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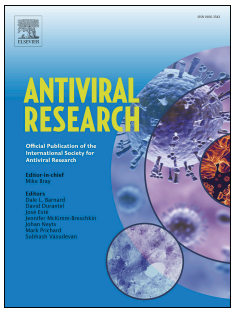

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Immunomodulatory effect of baculovirus in chickens: how it modifies the immune response against Infectious Bursal Disease Virus

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Abstract

Several reports have shown that baculoviruses (BVs) have strong adjuvant properties on the mammalian immune system. Recent studies of our group demonstrated the ability of BV to stimulate the innate immunity in chickens. In this investigation, we aimed to assess the potential antiviral effect of BV given both, before and after infectious bursal disease virus (IBDV). In the first case, specific pathogen free chickens were intravenously inoculated with $5x10^7$ pfu of *Autographa californica nuclear polyhedrosis virus* and three hours later were orally administered $2.5x10^5$ egg infectious doses $_{50}$ of IBDV. In the second case, chickens received IBDV three hours before BV inoculation. Five days later, chickens were bled and euthanized. RNA from the bursa was analyzed for cytokine production. Also, bursae were used for virus recovery, and processed for lymphocyte isolation. The results showed that the administration of BV 3h after the inoculation with IBDV produced important changes in the effect that IBDV causes in the bursa. BV reduced the infiltration of T lymphocytes, decreased the expression pattern of IL-6 and IFN-γ and inhibited IBDV replication. The results herein presented demonstrate that this Lepidopteran virus shows antiviral activity in chickens under experimental conditions. Investigations under field conditions have to be done to probe this strategy as a valuable sanitary tool for the treatment and prevention of chicken diseases.

Keywords: avian innate immunity, baculovirus, infectious bursal disease virus

Poultry has become the most consumed meat worldwide; and thus, the sanitary condition of chickens is relevant. Although chicken viral diseases are normally prevented by vaccination, chickens may be exposed to viral pathogens before a vaccine induces complete protection. Therefore, alternative strategies to reduce the chance of infection are essential.

The innate immune system constitutes the first line of defense against pathogens and is crucial against viral infections. Hence, the stimulation of early innate defense mechanisms could contribute to early immunity.

Baculoviruses (BVs) infect insects and have strong adjuvant properties in animals. BV *Autographa californica nuclear polyhedrosis virus* (AcNPV) activates early innate immune responses in mice, with induction of inflammatory cytokines and type I and II interferon (IFN) (Tjia et al., 1983). After 24 hours these cytokines return to basal levels (Abe et al., 2003; Kitajima et al., 2006; Kitajima and Takaku, 2008). Adjuvant properties are primarily mediated by IFN-α and IFN-β, although mechanisms independent of type I IFN signaling are also involved (Hervas-Stubbs et al., 2007).

BVs were also studied in avian cells and chickens. BV enhances inflammatory cytokines in macrophages and peripheral blood mononuclear cells (Niu et al., 2008). Niu and coworkers demonstrated protection of neonatal chickens against Infectious Bronchitis Virus. Also, we proved that the inoculation of BV in chickens produced a strong pro-inflammatory immune response and modifications in mononuclear cell patterns in different organs; and as in mammals, within few hours all cytokines returned to basal levels (Chimeno Zoth et al., 2012).

Infectious bursal disease virus (IBDV) is an endemic agent in poultry. IBDV causes a highly contagious immunosuppressive disease in chickens (Eterradossi and Saif, 2008), that destroys dividing IgM bearing B-lymphocytes in the bursa of Fabricius.

To investigate if BV can prevent or treat IBDV infection in chickens, we inoculated birds with AcNPV before or after IBDV administration. Animal experiments were approved by the Institutional Committee for the care and use of experimental animals. Specific-pathogen-free White Leghorn chickens (embryonated eggs from Rosenbusch S.A., Argentina) were divided in 5 groups of 6 animals each one and were inoculated as described in Table 1. The experiment was performed twice and results from experiments 1 and 2 are presented. The prophylactic effect analysis of BV on IBDV infection consisted of a treatment with BV followed by IBDV administration [Laboratorios Inmuner (Entre Ríos, Argentina)] 3 hours later (Table 1, G4). Simultaneously, the post inoculation effect analysis consisted of IBDV administration and BV inoculation 3 hours later (Table 1, G5). The time interval between BV and IBDV inoculation was chosen based on previous studies which demonstrated that 3 hours was the optimal time for BV to induce an antiviral state (Chimeno Zoth et al., 2012). AcNPV was produced as previously described (Chimeno Zoth et al., 2012) and the titre was calculated by an end-point dilution assay and converted to plaque forming units (pfu)/ml (O'Reilly et al., 1994). Each bird received BV (500 µl, $5x10^7$ pfu) in the wing vein and/or IBDV [$2.5x10^5$ egg infectious $dose_{50}$ (EID_{50}) of LZD (Delvax Gumboro LZD) intermediate strain from Laboratorios Immuner, Argentina] by the oral route. Both viruses' doses were determined in previous studies (Chimeno Zoth et al., 2012, Gómez et al., 2013). Five days after IBDV inoculation, animals were euthanized. Pieces (30 mg) of each bursa were placed in RNAlater solution (QIAGEN, Hilden, Germany) and stored up to 30 days at 4ºC, until RNA extraction. Remaining bursae were processed for lymphocyte and virus isolation.

