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An experimental subunit vaccine based on Bluetongue virus 4 VP2 protein fused to an antigen-presenting cells single chain antibody elicits cellular and humoral immune responses in cattle, guinea pigs and IFNAR(−/−) mice

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ABSTRACT

Bluetongue virus (BTV), the causative agent of bluetongue disease (BT) in domestic and wild ruminants, is worldwide distributed. A total of 27 serotypes have been described so far, and several outbreaks have been reported. Vaccination is critical for controlling the spread of BTV. In the last years, subunit vaccines, viral vector vaccines and reverse genetic-based vaccines have emerged as new alternatives to conventional ones. In this study, we developed an experimental subunit vaccine against BTV4, with the benefit of targeting the recombinant protein to antigen-presenting cells. The VP2 protein from an Argentine BTV4 isolate was expressed alone or fused to the antigen presenting cell homing (APCH) molecule, in the baculovirus insect cell expression system. The immunogenicity of both proteins was evaluated in guinea pigs and cattle. Titers of specific neutralizing antibodies in guinea pigs and cattle immunized with VP2 or APCH-VP2 were high and similar to those induced by a conventional inactivated vaccine. The immunogenicity of recombinant proteins was further studied in the IFNAR(−/−) mouse model where the fusion of VP2 to APCH enhanced the cellular immune response and the neutralizing activity induced by VP2.

1. Introduction

Bluetongue virus (BTV) is the causative agent of bluetongue disease (BT) of domestic and wild ruminants. Among domestic species, sheep is the most susceptible and most severely affected host, but cattle and wild ruminants serve as reservoirs for the virus.

The BTV genome is composed of 10 segments of double-stranded RNA which encode for seven structural proteins (VP1–VP7) and five non-structural proteins (NS1, NS2, NS3/3a, and NS4) [1]. Among the structural proteins, VP2 is the most variable one, the determinant of serotype, responsible for hemagglutination, receptor binding and induction of serotype-specific neutralizing antibodies (NAs) [2].

Vaccination against BTV is an effective mean to control BT since it minimizes direct losses, reduces virus circulation and enables safe movement of animals [3]. Requirements for an ideal BTV vaccine include low cost, the possibility to use DIVA (differentiate infected and vaccinated animals) strategy, and induction of immunity against several serotypes. Both inactivated and attenuated vaccines are effective. However, there are concerns with regard to each; attenuated vaccines could cause teratogenic effects, decreased fertility and potential reassortment with reversion of the vaccine strain. Whereas inactivated vaccines represent a safer alternative, some concerns exist over incomplete inactivation and cost productions [3–8]. Regarding the delivery of BTV antigens, promising results have been obtained using poxvirus and other viral vectors [9–13]. In addition, subunit vaccines based on BTV virus-like particles (VLPs) or VP2, VP7, or NS1 proteins of BTV4 incorporated into avian reovirus muNS-Mi microspheres have been found to inhibit virus replication and disease signals after a challenge with the homologous virus [14–17]. Prime-boost vaccination

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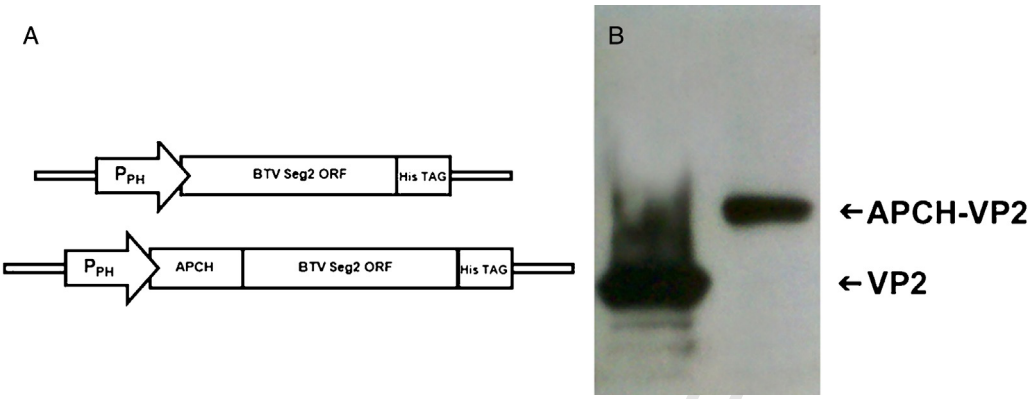

