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*Article*

# Recombinant BoHV-5 glycoprotein elicits long-lasting protective immunity in cattle

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**Abstract:** Bovine herpesvirus (BoHV)-5 is a worldwide distributed pathogen usually associated with a lethal neurological disease (meningoencephalitis) in dairy and beef cattle resulting in important economic losses due to the cattle industry. Using recombinant glycoprotein D of BoHV-5 (rgD5), we evaluated the long-duration humoral immunity of the recombinant vaccines in a cattle model. Here we report that two doses of intramuscular immunization, particularly with the rgD5ISA vaccine, are superior to iBoHV-5ISA immunization in the induction of long-lasting antibody responses. Recombinant gD5 antigen elicited tightly mRNA transcription of the *Bcl6* and the chemokine receptor *CXCR5* which mediate memory B cells and long-lived plasma cells in germinal centers (GCs). In addition, using an in-house Enzyme-Linked Immunosorbent Assay (ELISA) we observed higher and earlier responses of rgD5-specific IgG antibody and the upregulation of mRNA transcription of *IL2*, *IL4*, *IL10*, *IL15* and *IFN-γ* cytokines in rgD5 vaccinated cattle, indicating a mixed immune response. We further show that rgD5 immunization provides protection against both BoHV -1 and -5. Our findings indicate that the rgD5-based vaccine represents an effective vaccine strategy to induce an efficient control of alpha-herpesviruses.

**Keywords:** Germinal Center; Herpesvirus; Recombinant protein; rgD5; Vaccine; Long-Lasting

## 1. Introduction

Bovine Herpesvirus 5 (BoHV-5) is a highly contagious disease known to be responsible to cause the meningoencephalitis [1–3]. The infection is distributed worldwide but particularly in South America, been associated with significant economic losses [4–6]. To date, the disease is controlled by killed, conventional modified-live vaccines and subunit vaccines. However, the killed and live vaccines have several disadvantages regarding safety and efficacy, make unsuitable for vaccination of some targets [7]. Advances in molecular biotechnology have led to efforts to overcome the safety problems of vaccines generated from live viruses by protein-based subunit vaccines development, including the vaccines generated from viral envelop glycoprotein subunits. One of them is the glycoprotein D (gD), an immunodominant protein that is essential for viral penetration into host permissive cells [8,9]. The anti- and immunogenic properties of rgD5, as well as its ability to produce neutralizing antibodies (nAbs) in mice and cattle, provide the adaptive immune system's first line of defense. [9–11].

The production of long-lived antibody responses to protect against future infection is one of the criteria for effective vaccinations, which is dependent on a robust germinal center (GC) response [12]. Follicular helper T ($T_{FH}$) cells have recently been highlighted for their essential role in the formation of GCs and selection of mutated GC B cells [13,14]. $T_{FH}$ cells are $CD4^+$ T cells marked by high levels of CXCR5, PD-1, and Bcl6 expression and are specialized to support B-cell response in GCs that are essential for somatic hypermutation, and class switching for the generation of high-affinity antibody-producing plasma cells

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and memory B cells and long-lasting humoral immunity [13–15]. The expression of the transcription factor B cell lymphoma-6 (Bcl6), which is the master regulator of $T_{FH}$ cell differentiation, drives the upregulation of CXCR5, the receptor of CXCL13 and canonical feature of $T_{FH}$ cells, on the cell surface of pre-$T_{FH}$ cells that are now allowed to migrate to the border between the B- and T-cell area [15]. In the site, CXCR5 drives $T_{FH}$ cell migration into B cell follicles culminating in antibody production and the formation of GCs [14,16].

Most antigens require T-cell help to induce B-cell responses, and understanding the mechanisms involved in their recognition by which memory T and B cells are generated and maintained is important for understanding the generation of long-term immunity, as well as how their swift activation is executed is of fundamental importance for vaccine development [14,17]. Here, we explored the potential of taking rgD5-based vaccines regarding the long-term immune response and other points that can be limited to their commercial application. In the present study, we investigated the effect of Bcl6 and CXCR5 in regulating B cell activation in the setting of GC reactions, IgG class switching, long-lived humoral immunity, and immunological memory in the context of an experimental vaccine using the rgD5 antigen in cattle vaccinated against BoHV-5.

## 2. Materials and Methods

### *2.1. Expression of recombinant glycoprotein D from BoHV-5*

A previously reported *P. pastoris* strain KM71H Mut$^S$ [18], in which the recombinant gD5 was cloned and expressed by methanol induction in an orbital shaker was used in this study. After the induction phase, the culture supernatant was directly concentrated and used for vaccine formulation, whereas the purified recombinant gD5 was employed in the indirect ELISA.

### *2.2. Cells and viruses*

The BoHV-1 (Los Angeles strain) and -5 (Riopel – RP strain) were propagated in Madin Darby Bovine Kidney cells (MDBK, ATCC CCL22) as previously described by Araujo et al. [9]. The Virology and Immunology Laboratory of the Federal University of Pelotas (UFPel) – Brazil, provided the virus strains. Prior serum neutralization test, the virus stock was tittered and stored at -70 °C.

