Recombinant adenovirus as a multiserotype vaccine for Bluetongue Virus in the IFNAR mouse model

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MASTER'S FINAL DISSERTATION

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Abbreviation index

- ANOVA: Analysis of variance
- BCIP/NBT: 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium
- BEI: Binary ethyleneimine
- BHK: Baby Hamster Kidney
- BSA: Bovine Serum Albumin
- BT: Bluetongue
- BTV: Bluetongue virus
- CTL: Cytotoxic T Lymphocyte
- DIVA: Differentiation between infected and vaccinated animals
- DMEM: Dulbecco's Modified Eagle Medium
- ELISA: Enzyme-Linked ImmunoSorbent Assay
- ELISPOT: Enzyme-Linked ImmunoSpot Assay
- FBS: Fetal Bovine Serum
- FCS: Fetal Calf Serum
- GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
- HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
- HRP: Horseradish peroxidase
- IFN: Interferon
- IFNAR: Interferon-α/β receptor
- IgG: Immunoglobulin G
- IU: Infectious unit
- MOI: Multiplicity of infection
- OD450: Optical density at 450 nm
- PBS: Phosphate buffered saline
- PFU: Plaque-forming unit
- PI: Propidium iodide
- PVDF: Polyvinylidene difluoride
- RFP: Red Fluorescent Protein
- SDS: Sodium dodecyl sulfate
- SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
- TBS-T: Tris buffered saline - Tween
- TMB: 3,3',5,5'-Tetramethylbenzidine
- βGal: β-Galactosidase

Abstract

Bluetongue virus (BTV) is the prototype member of the genus *Orbivirus*, belonging to the *Reoviridae* family. This virus is the causal agent of Bluetongue (BT) disease, which causes high morbidity and mortality in ruminants, particularly in sheep. BTV is a widely-spread virus that displays a high variability, since 27 different serotypes are currently described. BT protection is serotype specific and re-infections with heterologous serotypes are possible. Current strategies for BTV control are based on vaccination with attenuated or inactivated viruses, which are effective but present drawbacks such as serotype-specificity and inability to induce long-term immunity. Therefore, improved vaccines are required to effectively control the disease. Recombinant vaccines expressing BTV protein subunits can represent an alternative to traditional vaccines. Recombinant adenovirus can typically elicit humoral and cellular immune responses, and thus can potentially grant protection against multiple serotypes of BTV.

In this work, we assessed the efficacy of replication-defective recombinant human adenovirus serotype 5 expressing BTV proteins (VP2 and VP7) as a vaccine.

IFNAR$^{(-/-)}$ mice inoculated with adenoviruses expressing the proteins VP2 from BTV1 and VP7 from BTV8 survived challenges with homologous and heterologous (with BTV4) viruses. Vaccination induced significant humoral and cellular immune responses. Additionally, we tested the efficacy of a polycistronic construct expressing VP2 from BTV4 and VP7 from BTV8 in the same mouse model. This adenovirus was able to induce the formation of antibodies against BTV4 and BTV8 and to elicit BTV-specific cellular immune response against BTV4, but not against BTV8. Vaccination with this recombinant adenovirus protected mice against BTV4 challenge but not against BTV8 challenge.

Our results show that replication-defective recombinant adenoviruses are a suitable method for the control of BTV. Vaccination with recombinant adenoviruses expressing BTV proteins VP2 and VP7 elicited humoral and cellular immune responses and protected against multiple serotypes of the virus.

Introduction

Bluetongue (BT) disease is a hemorrhagic disease, with high morbidity and mortality indexes, which mainly affects ruminants. Bluetongue Virus (BTV) is the causal agent of BT, and belongs to the *Reoviridae* family, being the prototype member of the genus *Orbivirus*. This virus is transmitted to animals by biting midges of the genus *Culicoides*. The virion is formed by a triple proteic layer which encapsulates the genomic material, composed by 10 segments of double-stranded RNA that encodes for 7 structural proteins (VP1 – VP7) and 5 non-structural proteins (NS1 – NS5) (Figure 1). The inner layer or core is constituted by the protein VP3, the

intermediate layer is formed by the protein VP7, which is a highly-conserved protein between serotypes, and the outer layer is composed by VP2 and VP5. VP2 shows a wide variability between the 27 described BTV serotypes described so far and it is responsible for eliciting serotype-specific neutralizing antibodies, usually correlated with protection against BTV (Maan et al., 2007).

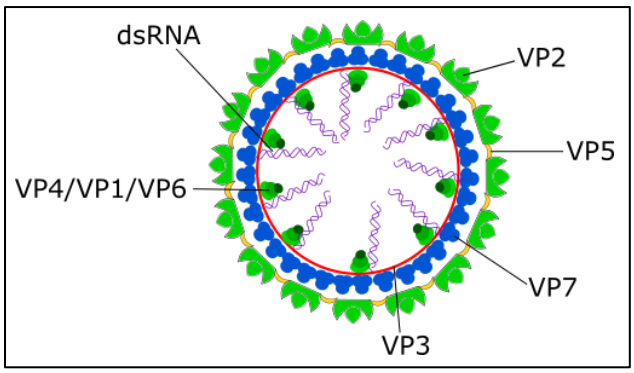

Figure 1: Scheme of the BTV structure. The virion is formed by 10 segments of double-stranded RNA encapsulated by an inner core formed by the protein VP3, an intermediate layer formed by the protein VP7, and an outer layer formed by the proteins VP2 and VP5.

Animals which overcome the disease are able to develop a long-lasting immunity, generating neutralizing antibodies (Jeggo et al., 1984a) and cytotoxic T lymphocytes (CTL) (Jeggo et al., 1984b). Both components of the immune response are crucial for an effective protection against BTV. Cellular immunity could also potentially provide some protection across serotypes since cross-reaction between different BTV serotypes has been shown (Umeshappa et al., 2010).

