Alteration of immunological parameters in infectious bronchitis vaccinated–specific pathogen-free broilers after the use of different infectious bursal disease vaccines

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ABSTRACT The vaccines currently available to control infectious bursal disease (**IBD**) include live-attenuated and inactivated vaccines, immune-complex vaccines, and vaccines consisting of viral constructs of herpesvirus of turkeys genetically engineered to express VP2 surface protein. To evaluate the impact of vaccines on the chicken immune system, 2 animal trials were performed in specific pathogen-free broiler chickens. In trial 1, birds were either vaccinated when they are one-day old with a dual recombinant herpes virus of turkey construct vaccine, expressing VP2 protein of (**IBDV**) and F protein of Newcastle disease virus, or an immune-complex IBDV vaccine or birds were not vaccinated. At 14, 28, and 35 D, the bursa of Fabricius was collected for bursa:-body weight (**B:BW**) ratio calculation. In trial 2, birds were vaccinated when they were 1-day old according to the same protocol as trial 1, but at day 14, all groups also received a live infectious bronchitis (**IB**) vaccine. At 0, 7, 14, 21, and 28 days after IB vaccination, birds were tested by ELISA for IB serology and, soon after the last blood sampling, they were euthanized for collection of Harderian glands, trachea, and spleen and testing by flow cytometry for characterization of mononuclear cells. The immune-complex vaccine groups showed significantly lower B:BW ratio, lower IBV antibody titers, and higher mean percentage of CD8+ T cells in the spleen, trachea, and Harderian glands than those in the other experimental groups. The results of the in vivo trials coupled with a depth analysis of the repertoire of parameters involved in the immune response to IBD and IB vaccinations show one vaccine may influence the immune response of other vaccines included in the vaccination program.

Key words: avian coronavirus, vectored vaccine, immune-complex vaccine, infectious bursal disease virus

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INTRODUCTION

Infectious bursal disease virus (**IBDV**) belongs to the family *Birnaviridae*, within the genus *Avibirnavirus*. Two serotypes of IBDV are known: serotype 1, further categorized into classical, attenuated, very virulent, and antigenic variant strains, and serotype 2, including only avirulent strains. New genetic lineages of IBDV continuously emerge worldwide (Michel and Jackwood, 2017) and possess different antigenic and pathogenic characteristics (Lupini et al., 2016; Felice et al., 2017; Fan et al., 2019). Independent of the pathogenicity of the strain involved and the severity of caused clinical signs, IBDV infection in chickens is always associated with damage to the bursa of Fabricius and immunosuppression (Sharma et al., 2000; Rautenschlein et al., 2003; Silveira et al., 2019; Lupini et al., 2020) which is more severe if the infection occurs during the first 3 wk of age in birds with low levels of maternally derived antibodies.

Infectious bursal disease (**IBD**) has a relevant economic impact on poultry production around the world because of the immunosuppression caused in chickens leading to an increased susceptibility to secondary infections and a failure of the vaccination programs applied (Aricibasi et al., 2010; Prandini et al., 2016). As IBDV is a nonenveloped virus, very resistant to commonly used disinfectants, biosafety measures applied in poultry farms are normally not sufficient

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to prevent persistence of the virus in the environment. Therefore, IBD control relies mostly on vaccination. The vaccines currently commercially available include traditional live-attenuated and inactivated vaccines; immune-complex vaccines, which contains a combination of live-attenuated IBDV and specific antibody; and vaccines consisting of viral constructs of herpesvirus of turkeys (**HVT)** genetically engineered to express the IBDV surface protein VP2 (Michel and Jackwood, 2017). Live virus strains included in the formulation of certain IBD vaccines, although attenuated, are reported to be able to induce in vaccinated birds macroscopic and microscopic changes of the bursa of Fabricius similar to those caused by pathogenic viruses (Killian et al., 2017). The death of lymphocytes caused by the virus, also through apoptotic phenomena, can lead to the risk that the vaccine virus itself causes immunosuppression.

Infectious bronchitis (**IB**) is a highly contagious viral respiratory disease of chickens caused by an *avian coronavirus* (**IBV**) characterized by high substitution and recombination rates, which have led to the emergence of several genotypes and lineages over time (Franzo et al., 2015; Listorti et al., 2017; Torres et al., 2017; Fraga et al., 2018; Jakhesara et al., 2018; Ma et al., 2019). The disease is widely controlled by the application of multifarious vaccination protocols (Franzo et al., 2014) mainly based on live vaccines, which stimulate systemic and upper respiratory tract immunity (either humoral or cell-mediated). Immunity conferred by IBV vaccines can be impaired by immunodeficiency caused by viral agents such as IBDV (Toro et al., 2006).

The aim of this project was to investigate, through 2 animal trials, the impact of the administration of a dual recombinant herpes virus of turkey (**rHVT**) construct vaccine, expressing VP2 protein of IBDV and F protein of Newcastle disease virus, or an immune-complex IBDV vaccine on the immune system of day-old specific pathogen free (**SPF**) broiler chickens. In particular, the integrity of bursa of Fabricius (trial 1) and the immune response of the vaccinated birds to different IBDV vaccines and live IBV vaccines, through serology and flow cytometry (**FMC**) analysis of mononuclear cells in the Harderian glands, trachea, and spleen (trial 2), were evaluated.

MATERIALS AND METHODS

Birds

SPF broiler chicken eggs (Hybro genetic) were supplied by GD Animal Health (Deventer, the Netherlands) and hatched in the animal facility of the Avian Pathology Service of the Department of Veterinary Medical Sciences (University of Bologna). One-hundred SPF chicks were used and housed in pens or isolators for the duration of the study. Food and water were provided *ad libitum*.

