved in this process.

PRRSV strains are extremely diverse, even when they belong to the same genotype. Among the structural proteins, M protein is the most conserved one, while GP5 is the most variable one (Dea et al., 2000). This high antigenic variability represents a problem for the development of universal vaccines against PRRSV, as shown by the low efficacy of current vaccines against heterologous challenge. To solve this problem, common critical B and T cell epitopes must be identified (Mateu and Diaz, 2008). Nevertheless, it has been reported that the ability of a vaccine to induce a strong cellular immune response may be more important for protection than the genetic similarity with the challenge strain (Diaz et al., 2006). An additional problem is the difference in the immune responses elicited by PRRSV in animals with different host genetic background (Lewis et al., 2007). Therefore, the knowledge of host responses to PRRSV infection is required for the development of an efficient vaccine.

2. Vectored vaccines

As mentioned above, both modified live and inactivated vaccines have been developed for PRRSV. Live vaccines led to better results than killed-virus based vaccines. Nevertheless, the live attenuated vaccines have several problems such as incomplete protection, virus shedding and possible reversion to virulence (Kimman et al., 2009). This problem was increased by the use of potentially hazardous methods to control the disease, such as the use of live field virus to vaccinate pigs. Vector-based vaccines could represent an advantage to stimulate both humoral and cell immune responses against PRRSV, and for the design of a marker vaccine. Nevertheless, the results reported to date using viral vectors are not fully satisfactory and new vectors, or antigenic combinations, must be explored.

2.1. Pseudorabies virus (PRV)

PRV, also known as Aujeszky's disease virus (ADV) is an alphaherpesvirus, classified within the family *Herpesviridae*. PRV is the causing agent of pseudorabies that was a worldwide-spread economically important disease. Swine is the natural host of PRV, but the virus also infects a broad range of vertebrates, including farm animals (Pomeranz et al., 2005). In order to eradicate the virus, modified live vaccines have been successfully used. All the vaccine strains were gE⁻ phenotype, i.e., have a gE gene deletion. The elimination of gE causes virus attenuation by reducing the virus transmission, but does not reduce virus production in cell culture nor the induction of protective immunity (Nauwynck et al., 2007). These live attenuated PRV have been used as vectors to protect against swine infectious diseases, such as classical swine fever (Hooft van Iddekinge et al., 1996), or porcine circovirus (Ju et al., 2005).

A recombinant PRV, based on the attenuated Bartha strain, was constructed expressing PRRSV GP5 protein (Qiu et al., 2005). Protection was evaluated by inoculation of 4-week-old piglets and homologous challenge with PRRSV CH-1 strain. None of the animals, even those inoculated with a commercially available inactivated vaccine, developed anti-GP5 antibodies before challenge. After challenge, the production of anti-GP5 antibodies was detected

in all animals. Nevertheless, none of them produced neutralizing antibodies against PRRSV (Qiu et al., 2005). Reduced lung lesions and viremia, and faster virus elimination from tissues was observed in animals inoculated with PRV vaccine vector, similar to that found in animals inoculated with the commercial vaccine (Qiu et al., 2005).

Alternative recombinant attenuated PRV vaccine vectors expressing different combinations of PRRSV antigens have also been generated. These vectors expressed GP5 alone or together with M protein, or modified GP5 (GP5m), containing a Pan DR T-helper cell epitope (PADRE) between the decoy epitope and the ECN, recognized by NAbs (Fang et al., 2006), alone or co-expressed with M protein. The GP5-M heterodimer was detected in the recombinant PRVs co-expressing both proteins, suggesting that PRRSV antigenic structures were not changed (Jiang et al., 2007c). The PRV co-expressing GP5m and M proteins was the most promising candidate in the induction of neutralizing antibodies and lymphocyte proliferation, as tested in the mouse model. As a consequence, the protection conferred by this vector was evaluated in the porcine respiratory model, in relation to the protection provided by a commercially available PRRSV killed vaccine. Animals inoculated with the recombinant PRV expressing PRRSV proteins developed NAbs before the challenge. Furthermore, after challenge, the NAb titer was up to 4-fold higher in animals inoculated with recombinant PRRSV compared with those inoculated with the killed vaccine. None of the animals inoculated with the empty PRV developed neutralizing antibodies at any time during the experiment (Jiang et al., 2007c). Lymphocyte proliferative responses were also higher in animals inoculated with the recombinant PRV expressing GP5m and M proteins. Accordingly, lung lesions and viremia were lower in these animals, indicating a certain protection against the homologous challenge (Jiang et al., 2007c).

2.2. Adenovirus

Adenoviruses are currently one of the most extended systems for gene delivery. As vectors, they have high capacity for the insertion of foreign genes (from 5 Kb up to 36 Kb, depending on the system), and are able to transduce a broad range of cell types (Bantounas and Uney, 2007). Different replication-defective recombinant adenoviruses (rAd) have been used as vectors for PRRSV, both for vaccine development and for analysis of immunogenic properties of PRRSV wt or modified structural proteins.

A set of rAds expressing PRRSV GP5, M and a M-Gly-Thr-Thr-GP5 fusion protein were generated. These rAds were tested in the mouse model. The rAd expressing M-GP5 fusion protein induced and increased neutralizing antibodies humoral immune response, compared with mice inoculated with rAd expressing GP5 and M proteins independently or empty adenovirus vector (Jiang et al., 2006). The rAd expressing M-GP5 fusion protein also induced enhanced lymphocyte proliferation and cytotoxic T-lymphocyte (CTL) responses (Jiang et al., 2006). Unfortunately, protection conferred by these vectors was not evaluated in the porcine system.

The same authors also generated a set of rAds expressing other PRRSV structural protein combinations, such as GP3, GP4 or GP5 alone, and GP3-GP5, GP4-GP5 or GP3-GP4-GP5 fusion proteins (Jiang et al., 2008). Mice inoculated with rAds expressing fusion proteins developed higher NAb titers and lymphocyte proliferation responses than those inoculated with rAds expressing independent PRRSV proteins. Interestingly, specific CTL responses were higher in mice inoculated with rAds expressing GP3-GP5 or GP3-GP4-GP5 fusion proteins (Jiang et al., 2008). In fact, authors selected the recombinant rAd GP3-GP5 as the best vaccine candidate for testing protection in pigs. This recombinant induces NAbs in vaccinated piglets before challenge, and higher lymphocyte proliferation responses, IL-4 and IFN-γ production. Nevertheless, the

rAd expressing GP3-GP5 fusion protein did not fully protect against homologous challenge, as only a moderate decrease in lung lesions and viremia was observed (Li et al., 2009; Wang et al., 2009).