Currently, the main methods for BTV control involve immunization with inactivated or live-attenuated vaccines. These approaches have demonstrated efficacy in producing a strong immune response against homologous BTV infection by inducing humoral and cellular immunity (Savini et al., 2008). However, these vaccines have several drawbacks such as teratogenic effects, residual virulence, and, more importantly, possibility of reassortment with wild-type viruses (Batten et al., 2008). In addition, none of the commercially available vaccines allow the differentiation between infected and vaccinated animals (DIVA).

Ongoing research efforts are orientated towards obtaining a vaccine which protects against the infection of multiple BTV serotypes in all ruminant species and, additionally, allows for DIVA control strategies. Among these new vaccines in development, approaches based on recombinant viral vectors which express BTV proteins have shown promising results. For instance, a canarypox virus expressing VP2 and VP5 induced protection against BTV in sheep (Boone et al., 2007). Overall, these vaccines often produce a humoral response but fail to elicit a

significant T-cell response. Recombinant adenoviruses vaccines have nonetheless the potential to trigger strong humoral and cellular immune response to the transgene (Tatsis & Ertl, 2004).

Vaccination with replication-defective recombinant human adenoviruses serotype 5 expressing BTV-VP2 or VP7 proteins resulted in non-sterile BTV protection in sheep and total protection in the IFNAR mouse model. These vaccines activated both humoral and cellular immune responses. Interestingly, protection in sheep occurred even in the absence of neutralizing antibodies, but vaccination triggered strong BTV-specific $CD8^+$ T cell responses which could contribute to vaccine efficacy (Martín et al., 2015). These recombinant adenovirus vaccines could therefore potentially protect against heterologous BTV challenge, since they trigger cellular immunity.

The main objective of this project is to obtain a vaccine which is able to effectively elicit cellular and humoral immune responses and protect against multiple BTV serotypes using the above described adenoviral platform.

To achieve these objectives several vaccine candidates were assessed. Firstly, we use adult $IFNAR^{(-/-)}$ mice to test the efficacy of an adenovirus candidate vaccine, denominated Ad-BI (VP2-4-VP7-8), which express a polycistronic construct that encodes for VP2 from BTV4 fused to an HA tag, VP7 from BTV8 fused to a FLAG tag and separated by the foot and mouth disease 3A protease, which produces a cleavage between VP2 and VP7. Secondly, we assessed the efficacy of the simultaneous vaccination with adenoviruses that express VP2 from BTV1 (Ad-VP2-1) and VP7 from BTV8 (Ad-VP7-8) when animals are challenged with homologous or heterologous BTV serotypes.

Materials and Methods

Cells and viruses

Vero cells (ATCC CCL-81) and Baby Hamster Kidney (BHK, ATCC CRL-6281) cells were grown in Dulbecco' modified Eagle's medium (DMEM), supplemented with 5% FBS, L-Glutamin (2mM) and 100U/ml Penicillin /100µg/ml Streptomycin. Virus stocks were obtained in BHK cells infected with BTV1 (FJ969720), BTV4 (KJ700442) or BTV8 (NET2006/04) at multiplicity of infection (MOI) of 0.1 and supernatants were collected at 48 hours post-infection. After 3 freeze/thaw cycles, the supernatants were sonicated for 5 minutes, centrifuged at 3000 RPM for 5 minutes and stored at -80ºC until use. Viruses were titrated by plaque assays using Vero cells as described by Baer & Kehn-Hall (2014). Inactive virus was obtained by incubation with 3 mM BEI for 24 h at 37 °C, followed by the addition of 0.02 M sodium thiosulphate.

Recombinant adenovirus production

The genes of interest were cloned into the pSIREN-EF1α plasmid, which functions as a donor vector to transfer the gene of interest to the acceptor vector pLP-Adeno-X-PLRS (Clontech) by Cre-loxP mediated recombination (Figure 2). To generate the replication defective adenoviruses Ad-βGal, Ad-BI (VP2-4-VP7-8), Ad-VP2-1 and Ad-VP7-8, HEK293T cells were transfected with the plasmid generated by the recombination through the Cre-loxP system, and the recombinant viruses were amplified, purified and titrated using standard protocols and commercial kits (Clontech, 631533).

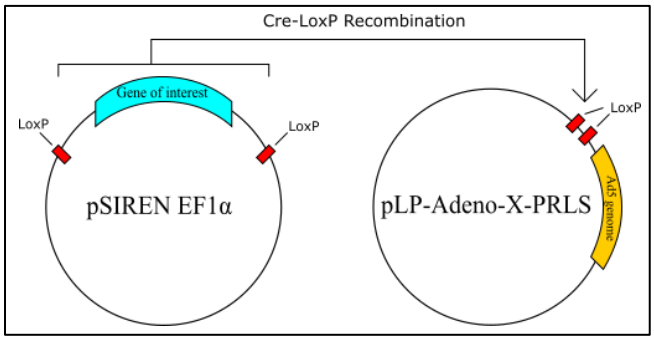

Figure 2: Schematic representation of the strategy used to produce the recombinant adenoviruses. The gene of interest is transferred from the donor vector pSIREN EF1α to the acceptor vector pLP-Adeno-X-PLRS (Clontech) through Cre-LoxP recombination.