### *2.3. Study vaccine*

The immunobiologics were prepared using inactivated BoHV-5 (iBoHV-5) harvested at a concentration of 1 x $10^{5,5}$ TCID50/mL and inactivated with 20 mM bromoetilamina (BEI) ($C_2H_7Br_2N$ – Merck) and recombinant gD5 (100 mg/dose). The formulations were adjuvanted with 50% (v/v) Montanide ISA 50 V2 (90% Marcol 52 e 10% Montanide 888, Seppic Adjuvants, France). The rgD5iBoHV5ISA formulation was prepared using 100 mg rgD5, 1.5 mL of iBoHV-5 at concentration of 1 x $10^{5,5}$ TCID50/mL and ISA 50 V2 (per dose). The control group (sentinel) received Phosphate Buffered Saline (PBS) only. The vaccines were prepared under aseptic conditions and stored at 4 °C during the experimental period. Twenty-eight 8-12-month-old calves were kindly provided by a producer from a local farm located in the south of Brazil (31°51’51’’S-52°49’24’’W) and were not submitted to food or water restrictions. The animals serologically negative to BoHV-1 and -5 confirming the negative history of IBR were randomically allotted into 4 groups of 8 animals (control group = 4 animals). The animals received a dose of 3 ml of the corresponding immunobiological preparations three times: 0, 26, and 357 days from *primo* vaccination (Figure 1). Importantly, unvaccinated animals were kept for the control purpose of antibodies that were generated only as the result of vaccinations [9]. Whole blood for serum assessment was collected on days 0, 26, 33, 56, 111, 160, 186, 357, and 367 through jugular vein puncture (Figure 1). All procedures were performed following the Brazilian National Council for Animal Experimentation Control (COBEA) guidelines and approved by the UFPel Ethics Committee for animal research (CEEA No. 002129).

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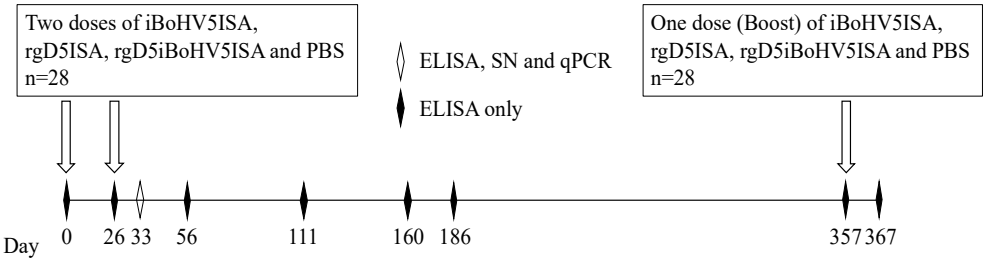


**Figure 1.** A schematic presentation of the study design. The study included 28 healthy calves aged 8-12-months old. The animals received vaccinations on days 0, 26, and 357, and follow-up samples were collected on days 0, 26, 33, 56, 111, 160, 186, 357, and 367 (one year). The black dots indicate ELISA testing only, and the white dots indicate Virus Neutralization (VN), ELISA, and qPCR testing.

2.4. Total IgG kinetics anti-rgD5

In-house ELISA was performed as described by Dummer et al. [19]. Briefly, 96-well microtiter plates were covered overnight at 4 °C with 50 ng/well of purified rgD5 and blocked by blocking solution (5% skim powdered milk + 3% casein in PBS-T) at 37 °C for 1 h; individual serum samples were diluted 1:400 were added in triplicate and incubated at 37 °C for 1 h and HRP-conjugated rabbit anti-bovine IgG (Sigma-Aldrich) diluted 1:5.00 was incubated at 37 °C for 1.5 h. Plates were washed three times between each step with PBS containing 0.5 percent Tween® 20. The reaction products are visualized through o-Phenylenediamine dihydrochloride (OPD) (Sigma-Aldrich). Plates were incubated for 15 min in the dark and the reaction stopped with 2 N $H_2SO_4$. The absorbances were determined on an ELISA TP-Reader plate spectrophotometer (Thermo Plate) at 492 nm.

2.5. IgG isotyping anti-rgD5

The IgG isotype levels were evaluated by ELISA using individual sera in triplicate from samples collected on days 26 and 33 of the experiment. Serum was diluted with PBS-T, starting at 1:100 and continuing in 2-fold dilutions [9] and IgG was used at 1:5.000 according to the instructions for isotyping for IgG1 (Abcam, ab112754) and the IgG2 (Abcam, ab112620) detection. The reciprocal of the highest dilution that had two times the absorbance of the control group was taken as a positive rgD5-specific antibody titer.

2.6. Virus Neutralization test (VNT) anti-BoHV-1 and -5

Serum samples were titrated in quadruplicate in 2-fold serial dilutions (starting dilution 1:2 until 1:256 in MEM). Subsequently, a 96-well tissue culture microtiter plate (TPP, Switzerland) with BoHV-5 virus suspension containing 100 $CCID_{50}$% was added following incubation at 37 °C and 5% $CO_2$. After 1 h of incubation, approximately $3x10^4$ MDBK cells/well were added and the microplates were incubated under the same conditions until the time of the 100 $TCID_{50}$ expressions. The microplates were observed by light microscope for the absence of cytopathic effect (CPE). The highest serum dilution inhibiting at least 90% of the CPE was indicated as the neutralization titer, while its presence resulted from the absence of neutralizing antibodies. The neutralizing antibody titer was calculated by the Behrends and Kärber method [20] and expressed as the reciprocal of the highest dilution.