To improve the efficacy of the rAd-based vaccine, heat shock protein (HSP) 70 and granulocyte-macrophage colony stimulating factor (GM-CSF) were co-expressed as genetic adjuvants (Li et al., 2009; Wang et al., 2009). A set of rAds was obtained, expressing HSP70-5xGly-GP3-GP5 and HSP70-2A-GP3-GP5 fusion proteins, with a five glycine or a 2A protease linker, respectively. Piglets inoculated with rAd expressing fusion proteins induced higher NAb titers, produced higher IFN-γ levels, and presented reduced lung lesions, than those inoculated with rAd expressing GP3-GP5 protein (Li et al., 2009). Introduction of 2A protease between the HSP70 and PRRSV fusion protein resulted in a better production of IL-4 by inoculated animals, and also lower viremia. This could be due to the release of native HSP70 with higher adjuvant activity (Li et al., 2009).

GM-CSF has been widely used as an effective mucosal adjuvant (Toka et al., 2004). Intranasal inoculation of vectors expressing GM-CSF stimulates IFN-γ and IL-12 production in lung tissues (Bukreyev et al., 2001). A rAd expressing GM-CSF-Leu-Glu-GP3-Lys-Leu-GP5 fusion protein was generated. A moderate increase in NAb levels was observed before challenge in piglets inoculated with this rAd vector, compared with animals inoculated with empty rAd or rAd expressing GP3-GP5 fusion protein alone. After challenge, animals inoculated with rAd expressing the fusion protein containing the adjuvant developed significantly higher NAbs than the control animals (Wang et al., 2009). Lymphocyte proliferation responses and IFN-γ and IL-4 production were also enhanced in those animals. These enhanced immune responses correlated with a significant decrease in the viremia and lung lesions, indicating that GM-CSF enhanced the immunogenicity of rAd-based GP3-GP5 vaccine (Wang et al., 2009).

Adenovirus vectors have also been used to evaluate the antigenicity of PRRSV structural proteins, such as GP3 and GP5, and the role of GP5 glycosylation on immune responses (Jiang et al., 2007a,b). Unfortunately, these studies have been performed using the mouse model. The effect of IFN-α for protection against PRRSV has recently been analyzed using rAd. Piglets were inoculated with a rAd expressing porcine IFN-α and challenged with PRRSV (Brockmeier et al., 2009). Results obtained indicate that the presence of IFN-α has a moderate protective effect against PRRSV infection.

2.3. Poxvirus

Poxviruses are the largest known animal DNA viruses. They have been extensively used as expression vectors for vaccination, allow expression of large foreign genes, induce strong cell mediated and humoral immune responses, and safe poxvirus vectors are available (Paoletti, 1996; Wang et al., 2007).

Fowlpox was the first poxvirus used as vaccine vector for PRRSV (Guoshun et al., 2007). Fowlpox virus (FPV) belongs to the Avipoxvirus genus, and its replication is restricted to avian species. Nevertheless, attenuated strains of FPV have been used as vectors for poultry and mammals, resulting in strong and protective immune responses (Paoletti, 1996; Wang et al., 2007). A GP5-Pro-Pro-Ser-GP3 fusion protein, alone or combined with porcine IL-18, was expressed using recombinant FPV. Piglets inoculated with recombinant FPV expressing PRRSV antigens induced neutralizing antibodies at 42 dpi, and higher lymphocyte proliferation response, than those inoculated with the empty vector (Guoshun et al., 2007). Vaccinated animals also showed increased IFN-γ production, compared with non-vaccinated ones. Piglets vaccinated with FPV co-expressing PRRSV antigens and IL-18 produced higher IFN-γ amount than those inoculated with FPV expressing GP5-GP3

fusion protein alone (Guoshun et al., 2007). Partial protection was also observed after challenge with an homologous strain, as viremia was decreased in vaccinated animals (Guoshun et al., 2007).

Modified vaccinia virus Ankara (MVA), a member of the Orthopoxvirus genus, has also been used as a vector for PRRSV (Zheng et al., 2007). MVA was used as the vaccine agent for the prevention of smallpox, and has been extensively used as viral vector for infectious diseases and cancer. MVA is highly attenuated, even in immunosuppressed animals, but induces strong humoral and cellular immune responses (Wang et al., 2007). Four recombinant MVA viruses expressing PRRSV antigens were constructed, expressing GP5 or M proteins alone, GP5-M fusion protein, or co-expressing GP5 and M proteins (Zheng et al., 2007). These vectors were tested in the mouse model. Mice inoculated with recombinant MVA expressing heterologous antigens developed PRRSV neutralizing antibodies, with the highest antibody titers found in mice inoculated with the recombinant MVA co-expressing GP5 and M proteins. Similar results were obtained when IFN-γ and IL-2 production was analyzed, indicating a Th1 type cellular immune response (Zheng et al., 2007). Unfortunately, authors did not perform protection experiments in piglets. Therefore, the usefulness of MVA as vector for PRRSV vaccination remains to be determined.

2.4. Virus replicons

Expression vectors have been engineered using different viral replicons, by replacing the virus structural genes by heterologous ones. These RNA vectors, or replicons, express high levels of the foreign proteins, and replicate but are not packaged into virus-like particles unless structural proteins are provided in trans. Therefore, replicons do not spread into neighbor cells and are safe for their use as vaccines (Nagai et al., 2007; Rayner et al., 2002).

An alphavirus replicon derived from Venezuelan equine encephalitis virus (VEEV) has been successfully used as a vaccine against different pathogens, including swine influenza (Vander Veen et al., 2009). VEEV-derived vaccines induce robust humoral, mucosal and cellular immunity (Rayner et al., 2002). It has been recently described that a VEEV replicon expressing PRRSV GP5 and M proteins reduced viremia after PRRSV challenge and provides partial protection (Mogler et al., 2008, 2009).

Replicons from classical swine fever virus (CSFV) and vesicular stomatitis virus (VSV) expressing GP5 and M proteins have also been generated, expressing high levels of PRRSV antigens (N. Ruggli, personal communication).