Western blot

Vero cells were infected with Ad-βGal or Ad-BI at a MOI of 1. Twenty-four hours post-infection, cells were lysed in Laemmli buffer, proteins resolved by SDS-PAGE (10% polyacrylamide) and transferred to a PVDF membrane (0.45 µm pore size). The membrane was blocked with 3% BSA in TBS-T buffer (Tris-buffered saline + 0.1% Tween 20) and incubated with a 1:1000 dilution of antibodies against HA (Cell Signaling, 37245), FLAG (Sigma-Aldrich, F3165) or GAPDH (Cell Signaling, 97166S). The proteins of interest were detected after incubation with a 1:10000 dilution of a peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies and the subsequent use of an ECL substrate system (ThermoFisher, 32132) according to the manufacturer´s recommendations.

IFN-γ ELISPOT assays

Splenocytes were obtained by mechanical disruption of mouse spleens and cultured in T Cell Media (RPMI + 10%FCS + 4 mM L-glutamine + 10 mM HEPES + 1% 100X non-essential amino-acids + 1 mM sodium pyruvate + 100 U/ml penicillin/100 µg/ml streptomycin + 50 nM 2-mercaptoethanol). Splenocytes were plated at a density of 2 x 10^5 cells per well in a MSIPS4510 (Millipore) plate coated with anti-IFN-γ antibody in the presence of the following stimulus: VP7-283 peptide, VP7-139 peptide, NS1-152 peptide, BEI-inactivated BTV (Serotypes 1, 4 or 8), BHK mock lysate or Concanavalin A. Subsequently, membranes were incubated with a biotin-labeled

anti IFN-γ and developed using streptavidin conjugated to alkaline phosphatase. Membranes were revealed using BCIP/NBT and the result was read using an ELISPOT Reader from AID.

Mice vaccination

Two experiments with mice were performed. In the first experiment, female IFN α/βR$^{o/o}$ IFNAR$^{(-/-)}$ mice on a C57BL/6 genetic background (n = 35) were immunized intramuscularly twice (at two weeks interval) with 10^9 infectious units (IU) of Ad-βGal or Ad-BI. 3 mice from each group were euthanized for splenocyte extraction, and the rest of the animals were challenged subcutaneously with 10^3 PFUs of BTV4 or BTV8 two weeks after the last immunization. Prior to the challenge, mice were divided in the following groups: Ad-βGal + BTV4: n = 6 / Ad-βGal + BTV8: n = 6 / Ad-BI + BTV4: n = 8 / Ad-BI + BTV8: n = 8.

In the second experiment, male (n = 6) and female (n=35) IFN α/βR$^{o/o}$ IFNAR$^{(-/-)}$ mice were immunized as described with 10^9 infectious units (IU) of Ad-βGal + Ad-RFP or Ad-VP2-1 + Ad-VP7-8. 3 mice from the vaccinated group were euthanized for splenocyte extraction, and the rest of animals were challenged subcutaneously with 10^3 PFUs of BTV1, BTV4 or BTV8 two weeks after the last immunization. Prior to the challenge, mice were divided in the following groups: Ad-βGal + Ad-RFP + BTV1: n = 6 / Ad-βGal + Ad-RFP + BTV4 n = 6 / Ad-βGal + Ad-RFP + BTV8 n = 6 / Ad-VP2-1 + Ad-VP7-8 + BTV1: n = 7 / Ad-VP2-1 + Ad-VP7-8 + BTV4: n = 7 / Ad-VP2-1 + Ad-VP7-8 + BTV8: n = 6.

In both experiments, sera were obtained before the first immunization, one week after the second immunization, and 15 days after challenge in surviving mice. Mouse general health and weight was monitored daily after challenge.

Flow cytometry cytotoxicity assay

Splenocytes from adenovirus inoculated mice were expanded one week in vitro with VP7-283 peptide, and then used as effector cells against RMA-s cells pulsed with peptides VP7-283 or NS1-152 (as control) as described by Rojas et al. (2016). Dead cells were labeled with propidium iodide (PI) (2 µg/ml) and immediately analyzed by flow cytometry. Positive maximum cell death (PBS + 0,2% saponin) and spontaneous cell death controls were added to the experiment. Specific target cell lysis was calculated following the formula: % specific lysis = 100 × (% PI+ target – % spontaneous death)/(% maximum death − % spontaneous death).

Anti-BTV IgG ELISA

MaxiSorp plates were coated, overnight at 4º C, with the equivalent to 10^4 PFUs/well of BEI-inactivated BTV1, BTV4 or BTV8. Plates were washed with PBS 0.1% Tween 20 (PBS-T) and blocked with 5% non-fat milk in PBS-T. Subsequently, sera obtained from the mice was diluted at 1:200, 1:400 and 1:800 in 2% non-fat milk in PBS-T, plated and incubated for 2 hours. Signal was detected through the incubation with a secondary HRP-conjugated goat anti-mouse

IgG and the addition of TMB substrate after extensive washing. Absorbance at 450 nm was measured on an ELISA plate reader after stopping the reaction with 3 N sulfuric acid.

Data analysis

The software used for the analysis was Graphpad Prism 6. Mice weight data was normalized to day 0 (pre-challenge with BTV) and analyzed through two-way ANOVA with Tukey multiple comparison test. ELISPOT results were analyzed through one-way ANOVA followed by a Tukey multiple comparison test.

Results

Characterization of the recombinant adenoviruses

To detect the expression of BTV proteins, Vero cells were infected with the produced adenoviruses (Table 1), and infected cell lysates were assessed for recombinant protein expression by Western Blot.

Adenovirus	Transgene
Ad-βGal	β-Galactosidase
Ad-RFP	Red fluorescent protein
Ad-BI	VP2-HA (From BTV1) FMDV 3A Protease VP7-FLAG (From BTV8)
Ad-VP2-1	VP2 from BTV1
Ad-VP7-8	VP7 from BTV8 (Used by Martín et al., 2015)

Table 1: Summary of the recombinant adenoviruses used in the study.