Vaccines

The following commercially available vaccines were administered in accordance to the guidelines of the manufacturers: a dual rHVT construct vaccine (designated **rHVT-ND-IBD**) which contains IBDV-VP2 and NDV-F genes (Innovax-ND-IBD, MSD Animal Health); an immune-complex IBDV vaccine (designated **IMMUNE-IBDV**) consisting a live intermediate plus IBDV strain (2512-IBDV) conjugated with hyperimmune serum against IBDV (Cevac Transmune, Ceva Salute Animale); a cell-associated HVT-FC126 strain (designated HVT) (Nobilis Marexine CA 126, MSD Animal Health); an IBV live vaccine based on the MA5 strain (Nobilis IB Ma5, MSD Animal Health); and an IBV live vaccine based on the 4/91 strain (Nobilis IB 4-91, MSD Animal Health).

Trial 1

Experimental plan Sixty male birds were randomly divided in groups, housed in pens, and vaccinated as reported in Table 1. Briefly, chickens were vaccinated via the subcutaneous route at 1 D of age with rHVT-ND-IBD (designated rHVT-ND-IBD group, n = 24 birds), IBD immune complex vaccine and HVT vaccine (designated IMMUNE-IBDV group, n = 24 birds), or vaccinated with HVT vaccine and not vaccinated for IBDV (designated control group, n = 12 birds). From each of the groups rHVT-ND-IBD and IMMUNE-IBDV, 14, 5, and 5 birds, were euthanized, at 14, 28, and 35 D of age, respectively, weighed and subjected to postmortem examination. Bursa of Fabricius of the birds were collected and weighed for the subsequent calculation of the bursa:body weight (**B:BW**) ratios. From the control group, 4 birds for each sampling date were analogously processed.

Bursa:body weight ratio B:BW ratios were calculated according to the following formula: B:BW ratio = [bursa weight (g)/body weight (g)] × 1,000.

Trial 2

Experimental plan Forty female birds were randomly divided in groups, housed in poultry isolators, and vaccinated as reported in Table 1. Briefly, chickens were inoculated at one day of age via subcutaneous route with rHVT-ND-IBD (designated rHVT-ND-IBD group, n = 17 birds), with IBD immune-complex and HVT vaccines (designated IMMUNE-IBDV group, n = 17 birds), or not IBDV vaccinated (designated control group, n = 6 birds). At 14 D of life, all the experimental groups were eye-drop vaccinated for IBV with MA5 and 4/91 vaccines. At 0, 7, 14, 21, and 28 D after IBV vaccination, birds were bled, and their sera were tested for IBV antibody. At 28 D after IBV vaccination, birds were euthanized and necropsied for collection of Harderian glands, trachea, and spleen (0.3 g ±10%). Tissues were individually stored in MACSTissue Storage Solution (Miltenyi Biotec, Bergisch Gladbach, Germany)

Table 1. Vaccination plans applied to birds in trial 1 and trial 2.

Groups	No. of birds	Vaccinations	
		At 1-day-old	At 14 D of life
Trial 1			
Control	12	HVT	Not done
rHVT-ND-IBD	24	rHVT-ND-IBD	Not done
IMMUNE-IBDV	24	IMMUNE-IBDV + HVT	Not done
Trial 2			
Control	6	HVT	IBV Ma5+4/91
rHVT-ND-IBD	17	rHVT-ND-IBD	IBV Ma5+4/91
IMMUNE-IBDV	17	IMMUNE-IBDV + HVT	IBV Ma5+4/91

at + 5°C, for a maximum of 24 h, until processing for mononuclear cells isolation and FMC analysis.

Serology Anti-IBV antibody titers were determined in serum samples using a commercial kit (IDEXX IBV Ab Test-IDEXX), following the manufacturer's instructions.

Mononuclear cells isolation To obtain mononuclear cells for subsequent FMC analysis, tissues were dissociated using the gentleMACSTMOcto Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany). Spleen samples and whole Harderian glands were first suspended in 5 mL of PBS and subsequently dissociated for 30 s (program m_spleen_01_01) and 28 s (program MultiA_01), respectively. Tracheas were cut into 2- to 3-mm slices, suspended in 8 mL of Collagenase type IV solution (RPMI-1640, 1% HEPES, 1% Penicillin-Streptomycin, 200 Collagen Digestion Units/mL; Sigma Aldrich, Hamburg, Germany), and then dissociated for 45 min at 37°C (program 37C_MULTIA_01). Dissociated cells were filtered (70-µm mesh) and centrifuged in PBS at 300 *g* for 10 min. The supernatants were discarded, and cell pellets were resuspended in 4 mL of PBS. Mononuclear cells were collected from interphase after density gradient centrifugation over Ficoll Paque Plus (GE Healthcare, Little Chalfont, UK) at 400 g for 30 min, washed twice, resuspended in 1 mL of PBS, and counted. For each subsequent FMC analysis, 10^6 cells were used.

Flow cytometry In the whole, FCM analyses were performed on mononuclear cells obtained by 40 spleens, 40 Harderian glands, and 40 tracheas. Mononuclear cells were stained with antichicken undiluted appropriate fluorochrome-conjugated antibodies, for 30 min at 4°C in the dark, against CD45 leukocytes (CD45-APC), CD8 cytotoxic T cells (CD8-FITC), CD4 T-helper cells (CD4-PE), and Bu1A B-cells (Bu1A-FITC) (Table 2) (Smialek et al., 2016). A multiparametric experiment was carried out as reported in Table 3. Unstained controls to evaluate inherent background or autofluorescence were obtained omitting primary antibodies but adding only Viability 405/452 Fixable Dye (VioBlue, Miltenyi Biotec, Bergisch Gladbach, Germany) used to distinguish viable and dead cells. To compensate for spectral overlap, single staining for each antibody and unstained cell samples was used. After incubation, cells were washed twice and resuspended in 200 µL of PBS then analyzed by using MacsQuant Analyzer10 (Miltenyi Biotec, Bergisch Gladbach, Germany) equipped with 3 lasers (405, 488, and 638 nm). This flowcytometer quickly performs absolute cell counting without the need for counting beads or cell staining. Data were analyzed using the Flowlogic software (Miltenyi Biotec, Bergisch Gladbach, Germany), and gates applied for populations discrimination were set manually based on control samples. A uniform hierarchical gating strategy was used throughout all samples. To start, cellular events were discriminated from debris using forward (**FSC-A**) and side scatter (**SSC-A**). Doublets have been excluded for analysis by FSC-area and height (FSC-A/FSC-H), and dead cells were kept out on the basis of gate set in samples stained with VioBlue. The single cells were further analyzed for expression of CD45 marker to select leukocyte from other events. CD8, CD4, and Bu1A positive cells were identified among CD45-positive population.