Fig. 1. pFastBac Dual schematic representation of the constructs for recombinant fusion protein expression (A). Production and detection of recombinant fusion proteins VP2 (110 kDa) and APCH-VP2 (140 kDa) in SF9 cells (B). Cell culture was infected at MOI 5 with recombinant baculoviruses. Infections progressed for 96 h for VP2 expression and 144 h for APCH-VP2 expression and total protein extracts were collected at the times indicated. Crude preparations were resolved by 8% SDS-PAGE and analyzed by Western blot using monoclonal antibody anti-Penta-His.

strategies have also been evaluated using naked DNA and recombinant modified vaccinia virus Ankara (rMVA) expressing BTV4 proteins [18,19]. In those studies, IFNAR(−/−) mice inoculated with DNA/rMVA expressing VP2, VP5, and VP7 generated a high level of NAs and complete protection against a homologous BTV4 challenge and the inclusion of NS1 in the DNA/rMVA vaccine composition conferred cross-protection against heterologous BTV1 and BTV8 challenges [18,19].

Subunit vaccines provide an opportunity to develop safe and rational vaccines, with the possibility of differentiating between vaccinated and infected animals. However, the challenge is to produce a vaccine capable of eliciting an efficient immune response with an affordable cost for veterinary applications. In this regard, considerable effort is being made to develop methods to enhance the immunogenicity of such vaccines. One of the most successful strategies under study is based on targeting the encoded antigens to specific sites of the immune cells. Antigen presenting cell homing 1 (APCH1), a single-chain variable fragment (scFv) that specifically recognizes an invariant epitope of the MHC II DR molecule on the surface of antigen-presenting cells, has been previously reported. This strategy has been demonstrated to be very efficient in improving the immune responses induced against many different antigens, using either recombinant subunit proteins or DNA vaccination [20–24].

In this work, the VP2 protein from an Argentine BTV4 isolate was expressed either alone or fused to APCH, a molecule that targets antigen-presenting cells (APCH-VP2) in the baculovirus insect cell expression system. The immunogenicity of both proteins was evaluated in guinea pigs, IFNAR(−/−) mice, and cattle.

2. Materials and methods

2.1. Construction of recombinant baculoviruses

BTV serotype 4 (BTV4) strain used in this study was an Argentine isolated named 4/ARG/829/2001, described by Legisa [25,26]. Segment 2 (Seg2) open reading frame was cloned into pFastBac-Dual plasmid (Invitrogen). Two constructs were generated: Seg2 ORF alone (VP2) and Seg2 tagged in its 5′ terminus to APCH (APCH-VP2) (Fig. 1 A). Recombinant baculoviruses were generated by using Bac-to-Bac Baculovirus Expression System (Invitrogen), according to the method recommended by the manufacturer. Briefly, Bacmids were generated transforming DH10Bac *E. coli* competent cells with 1 ng of plasmid. Positive clones were selected by color detection in LB Agar plates containing antibiotics. Bacmids were characterized by sequencing. *Spodoptera frugifera* cells (SF9) were transfected using Cellfectin II reagent and 2 μg of Bacmid. Supernatant were harvested at 72 h post-infection (hpi). This first supernatant containing recombinant baculovirus (P1) was used to infect SF9 cells at MOI 0.1 to increase baculovirus stock titter (P2).