3. TGEV as a vector

Coronaviruses have several advantages as vectors over other viral expression systems: (i) they are single-stranded RNA viruses that replicate in the cytoplasm without a DNA intermediary, making integration of the virus genome into the host cell chromosome unlikely (Lai and Cavanagh, 1997); (ii) these viruses have the largest RNA virus genome and, in principle, have room for the insertion of large foreign genes (Enjuanes et al., 2001, 2005); (iii) a pleiotropic secretory immune response is best induced by the stimulation of gut associated lymphoid tissues. Since coronaviruses in general infect both respiratory and enteric mucosal surfaces, these viruses may be used to target the antigen to the enteric and respiratory areas to induce a strong secretory immune response; (iv) the tropism of coronaviruses may be engineered by modifying the S gene (Ballesteros et al., 1997; Kuo et al., 2000; Sanchez et al., 1999); (v) non-pathogenic coronavirus strains infecting most species of interest (human, porcine, bovine, canine, feline, and avian) are available and therefore are suitable to develop safe virus vectors; and (vi) infectious coronavirus cDNA clones are available to design expression systems.

Our group obtained the first infectious coronavirus cDNA clone, for TGEV. This cDNA was propagated as a bacterial artificial chromosome (BAC) (Almazán et al., 2000; Gonzalez et al., 2002). Vectors based on this infectious cDNA were engineered by cloning foreign genes in the place previously occupied by non-essential genes 3a and 3b, leading to high (>50 μg/10⁶ cells) and stable (>30 passages) expression levels of specific heterologous genes (Enjuanes et al., 2005; Ortego et al., 2003; Sola et al., 2003). Foreign gene expression levels were optimized by the study of the transcription-regulating sequences (TRSs), involved in coronavirus gene expression. Our group has generated a set of TRSs ranging from intermediate to high foreign gene expression levels (Alonso et al., 2002), a combination of these TRSs could be used to drive the expression of two or three heterologous genes from just one infectious cDNA (i.e., dicistronic or tricistronic vectors). TGEV derived vector biosafety was improved by the generation of replication-competent, propagation-deficient viruses (Ortego et al., 2002).

Porcine respiratory coronavirus (PRCV) is a mutant of TGEV that replicates in the respiratory tract and causes no or mild clinical signs. PRCV is spread worldwide and induces antibodies that can also neutralize TGEV (Saif et al., 1994). Therefore, preexisting immunity against the TGEV vector could have been a problem. Nevertheless, in vivo experiments showed that antibody titers against TGEV increased even after two re-infections of pigs with rTGEV vector (Alonso S., Sola I. and Enjuanes L., unpublished results). One of the main advantages of recombinant TGEV (rTGEV) as a vector for PRRSV is that TGEV is a potent inducer of IFN-α in a process that is mediated by the virus transmembrane (M) protein (Calzada-Nova et al., 2010; Charley and Laude, 1988). In addition, as mentioned above, TGEV vectors may present antigens at mucosal sites, eliciting mucosal and systemic immune responses. Therefore, rTGEV vectors will represent a novel strategy to study the induction of protection against PRRSV.

4. Engineered TGEV vectors expressing PRRSV antigens

4.1. The GP5-M antigenic platform

PRRSV structural proteins GP5 and M accumulate in the endoplasmic reticulum of infected cells, where they form disulfide-linked heterodimers that are incorporated into the virion. M protein
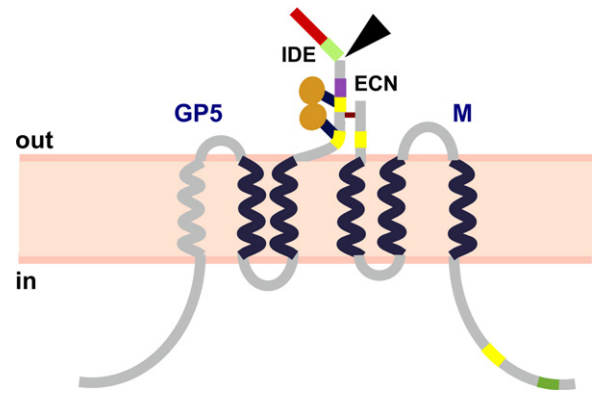

Fig. 1. Predicted GP5-M heterodimer topology. The PRRSV GP5-M heterodimer may be anchored in membranes, with both proteins exposing to the surface a short N-terminal ectodomain. The GP5 protein ectodomain contains the protein motives relevant in antigenicity, such as the epitope critical in neutralization (ECN, purple) and the decoy immunodominant epitope (IDE, green). Signal peptide (red) cleavage is represented by a black arrowhead. Both GP5 and M proteins contain predicted glycosylation sites (yellow), although only GP5 protein is glycosylated (represented by orange circles). M protein contains in its C-terminal an endoplasmic reticulum retention signal (dark green).

homodimers are also detected in infected cells, but are not incorporated into the virus particle (Dea et al., 2000; Meulenberg, 2000). GP5 and M proteins are essential for the production of viral particles, although additional minor envelope proteins are required for virion infectivity (Wissink et al., 2005). According to the accepted topology of the GP5-M heterodimer (Fig. 1), both GP5 and M proteins expose a short ectodomain on the virion surface, being involved in receptor recognition. GP5 ectodomain contains several glycosylation sites, depending on the viral strain. It has been described that GP5-M protein heterodimer formation is previous to GP5 glycosylation (Mardassi et al., 1996). GP5 glycosylation sites are close to the ECN epitope, and it has been proposed that the steric hindrance caused by the glycosylation is one of the causes for the potential delay in the production of NAbs after PRRSV infection (see below). It has been recently described that GP5-M heterodimer interacts with the PRRSV receptor, porcine
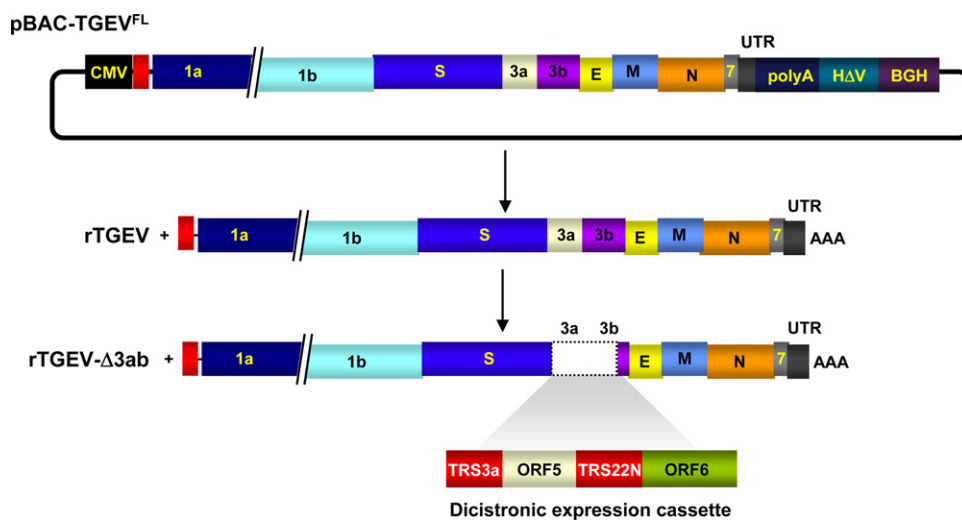