2.7. Collection, preparation, and culture of PBMCs

Blood samples were collected in heparinized tubes on the 33rd day of the follow-up study. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll Histopaque (1.083 density, Sigma-Aldrich). Approximately 1 X $10^7$ pooled cells were cultured in 24-well plates (Kasvi, China) containing 1 mL of RPMI

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1640 medium (Gibco, USA) with 10% fetal bovine serum (FBS; Gibco), 10,000 IU/ml penicillin, 10 mg/ml streptomycin, and 25 mg/ml amphotericin B (Gibco) for 24 h at 37 °C in a 5% $CO_2$ atmosphere. After a change of the medium, PBMCs were restimulated with 10 μg/mL of rgD5, and 5 μg of concanavalin A (ConA) (Sigma-Aldrich) as a positive control, whereas RPMI 1640 was a negative control. The plates were incubated for 12 h under the same conditions. Cells were then harvested with TRIzol Reagent (Life Technologies, SP, Brazil) according to the manufacturer's instructions for total RNA isolation and pooled lysates were frozen at -80 °C until further processing.

2.8. Real-time PCR

Reverse transcription was carried out using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies, SP, Brazil) using approximately 1 μg of RNA. Quantitative real-time PCR was performed using the Stratagene Mx3005P qPCR system (Agilent Technologies, Santa Clara, USA) to quantify gene expressions. The primers used in the PCR were synthesized by GenOne Biotech (Brazil) and approximately 500 ng of the resulting cDNA was subjected to qPCR to analyze of transcription of the following five cytokines: *IL2*, *IL4*, *IL10*, *IL15*, and *INF-γ*; the chemokine receptor CXCR5 and the transcription factor Bcl6 (Table 1). Pooled samples were analyzed in triplicate using the comparative threshold cycle ($\Delta\Delta C_t$) method to determine the relative mRNA expression compared to *GAPDH* was used as endogenous reference genes and animals in pre-immune conditions were used as calibrators.

**Table 1. Target genes, primers sequences, product size, melt temperature (MT), and efficiency used for real-time quantitative PCR.**

| Gene | Forward (5′- 3′) | Reverse (5′- 3′) | Product Size | MT | Efficiency | |
|---|---|---|---|---|---|---|
| | | | | | $R^2$ | E |
| GAPDH | GCATCGTGGAGGGACTTATGA | GGGCCATCCACAGTCTTCTG | 67 pb | 60 °C | 0,98 | 1,93 |
| IL2 | CCTCGAGTCCTGCCACAATG | CCGTAGAGCTTGAAGTAGGTGC | 89 pb | 60 °C | 0,99 | 2,00 |
| IL4 | GCCACACGTGCTTGAACAAA | TCTTGCTTGCCAAGCTGTTG | 66 pb | 60 °C | 0,99 | 1,97 |
| IL10 | GTCTGACAGCAGCTGTATCCACTTG | AACCCTGGATTGGATTTCAGAGGTC | 551 pb | 60 °C | 0,99 | 1,89 |
| IL15 | CATATTTGAGAAGTACTTCCATCCAG | GAAGTGTTGATGAACATTTGCAC | 468 pb | 60 °C | 0,98 | 1,93 |
| IFN-γ | CAGAAAGCGGAAGAGAAGTCAGA | CAGGCAGGAGGACCATTACG | 70 pb | 60 °C | 0,96 | 2,05 |
| CXCR5 | TACCCTCTCACTCTGGACATGG | CTCCGTACTGTCATTGTAGCTCC | 87 pb | 60 °C | 0,99 | 2,15 |
| BCL6 | GTATCCAGTTCACCCGCCAT | ACATCAGTCAAGATGTCACGGC | 80 pb | 60 °C | 1,00 | 2,07 |

Primers were designed by Dr. Alceu Gonçalves dos Santos Junior in the Microbiology Laboratory – CDTec – UFPel, Brazil.

2.9. Statistical analysis

Differences in individual serum ELISA antibody titers presented in Figures 1 and 2 were performed on $\log_2$-transformed titer data, whereas serum neutralizing antibodies analysis presented in Figure 3 was performed on $\log_{10}$-transformed. ELISA and VNT results were analyzed by two-way ANOVA followed by Tukey's Multiple Comparisons Test. Expression levels of all target genes were calculated relative to the housekeeping gene (GAPDH). Two-way ANOVA followed by Dunnett's Multiple Comparisons test was used to analyze relative gene expression presented in Figures 4 and 5. All statistical analysis was performed in GraphPad Prism version 9.1.1 for macOS (GraphPad Software, USA) and a $p$-value of ≤ 0.05 was considered statistically significant. Error bars represent mean value ± standard error.

