Research paper

Modulation of innate immunity in chickens induced by *in vivo* administration of baculovirus

Silvina Chimeno Zoth [a,b,*], Juan Manuel Carballeda [a,b], Evangelina Gómez [a], María José Gravisaco [a], Elisa Carrillo [a,b], Analía Berinstein [a,b,**]

[a] *Instituto de Biotecnología, CICVyA, INTA, Castelar, Cc 25, B1712WAA Buenos Aires, Argentina*
[b] *Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Rivadavia 1917, C1033AAJ, Ciudad de Buenos Aires, Argentina*

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ABSTRACT

Baculoviruses stimulate cytokine production in mammalian cells. They induce a strong innate immune response in animals and have adjuvant properties. The purpose of this work was to study the *in vivo* effect of baculovirus on chicken innate immune response. SPF chickens were inoculated intravenously with Autographa californica nuclear polyhedrosis virus (BV). Three hours later, chickens were bled, euthanized and their spleen, duodenum and cecal tonsils were excised in order to take samples for RNA extraction and real time PCR, and to isolate lymphocytes, which were stained and analyzed by flow cytometry. The results obtained showed that baculovirus inoculation up-regulates the expression of IFN-γ, IL-6 and LITAF in spleen cells. This result (IFN-γ) correlated with that obtained by ELISA which showed a very strong increase of IFN-γ in chicken plasma. Flow cytometry analysis revealed that BV inoculation induced in spleen an increase in the percentage of monocyte/macrophage population together with an increase in CD3$^+$CD4$^+$ T lymphocytes. On the other hand, BV inoculation decreased the percentage of CD3$^+$CD4$^+$ T lymphocytes and increased the percentage of NK cells in cecal tonsils. However, intraepithelial lymphocytes of the gut did not show differences between BV and control treated animals. Even though further studies in order to understand the mechanisms by which BVs affect the avian immune response are needed, results obtained in the present work demonstrate the ability of BVs to stimulate the innate immunity in chickens, modifying the expression pattern of related genes and the profile of the immune cells involved.

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1. Introduction

Production and consumption of poultry products have increased significantly in the last years. The chicken is an economically relevant livestock animal worldwide, poultry meat accounting for almost 40% of global meat consumption. In this context, sanitary conditions are of outstanding importance. Almost all chicken viral diseases are prevented by vaccination. Nevertheless, there is a chance of exposure to viral pathogens before the appropriate vaccine induces complete protection. Consequently, the identification of different ways to provide a protective status during the "window of susceptibility" is essential.

The innate immune system constitutes the first line of host defense against infection and is presently recognized as an essential component of the immune response to viral infections in chicken. Therefore, finding an alternative

* Corresponding author at: Instituto de Biotecnología, CICVyA, INTA, Castelar, Cc25, B1712WAA, Buenos Aires, Argentina.
Tel.: +54 11 4621 1278.
** Corresponding author at: Instituto de Biotecnología, CICVyA, INTA, Castelar, Cc25, B1712WAA, Buenos Aires, Argentina.
Tel.: +54 11 4621 1278; fax: +54 11 4621 0199.
E-mail addresses: schimeno@cnia.inta.gov.ar (S. Chimeno Zoth), aberinstein@cnia.inta.gov.ar (A. Berinstein).

means to stimulate early innate mechanisms of defense would be a valuable achievement, and could be a suitable tool to deal with early immunity.

Baculoviruses (BVs) are dsDNA viruses which infect insects. They have been widely used as selected bioinsecticides (Thiem, 1997) and for the expression of recombinant proteins in insect cells and larvae (Bai et al., 2008; Chimeno Zoth et al., 2009, 2011; Facciabene et al., 2004; Lu et al., 2007; Strauss et al., 2007; Wu et al., 2009). BVs cannot replicate in mammalian or other vertebrate animal cells (Via et al., 1983). However, recent studies showed that BVs have strong adjuvant properties in mice, promoting potent humoral and CD8$^+$ T cell adaptive responses against coadministered antigens (Abe et al., 2003; Gronowski et al., 1999). BVs also induce the *in vivo* maturation of dendritic cells and the production of inflammatory cytokines. 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).