Statistical Analysis

Data were analyzed using R version 3.5.1 (2018-07-02) (2018 The R Foundation for Statistical Computing). Data are expressed as mean ± standard deviation; Shapiro Wilk test was used to test normality. One-way ANOVA and Tukey post hoc test were performed to assess the difference between groups. Significance was set at $P < 0.05$.

Table 2. Details of antichicken fluorochrome-conjugated antibodies used in the FMC analysis on mononuclear cells.

Name	Specificity	Clone	Fluorochrome	Stained cells	Supplier
CD45-APC	chCD45	LT40	APC	Leukocytes	Invitrogen
CD8-FITC	chCD8	11-39	FITC	Cytotoxic T cells	Biorad
CD4-PE	chCD4	2-35	PE	T-helper cells	Thermo Fisher Scientific
Bu-1A-FITC	Bu1	L22	FITC	B-cells	Invitrogen

Table 3. Multicolor panel design and pipetting scheme for staining—fluorochromes and filters details.

Sample	CD45-APC	CD8-FITC	CD4-PE	Bu1A-FITC	VioBlue	Exitation max/emission max (nm)	Filter (nm)
Control	/	/	/	/	10 µL	405/452	450/50 V1
Single color staining	0.5 µL	/	/	/	/	651/662	655-730 R1
Single color staining	/	0.5 µL	/	/	/	490/525	525/50 B1
Single color staining	/	/	0.5 µL	/	/	565/575	585/40 B2
Single color staining	/	/	/	0.5 µL	/	490/525	525/50 B1
Multicolor panel A	0.5 µL	0.5 µL	0.5 µL				
Multicolor panel B	0.5 µL	/	/	0.5 µL	/		

Ethical Statement

The experimental trials were performed in agreement with the European regulations on animal experiments and animal welfare (EU Directive 2010/63/EU) and after obtaining approval for the animal procedures from the Italian Ministry of Health (permit number 478/2016-PR).

RESULTS

Trial 1

B:BW ratio Mean B:BW ratios per experimental group and day of sampling are reported in Figure 1. At 14, 28, and 35 D after IBDV vaccination, the mean B:BW ratios of the group of birds vaccinated with the immune-complex vaccine (IMMUNE-IBDV group) were significantly lower than the mean B:BW ratios of the group of birds vaccinated with rHVT-ND-IBD vaccine (rHVT-ND-IBD group) and the group not IBDV vaccinated (control group) ($P < 0.05$).

Post-mortem examination At postmortem examination, birds of the IMMUNE-IBDV group have shown a macroscopically evident atrophy of the bursa of Fabricius from 14 D after IBDV vaccination until the end of the trial compared with the birds of the other experimental groups.

Trial 2

Serology ELISA anti-IBV antibody titers detected in experimental groups are graphically reported in Figure 2. From 7 D after IBV vaccination, anti-IBV antibodies have been detected in all groups with increasing titers up to the end of the trial. At 14 and 21 D after IBV vaccination, birds of the group IBDV vaccinated with immune-complex vaccine (IMMUNE-IBDV group) showed significantly lower mean IBV antibody titers than birds of the rHVT-ND-IBD group and of the not–IBDV vaccinated group (Control group) ($P < 0.05$). At 28 D after IBV vaccination, differences were statistically significant between groups rHVT-ND-IBD and IMMUNE-IBDV, showing the last group a lower mean antibody titer ($P < 0.05$).

Flow cytometry analysis At 28 D after IBV vaccination, mononuclear cells from the spleen, Harderian gland, and trachea of birds of all groups were analyzed by FMC using different staining panels (Table 3) and a uniform hierarchical gating strategy as shown in Figure 3. On average 144,234; 29,879; and 68,383 events were analyzed for spleens, Harderian glands, and tracheas, respectively. Percentage of leukocytes was

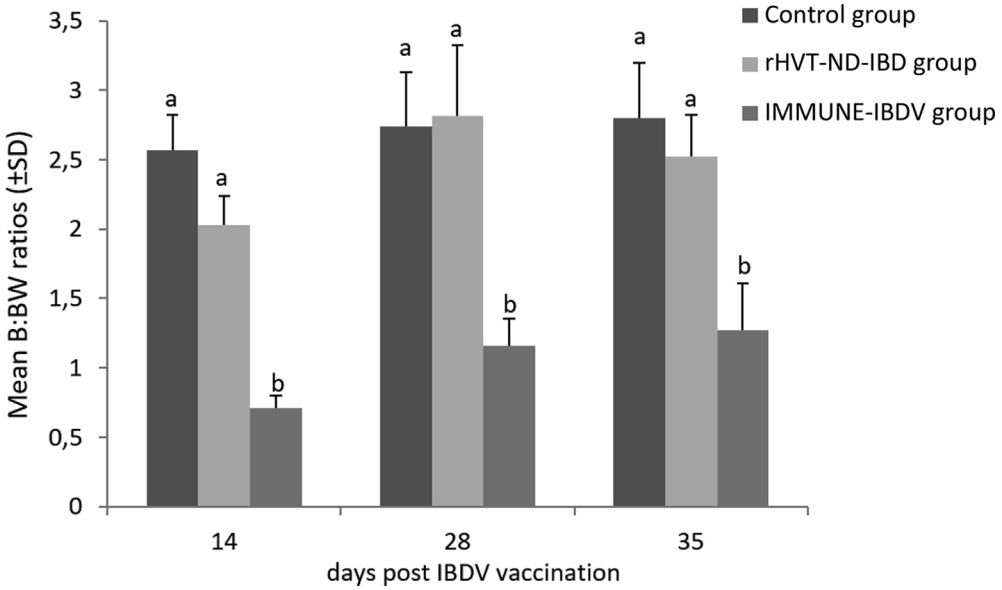

Figure 1. Bursa:body weight ratios analysis—trial 1. Mean bursa:body weight ratios (±standard deviations) of the not-IBDV vaccinated group (control group, n = 12), of the rHVT-ND-IBD vaccinated group (rHVT-ND-IBD group, n = 24 birds), and of the immune-complex vaccinated group (IMMUNE-IBDV group, n = 24 birds), by day of sampling. Different letters indicate the difference is statistically significant ($P < 0.05$).