Fig. 2. Design of rTGEV expressing PRRSV antigens. Scheme of the TGEV infectious cDNA clone, cloned in a BAC (pBAC-TGEVᶠᴸ). After transfection of cells, a full-length virus genome is generated (rTGEV). CMV, cytomegalovirus immediate-early promoter; polyA, tail of 24 A residues; HDV, hepatitis delta virus ribozyme; BGH, bovine growth hormone termination and polyadenylation sequences. The TGEV derived vectors are based on a TGEV genome in which non-essential 3ab genes were deleted (rTGEV-Δ3ab). Genes encoding PRRSV heterologous proteins were cloned in this position. Expression of the foreign genes was driven by transcription regulatory sequences (TRSs) from genes 3a and N.
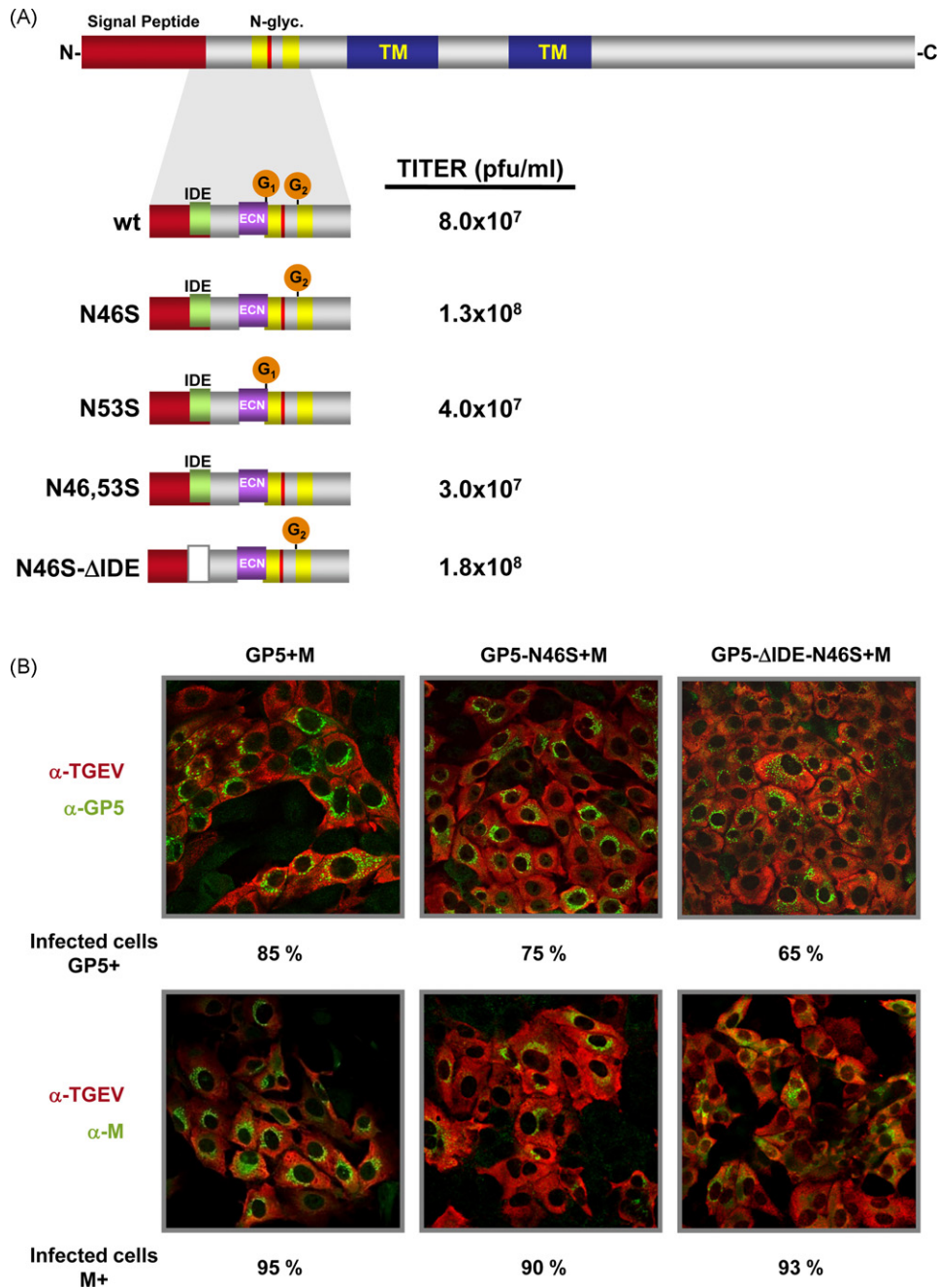
Fig. 3. Generation of rTGEV co-expressing GP5 and M proteins. (A) Schematic representation of PRRSVOlot91 GP5 domains. A detail of the domain containing the epitopes inducing non-neutralizing (IDE) and neutralizing (ECN) antibodies is shown. Two N-glycosylation sites, N46 (G1) and N53 (G2), are located within this domain. Three different mutants were generated, substituting Asn 46 and 53 by Ser, avoiding the glycosylation at these positions (N46S, N53S, and N46,53S). An additional mutant, lacking N46 glycosylation site and decoy epitope, was obtained (N46S-ΔIDE). In all cases, rTGEV viruses were recovered with high titers. (B) ST cells were infected with the rTGEVs and double immunofluorescence staining was performed. TGEV N protein specific monoclonal antibodies and a secondary antibody staining red were used to identify virus-infected cells. Expression of GP5 was detected with rabbit antiserum specific for a GP5 peptide coupled to a secondary antibody staining green (upper panels). Expression of M protein was detected with a rabbit antiserum specific for an M protein peptide, coupled to a secondary antibody staining green (lower panels). The percentage of infected cells expressing PRRSV antigens was estimated by the analysis 10 different microscopic fields.