Samples from Ad-βGal and Ad-BI were incubated with both α-HA and α-FLAG antibodies. A specific band corresponding to VP2 size was detected in the extract from cells infected with Ad-BI but not in samples from Ad-βGal (Figure 3). However, VP7 could not be detected by incubation with α-FLAG (Result not shown). Due to the lack of specific antibodies against VP2, the expression of the transgene in Ad-VP2-1 could not be detected by Western Blot. Expression levels of VP7, from Ad-BI, and VP2, from Ad-VP2-1, are currently being determined at the messenger level. VP7 expression in Ad-VP7-8 infected cells has been previously characterized by Martín et al. (2015).

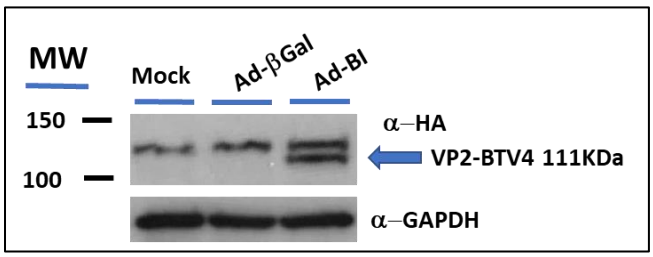

Figure 3: VP2 expression in AdBI transduced cells. Samples from Vero cells, infected with Ad-βGal or Ad-BI, were analyzed through Western Blot using a specific antibody against the HA tag.

Immunization with Ad-BI protects against BTV4, but not BTV8 challenge

Adult female IFNAR$^{(-/-)}$ mice were immunized with Ad-βGal or Ad-BI, followed by a boosting dose after 15 days. At day 15 post-boost, mice were challenged with 10^3 PFUs of BTV4 or BTV8 and monitored daily after the infection (Figure 4A).

Mice in both control (Ad-βGal) and vaccine (Ad-BI) groups developed signs of the disease: A significant reduction in the weight of the animals was detected from day 4 post-challenge for BTV4 infected mice and from day 5 post-challenge for BTV8 infected mice. Weight loss in Ad-BI vaccinated mice challenged with BTV4 was significantly less pronounced than in their Ad-βGal control counterparts. Mice which overcame the disease recovered their initial weight (Figure 4B).

All mice inoculated with the adenovirus Ad-βGal succumbed to the disease, as predicted. In contrast, mice inoculated with Ad-BI obtained were protected against BTV4 challenge, but did not survive BTV8 challenge. (Figure 4C).

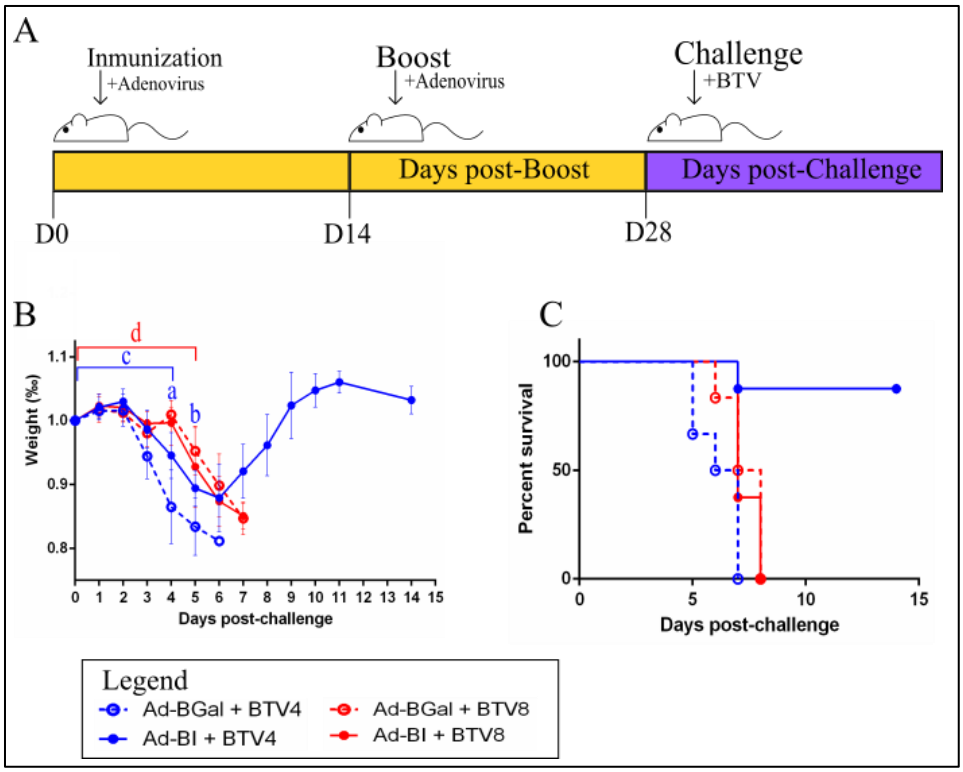

Figure 4: Ad-BI protected against BTV4 but not against BTV8 challenge. (A) Scheme of the experimental procedure performed in mice. **(B)** Weight measures in mice after BTV challenges. (a and b) Significant differences between Ad-βGal + BTV4 and Ad-BI + BTV4 at days 4 and 5 post-challenge ($p < 0.0001$ and $p < 0.01$ respectively). (c) Significant differences between days 4 and 0 in BTV4 challenged animals ($p < 0.0001$) (d) Significant differences between days 5 and 0 (In Ad-βGal + BTV8 and Ad-BI + BTV8 groups, $p < 0.0001$) (Sample sizes are described in the materials and methods section). Error bars represent ±SD **(C)** Mice survival after BTV challenge.