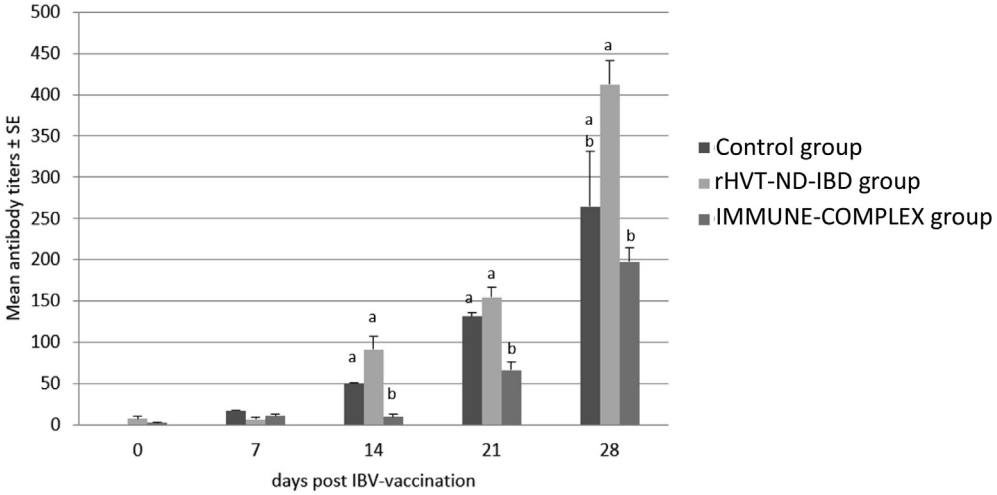

Figure 2. IBV Serology—trial 2. Mean IBV antibody titres (±standard errors) detected in the not-IBDV vaccinated group (control group, n = 6), in the rHVT-ND-IBD vaccinated group (rHVT-ND-IBD group, n = 17 birds), and in the immune-complex vaccinated group (IMMUNE-IBDV group, n = 17 birds), according to days post-IBV vaccination. Different letters indicate that the difference among groups at each experimental point is statistically significant ($P < 0.05$).

expressed as $CD45^+$/total cells (Figure 4A). CD8, CD4, and Bu1A positive cells were identified among CD45-positive population (expressed as percentage of CD4+/CD45+, CD8+/CD45+, and Bu-1A+/CD45+ cells) (Figures 4B, 5A and 5B).

Results showed that the mean relative number of Bu-1A and CD4 positive cells did not vary significantly between IBDV-vaccinated and not–IBDV vaccinated groups ($P > 0.05$) in all the examined tissues (Figures 3B and 4A). Conversely, statistically significant differences were observed between the groups in the mean relative number of CD8+ cells, which was higher in the IMMUNE-IBDV group, in spleen and trachea, than in the rHVT-ND-IBD group ($P < 0.05$), and also in the Harderian gland compared to the control group ($P < 0.05$) (Figure 4B). Moreover, the mean CD4+/CD8+ ratio was calculated and resulted to be significantly lower in the tracheas of the IMMUNE-IBDV group than in the tracheas of the control group ($P < 0.05$) (Figure 4C). No other statistically significant differences in the mean CD4+/CD8+ ratios were observed between groups ($P > 0.05$).