sialoadhesin, and that this interaction is dependent on GP5 glycosylation, most likely at the glycosylation site overlapping with the epitope recognized by neutralizing antibodies (Van Breedam et al., 2010). As described above, GP5 and M proteins have been involved in the induction of PRRSV neutralizing antibodies and a strong cellular immune response, respectively (Bautista et al., 1999; Ostrowski et al., 2002). These data indicate that the GP5-M heterodimer is the most promising antigenic structure that could be used in the construction of an efficacious vaccine against PRRSV.

4.2. rTGEV expressing GP5 and M proteins

A dicistronic TGEV cDNA encoding PRRSV GP5 and M proteins was engineered (Fig. 2). PRRSV genes were cloned in the place of non-essential genes 3a and 3b. GP5 expression was driven by the transcription-regulating sequence of gene 3a (TRS3a), while M protein was expressed from an optimized TRS partially derived from gene N (TRS22N) (Alonso et al., 2002). Therefore, PRRSV genes were expressed from independent subgenomic mRNAs. The recovered virus expressed GP5 and M proteins in 85% and 95% of the
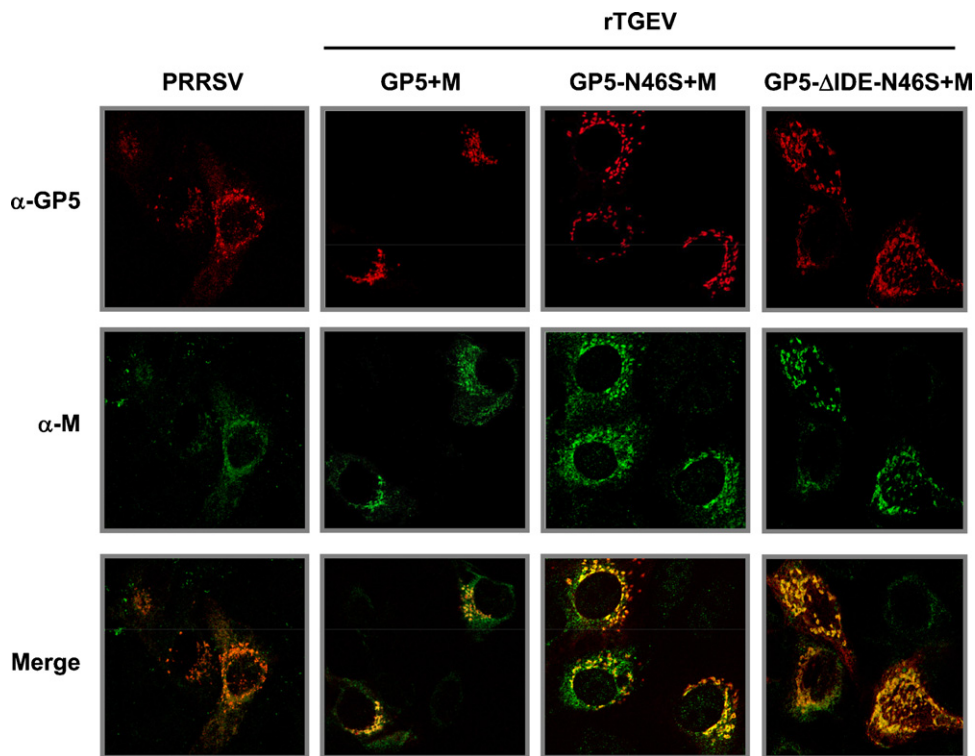
Fig. 4. Colocalization of GP5 and M proteins. To study if GP5 and M proteins expressed by rTGEVs also colocalize, confocal microscopy analysis was performed. MA-104 or ST cells were infected with PRRSV and the rTGEVs, respectively, and double immunofluorescence staining was performed. Expression of GP5 was detected with a monoclonal antibody specific for GP5, coupled to a secondary antibody staining red (upper panels). Expression of M protein was detected with a rabbit antiserum specific for an M protein peptide, coupled to a secondary antibody staining green (medium panels). As shown in the merge, colocalization of GP5 and M proteins was observed both in the PRRSV and rTGEV infected cells (lower panels). Mutant GP5 proteins (GP5-N46S and GP5-ΔIDE-N46S) expressed by rTGEVs also colocalized with M protein.

rTGEV infected cells, respectively (Fig. 3(B)). Expression levels were maintained even in virus recovered from tissues after infection of piglets with the rTGEV. This result substantially advanced the efficacy of previous rTGEVs expressing individually PRRSV antigens, showing high expression levels of GP5, but with limited stability. Co-expression of M protein with GP5 reduced GP5 toxicity and probably will elicit a better T cell immune response. The protection conferred by this vector was tested in vivo. One-week-old piglets were inoculated with 1×10^8 pfu of the rTGEV by three routes: oral, nasal and intragastric. Nine weeks later, a challenge was perfomed with 1×10^7 TCID$_{50}$ of a virulent European PRRSV strain. Blood samples were collected at different times post-inoculation, and humoral immune responses were evaluated by ELISA. All animals presented a high antibody response against TGEV, therefore, the vector infected target tissues as expected. Vaccinated animals also showed a clear humoral response against PRRSV GP5 and M proteins. A fast recall of the immune response was observed after the challenge, as vaccinated animals induced higher antibody titers against PRRSV antigens and earlier than control ones. Nevertheless, the immune response elicited by this rTGEV provided very limited protection, and antibody titers decreased before challenge. The lack of protection against challenge was likely due to the relatively low levels of neutralizing antibodies produced before challenge. Nevertheless, results using rTGEV as a platform were promising, as a humoral immune response against PRRSV antigens was elicited.

4.3. Generation of rTGEV based vaccines expressing modified GP5 protein

GP5 antigenicity may be a problem for the obtention of efficient vaccines. Therefore, several strategies to change GP5 antigenic structure were performed (Fig. 3(A)). In all cases, the GP5 mutants were co-expressed with M protein using a dicistronic vector, to minimize toxicity problems due to GP5 production.