RNA was obtained with RNeasy kit (QIAGEN, Hilden, Germany) and treated with DNase I. Reverse transcription was performed using SSIII Reverse transcription kit

(Invitrogen, Carlsbad, CA). Oligonucleotides to amplify regions of cytokines and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, control) genes were previously described (Chimeno Zoth et al., 2012). Amplifications and detections were performed as previously described (Haghighi et al., 2008, Carballeda et al., 2011). Quantification was performed with SYBR®Green Master Mix Kit (Applied Biosystems, Warrington, UK). Cycle threshold (CT) values were used to plot a standard curve. Sample CTs were extrapolated from the standard curve to determine the initial amount of cytokines and GAPDH mRNA. IFN-γ, interleukin (IL)-6, IFN-α and IL-8 mRNA of each individual sample was normalized with the own GAPDH measure. In the control group (G1) the same procedure was applied and, after that, the mean value of each cytokine was calculated. For the other groups (G2, G3, G4 and G5) each individual value of each cytokine (already normalized to its own GAPDH value, as mentioned before) was normalized to the corresponding G1 mean cytokine value. These values were compared between treatments by ANOVA and Bonferroni´s test (GraphPad Prism 5, $p<0.05$ as statistically significant) and their means are showed in Figure 1. Results from Experiment 1 (Fig. 1A) revealed that, in general, cytokine mRNA levels increased in G2 because of IBDV inoculation. Particularly, IFN-γ and IL-6 mRNA expression was upregulated in G2 ($p<0.001$). IL-8 mRNA levels were also higher in G2 ($p<0.05$). IBDV inoculation did not seem to affect IFN-α mRNA levels, nor was IFN-α mRNA expression altered in any of the BV treated groups at 5 dpi. Results obtained in Experiment 2 showed similar results (Fig. 1B).

We also evaluated the effect on immune cell frequency in bursa. Bursae were cut in small pieces and mechanically disrupted. Cellular suspensions were passed through a mesh (Cell Strainer, BD) and mononuclear cells were isolated by centrifugation over Histopaque density gradients (1.077 g/ml; Sigma, St. Louis, MO). Cells were isolated

from the interface and washed. For flow cytometry analysis, cells were resuspended in Staining Buffer [phosphate saline buffer (PBS) 1x, 10 % fetal bovine serum (FBS), 0.1 % Sodium Azide] and $1x10^6$ cells/well were seeded on 96-well plates (V-shape). Staining was performed using different antibody combinations [Monoclonal antibodies: CD3-SPRD, CD4-PE, CD8α-FITC, CD8β-PE and Bu1-PE (Southern Biotech, Birmingham, AL)], as previously described (Chimeno Zoth et al., 2012). Positive cells were analyzed [FACS Calibur flow cytometer (BD Biosciences, San Jose, CA) and CellQuest software]. Each sample value was normalized to the mean value of G1 group and expressed as the fold change (Fig. 2). Mean values of each group were calculated and compared by Student´s t test ($p< 0.05$, as statistically significant). Results from Experiment 1 showed that, as previously described (Carballeda et al., 2011; Kim et al., 2000), IBDV inoculation of chickens induced T-lymphocyte infiltration in bursae at 5 dpi (days post infection), together with a decrease in Bu1+ cells (G2, Fig. 2A). Indeed, the proportion of $CD4^+$ and $CD8\alpha\beta^+$ cells in G2 was significantly higher than in G3 and G5, but not than in G4. Conversely, G3 showed no T-cell infiltration and B-cell proportions (Bu1+ cells) remained unaltered compared with G1. Regarding BV treatment, flow cytometry results demonstrated the attenuation of T-cell infiltration induced by IBDV. When BV was given before IBDV, 3 out of 6 animals maintained normal values of T-cells in bursae, giving values similar to G1 and G3. On the other hand, the remaining 3 animals showed a marked increase of T-cells, mainly $CD8\alpha\beta^+$. Altogether, $CD8\alpha\beta^+$ and $CD4^+$ levels from BV/IBDV treatment were similar to those of IBDV alone. When given after IBDV (G5), the effect of BV resulted more homogeneous and indistinguishable from the results with BV alone: no increase of T-cell populations and significantly fewer infiltrated T-cells than in G2. Thus, BV