## 3. Results

3.1. A long-term follow-up study of anti-rgD5 IgG

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The titers were first detected 26 days after the first dose of the vaccine and then increased up to day 33 (Figure 2). After the second dose, titers reached a maximum level at days 7–357, and antibodies against the rgD5 were measured by ELISA, with all groups responding to the vaccines compared with the control group. Seventh days after the boost the group vaccinated with both antigens had significantly higher IgG titers (1.5-fold increase) compared with the iBoHV5ISA-vaccinated group (Figure 2). On the 56th day of the experiment, both groups containing the recombinant antigen had significantly ($p<0.05$) IgG titers than the iBoHV5ISA group (Figure 2). In addition, on the same 56th day, the mixed antigen formulation vaccine presented significantly higher IgG titers (1.5-fold increase) than the rgD5ISA group. Titers levels gradually declined on day 56 but remained higher than after the first dose of the vaccine. After the third vaccine shot (day 357), all groups responded to the vaccines (day 367). The control group did not increase the antibody levels during the experimental period, which was like the levels observed before the *primo* vaccination, on day 0 (Figure 2).

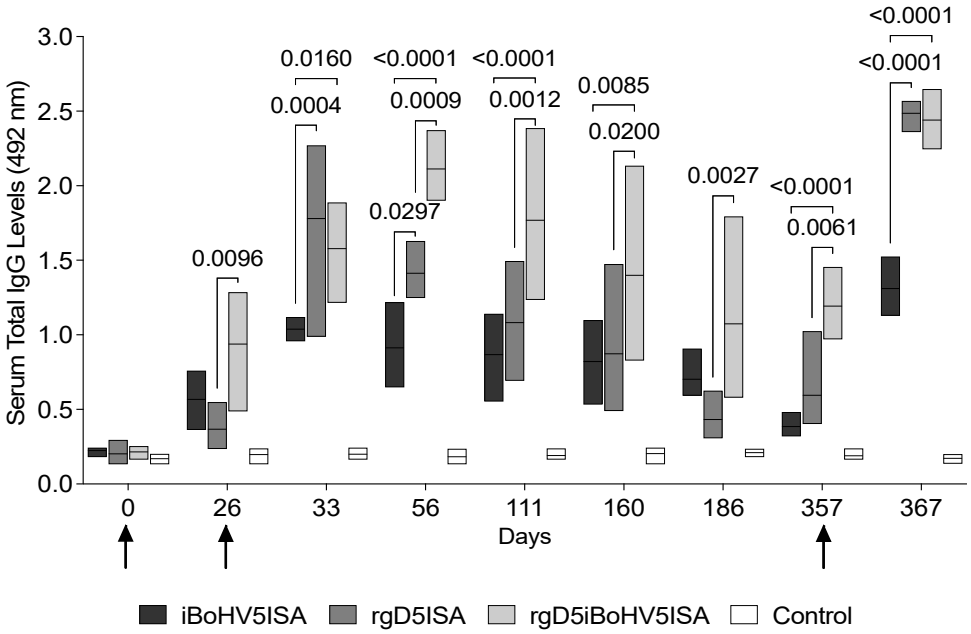


**Figure 2.** A long-term follow-up study to evaluate the immunogenicity of the rgD5 in cattle vaccinated with subunit vaccine against BoHV-5. Total serum anti-rgD5 IgG levels from day zero to day 367 of the experiment. The curves represent the average values of the measured optical density (OD) at 492 nm over time. Statistical analysis by ordinary two-way ANOVA with a *post hoc* Turkey Test. *p*-values less than 0.05 were not included in the figure. n=8 animals/group (control=4 animals). Arrows indicate vaccination and the solid line represents the mean titer of each group. Significant differences compared with the control group are not shown.

3.2. Mixed IgG isotype response in vaccinated animals

The IgG subclasses were detected 26 days after the first dose of the vaccine and then increased up to day 33, seven days after the second dose (Figure 3). Our results showed that the immune response elicited a mixed Th1/Th2 profile, with higher titers of anti-rgD5 IgG1 antibodies after the first and second doses of the vaccine.

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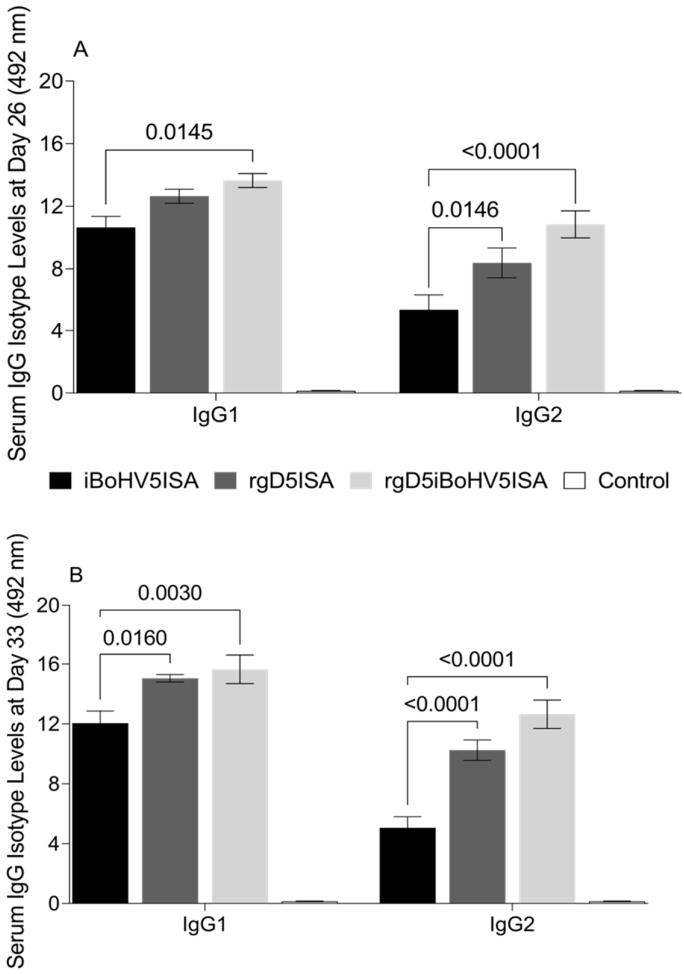