4.3.1. Expression of GP5 mutants with a modified glycosylation pattern

The ectodomain of GP5 protein is N-glycosylated. There are three or four predicted glycosylation sites in the GP5 from the North American strains of PRRSV, whereas there are only two sites in the GP5 protein from European strains (wt, Fig. 3(A)). The relevance of the N-glycans in GP5 antigenicity is not clear. It has been proposed that the removal of the glycosylation sites could lead to the improvement of the immune response against PRRSV, due to the elimination of the steric hindrance raised by the carbohydrate on the epitope inducing NAbs (Ansari et al., 2006). Elimination of the glycosylation sites present only in the North American strains, both in engineered and natural PRRSV mutants, led to an increase in the levels of NAbs induced by the mutant viruses (Ansari et al., 2006; Faaberg et al., 2006). Nevertheless, it is worth noting that these sites are not present in European PRRSV strains. Although the elimination of the glycosylation site overlapping the epitope critical for neutralization (G1) (Fig. 3(A)) often leads to non-infectious viruses (Ansari et al., 2006; Wissink et al., 2005), natural North American strain mutants lacking this glycosylation site were found (2.1% of the sequenced GP5 proteins). Surprisingly, one of this natural mutants elicited lower neutralizing antibody response than the wild-type PRRSV strain (Faaberg et al., 2006). This is in contrast with the data obtained with lactate dehydrogenase-elevating virus (LDV), where deletion of the N-glycan enhanced the NAbs response (Plagemann and Moenngin, 1997). Elimination of the most conserved N-glycosylation site (G2, Fig. 3(A)) (only 0.2% of the sequenced North American GP5 proteins lack this motif) led to higher levels of neutralizing antibodies compared with the response elicited by the wild-type virus (Ansari
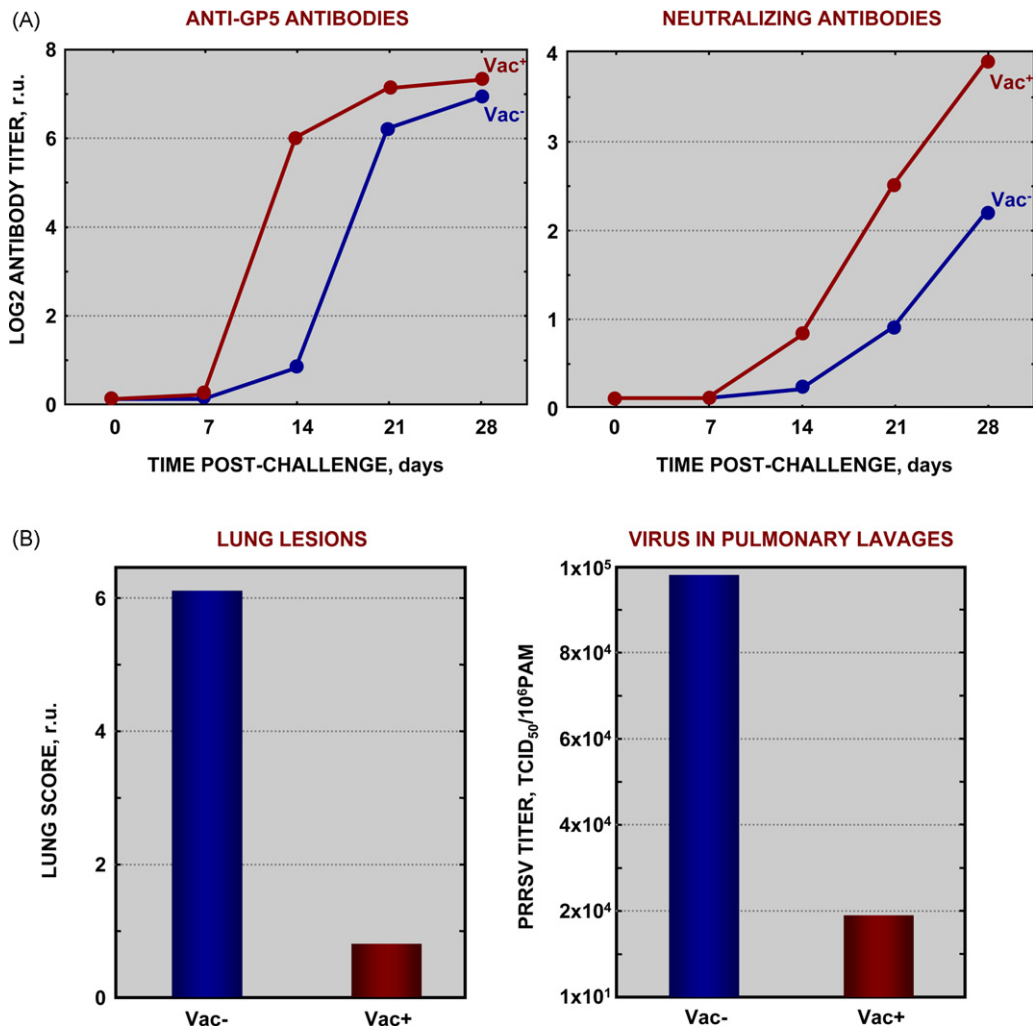
(A)

(B)

Fig. 5. Protection conferred by rTGEV based inactivated vaccine expressing GP5 with altered glycosylation pattern. (A) Killed vaccine was formulated from rTGEVs expressing GP5 with altered glycosylation pattern. Protection was analyzed and blood samples of animals were collected at indicated times post-challenge. Samples were analyzed by immunoperoxidase monolayer assay (IPMA) specific to detect antibodies against GP5 (left panel). Cells expressing recombinant GP5 were used as antigens for the IPMA assay. Neutralizing antibodies titers were calculated from neutralization assays of PRRSV Olot91 strain infecting MA-104 cells (right panel). (B) Lung damage caused by PRRSV infection (left panel). The lungs from vaccinated and non-vaccinated animals were analyzed. Lung lesions observed in all the pigs, with different degree of severity, included a craneo-ventral consolidation of apical and medial lung lobes. Viremia was also analyzed (right panel) by PRRSV quantification in samples, using Q-RT-PCR. Results were expressed as PRRSV $TCID_{50}$ per million of pulmonary lavages (PAM).

et al., 2006). As glycosylation of GP5 is probably also involved in virus infectivity, it is difficult to analyze the influence of N-glycans on the immunogenicity of the protein. In the rTGEV system, PRRSV GP5 and M are not involved in infectivity and, therefore, the relevance of these proteins antigenicity in protection could be analyzed in our laboratory using the rTGEV vector.