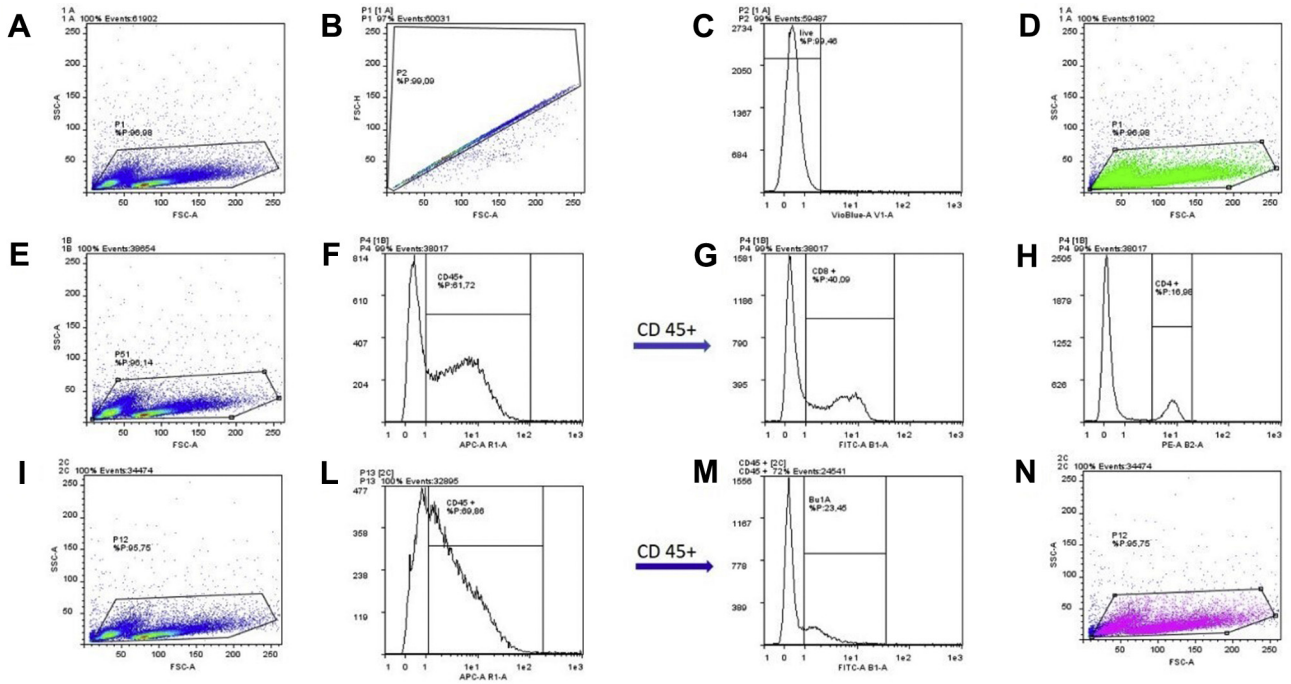

Figure 3. Gating strategy in multicolor flow cytometry analysis. Representative cytograms (A, E, I, D, N), FSC-A/FSC-H plot (B), and single parameter histograms (C, F, G, H, L, M) concerning mononuclear cells in spleen samples. Debris were discarded using forward (FSC-A) and side scatter (SSC-A) (A, E, I). Doublets were excluded by FSC-A/FSC-H discrimination gate (B). Live cells were selected based on gate set in samples stained with VioBlue for further analysis (C). Live cells showed in green on the cytogram (D). Singlets were analyzed for CD45 (F, L), CD8 (G), CD4 (H), and Bu-1A (M) expression. CD45-positive cells showed in pink on the cytogram (N).

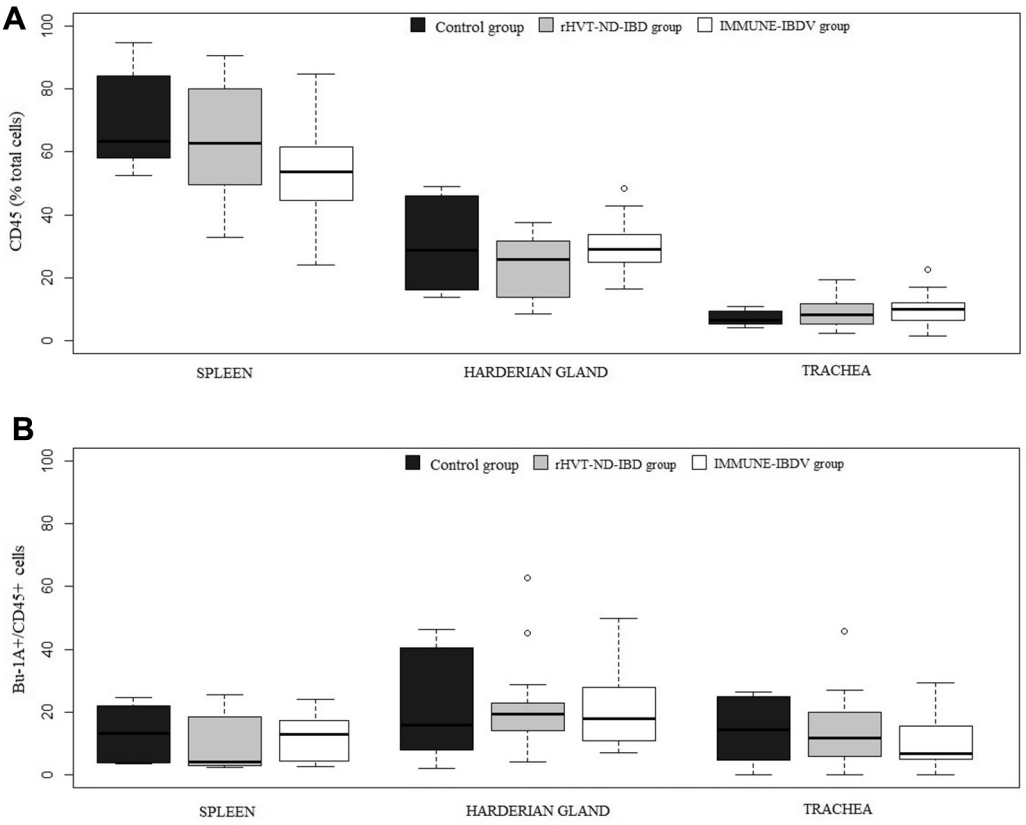

Figure 4. Mononuclear cells characterization (CD45+ and Bu1-A+) by flow cytometry analysis—trial 2. Percentage of CD45+/total cells (A) and Bu-1A+/CD45+ cells (B) in the spleen, Harderian gland, and trachea at 28 D after IBV vaccination in the not–IBDV vaccinated group (control group, n = 6), in the rHVT-ND-IBD vaccinated group (rHVT-ND-IBD group, n = 17 birds), and in the immune-complex vaccinated group (IMMUNE-IBDV group, n = 17 birds). Horizontal lines represent median; boxes represent 25-75 percentile; whiskers represent min-max; dots represent outliers.

DISCUSSION

In the present study, we compared, in 2 animal trials, the effects of vaccination of SPF broiler chickens with different IBD vaccines, namely an rHVT-ND-IBD construct vaccine or an IBDV immune-complex vaccine, on the integrity of the bursa of Fabricius and the immune response to live IBV vaccination.

In the first experiment, the impact of the different IBDV vaccines was evaluated at 14, 28, and 35 D after IBDV vaccination, by B:BW ratios comparison between groups. Birds of the group vaccinated with the immune-complex vaccine showed bursal atrophy represented by significantly lower B:BW ratios, at all time points of sampling, when compared to the non–IBDV vaccinated birds or to the group vaccinated with the rHVT-ND-IBD construct. These results confirm previously published reports where different live IBDV vaccines are shown to cause bursal atrophy and moderate to severe bursal lesions under experimental or field conditions (Thangavelu et al., 1998; Killian et al., 2017; Jahromi et al., 2018).

Moreover, no evidence of alteration of the bursa of Fabricius was shown during trial 1 in birds vaccinated with the rHVT-ND-IBD construct; these data expand the already reported evidence about the safety of HVT vaccine constructs developed to express foreign antigens (Tsukamoto et al., 2002; Roh et al., 2016; Dey et al., 2017).