**Figure 3.** Mixed Th1/Th2 profile as measured by IgG subclasses. **(A-B)** At 26 days post-first vaccination and 7 days post-second vaccination the levels of IgG1 and IgG2 were measured by ELISA. Statistical analysis by ordinary two-way ANOVA with a *post hoc* Turkey Test. *p*-values less than 0.05 were not included in the figure. n=8 animals/group.

3.3. Neutralizing antibody responses against BoHV-1 and -5

Neutralizing antibody (nAb) levels were investigated 33 days after the first vaccination and all groups already had titers of nAb against BoHV-1 and -5 with a significant ($p < 0.05$) increase compared with the control group as shown in Figure 4. No significant differences in cross-neutralizing serum titers were present between both alpha-herpesviruses tested. Furthermore, all vaccine groups showed nAb titer above 3log2 (1:8, indicated by the dashed lines), levels considered as protected a herd [21]. The rgD5iBoHV5ISA formulation (light grey bars) had elevated but not significantly nAb response against both viruses when compared with the other groups (Figure 4).

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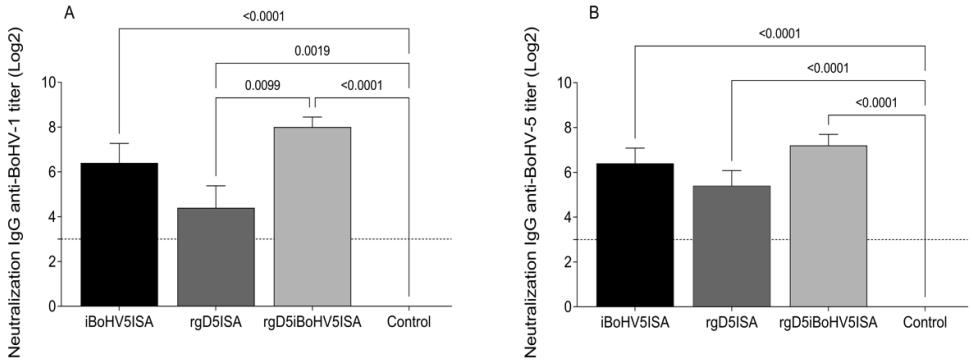


**Figure 4.** Recombinant gD5-specific neutralizing antibody titers in vaccinated cattle. Individuals nAb responses to BoHV-1 **(A)** and BoHV-5 **(B)** in serum of vaccinated cattle 7 days post-second vaccination. Data are mean ± SEM of log2 transformed and expressed as the reciprocal of the highest dilution that completely inhibited virus-induced CPE. Statistical analysis by ordinary two-way ANOVA with a *post hoc* Turkey Test. *p*-values less than 0.05 were not included in the figure. n=8 animals/group. Dashed lines mean neutralizing titer 3log2 (1:8).

3.4. Th1, Th2, and Treg cytokine transcription

Polled PBMCs restimulated with 10 μg of rgD5 from all groups (Figure 5) upregulated the mRNA transcription of *IL2, IL4, IL10, IL15, and IFN-γ* when compared with the control group (white columns). The association of inactivated BoHV-5 and recombinant gD5 antigens (light grey columns) downregulated the mRNA transcription of *IL2*, *IL4*, *IL10*, *IL15*, and *IFN-γ*, compared with the group that received rgD5 alone (dark grey columns). However, the antigens association (light grey columns) upregulated the mRNA transcription levels of *IL2*, *IL4*, *IL15*, and *IFN-γ*, while downregulated the *IL10*, relative to the inactivated BoHV-5 antigen alone (black columns). Finally, the inactivated BoHV-5 antigen (dark columns) downregulated the mRNA transcription of *IL2*, *IL4*, *IL10*, *IL15*, and *IFN-γ*, compared with the recombinant gD5 antigen (dark grey columns). Table 2 show the data of all vaccine group comparison, including fold change statistics from the panel of cytokines.