Immunization with Ad-VP2-1 and Ad-VP7-8 induces protection against BTV

IFNAR$^{(-/-)}$ mice were inoculated with 2 different combinations of adenoviruses: Ad-βGal + Ad-RFP, for the control group and Ad-VP2-1 + Ad-VP7-8 for the vaccinated group. These animals were then challenged with either BTV1, BTV4 or BTV8 (Figure 4A).

Similar to the previous result, a significant decrease in the weight of the animals was detected after BTV challenge (except for vaccinated animals challenged with BTV8, in which case, the weight difference is not statistically significant) Surviving animals are able to recover their initial weight after overcoming the infection (Figure 5A).

As predicted, all but one mouse in the control groups perished to the different serotype BTV challenge. In contrast, vaccinated mice (Ad-VP2-1 + Ad-VP7-8) were partially protected against both homologous and heterologous BTV challenge, with a survival percentage of 85, 70 and 50 for serotype 1, 4 and 8, respectively (Figure 5B).

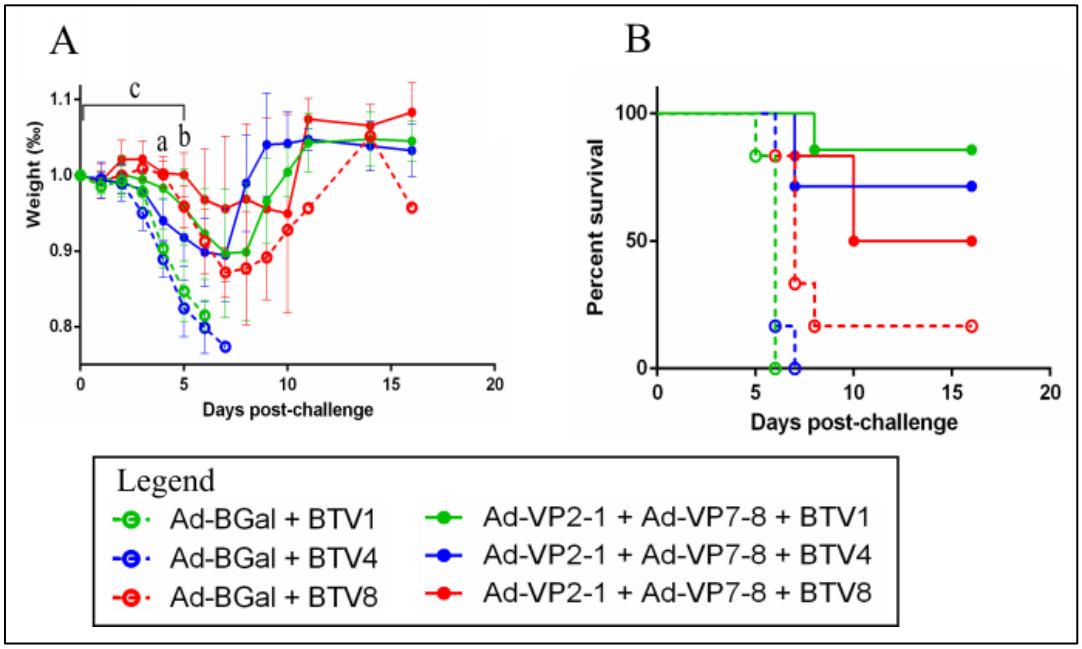

Figure 5: Vaccination with Ad-VP2-1 and Ad-VP7-8 protects against homologous and heterologous BTV challenge. (A) Weight measures observed in mice after BTV challenges. (a) Significant differences were observed between vaccinated and control groups (BTV1 challenge, $p < 0.0001$; BTV4 challenge, $p = 0.0015$; BTV8 challenge, ns). (b) Significant differences were observed between vaccinated and control groups (BTV1 challenge, $p < 0,0001$; BTV4 challenge, $p < 0.0001$; BTV8 challenge, $p = 0.0231$). (c) Significant differences between days 5 and 0 in every group (Ad-βGal + BTV1 $p < 0.0001$ / + BTV4 $p < 0.0001$ / + BTV8 $p = 0,0025$ / Ad-VP2-1 + Ad-VP7-8 + BTV1 $p = 0.0006$ / +BTV4 $p < 0.0001$) except Ad-VP2-1 + Ad-VP7-8 + BTV8 (ns) (Sample sizes are described in the materials and methods section). Error bars represent ±SD **(B)** Survival in mice after challenge.

Immunization with recombinant adenoviruses induce BTV-specific antibody production

The production of BTV-specific antibodies was detected by ELISA. Sera obtained from mice at day 0, day 7 post-boost and day 14 post-challenge were incubated in ELISA plates coated with BEI-inactivated BTV from serotypes 1, 4 and 8.

Animals inoculated with Ad-βGal or Ad-βGal + Ad-RFP did not produce significant levels of antibodies against none of the analyzed serotypes (Figures 6A to 6F). Regarding the vaccinated groups, mice immunized with Ad-BI were able to produce antibodies against both BTV4 and BTV8 (Figures 6A, 6B respectively), while mice inoculated with Ad-VP2-1 + Ad-VP7-8 showed humoral response against BTV1, BTV4 and BTV8 (Figures 6C, 6D and 6E respectively)