administration 3h after IBDV inoculation diminishes the effects of IBDV infection, regarding the infiltration of T-cells in bursae. Similarly, G5 bursae showed no changes in B-cell proportion. Similar results were observed in Experiment 2 (Fig. 2B). BV effect was exclusively observed 3 hours after IBDV administration. Other times (16, 24, 72 h) were evaluated but BV could not avoid IBDV-induced infiltration (not shown). Finally, to evaluate BV antiviral activity, we performed viral isolation assays. Bursae were mechanically disrupted in PBS and 3 freeze/thaw cycles were performed to recover IBDV. Homogenates were ten-fold serially diluted and the dilutions were used to infect monolayers of chicken embryo fibroblasts. After 4 days at 37 °C, cytopathic effect was evaluated. Viral titres were expressed as tissue culture infectious dose 50 ($TCID_{50}$)/ml (Reed and Muench, 1938). As expected, IBDV was undetectable in bursae from G1 and G3, whereas bursae from G2 showed high IBDV titres in both experiments (Table 2). We could determine the viral titre on 3 out of the 6 samples of G4; in this case the results are inconclusive as only one of the three samples analyzed showed a reduced viral load. When bursae from Experiment 2 were analyzed, 3 out of 6 samples showed a reduction in viral load and the other 3 revealed high viral load. On the hand, results were more determinative in G5 of both experiments. Most animals (4/6 in Exp. 1 and 3/6 in Exp. 2) revealed a marked decrease in viral load titres, whereas the remaining animals had undetectable IBDV (Table 2). In every case, the level of histological damage was in accordance with the viral load (data not shown).

Altogether, the analysis of the cytokine profile, the presence of T cells in bursae, and the viral recovered from bursae after infection revealed a strong antiviral effect of BV when given after IBDV (G5).

Defense against viral infections in poultry consists of innate and adaptive mechanisms (Jeurissen et al., 2000). The innate immune system provides an important

initial response to pathogens, which can limit or prevent infection. The innate defense is mainly given by natural killer cells, granulocytes and macrophages and secreted products (e. g. nitric oxide and cytokines) (Jeurissen et al., 2000). Our group previously demonstrated that BV can stimulate chicken innate immunity *in vivo* by modifying the profile of immune cells (Chimeno Zoth et al., 2012). Furthermore, several reports described its antiviral activity in mice. Gronowski et al. (1999) demonstrated that BV protects mice against encephalomyocarditis virus and Abe et al. demonstrated its protection against influenza H1N1 (Abe et al., 2003). In addition, Molinari et al. (2010) demonstrated BV protection against foot and mouth disease virus (FMDV).

This study reports, for the first time, BV antiviral effect against IBDV in chickens. We showed that BV, administered after IBDV inoculation, produced important changes in the effect that IBDV causes in the bursa, reducing its ability to replicate in this organ.

Although further investigations to explore the applicability of BV under field conditions are needed, results obtained in the present work indicate that this Lepidopteran virus has antiviral activity in chickens under experimental conditions. Some of the issues that would be interesting to address are the evaluation of more suitable BV inoculation routes and the effect of BV on IBDV infection at different time points.

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Conflict of interest statement

We disclose any financial and personal relationship with other people or organization that could inappropriately influence our work.

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Figure Captions

Figure 1: Transcriptional pattern of cytokine genes in the bursa at 5 dpi. Total RNA was extracted from bursae of chickens from G1 (negative control), G2 (IBDV), G3 (BV), G4 (BV/IBDV) and G5 (IBDV/ BV) and cDNA was synthesized in both experiments: A: Experiment 1, B: Experiment 2. mRNA levels were determined by Quantitative Real Time PCR using specific primers and SYBR®Green method. Each mRNA (IL-6, IFN-γ, IL-8 and IFN-α) expression level was calculated in relation to GAPDH expression level. Each bar represents the mean value of the fold change of each group compared to G1 mean (negative control) ± SE. (*) indicates significant differences between treatments ($p<0.001$ for IFN-γ and IL-6 and $p<0.05$ for IL-8).