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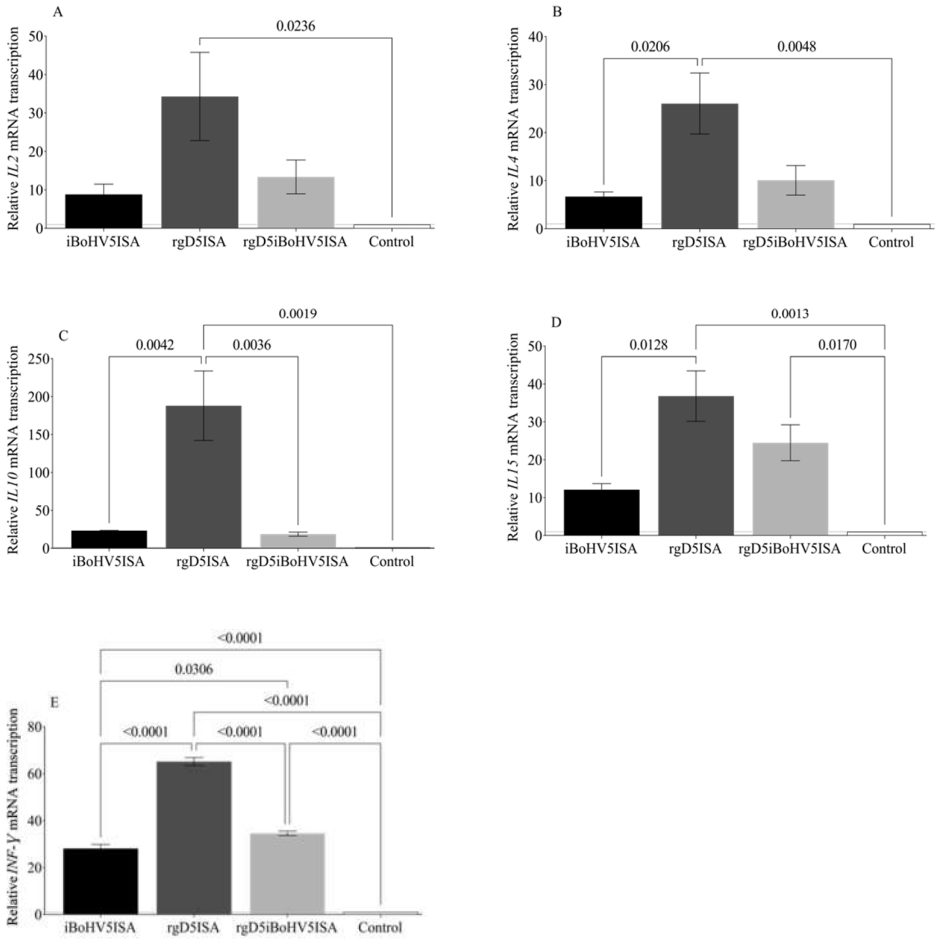


**Figure 5.** Th1, Th2, and Treg cytokine transcription. Seven days after the second vaccination, PBMCs were restimulated *in vitro* with 10 μg of rgD5 for 18 h. Total mRNA was extracted, and the corresponding cDNA was subjected to quantitative PCR. The fold changes for *IL2* **(A)**; *IL4* **(B)**; *IL10* **(C)**; *IL15* **(D)** and *IFN-γ* **(E)** transcription were calculated from the threshold cycle (Ct) values normalized to Ct values obtained from PBMCs of non-vaccinated animals. Data are generated from PBMCs pooled (n=8/group) collected on day 33 of the experiment. Statistical analysis by ordinary two-way ANOVA with a *post hoc* Dunnett's test. *p*-values less than 0.05 were not included in the figure. n=8 animals/group (control=4 animals).

3.5. Bcl6 and CXCR5 transcription underlie the germinal center formation

Polled PBMCs restimulated with 10 μg of rgD5 from all groups (Figure 6) were upregulated the mRNA transcription of *Bcl6* and *CXCR5* when compared with the control group (white columns). The group iBoHV5ISA (black columns) upregulated the mRNA transcription of *Bcl6* and *CXCR5*. The rgD5ISA (dark grey columns) upregulated the mRNA transcription of *Bcl6* and *CXCR5*. The group rgD5iBoHV5ISA (light grey columns) upregulated the mRNA transcription of *Bcl6* and *CXCR5*. The association of inactivated BoHV-5 and recombinant gD5 antigens (light grey columns) downregulated the mRNA transcription of *Bcl6* and *CXCR5*, compared with the rgD5 alone (dark grey columns). However, this antigen association (light grey columns) upregulated the mRNA transcription levels of Bcl6 and CXCR5, relative to the inactivated BoHV-5 alone (black columns). Finally, the inactivated BoHV-5 antigen (dark columns) downregulated the mRNA transcription of Bcl6 and CXCR5, compared with the recombinant gD5 antigen (dark grey

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columns). Table 3 show the data of all vaccine group comparison, including fold change statistics from the Bcl6 and CXCR5.

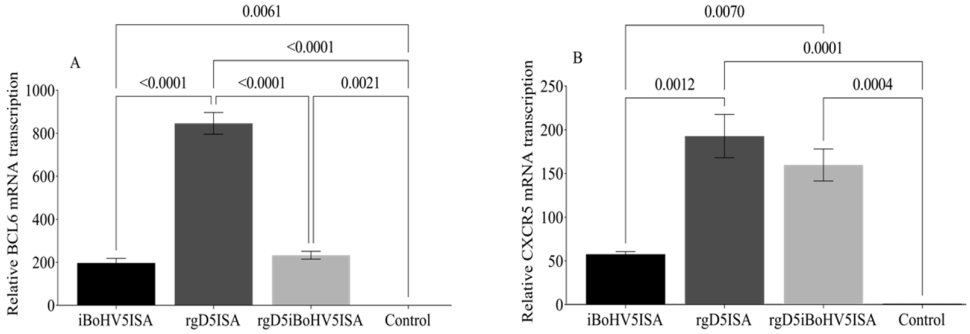


**Figure 6.** Bcl6 and CXCR5 mRNA transcription underlie germinal center formation. Seven days after the second vaccination, PBMCs were stimulated *in vitro* with 10 µg of rgD5 for 18 h. Total RNA was extracted, and the corresponding cDNA was subjected to quantitative PCR. The fold changes for Bcl6 **(A)** and CXCR5 **(B)** transcription were calculated from the threshold cycle (Ct) values normalized to Ct values obtained from PBMCs of non-vaccinated animals. Data are generated from PBMCs pooled (n=8/group) collected on day 33 of the experiment. Statistical analysis by ordinary two-way ANOVA with a *post hoc* Dunnett's test. *p*-values less than 0.05 were not included in the figure. n=8 animals/group (control=4 animals).