2.2. Protein production

Optimal infection conditions were assessed for each recombinant baculovirus. For VP2 expression, Sf9 cells were infected at a multiplicity of infection (MOI) of 5 and harvested 96 hpi. For APCH-VP2, Sf9 cells were infected at a MOI of 5 and harvested 144 hpi. Protein samples were analyzed by 8% SDS-PAGE and western immunoblot using monoclonal antibody anti-Penta-His (QIAGEN).

2.3. Vaccine formulations

Vaccines were formulated with oil adjuvant Montanide ISA50 in a 60:40 adjuvant: antigen proportion. As positive control, BEI-inactivated BTV4 (4/ARG/829/2001) containing 8×10^6 $TCID_{50}$/ml was included. As a negative control, the same formulation was used with non-related recombinant baculovirus.

2.4. Immunization strategies

2.4.1. Immunization of guinea pigs

Eight- to 12-week-old guinea pigs (5 animals per group) were immunized either with (i) 0.2 or (ii) 2.4 μg of VP2 or with (iii) 0.15 or (iv) 0.6 μg of APCH-VP2, containing similar total protein mass. Guinea pigs were immunized on days 0 and 30. Immunogens were administered by the intramuscular route (i.m.). Sera were sampled at 0, 30 and 60 days post-inoculation (dpi).

2.4.2. Immunization of cattle

Aberdeen Angus cattle (6–8 months old) (5 animals per group) were used. Vaccine doses were: (i) 3.6 μg of VP2 and (ii) 0.9 μg of APCH-VP2. Groups were immunized on days 0 and 30 by i.m inoculation. Sera were collected at 0, 30, 60 and 90 dpi. Vaccine safety was evaluated throughout the assay.

2.4.3. Immunization of IFNAR(−/−) mice

In context of the IFNAR(−/−) animal model, eight-week-old IFNα/β$^{-/-}$ 129/sv mice (IFNAR(−/−) were used. Groups of five IFNAR(−/−) mice were immunized by prime-boost vaccination with 10 μg of each recombinant purified protein administered 3 weeks apart. Virus challenge was conducted at 35 dpi. Mice were subcutaneously inoculated with 10^3 PFUs of BTV4. Mice were bled

before each immunization and virus challenge. Sera were tested for BTV4 NAs by the standard virus neutralization Test (VNT) [27].

2.5. Animal welfare

Guinea pigs and cattle handling, inoculation, and sample collection were performed by trained personnel under the supervision of a veterinarian and in accordance to protocols approved by the Ethical Committee of Animal Welfare of INTA (CICUAE 20/2010, 40/2013 for guinea pigs assays and 45/2013 for cattle assays). Mice were maintained under pathogen-free conditions and allowed to acclimatize to the biosafety level 3 animal facilities at the CISA, INIA, Spain, for 1 week before use in our experiments. All experiments with IFNAR(−/−) mice were performed under the guidelines of the European community (86/609) and approved by the ethical review committee at CISA (Permit number: CEEA 2010-034). All efforts were made to minimize suffering.

2.6. Serum neutralization test

Serum NAs were detected by the virus neutralization assay [27]. Differences in antibody titers among experimental groups were evaluated by ANOVA under a model of repeated measures throughout time, followed by a general contrast post-ANOVA test. Statistical significance was assessed at $p < 0.05$ for all comparisons, using Statistix 8.

2.7. Competitive ELISA

Serum samples from 90 dpi were analyzed for anti-BTV antibodies, using C-ELISA kit BTV Antibody Test Kit (VMRD, Pullman, USA). This commercial kit detects anti VP7 antibodies.