Figure 2: Evaluation of mononuclear cell populations by flow cytometry. Chicken leukocytes were isolated from bursa of chickens from G1 (negative control), G2 (IBDV), G3 (BV), G4 (BV/IBDV) and G5 (IBDV/ BV) of both experiments (A: Experiment 1, B: Experiment 2). Cells were stained with different combinations of antibodies and analyzed by flow cytometry. The gating strategy consisted of the location of the lymphocytes in a forward/side scatter-defined gate and 30,000 events were analyzed for sample. Results are expressed as the mean value of individual fold changes (obtained in comparison to G1 mean) of each group ± SE. [a, b] Different letters represent significant differences between treatments ($p<0.05$).

Group	Treatment	
	(T: 0h)	(T: 3h)
G1	Sf9 culture medium	PBS
G2	Sf9 culture medium	IBDV $2.5x10^5$ EID_{50}
G3	AcNPV $5x10^7$ pfu	PBS
G4	AcNPV $5x10^7$ pfu	IBDV $2.5x10^5$ EID_{50}
G5	IBDV $2.5x10^5$ EID_{50}	AcNPV $5x10^7$ pfu

Table 1: Experimental design. Thirty 4 week-old specific-pathogen-free White Leghorn chickens were divided in five groups of six animals each, and inoculated with BV (AcNPV $5x10^7$ pfu) and/or IBDV ($2.5x10^5$ EID_{50}), or with the corresponding controls (Sf9 culture medium or PBS).

	Experiment 1		Experiment 2	
Group	**Sample**	**Viral Titre ($TCID_{50}$/ml)**	**Sample**	**Viral Titre ($TCID_{50}$/ml)**
G1 (Negative)	**1.1**	**Neg.**	**1.1**	**Neg.**
	1.2	**Neg.**	**1.2**	**Neg.**
	1.3	**Neg.**	**1.3**	**Neg.**
	1.4	**Neg.**	**1.4**	**Neg.**
	1.5	**Neg.**	**1.5**	**Neg.**
	1.6	**Neg.**	**1.6**	**Neg.**
G2 (IBDV)	**2.1**	**> 1.26×10^9**	**2.1**	**> 1.26×10^9**
	2.2	**> 1.26×10^9**	**2.2**	**> 1.26×10^9**
	2.3	**> 1.26×10^9**	**2.3**	**> 1.26×10^9**
	2.4	**> 1.26×10^9**	**2.4**	**> 1.26×10^9**
	2.5	**> 1.26×10^9**	**2.5**	**> 1.26×10^9**
	2.6	**> 1.26×10^9**	**2.6**	**> 1.26×10^9**
G3 (BV)	**3.1**	**Neg.**	**3.1**	**Neg.**
	3.2	**Neg.**	**3.2**	**Neg.**
	3.3	**Neg.**	**3.3**	**Neg.**
	3.4	**Neg.**	**3.4**	**Neg.**
	3.5	**Neg.**	**3.5**	**Neg.**
	3.6	**Neg.**	**3.6**	**Neg.**
G4 (BV/IBDV)	**4.1**	**5.76×10^6**	**4.1**	**1.83×10^5**
	4.2	**> 1.26×10^9**	**4.2**	**1.26×10^5**
	4.3	**> 1.26×10^9**	**4.3**	**5.76×10^6**
	4.4	**Not done**	**4.4**	**>1.26×10^9**
	4.5	**Not done**	**4.5**	**>1.26×10^9**
	4.6	**Not done**	**4.6**	**>1.26×10^9**
G5 (IBDV/BV)	**5.1**	**1.26×10^4**	**5.1**	**Neg.**
	5.2	**8.55×10^3**	**5.2**	**5.76×10^3**
	5.3	**1.26×10^4**	**5.3**	**Neg.**
	5.4	**Neg.**	**5.4**	**Neg.**
	5.5	**1.26×10^5**	**5.5**	**6.79×10^4**
	5.6	**Neg.**	**5.6**	**3.16×10^4**

Table 2: Viral isolation from chicken bursae. Pieces of bursae from chickens from G1 (negative control), G2 (IBDV), G3 (BV), G4 (BV/IBDV) and G5 (IBDV/ BV) were mechanically disrupted in PBS and 3 frost/thaw cycles were performed. Homogenates were ten-fold serially diluted and the dilutions were used to infect monolayers of chicken embryo fibroblasts seeded in 96-well plates. After 4 days at 37 °C, the presence of cytopathic effect was evaluated in each well. Viral titres obtained in Experiment 1 and 2 were expressed as $TCID_{50}$/ml, using the Reed and Muench method. Neg.: Negative result corresponds to a viral titre lower than 1.26×10^3 $TCID_{50}$/ml.