## 4. Discussion

The induction of long-lasting immune response, mixed Th1/Th2 response, and cytotoxic T lymphocytes are essential in the most effective vaccines that are in use today. The identification and selection of antigens and their adjuvant association in a vaccine should achieve the necessary modulation of the immune response to improve the type of immunity necessary to protect the host. Subunit vaccine candidates against alpha-herpesviruses have been based on the viral major envelope glycoproteins as they act in the initial attachment and virus penetration to the host cell surface receptors as well as mediate the immune responses of the host to the infection [22–27]. Although immunization with purified protein antigens normally induces a small antibody response with little T cell response, several immunizations may be necessary to elicit adequate antibody responses. [28]. Nevertheless, the glycosylation technology plays an important role to overcome the lower antigenicity generated by ultra-purified recombinant antigens. For instance, Dummer et al. 2009 [18] demonstrated firstly that the rgD5 expressed in *Pichia pastoris* has been capacity to conduct post-transcriptional modifications that are important for antigenicity. Thus, this truncated form of rgD5 generated has antigenic and immunogenic characteristics similar to the corresponding native glycoprotein that allow its recognition and ability to induce cellular and humoral immune responses in mice and cattle [9,10,18,25]. The B cell help provided by $T_{FH}$ cells represents a key aspect of adaptive immunity to many pathogens and vaccines. $T_{FH}$ and B cells provide signals by expressing co-stimulatory molecules and secreting cytokines, which are essential for GC B cells to undergo class switch recombination, somatic hyper-mutation, affinity maturation, and differentiation of plasma cells and memory B cells in GCs [13,14,16,29]. The expression of master regulator Bcl6 and the chemokine receptor CXCR5 by the $T_{FH}$ cells are essential for the T cell's migration into the B cell follicle in lymphoid organs [30–32]. In this study, both Bcl6 and CXCR5 transcription was tightly upregulated in PBMCs re-stimulated *in vitro* with rgD5 at 7 days after the 2$^{nd}$ vaccination. We found a 4.3 and 3.34 -fold increase of CXCR5 and Bcl6, respectively, in the vaccine that received only the recombinant antigen (Figure 6A-B, dark grey columns) compared with the group that received only the inactivated BoHV-5 antigen (Figure 6A-

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B, black columns). In parallel, we observed one week after the boost at day 367 from *primo vaccination* the highest levels of total anti-rgD5 IgG were generated by the groups that received the rgD5 in their vaccine formulation (Figure 2, dark and light grey columns) [9]. These findings suggest that the presence of rgD5 provides a critical early signal to induce Bcl6 and CXCR5, contributing to the generation of memory B cells by GCs.

Furthermore, Bcl6 is regulated by a complex signaling circuitry in $T_{FH}$ differentiation, which may exert anti- or pro-inflammatory activities depending on cell types and/or stimuli [33–35]. Accordingly, with the high levels of *IFN-γ*, notably by the rgD5ISA group (Figure 5E, dark grey column), the Bcl6 transcription was substantially elevated (Figure 6A, dark grey column), indicating that IFN-γ is required for Bcl6 upregulation and subsequent Bcl6 control of the $T_{FH}$ differentiation program. Besides IFN-γ, IL-2 is an additional cytokine produced by Th1 cells, which stimulates both Th1 cells and cytotoxic T lymphocytes. *IFN-γ* induces expression of the *IL2* (Figure 5A, dark grey column) that does not directly inhibit GC formation, but instead suppressed the differentiation of $T_{FH}$ cells [36]. The formation of $T_{FH}$ cells since IL2 signaling induces STAT-5 phosphorylation and expression of BLIMP-1, a mutual repressor of Bcl6 and CXCR5 [37,38]. Nonetheless, after cell restimulation, we observed an upregulation of both *IFN-γ* and *IL2* cytokine levels by the g rgD5ISA vaccine group, compared to controls (Figure 5A and 5E, dark grey columns). However, we found high levels of *CXCR5* mRNA transcription in the same rgD5ISA group (Figure 6B, dark grey column). Under the CXCR5 signaling circuitry in $T_{FH}$ differentiation, beyond the high levels of CXCR5, we found the *IL4* mRNA transcription upregulated (Figure 5B, dark grey column), suggesting that the rgD5ISA vaccine by a complicated signaling circuitry allows $T_{FH}$ cells migration into the GCs [39]. The induced-up modulation of *IL2* by rgD5 stimulation suggests an enhanced T-cell response to rgD5 (Figure 5A) and is important for the generation of fully functional memory $CD8^+$ T cells [40,41]. However, upregulation levels observed for *IL4* and *IL10* (Figure 5B-C, light grey columns) may have played a role in the downmodulation of IgG2 (Figure 3, light grey column), since these cytokines have an antagonist effect on IFN-γ [42]. Noteworthy, IFN-γ induction always precedes IL10 secretion, even in high IL2 conditions. IL2 and IL15 have several similar functions with both cytokines stimulating the proliferation of T cells; inducing the generation of cytotoxic T lymphocytes and facilitating the proliferation of B cells [43,44]. Here, we found a similar mRNA transcript level of *IL15* (Figure 4D). In addition, IL15 also supported robust $CD8^+$ T cell-mediated long-term immunity [45,46]. These data suggest that the IL2 signalization during $CD4^+$ and $CD8^+$ T cell activation/expansion as well as the role of IL15 in the generation and maintenance of memory T cells is necessary to induce long-lasting cellular immunity [45,47].