2.8. Isotype-specific antibody ELISA

Specific IgG_1 and IgG_2 were detected by an indirect ELISA. Briefly, 96-well Plates 1B (Maxisorp, NUNC) were coated with 100 μl of cell culture supernatant from BHK21 cells either infected with BTV4 or non-infected. After each incubation period, three washes were made using PBS pH 7.4 Tween-20 (0.1%). The plate was blocked and samples were added in serial four-fold dilutions (1:8 to 1:128). Anti-IgG1 and anti-IgG2 monoclonal antibodies were added at 1:10,000 and 1:4000 dilutions, respectively. Horseradish peroxidase-conjugated anti-mouse serum was added and *O*-phenylenediamine–H_2O_2 was used as substrate. Absorbance was recorded at 492 nm.

2.9. Detection of epitope-specific $CD4^+$ and $CD8^+$ T cell responses by Intra-Cellular Cytokine Staining (ICCS)

Immunized mice were sacrificed 14 days post-booster and their spleens were harvested. ICCS was performed as described by Marín-López et al. [17]. Briefly, splenocytes from subunit vaccine-immunized mice were re-stimulated with 15 μg of recombinant BTV4 VP2 protein for 24 h and intracellular IFNγ production by $CD8^+$ T and $CD4^+$ T cells was determined by flow cytometry upon treatment with brefeldin A. Data were acquired by FACS analysis on a FACS Scalibur (Becton Dickinson) and analyzed with CellQuest Pro software.

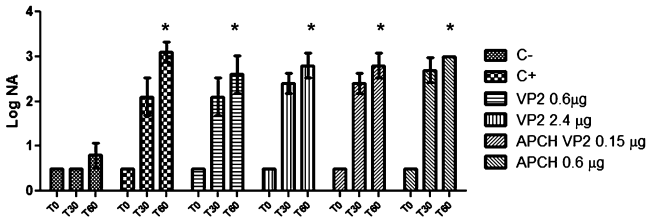

Fig. 2. Neutralizing antibody response to recombinant proteins VP2 and APCH-VP2 in guinea pigs. Animals were immunized with an oil adjuvant formulation of each protein. Each bar represents the geometric mean of NA titers of individual serum samples collected on day 0, 30 and 60 post-immunization. Error bars represent the standard error within the samples. Asterisks represent significant difference detected between T0 and T60 into each group (VP2; APCHVP2 or C+). At T30 and T60 significant differences were detected between negative control group and either subunit or BEI inactivated vaccines.

3. Results

3.1. Humoral response to the experimental subunit vaccine in guinea pigs and cattle

Seroconversion in guinea pigs was evident at 30 dpi in all immunized groups, while the negative control group remained negative throughout the experiment. After booster (60 dpv), animals immunized with VP2, APCH-VP2, or BEI-inactivated vaccine showed high NA titers which differed significantly from those at 0 dpv. There were no significant differences between the doses evaluated for each vaccine or between vaccines (Fig. 2).

Based on the results obtained in this experimental model, we next evaluated the immunogenicity of vaccines in cattle. Experimental groups included five animals each, which were immunized with either 3.6 μg of VP2 or 0.9 μg of APCH-VP2. The assay also included a positive control group (immunized with a BEI-inactivated BTV4) and a negative control group. At 30 dpi, after one immunization, specific NAs were detected in animals vaccinated with the recombinant proteins and with the inactivated vaccine (Fig. 3). Following the second vaccination, NA titers increased to very high levels in the vaccinated groups. In contrast, no NAs were detected in the control group at any of the time points analyzed (Fig. 3). After vaccination, none of the animals showed local reactions or adverse effects. It is important to note that the animals immunized with APCH-VP2 showed no significant differences with those immunized with either the VP2- or BEI-inactivated vaccines, although they received a four-fold lower dose of antigen.

To verify DIVA compliance of the experimental vaccines, a competition commercial ELISA was used to test cattle sera at 90 dpi. Animals immunized with BEI-inactivated BTV4 were positive for the test, while non-immunized animals and animals immunized with subunit vaccines were negative (data not shown).