The IBDV immune-complex vaccine effect on bursa of Fabricius may correlate with a decrease in the ability to mount an adequate humoral immune response and with an impairment of the immune response to vaccinations as previously reported after live IBDV vaccination application (Saif, 1991; Kim et al., 1999; Rautenschlein et al., 2007; Prandini et al., 2016).

To confirm this hypothesis, in the second trial, antibody titers after IBV vaccination had a significantly lower increase in the group vaccinated with the IBDV immune-complex vaccine than in the other experimental groups. Controversially, in this group, despite the significantly lower level of circulating IBV-induced antibodies, the relative amount of Bu-1A–positive lymphocytes, measured by cytofluorimetric analysis, was not impaired in all the examined organs. This feature could be attributed to postinfection repopulation of bursal follicles with B lymphocyte unable to mount an appropriate antibody response, which has been reported at early age of chicken after infection with virulent IBDV (Withers et al., 2005).

Interestingly, birds belonging to the group vaccinated with immune-complex vaccine showed a significantly

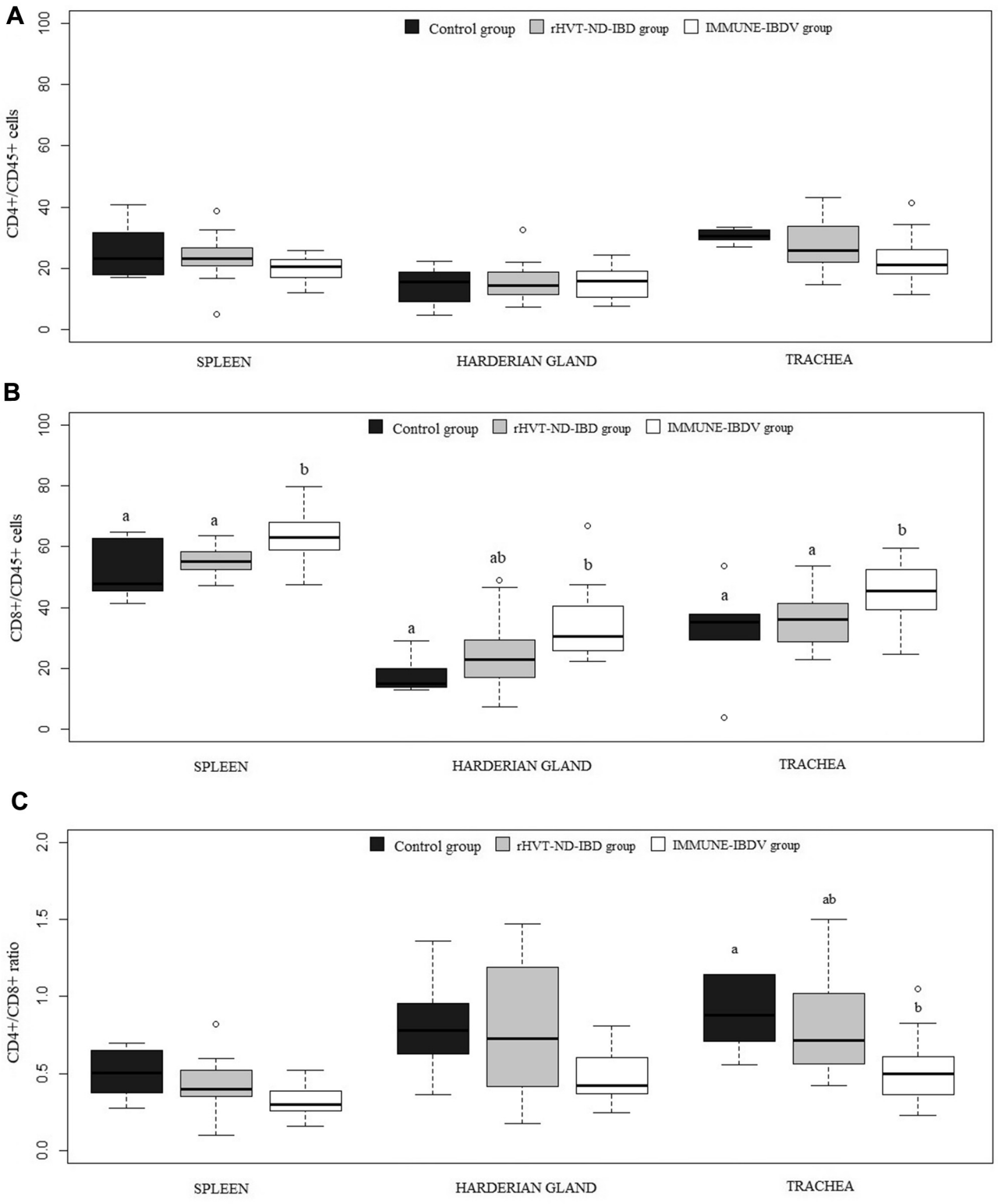

Figure 5. Mononuclear cells characterization (CD4+ and CD8+) by flow cytometry analysis—trial 2. Percentage of CD4+/CD45+ cells (A), CD8+/CD45+ cells (B), and CD4+/CD8+ ratio (C) obtained by FMC analysis, in the spleen, Harderian gland, and trachea at 28 D after IBV-vaccination in the not–IBDV vaccinated group (control group, n = 6), in the rHVT-ND-IBD vaccinated group (rHVT-ND-IBD group, n = 17 birds), and in the immune-complex vaccinated group (IMMUNE-IBDV group, n = 17 birds). Horizontal lines represent median; boxes represent 25-75 percentile; whiskers represent min-max; dots represent outliers. Different superscripts indicate significant difference for $P < 0.05$.