As suggested in Figure 3, the bias of the immune response showed after the first vaccine administration is Th2 [9,10], however, after subsequent vaccination, an increase in the levels of IgG2 indicated a trend toward a mixed Th1/Th2 immune response. However, in our previous study, we do not observe upregulation of the *IL4* in *female* outbred Swiss albino mice splenocytes stimulated for 18 h with 10 μg of rgD5 [10]. Nevertheless, a similar effect toward a Th1 response using an oil-based adjuvant was reported [48,49], when a TLR ligand agonist was used. Except for TLR-9, significant upregulation of all TLRs has been detected following primary infection of neural tissues by both BoHV-1 and -5 herpesviruses, but the role of gD in this mechanism is not totally known [50]. Here, we showed that the IgG kinetics for the rgD5ISA and rgD5iBoHV5ISA groups were similar, with the last group responding with higher IgG levels after the first vaccine boost and keeping superior to the other groups until the end of the experiment on day 367 (Figure2, dark and light grey bars).

Cytokines like IL4 and IFN-γ have been shown to regulate Ig class switching in cattle by positively or negatively regulating germline transcription via various modes of B cell activation and cytokine combinations. The IgG antibody subclass distribution elicited after vaccination is also indicative of the type of immune response, as the IgG1 subclass is believed to signal a Th2 response whilst the IgG2 subclass indicates more of a Th1 profile [51,52]. We found a significant increase in *IFN-γ* mRNA transcription in cattle vaccinated

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only with the rgD5 antigen (Figure 5E, dark grey column), characterizing a Th1-type cell response, more favorable for viral infection control with the IgG2 participating in opsonization of the pathogen to facilitate phagocytosis [53]. The presence of IL4 facilitates antigen presentation through increased expression of major histocompatibility complex (MHC) II molecules and proliferation of T lymphocytes, in addition to inducing exchange of the IgG isotype to IgG1 [54,55]. The crosstalk between B cells and $T_{FH}$ cells leads to class switch recombination and affinity maturation in GC B cells with underlying potent nAb formation in persistent viral infection [13,56]. The virus neutralization results are quite significant since vaccine protection can be evaluated by the nAbs induced against the BoHV-1 and -5 (Figure 4A-B) but, the precise level of antibody required has been difficult to establish. Consistently studies found nAbs titers in the range of 1:4 to 1:8 to be associated with protection. However, titers of vaccine-induced neutralizing antibodies associated with protection have varied widely [21,57]. Here, 100% of animals vaccinated with rgD5 had nAbs significantly higher ($p < 0.05$) than 1:8 for BoHV-1 and -5 on the 33$^{rd}$ day of the experiment, suggesting that a vaccine for BoHV-5 might protect against both alphaherpesviruses (Figure 4A-B). These findings are quite interesting, even with a similarity of ~80% of identity between BoHV-1 and -5 genomes and similar biological properties between the two gD glycoproteins [58,59] the anti-rgD5 antibodies were able to recognize and neutralize the BoHV-1. Interestingly, nAbs, IgG2 isotype, and high levels of *IFN-γ* were found in mice vaccinated with recombinant vaccine formulated with rgD5 [10], demonstrating the immunogenicity and protective capacity of this recombinant vaccine. The application of Montanide ISA adjuvant, a W:O emulsion adjuvant carrier, associated with the rgD5 (rgD5ISA) increased the mRNA transcription of *IFN-γ* in cells stimulated by rgD5 (Figure 5E, dark grey column). Indeed, the Montanide ISA 50 V2 adjuvant has an immune stimulatory effect and enhances antigen-specific antibody titers and cytotoxic T lymphocyte responses [9,48,60]. The present study complements previous reports from our laboratory and from others, which have investigated immune responses at the onset of subunit vaccines on the family Alphaherpesviridae infection. During the past decade, a series of elegant studies have investigated the contribution of rgD5 as an interesting vaccine antigen and diagnostic tool [8–10,18,19,25,61–63]. We are continuing our efforts for this decade, expanding our strategies to increase the knowledge about vaccines and their potential application for the development of vaccine candidates and diagnostic techniques based on the conserved epitopes on gD or in combination with those of other herpesvirus glycoproteins, including test new adjuvants, combined or not with immunostimulators molecules, capable of improving the vaccine response effect.

**Supplementary Materials:** The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: Th1, Th2, and Treg cytokine transcription; Table S2: Bcl6 and CXCR5 transcription underlie the germinal center formation.

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