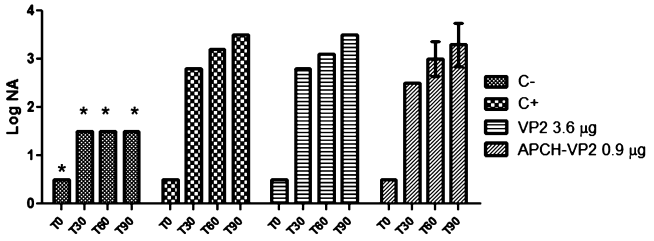

Fig. 3. Neutralizing antibody response to recombinant proteins VP2 and APCH-VP2 in cattle experiment. Animals were immunized with an oil adjuvant formulation of each protein. Each bar represents the arithmetic mean of NA titers of individual serum samples collected on day 0, 30, 60 and 90 post-immunization. Error bars represent the standard error within the samples. Asterisks represent significant difference detected between C- and treatments at T30, T60 and T90.

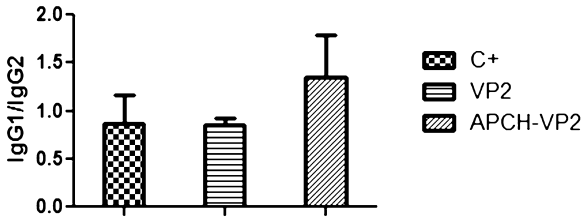

Fig. 4. Isotype-specific antibody ELISA. Specific IgG1/IgG2 ratio detected by an indirect ELISA. Serum samples from cattle at day 90 p.i. were analyzed and titer ratio were conducted. Bars represent the ratio between arithmetic means for titers measured by isotype ELISA in each treatment group.

3.2. IgG isotype profile induced by VP2 and APCH-VP2 in cattle

Sera from vaccinated cattle at 60 dpi were evaluated for the IgG isotype profile. IgG1 and IgG2 titers showed no significant differences both within and between vaccination treatments. However, the IgG1 titers tended to be higher than the IgG2 ones for APCH-VP2-vaccinated cattle (IgG1/IgG2 ratio near 1.4) (Fig. 4).

3.3. APCH-VP2 induces humoral and cellular immune response in IFNAR(−/−) mice

To evaluate the immunogenicity of the recombinant proteins VP2 and APCH-VP2, IFNAR(−/−) mice were inoculated intraperitoneally with 10 μg of each recombinant protein without adjuvant. Two weeks after the second immunization, immunized and control IFNAR(−/−) mice were challenged subcutaneously with 10^3 PFUs of BTV4.

Even when no adjuvant was used in the formulation of the vaccines, moderate NA response against BTV4 was elicited (Fig. 5A). Titers for APCH-VP2-immunized animals were higher than those for VP2-immunized animals.

To analyze whether APCH fused to VP2 improved the T-cell immune response elicited by VP2, the phenotype of the VP2 BTV4-specific IFNγ-producing T cells was analyzed by intracellular cytokine staining. Specific $CD4^+$ and $CD8^+$ T cells producing IFNγ following virus stimulation were observed in animals immunized with APCH VP2 meanwhile lower levels were recorded for animals immunized with VP2, showing that the cellular response in mice vaccinated with APCH-VP2 was enhanced (Fig. 5B and C).

4. Discussion

BT is included in the unified OIE list of notifiable terrestrial and aquatic animal diseases, then, major concerns are linked to viral presence and disease detection [28,29].

Historically, inactivated or attenuated vaccines have been used due to their low cost and easy production, despite the risk associated with their production. During the last decades, field isolates reporting reassortants comprising live vaccine strains, together with other biosafety issues, have raised concerns regarding the use of conventional vaccines and have encouraged the development of new generation vaccines.

The eradication of BTV from enzootic areas may not be easy, but, in non-enzootic areas, vaccination with inactivated vaccine incorporating the prevalent serotypes is advantageous [30]. However, inactivated vaccines are available in a few countries, including the European Union, India, the USA and China [30]. In this sense, development of recombinant vaccines in countries with the presence of only one or a few serotypes and with no local production of conventional vaccines becomes a promising strategy to combat BTV infection.