higher mean percentage of CD8+ T cells than both the other groups, in the spleen and trachea and, compared with the control group, also in the Harderian glands. During the immune response, CD8+ T cells first recognize the antigen peptides associated to major histocompatibility class I complex then proliferate and differentiate into armed antigen-specific cytotoxic T lymphocytes (**CTLs**). CTLs exert their protective effect via a range of effector mechanisms and cytolytic pathways that have been demonstrated to clear viruses infection (Dai et al., 2019). CTLs were suggested also to contribute to lesion development in the bursa after IBDV infection (Kim et al., 1999; Rauf et al., 2012) and in the late stage of the infection. Kim et al., 2000 (Kim et al., 2000) observed that IBDV exposed birds showed a higher lymphocyte subpopulation of CD8+ T cells than virus-free birds, in the bursa and in the spleen up to 22 D after IBDV infection. Furthermore, Li et al., 2018 (Li et al., 2018) reported an increased number of CD8+ T cells in the bursa, in the cecal tonsils, and in the lamina propria of the caecum up to 14 D after very virulent IBDV infection, suggesting that the CD8+ T cells are not only activated in the bursa but also in the gut. Our results showed a higher amount, after IBDV vaccination with the immune complex vaccine, of cytotoxic CD8+ T lymphocytes in further lymphoid districts such as the Harderian gland and the trachea. The CD8+ T cell increasing also resulted in an altered proportion in the trachea of the CD4+/CD8+ ratio, that tended to be significantly reduced in the immune-complex IBD vaccine group, compared to the not-vaccinated group. A reduction in the proportion of CD4+/CD8+ and an increase of CD8+, associated with immunological disfunctions, have been also reported in humans during prolonged infections with human immunodeficiency virus and cytomegalovirus (Ouyang et al., 2004; Papagno et al., 2004).

The CTL response has been reported to be critical in the protection of chickens from IBV acute infection (Seo et al., 2000); therefore, it could be speculated that the significant difference of the amount of CD8+ cells observed among groups could be attributed to an higher replication of the IBV vaccines in the immunologically impaired immune-complex vaccinated group.

The results of the in vivo trials performed in SPF broiler chickens coupled with a depth analysis of the repertoire of parameters involved in the immune response to IBDV vaccination suggest that more than one aspect should be considered when choosing the vaccination program, considering the mutual influence that can occur between the different vaccines applied.

Further studies involving commercial chickens could expand upon the results obtained, exploring how the maternally derived antibodies can influence the impact of IBDV vaccination on the chicken immune system.

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REFERENCES

Aricibasi, M., A. Jung, E. D. Heller, and S. Rautenschlein. 2010. Differences in genetic background influence the induction of innate and acquired immune responses in chickens depending on the virulence of the infecting (IBDV) strain. Vet. Immunol. Immunopathol. 135:79–92.

Dai, M., C. Xu, W. Chen, and M. Liao. 2019. Progress on chicken T cell immunity to viruses. Cell. Mol. Life Sci. 76:2779–2788.

Dey, S., M. Chellappa, D. Pathak, S. Gaikwad, K. Yadav, S. Ramakrishnan, and V. Vakharia. 2017. Newcastle disease virus Vectored Bivalent vaccine against virulent infectious bursal disease and Newcastle disease of chickens. Vaccines 5:31.

Fan, L., T. Wu, A. Hussain, Y. Gao, X. Zeng, Yulong Wang, L. Gao, K. Li, Y. Wang, C. Liu, H. Cui, Q. Pan, Y. Zhang, Y. Liu, H. He, X. Wang, and X. Qi. 2019. Novel variant strains of isolated in China. Vet. Microbiol. 230:212–220.

Felice, V., G. Franzo, E. Catelli, A. Di Francesco, M. Bonci, M. Cecchinato, G. Mescolini, D. Giovanardi, P. Pesente, and C. Lupini. 2017. Genome sequence analysis of a distinctive Italian. Poult. Sci. 96:4370–4377.

Fraga, A. P. de, T. Gräf, C. S. Pereira, N. Ikuta, A. S. K. Fonseca, and V. R. Lunge. 2018. Phylodynamic analysis and molecular diversity of the avian infectious bronchitis virus of chickens in Brazil. Infect. Genet. Evol. 61:77–83.

Franzo, G., V. Listorti, C. J. Naylor, C. Lupini, A. Laconi, V. Felice, M. Drigo, E. Catelli, and M. Cecchinato. 2015. Molecular investigation of a full-length genome of a Q1-like IBV strain isolated in Italy in 2013. Virus Res. 210:77–80.

Franzo, G., C. J. Naylor, C. Lupini, M. Drigo, E. Catelli, V. Listorti, P. Pesente, D. Giovanardi, E. Morandini, and M. Cecchinato. 2014. Continued use of IBV 793B vaccine needs reassessment after its withdrawal led to the genotype's disappearance. Vaccine 32:6765–6767.

Jahromi, M. Z., M. B. Bello, M. Abdolmaleki, S. K. Yeap, M. Hair-Bejo, and A. R. Omar. 2018. Differential activation of intra-epithelial lymphocyte-natural killer cells in chickens infected with very virulent and vaccine strains of. Dev. Comp. Immunol. 87:116–123.

Jakhesara, S. J., B. Nath, J. K. Pal, C. G. Joshi, and S. Kumar. 2018. Emergence of a genotype I variant of avian infectious bronchitis virus from Northern part of India. Acta Trop. 183:57–60.

Killian, M. P., J. D. Boviez, M. Gambarotta, and D. M. Lombardo. 2017. Induction of apoptosis in the bursa of Fabricius by vaccination against Gumboro disease. Avian Pathol. 46:526–534.

Kim, I. J., M. Gagic, and J. M. Sharma. 1999. Recovery of antibody-producing ability and lymphocyte repopulation of bursal follicles in chickens exposed to. Avian Dis. 43:401.

Kim, I.-J., S. K. You, H. Kim, H.-Y. Yeh, and J. M. Sharma. 2000. Characteristics of bursal T lymphocytes induced by infectious bursal disease virus. J. Virol. 74:8884–8892.

Li, L., T. Kubasová, I. Rychlik, F. J. Hoerr, and S. Rautenschlein. 2018. infection leads to changes in the gut associated-lymphoid tissue and the microbiota composition. PLoS One 13:e0192066.