GP5 mutants lacking glycosylation site G1 (N46S), G2 (N53S) or both (N46,53S) were generated (Fig. 3(A)). The mutation Asn by Ser was selected in all cases, as this substitution most likely introduced little secondary structure modifications. Also, some PRRSV field strains bear similar Asn by Ser aminoacid mutations in putative glycosylation sites. All rTGEV viruses were recovered with high titers (Fig. 3(A)). Nevertheless, only the N46S mutant, lacking the glycosylation site partially overlapping the ECN, was stable (Fig. 3(B)). This rTGEV vector expressed high levels of GP5-N46S and M PRRSV proteins in 75% and 90% of the infected cells, respectively (Fig. 3(B)).

4.3.2. Generation of GP5 mutants lacking the decoy epitope

Several B cell epitopes have been found in GP5 protein. An immunodominant epitope is located in the endodomain and, therefore, has probably limited effect on the antigenicity of the ECN epitope, as it is not exposed in the viral surface (Dea et al., 2000;

Oleksiewicz et al., 2002; Rodriguez et al., 2001). A second immunodominant epitope (IDE) was described in the ectodomain of GP5, close to the ECN (Fig. 3(A)) (Ostrowski et al., 2002). It has been suggested that this immunodominant site could be responsible for the delay in the production of NAbs against PRRSV acting as a decoy epitope. Antibodies against IDE and ECN epitopes were found in the sera of PRRSV infected pigs, appearing at different times post-infection. Furthermore, an increase in the titers against ECN correlates with a decrease in the level of antibodies specific for IDE (Lopez and Osorio, 2004; Ostrowski et al., 2002). An enhanced immunogenicity of a recombinant GP5 protein in which a synthetic sequence spacer has been introduced between IDE and ECN epitopes, to better display the neutralizing epitope has been reported. The data suggests that IDE is in fact acting as a decoy epitope (Fang et al., 2006).

rTGEV vectors were engineered expressing GP5 mutants lacking IDE, in order to clarify whether this epitope is acting as a decoy epitope, enhancing the production of PRRSV specific NAbs. This approach represents an advance over similar constructions made in a PRRSV infectious cDNA clone, as in this case the deletion of the decoy epitope prevents the recovery of the recombinant virus (Ansari et al., 2006). Two GP5 modifications were combined within

the same construct, expressing GP5 protein lacking the decoy epitope and the glycosylation site overlapping the epitope recognized by neutralizing antibodies (N46S-ΔIDE, Fig. 3(A)). The rTGEV virus was recovered with high titer (Fig. 3(A)), and expressed modified GP5 and M proteins in 65% and 93% of the infected cells, respectively (Fig. 3(B)).

4.4. Stability of PRRSV proteins expression in the rTGEV system

The data obtained in cultured cells suggest that rTGEV vectors expressing PRRSV antigens were not fully stable, mainly due to GP5 protein toxicity resulting in a significant lost of GP5 expression after 8–10 virus vector passages in cell culture. In contrast, M protein expression was fully stable, with at least 95% of infected cells expressing M protein for more than 10 passages in tissue culture. A decrease in GP5 expression was also observed after the introduction of modifications in this protein (upper panels, Fig. 3(B)). Again, M protein expression remained constant, independently of GP5 mutant co-expressed (lower panels, Fig. 3(B)). The reduction in GP5 expression could be responsible of the modest results in protection observed with the live rTGEV vectors, in comparison to the protection elicited with non-infectious antigens expressed using rTGEV vectors.

As described above, the rTGEV vector expressing PRRSV GP5 and M proteins represents a substantial advance on the efficacy of previous rTGEVs expressing PRRSV antigens (i.e., GP5 alone). We postulated that co-expression of M protein with GP5 reduces GP5 toxicity by the formation of GP5-M heterodimer. To clarify this issue, confocal microscopy analysis was performed (Fig. 4). MA-104 or ST cells were infected with PRRSV and the rTGEVs, respectively, and double immunofluorescence staining was performed. As shown in the merge (Fig. 4, lower panels), colocalization of GP5 and M proteins was observed both in the PRRSV and rTGEV infected cells. This result suggests that the GP5-M heterodimer is formed in both cases. The decrease in GP5 expression levels by the introduction of GP5 mutations suggested that the modifications could affect heterodimer formation. Colocalization of GP5 and M proteins was also observed when a mutant GP5 protein (i.e., GP5-N46S, or GP5-ΔIDE-N46S) was expressed by the rTGEV vector (Fig. 4), suggesting that a heterodimer was also formed by mutant GP5 proteins. Co-immunoprecipitation of GP5 and M proteins to fully demonstrated GP5-M heterodimer formation is in progress.