In this study, we developed an experimental subunit vaccine against BTV4 with the benefit of targeting the recombinant protein to antigen-presenting cells. In previous works, our group characterized Argentine isolates as a well-differentiated independent lineage [25]. However, the similarity analysis performed (data not shown) including amino acid sequences and comparing well-defined groups showed that the Argentine group and worldwide BTV4 isolates were highly similar and conserved the regions previously recorded as antigenic sites [31,32].

Huismans et al. first demonstrated in 1987 that VP2 was able to induce NAs and protection against homologous challenge in sheep [33]. After that, other reports in which VP2 was expressed using different expression systems have confirmed its role in protection [34–36].

In this study, high titers of specific NAs were induced in guinea pigs and cattle immunized with VP2 or APCH-VP2 expressed in the baculovirus system. Specifically, similar titers were reached for treatments including BEI-inactivated vaccine, VP2- and the APCH-VP2-based vaccines, although a four-fold lower antigenic mass was used in the APCH-VP2 group. The APCH molecule has been described as an immune response enhancer when it was fused to rabbit hemorrhagic disease virus, *Canine Parvovirus*, and Bovine viral diarrhea subunit vaccines [20,37,38]. In those reports, different expression systems as baculoviruses (sf9 cells and *Tricoplusia nii* larvae) or molecular farming (*Medicago sativa L.*) were used to express the recombinant antigens fused to APCH.

Recombinant vaccines comprising plasmid DNA or MVA virus encoding VP2, VP5 or VP7 proteins have been evaluated either alone or in combination. Whether the inclusion of VP5 and VP7 is critical for the induction of protection is still controversial. Some reports showed that complete protection is only achieved when VP2, VP5 and VP7 are used in combination in the vaccine composition [18,39,40]. However, other studies with BTV and other related

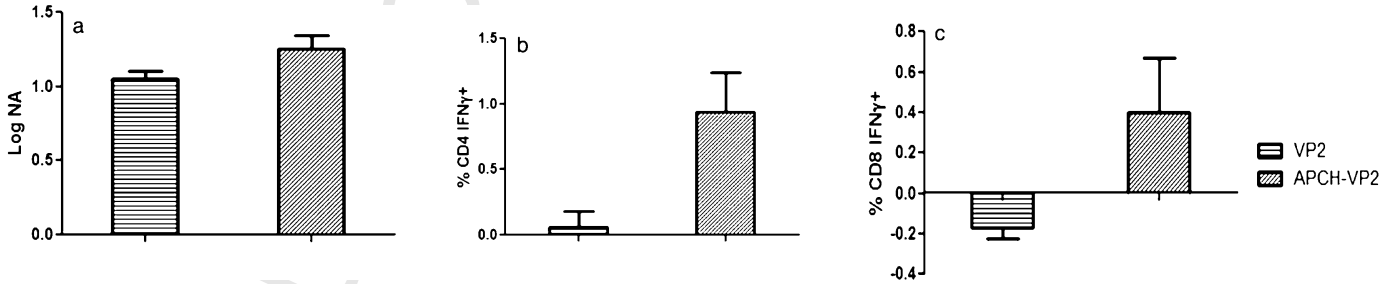

Fig. 5. IFNAR (−/−) experiments. (A) Humoral immune response observed in IFNAR(−/−) mice vaccinated with each vaccine. Specific neutralizing antibodies were analyzed in sera of immunized mice by VNT. Neutralization titers at day 14 post-booster treatment in sera of animals immunized with purified recombinant proteins means are presented as bars. (B and C) Cellular immune response detection of epitope-specific INFγ—CD4+ (B) and CD8+ T-cell (C) by Intra-cellular Cytokine Staining (ICCS). Specific Proliferation. were recorded in splenocytes obtained from mice immunized with recombinant proteins at 14 days post-booster. Splenocytes were stimulated in vitro with 15 μg of VP2 recombinant protein. The results represent the average of four mice after subtraction of background values ± SD.