Listorti, V., A. Laconi, E. Catelli, M. Cecchinato, C. Lupini, and C. J. Naylor. 2017. Identification of IBV QX vaccine markers: should vaccine acceptance by authorities require similar identifications for all live IBV vaccines? Vaccine 35:5531–5534.

Lupini, C., V. Felice, F. Silveira, G. Mescolini, G. Berto, V. Listorti, M. Cecchinato, and E. Catelli. 2020. Comparative in vivo pathogenicity study of an ITA genotype isolate (G6) of. Transbound. Emerg. Dis. 67:1025–1031.

Lupini, C., D. Giovanardi, P. Pesente, M. Bonci, V. Felice, G. Rossi, E. Morandini, M. Cecchinato, and E. Catelli. 2016. A molecular

epidemiology study based on VP2 gene sequences reveals that a new genotype of is dominantly prevalent in Italy. Avian Pathol. 45:458–464.

Ma, T., L. Xu, M. Ren, J. Shen, Z. Han, J. Sun, Y. Zhao, and S. Liu. 2019. Novel genotype of infectious bronchitis virus isolated in China. Vet. Microbiol. 230:178–186.

Michel, L. O., and D. J. Jackwood. 2017. Classification of into genogroups. Arch. Virol. 162:3661–3670.

Ouyang, Q., W. M. Wagner, W. Zheng, A. Wikby, E. J. Remarque, and G. Pawelec. 2004. Dysfunctional CMV-specific CD8+ T cells accumulate in the elderly. Exp. Gerontol. 607–613.

Papagno, L., C. A. Spina, A. Marchant, M. Salio, N. Rufer, S. Little, T. Dong, G. Chesney, A. Waters, P. Easterbrook, P. R. Dunbar, D. Shepherd, V. Cerundolo, V. Emery, P. Griffiths, C. Conlon, A. J. McMichael, D. D. Richman, S. L. Rowland-Jones, and V. Appay. 2004. Immune activation and CD8+ T-cell differentiation towards senescence in HIV-1 infection. PLoS Biol. 2:e20.

Prandini, F., B. Simon, A. Jung, M. Pöppel, S. Lemiere, and S. Rautenschlein. 2016. Comparison of infectious bursal disease live vaccines and a HVT-IBD vector vaccine and their effects on the immune system of commercial layer pullets. Avian Pathol. 45:114–125.

Rauf, A., M. Khatri, M. V. Murgia, and Y. M. Saif. 2012. Fas/FasL and perforin-granzyme pathways mediated T cell cytotoxic responses in infected chickens. Results Immunol. 2:112–119.

Rautenschlein, S., G. von Samson-Himmelstjerna, and C. Haase. 2007. A comparison of immune responses to infection with virulent (IBDV) between specific-pathogen-free chickens infected at 12 and 28 days of age. Vet. Immunol. Immunopathol. 115:251–260.

Rautenschlein, S., H. Y. Yeh, and J. M. Sharma. 2003. Comparative immunopathogenesis of mild, intermediate, and virulent strains of classic. Avian Dis. 47:66–78.

Roh, J.-H., M. Kang, B. Wei, R.-H. Yoon, H.-S. Seo, J.-Y. Bahng, J.-T. Kwon, S.-Y. Cha, and H.-K. Jang. 2016. Efficacy of HVT-IBD vector vaccine compared to attenuated live vaccine using in-ovo vaccination against a Korean very virulent IBDV in commercial broiler chickens. Poult. Sci. 95:1020–1024.

Saif, Y. M. 1991. Immunosuppression induced by. Vet. Immunol. Immunopathol. 30:45–50.

Seo, S. H., J. Pei, W. E. Briles, J. Dzielawa, and E. W. Collisson. 2000. Adoptive transfer of infectious bronchitis virus primed αβ T cells bearing CD8 antigen protects chicks from acute infection. Virology 269:183–189.

Sharma, J. M., I. J. Kim, S. Rautenschlein, and H. Y. Yeh. 2000. of chickens: pathogenesis and immunosuppression. Dev. Comp. Immunol. 24:223–235.

Silveira, F., V. Felice, G. Franzo, G. Mescolini, E. Catelli, M. Cecchinato, G. Berto, V. Listorti, and C. Lupini. 2019. Inoculation of specific pathogen-free chickens with an of the ITA genotype (G6) leads to a high and persistent viral load in lymphoid tissues and to a delayed antiviral response. Vet. Microbiol. 235:136–142.

Smialek, M., B. Tykalowski, D. Dziewulska, T. Stenzel, and A. Koncicki. 2016. Immunological aspects of the efficiency of protectotype vaccination strategy against chicken infectious bronchitis. BMC Vet. Res. 13:44.

Thangavelu, A., G. D. Raj, S. Elankumaran, B. M. Manohar, A. Koteeswaran, and A. T. Venugopalan. 1998. Pathogenicity and immunosuppressive properties of field isolates and commercial vaccines in India. Trop. Anim. Health Prod. 30:167–176.

Toro, H., V. L. Van Santen, L. Li, S. B. Lockaby, E. Van Santen, and F. J. Hoerr. 2006. Epidemiological and experimental evidence for immunodeficiency affecting avian infectious bronchitis. Avian Pathol. 35:455–464.

Torres, C. A., V. Listorti, C. Lupini, G. Franzo, M. Drigo, E. Catelli, P. E. Brandão, and M. Cecchinato. 2017. Molecular and cellular biology: Gamma and Deltacoronaviruses in quail and pheasants from Northern Italy. Poult. Sci. 96:717–722.

Tsukamoto, K., S. Saito, S. Saeki, T. Sato, N. Tanimura, T. Isobe, M. Mase, T. Imada, N. Yuasa, and S. Yamaguchi. 2002. Complete, long-lasting protection against lethal challenge by a single vaccination with an avian herpesvirus vector expressing VP2 antigens. J. Virol. 76:5637–5645.

Withers, D. R., J. R. Young, and T. F. Davison. 2005. -induced immunosuppression in the chick is associated with the presence of undifferentiated follicles in the recovering bursa. Viral Immunol. 18:127–137.