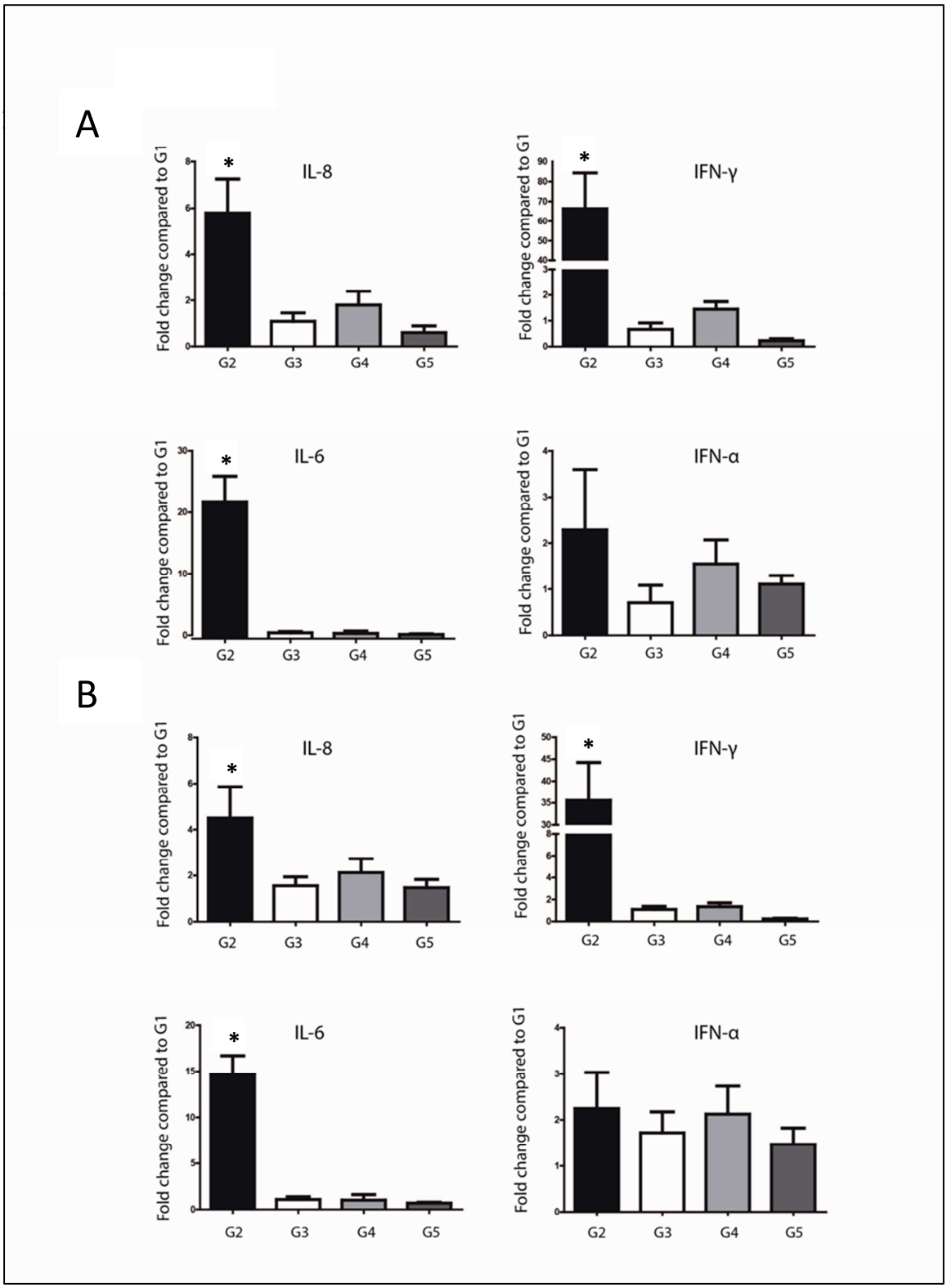
A
IL-8
IFN-γ
IL-6
IFN-α
B
IL-8
IFN-γ
IL-6
IFN-α
Fold change compared to G1
G2
G3
G4
G5
*