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orbivirus have shown that complete protection can also be achieved by subunit vaccines containing the VP2 protein alone [41–43].

Previous studies using VP2 as antigen have shown that a minimal dose of 100 μg VP2 is needed to protect sheep against the challenge; however, this dose could be decreased to 50 μg when VP2 is used in combination with VP5 [35,44]. Lower doses of VP2 were required when VP2 was present in the context of a virus-like particle. It has been reported that 10 μg of virus-like particles (containing 2.39 μg VP2) formulated with either incomplete Freund's adjuvant or incomplete Montanide ISA-50 adjuvant elicit protection against a virulent challenge [45]. In this work, we also observed that lower doses of APCH recombinant proteins, formulated with Montanide ISA-50 adjuvant, were needed to reach high antibodies titers in comparison with the recombinant protein alone. Specifically, a minimal dose of APCH-VP2 was needed in both guinea pigs and cattle (0.15 μg and 0.9 μg, respectively) to reach a specific antibody response similar to that obtained with VP2 (2.4 μg in guinea pigs and 3.6 μg in cattle) and the inactivated experimental vaccine. This result suggests that APCH could act as an effective enhancer for subunit vaccines and allow decreasing the antigen mass, which is a desirable feature for a subunit vaccine.

To characterize the immune response, an IgG isotype ELISA was conducted as a first approach. No significant differences between groups were found. However, the IgG1/IgG2 ratio showed a difference which suggests a special immune response pattern. The IgG1/IgG2 ratio obtained was similar to that of other reports using APCH fused to the E2t protein as a subunit vaccine for Bovine viral diarrhea virus [37]. These results could indicate that the APCH molecule slightly switches the isotype profile toward IgG1. This profile was also reported by Gil et al. [20]. This could be a desirable feature for an experimental vaccine used in the field since some studies have shown that colostrum containing NAs against BTV could protect against the virus infection [46,47].

In cattle, high NA titers were reached and no adverse effects were recorded. In addition, recombinant vaccines were tested to confirm their DIVA compliance. This DIVA feature of differentiate between infected and vaccinated animals is important, particularly in cattle, which are usually asymptomatic after BTV infection, but are able to spread the virus [44].

It has been reported that, in BT, the humoral response is the main component of the immune response against the virus and the disease. The cellular component is also important but, so far, how it works and how important its contribution is to the whole immune response are not well understood. To better understand the immunogenicity of both recombinant proteins, the IFNAR (−/−) mouse model was used to evaluate the immune response elicited by VP2 and APCH-VP2 proteins without adjuvant. Regarding the humoral response, moderate NA levels were recorded in animals vaccinated with APCH-VP2. Moreover, in the homologous challenge, a survival trend was recorded for both recombinant proteins (Supplementary Fig. 1). Since VP2 and APCH-VP2 were inoculated without adjuvant, a protective immune response was not expected. However, the net result of targeting the antigen to antigen-presenting cells could be observed without any masking effect of the adjuvant.

In the cellular immunity assays, APCH-VP2-vaccinated mice showed specific IFNγ $CD4^+$ and IFNγ $CD8^+$ cell proliferation, suggesting that APCH is an enhancer both of the humoral and cellular immune responses.

Summarizing, this work addressed the immunogenicity of a recombinant vaccine based on BTV4-VP2 protein fused to the molecule APCH, which enhances the immune response. As mentioned above, further experiments with APCH-VP2 should be performed to assess the effective dose capable of inducing the desired immune response. In addition, the T cell response in cattle should also be studied to understand the complete immune response induced.

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Conflict of interest statement

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2015.03.067.

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