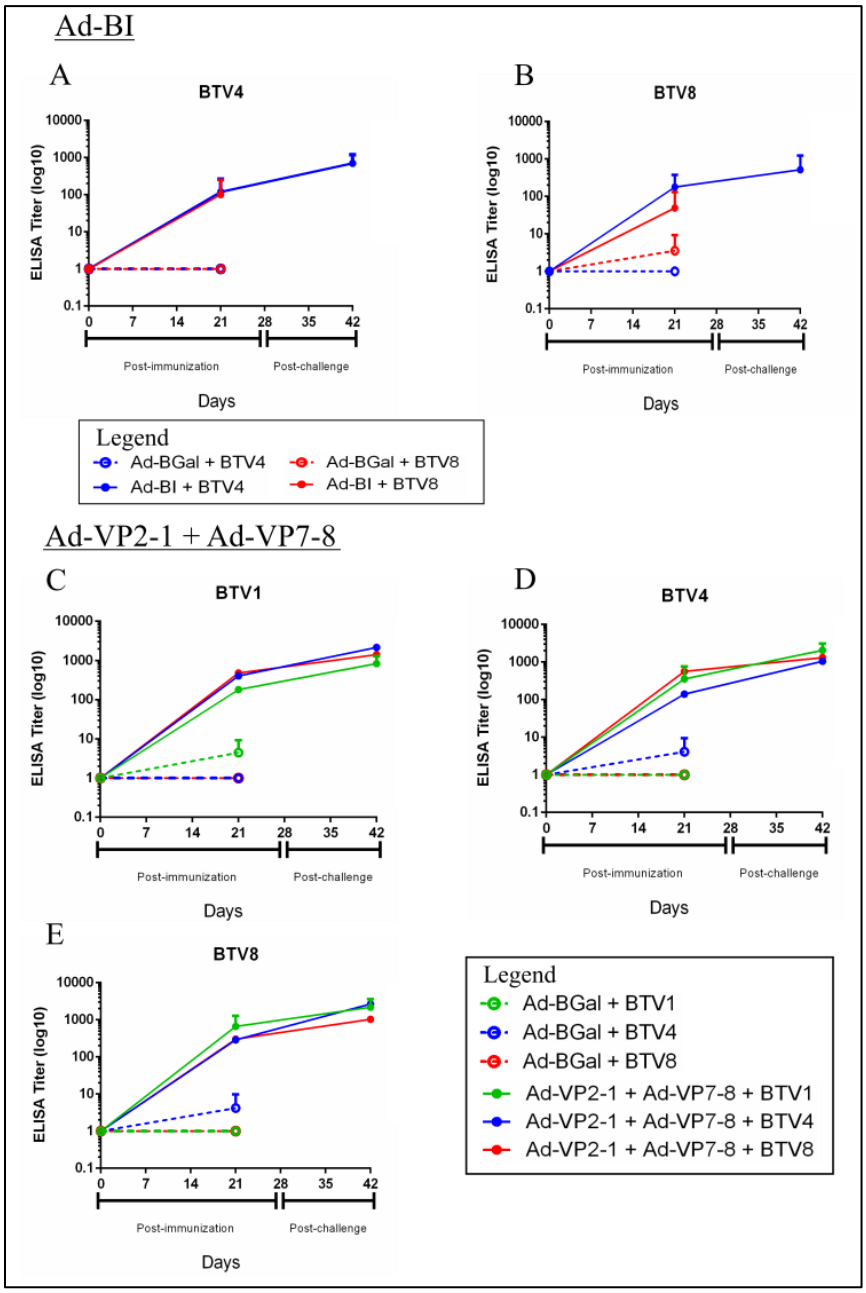

Figure 6: Serum IgG titer obtained by ELISA. Sera from (A and B) Ad-BI / Ad-βGal-or (C -E) Ad-VP2-1 + Ad-VP7-8 / Ad-βGal + Ad-RFP inoculated mice were titrated in plates coated with BEI-Inactivated BTV serotypes 1 (C), serotype 4 (A and D) and serotype 8 (B and E). Titers are expressed as the reciprocal of the highest dilution of serum (log10) that gives an ODA450 of twice the value obtained with the pre-immune serum of the corresponding animal. Error bars represent ±SD.

Immunization with recombinant adenoviruses induces T-cell responses against BTV

Splenocytes from adenovirus inoculated mice (Ad-βGal, Ab-BI or AdVP2-1 + AdVP7-8) were cultured in the presence of BTV peptides (NS1-152, as negative control, VP7-283 and VP7-139) or BEI-inactivated virus. The production of IFN-γ by T-cells was analyzed by ELISPOT assays.

Mice inoculated with Ad-βGal showed no significant response against the BTV peptides or the inactivated viruses (Figures 7A, 7B). In contrast, mice in the vaccinated groups (Ad-BI or Ad-VP2-1 + Ad-VP7-8) responded to BTV stimuli. Particularly, mice inoculated with Ad-BI showed a specific response against BTV4 (Figure 7D), while animals immunized with the adenoviruses Ad-VP2-1 and Ad-VP7-8 showed and specific T-cell response against VP7-283 peptide (Figure 7E) and against all the tested BTV serotypes (Figure 7F).