Figure 1

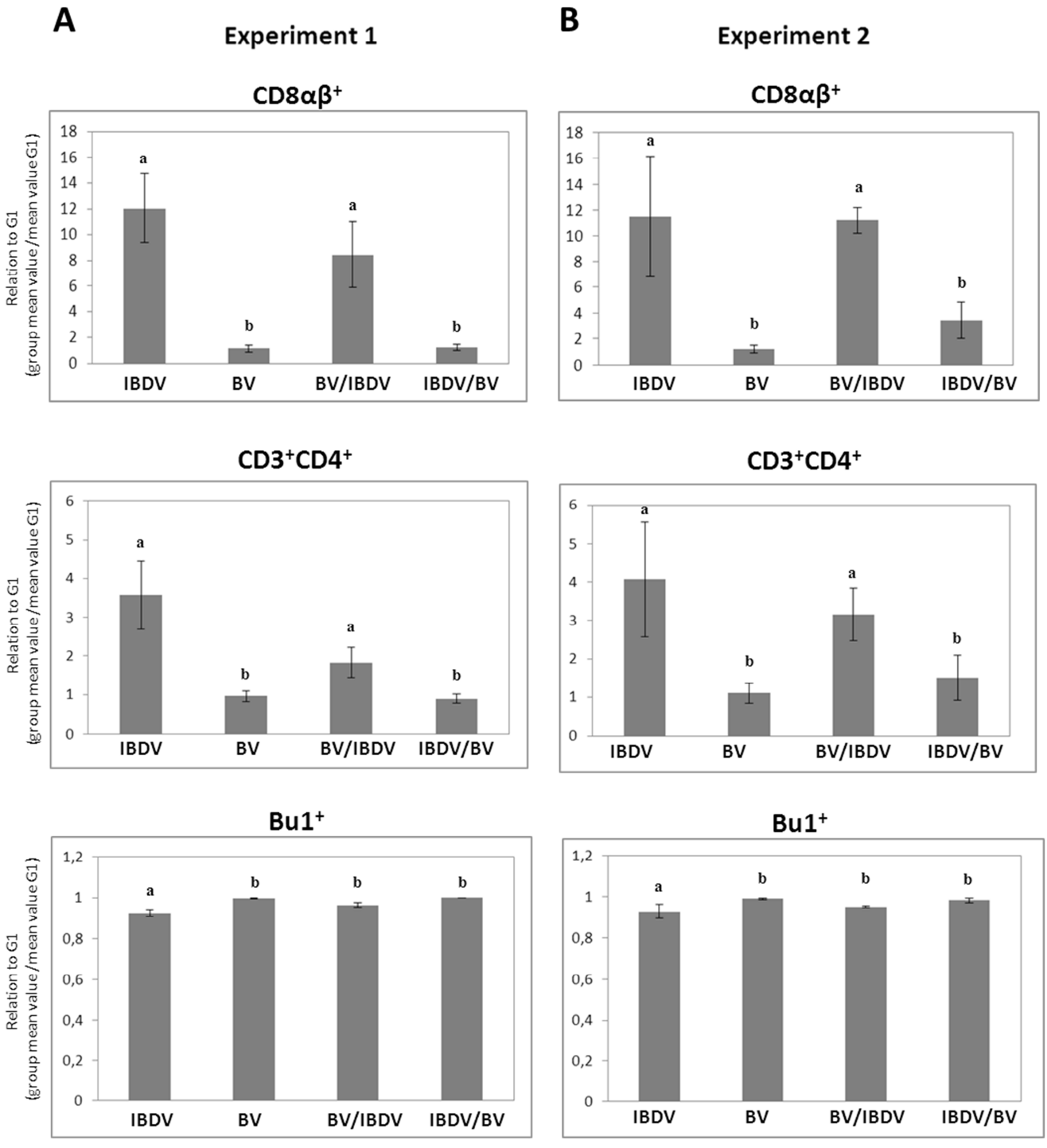
A
Experiment 1
B
Experiment 2
CD8αβ+
CD3+CD4+
Bu1+
Relation to G1
(group mean value /mean value G1)
IBDV
BV
BV/IBDV
IBDV/BV
a
b

Figure 2

The antiviral effect of BV was evidenced by its ability to inhibit IBDV replication

BV administration after IBDV inoculation diminishes infiltration of T-cells in bursa

With the therapeutic approach the effect of BV resulted more homogeneous in birds