4.5. Protection conferred by rTGEV derived vaccines

4.5.1. Formulation of a killed vaccine expressing GP5 mutants with alterations in the glycosylation pattern

As a complementary approach, a killed vaccine was developed based on the rTGEV-GP5-N46S-M virus, co-expressing GP5 lacking the first glycosylation site and M proteins. ST cells were infected with this rTGEV, and the culture medium was harvested at 48 hpi. Soluble antigens were inactivated by incubation with binary ethylenimine (BEI), and a vaccine was formulated. Groups of six 1-week-old piglets were intramuscularly inoculated with the formulation to evaluate the protection conferred by this vaccine. A boost was performed 3 weeks after inoculation. Six weeks after the first inoculation, animals were challenged by intranasal inoculation with 10^7 TCID$_{50}$ of PRRSV/Olot91 strain. Blood samples were collected at different times post-inoculation to determine the levels of specific antibodies by ELISA. Vaccinated animals induced higher and faster antibody titers against PRRSV antigens than control animals (Fig. 5(A), left panel). Neutralizing antibody titers were also higher in the vaccinated animals when compared with non-vaccinated animals (Fig. 5(A), right panel). Viremia, gross lesions, and histopathology in the lungs of vaccinated and non-vaccinated animals were analyzed. A clear degree of protection
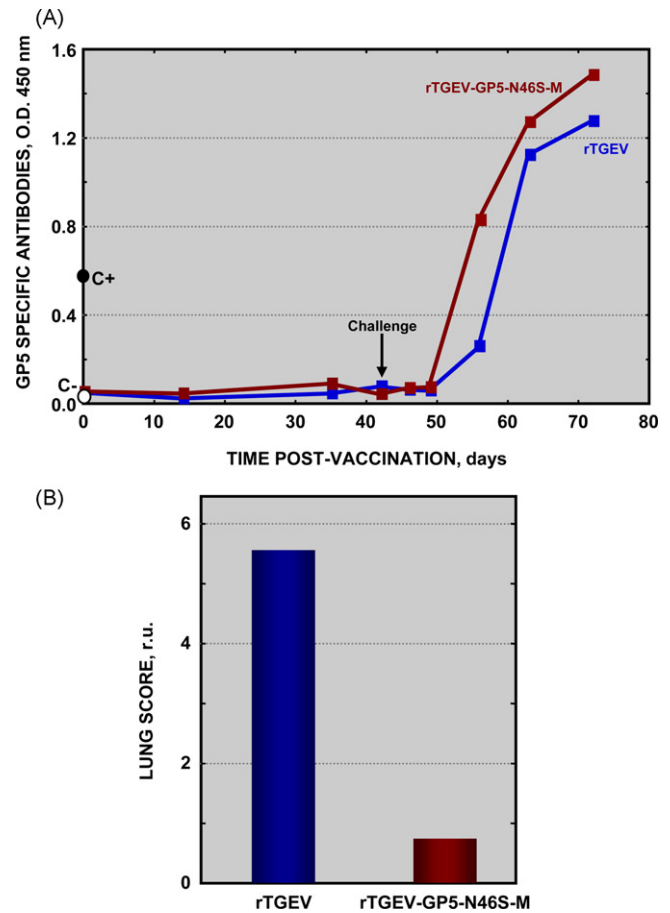

Fig. 6. Protection conferred by rTGEV based live vaccine expressing GP5 with altered glycosylation pattern. (A) Humoral immune response elicited by live rTGEV based vaccine. Blood samples of animals we collected at indicated times post-inoculation. Samples were analyzed by enzyme-linked immunosorbent assays (ELISAs) specific to detect antibodies against TGEV, GP5 and M. To evaluate response against GP5, GP5 protein from PRRSV Olot91 strain was expressed and purified from insect cells and used as antigen for the ELISA. (B) Lung damage caused by PRRSV infection. The lungs from animals inoculated with empty rTGEV vector, or rTGEV expressing GP5-N46S and M proteins, were analyzed. Lung lesions observed in all the pigs, with different degree of severity, included a craneo-ventral consolidation of apical and medial lung lobes.

was observed, as the lungs from vaccinated animals showed a significantly lower degree of lung damage than those from non-vaccinated ones (Fig. 5(B), left panel). Furthermore, a reduction in viremia was also observed in vaccinated animals (Fig. 5(B), right panel). Altogether, these data suggested that the elimination of the glycosylation site close to the neutralizing epitope improves protective immune response against PRRSV.

4.5.2. In vivo testing of rTGEV expressing PRRSV M protein and GP5 mutant with altered glycosylation

The protection conferred by rTGEV-GP5-N46S-M was tested in vivo. One-week-old piglets were inoculated with 1×10^8 pfu of the rTGEV by three routes: oral, nasal and intragastric. A boost was performed 3 weeks after inoculation. Six weeks later, a challenge was performed with 1×10^7 TCID$_{50}$ of PRRSV/Olot91 strain. Blood samples were collected at different times post-inoculation, and humoral immune responses were evaluated by ELISA. All the animals produced a high antibody response against TGEV (data not shown), therefore, the vector infected target tissues as expected. After challenge, vaccinated animals showed a clear humoral response against PRRSV antigens (Fig. 6(A)). A moderately faster recall response was observed, as vaccinated animals

induced higher antibody titers against PRRSV antigens and earlier than control animals (Fig. 6(A)).

The protection conferred by this TGEV based vaccine was also evaluated. A certain degree of protection was observed, as the lungs from vaccinated animals showed a lower degree of lung damage than those from non-vaccinated ones (Fig. 6(B)). Nevertheless, the immune response was not strong enough to provide full protection, probably because the levels of neutralizing antibodies were similar in vaccinated and non-vaccinated animals (data not shown).

4.6. Strategies for the improvement of TGEV derived vectors

To date, rTGEV expressing PRRSV antigens only provided partial protection. This could be due to the fact that the expression of PRRSV antigens by rTGEV vectors was not fully stable, mainly due to GP5 protein toxicity resulting in a significant lost of GP5 expression in 8–10 passages. In contrast, M protein expression was fully stable, with at least 95% of infected cells expressing M protein for more than 10 passages in tissue culture.

The lack of full protection using rTGEV expressing PRRSV antigens could also be due to the presence of domains in the expressed proteins inducing negative regulatory T cells (Treg). As the vector used in the immunization (rTGEV) efficiently induced the production of IFN, it is likely that either PRRSV GP5 or M proteins could contain negative signals inducing Treg. This negative regulation of the immune response elicited could also be a major cause for the delay in the development of a protective immune response against PRRSV.

To improve rTGEV vector stability different strategies can be developed, such as the expression of small domains of GP5 containing the epitopes relevant for protection but lacking domains responsible for instability in their expression. Alternatively, the generation of a library of point mutants in GP5 fragments in which the epitopes eliciting negative Treg have been eliminated may overcome what we consider the second most relevant limitation in the protection against PRRSV. These approaches are currently in progress in our laboratory.

5. Conclusions

An improvement of vaccination strategies against PRRSV is required, as current vaccines have limited efficacy. Best results have been obtained using modified live vaccines and virus vectored vaccines could represent an advantage to stimulate immune responses against PRRSV. The results reported to date using viral vectors are not fully satisfactory and new vectors must be explored. TGEV based vector vaccines expressing different PRRSV antigenic combinations represent a promising candidate to provide protection against two porcine viruses: PRRSV and TGEV. The use of rTGEV vectors led to promising results, similar to those obtained with other vectored vaccines. Nevertheless, as reported for other RNA viruses, data obtained indicate that heterologous protein expression stability was limited. Therefore, increase of PRRSV antigens expression stability, and removal of domains eliciting Treg, represent new avenues to improve the development of an efficient PRRSV vaccine.

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