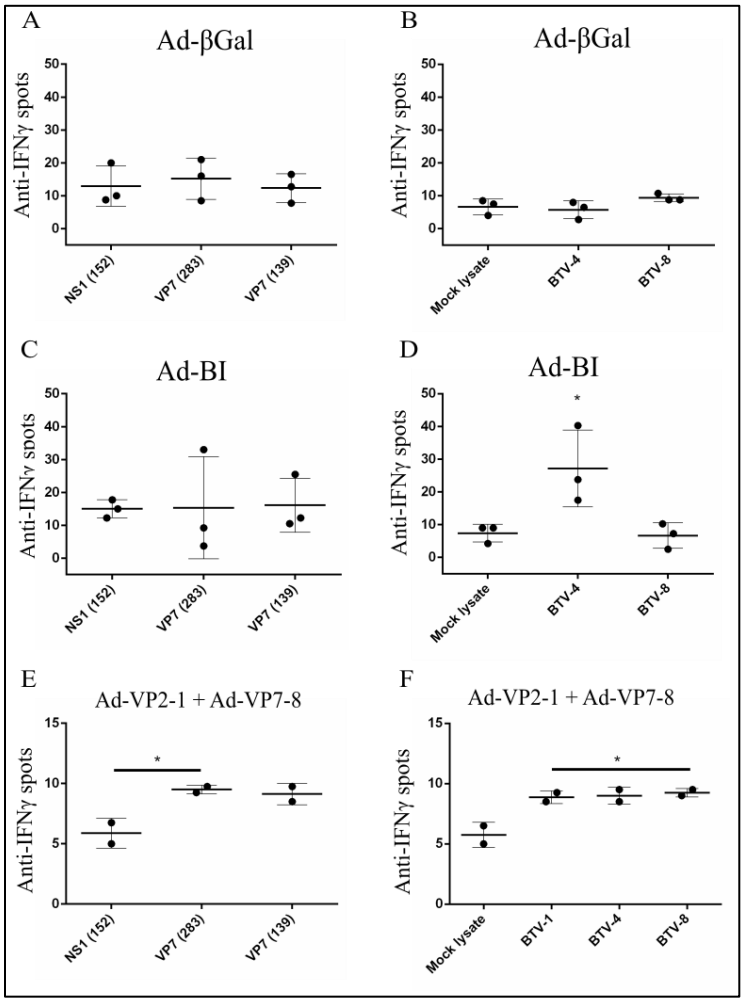

Figure 7: (A-F) Ad-Bi and Ad-VP2-1+Ad-VP7-8 vaccination induced T-cell responses. **(A-F)** Number of Anti-IFNγ spots for 2 x 10^5 splenocytes, obtained by ELISPOT assays performed as described in the materials and methods section. Splenocyte response to VP7 peptides for (A) Ad-βGal (C) Ad-Bi and (E) Ad-VP2-1+Ad-VP7-8 vaccinated mice. Splenocyte response to BTV serotypes for (B) Ad-βGal (D) Ad-Bi and (F) Ad-VP2-1+Ad-VP7-8 vaccinated mice. *Statistically significant values ($p < 0.05$). Error bars represent ±SD.

Additionally, splenocytes from immunized mice were used as effector cells in a flow cytometry-based cytotoxicity assay in which RMA-s cells pulsed with NS1-152, as negative control, or VP7-283 were targeted by splenocytes which had been expanded in vitro with VP7-283 peptide. This assay showed that CTL specific for the VP7-283 peptide were stimulated in Ad-VP2-1 + Ad-VP7-8 but not in Ad-βGal or Ad-BI immunized mice (Figure 8).

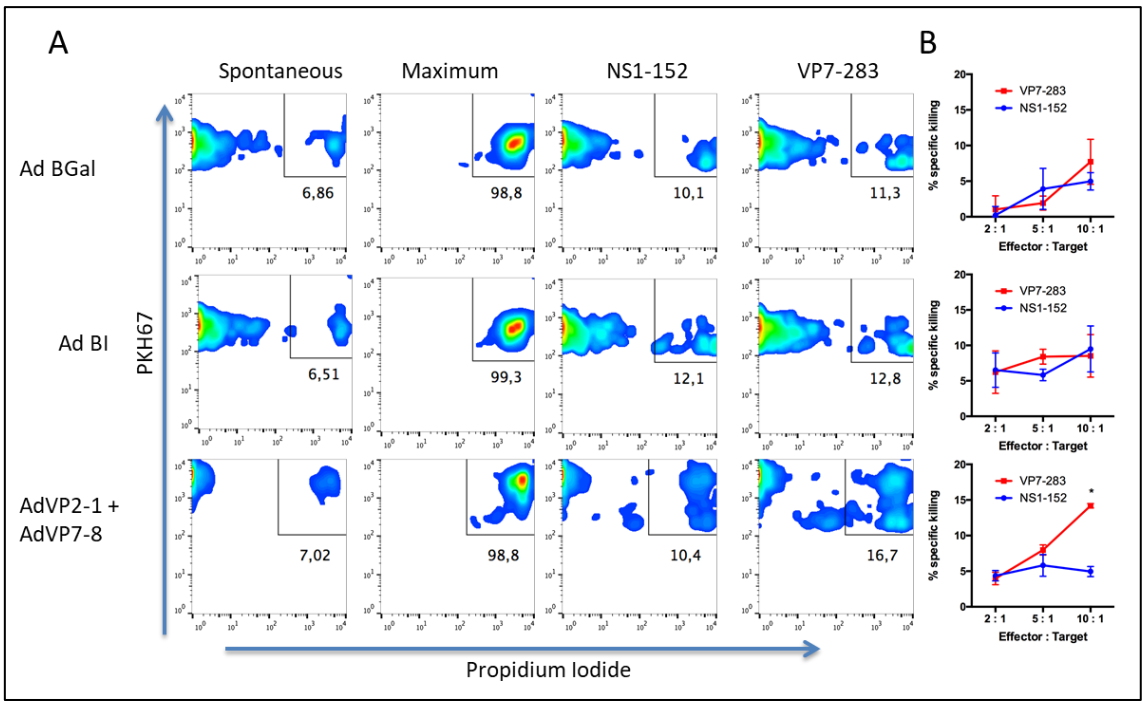

Figure 8: Cytotoxicity assay for peptide VP7-283 in splenocytes from vaccinated mice. **(A)** Examples of flow cytometry cytotoxicity assays presenting target cells (RMA-s) labelled with PKH67 showing spontaneous cell death, maximum cell death (saponin permeabilization) and when cultured with splenocytes isolated from the different recombinant adenovirus vaccinated mice and stimulated in vitro with VP7-283 peptide. **(B)** Specific killing was determined as described in material and methods. *Statistically significant values ($p < 0.05$). Error bars represent ±SD.

Discussion

In the present work, we assessed the efficacy of two vaccination strategies with replication-defective recombinant human adenovirus 5 expressing VP2 and VP7 BTV proteins to induce strong immune responses, looking for a cross-serotype BTV protection. VP2 from two serotypes (1 and 4) were chosen based on their content of the major neutralizing determinants of

BTV (White & Eaton, 1990) and VP7 from BTV8 was chosen based on its high T cell epitope content (Rojas et al., 2011) and its homology between serotypes.

Firstly, we tested a vaccine (Ad-BI) which could express both VP2 (from BTV4) and VP7 (from BTV8) in a single adenoviral construction. Upon characterization of the adenovirus by Western Blot, expression of VP7 could not be detected, but VP2 was correctly expressed.

When immunized IFNAR$^{(-/-)}$ mice were challenged with BTV4, they showed a reduced weight loss in comparison with the other 3 groups, which is probably related to the effect of the vaccine. These mice were able to overcome the disease. In contrast, vaccinated mice which were challenged with BTV8 showed a similar pattern to those of the control group and did not recover from the disease.

As described by Martín et al. (2015), VP7 from BTV8 is able to elicit BTV-specific humoral and cellular responses, granting protection against this serotype. Therefore, the inability of Ad-BI-immunized mice to survive BTV8 challenge could be due to insufficient VP7 expression as suggested by the Western Blot analysis in which VP7 was not detected. Interestingly, production of antibodies against BTV8 after the booster immunization were observed in these mice, which indicated that a low VP7 expression may be produced. However, the lower antibody titers compared to those produced against BTV4 together with the lack of a T-cell response, probably led to Ad-BI inefficient protection against BTV8.

Regarding the vaccinated group which was challenged with BTV4, a survival percentage of 90% was achieved. As showed by the ELISA and ELISPOT assays, Ad-BI could effectively produce BTV-specific antibodies against the virus and induce a BTV4-specific T-cell response at day 7 post-boost. We are currently evaluating whether the detected antibodies can neutralize BTV infection.

Secondly, we assessed the efficacy of the simultaneous vaccination with 2 adenoviruses which expressed VP2 (from BTV1) and VP7 (from BTV8). In this experiment, mice were challenged with BTV1, BTV4 or BTV8.

The ELISPOT assay performed with splenocytes obtained from mice at day 7 post-boost indicated that these adenoviruses were able to elicit specific T-cell responses against all the tested BTV serotypes, presumably, through VP7 recognition. These results were further confirmed with a cytotoxicity assay in which CTL specific for the VP7-283 peptide were detected. Vaccinated mice also produced specific antibodies against BTV1, BTV4 and BTV8.

Upon BTV challenge, weight loss in the control groups was more pronounced than in the vaccinated mice. In the case of the Ad-VP2-1 + Ad-VP7-8 vaccine, mice in every vaccinated group were able to overcome the disease. Remarkably, this vaccine achieved a 70% survival rate in the vaccinated group which was challenged with the heterologous BTV4 serotype. This result

means that this combination of BTV proteins is able to grant cross-protection against other serotypes. Presumably, VP7 is the main contributor to this effect as it is a conserved protein between serotypes and a T cell effector, while VP2 displays high variability being the protein that defines the serotype. This has been the main goal of this work.

Altogether, these results confirm that the use of recombinant adenoviruses is a suitable strategy for the effective control of BTV. This platform has the capability of eliciting both humoral and cellular immune responses without an adjuvant and, moreover, it has the potential to grant protection against multiple serotypes of BTV. These characteristics set recombinant adenoviruses as an ideal alternative to live-attenuated or inactivated BTV vaccines, as it overcomes most of the drawbacks of classical vaccine. Recombinant adenoviruses expressing BTV proteins could also help develop DIVA control strategies by differentiating vaccinated animals that only respond to the BTV protein expressed in the vaccine from infected animals that will respond to BTV proteins not included in the vaccine. Moreover, in comparison to other recombinant vaccines, adenoviruses are not only better at inducing immune responses, but also have a favorable safety record (Collins et al., 2017)

In order to fully characterize the potential vaccines used in this project, further assays are needed. Firstly, it is necessary to check whether VP7 is expressed by Ad-BI at the messenger level and, if confirmed, to determine the reason of the insufficient protein levels, resulting in the lack of protection. Additionally, neutralization assays will be necessary to determine if the antibodies produced by adenovirus vaccinated mice are able to block BTV infection, together with real time qPCR analysis, which are currently ongoing, in order to determine the viremia in blood of animals, allowing the evaluation of the type of protection (sterile or non-sterile).

Secondly, it is necessary to better characterize the main effector of the BTV protection in the experiment performed with Ad-VP2-1 and Ad-VP7-8. This can be achieved by coating ELISA plates with VP2 or VP7 to check the protein targeted by the antibodies produced by the mice, and further confirmed by inoculating mice with only Ad-VP2-1 or Ad-VP7-8 and challenging these animals with BTV4. Once the main effector of the observed cross-protection is elucidated, this vaccine could be further tested with additional BTV serotypes to establish the range of effectiveness.

Finally, once the vaccine candidate has performed as expected in all of these tests, experiments in sheep, which is the natural host of the virus, are necessary to confirm its effectiveness and viability in the field. In this project, we confirm that replication-defective recombinant adenoviruses are a suitable platform for BTV vaccination. Our results show that these vectors are able to elicit both cellular and humoral immune responses resulting in a protection against BTV across several serotypes.

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