Little information is available regarding the stimulatory effect of baculovirus in the chicken. Studying the effect of BV in chicken macrophage cell line HD 11 and in chicken peripheral blood mononuclear cells *in vitro*, it was shown that BV enhances inflammatory cytokine mRNA expression in both cell types and up-regulates nitric oxide production in HD 11 cells (Niu et al., 2008). Protection of neonatal chickens against Bronchitis Infectious Virus by BV was demonstrated (Niu et al., 2008), but no studies on the BV effect in the animal was reported. In this study, we report the effect of *in vivo* administration of BV on the chicken immune system regarding the enhancement of cytokine expression, nitric oxide production, and immune cell profile situation.

2. Materials and methods

2.1. Animals and viruses

Specific-pathogen-free White Leghorn embryonated eggs were purchased from Rosenbush SA (CABA) and hatched in an automatic incubator (Yonnar, Rosario, Argentina). One day old chickens were kept in individual cages. Chickens were provided food and water *ad libitum*.

Autographa californica nuclear polyhedrosis virus (AcNPV) was produced in *Spodoptera frugiperda* cell line (Sf9) and cultured in Sf900 medium with 2% of fetal bovine serum (FBS) at 27 °C. Virus titers were calculated by end point dilution assay and converted to PFU/ml as described by O'Reilly et al. (1994).

2.2. Experimental design

Twenty 3-week-old chickens were randomly designed in two groups. One group was intravenously inoculated in the wing with 300 µl of wild type AcNPV containing 1×10^8 PFU (Group: BV). Another group (negative control) was inoculated with an equal volume of supernatant of mock-infected Sf9 cells (Group: Mock).

Three hours post-intravenous inoculation, 5 chickens of each group were bled and euthanized. Complete animals and their spleens were weighed. Pieces of 30 mg of each spleen and duodenum were kept immediately in RNAlater solution (QIAGEN, Hilden, Germany) and stored up to 30 days at 4 °C, until RNA extraction. Remaining spleens and duodenums, together with the cecal tonsils, were processed (pool from 5 BV inoculated animals and pool from 5 Mock inoculated animals) for lymphocytes isolation. The same procedure was performed at 16 hpi with the 10 remaining animals.

2.3. RNA isolation and cDNA synthesis

RNA from each piece of tissue was individually obtained with RNAeasy kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The concentration and purity of the purified RNA were determined using the 260/280 nm absorbance ratio. RNA was stored at −80 °C until use. RNA was treated with DNAse I and reverse transcription was performed using SSIII Reverse transcription kit (Invitrogen, Carlsbad, CA) with random hexamers.

Table 1
Primers used to amplify cytokine and control coding regions.

Name	Sequence (5′–3′)	Accession number (Gene bank)	Size of PCR product (bp)
LITAF Fw	CCATCTGCACCACCTTCA	NM_204267.1	184
LITAF Rv	TTGCTGCACATACACAGT		
IL-8 Fw	ATGAACGGCAAGCTTGGA	NM_205018.1	190
IL-8 Rv	GCAGTGGGGGCCGCTTGG		
IL-15 Fw	ACAGCCATTTCTTTTGCC	NM_204571.1	179
IL-15 Rv	CTCGTATGTGTTTGCAGT		
IFNα Fw	CTCACGCTCCTTCTGAAA	NM_205427.1	174
IFNαRv	CAGGATGGTGTCGTTGAA		
IFNγ Fw	CAAAGCCGCACATCAAACA	Y07922	259
IFNγRv	TTTCACCTTCTTCACGCCATC		
IL-6 Fw	CAAGGTGACGGAGGAGGAC	AJ309540	254
IL-6 Rv	TGGCGAGGAGGGATTTCT		
TLR21 Fw	GATGATGGAGACAGCGGAGAAG	NM_001030558.1	80
TLR21 Rv	GCAGCAGCAGCCAGAGT		
TGFβ Fw	CGGGACGGATGAGAAGAAC	M31160	258
TGFβ Rv	CGGCCCACGTAGTAAATGAT		
GAPDH Fw	AGAACATCATCCCAGCGTCC	K01458	264
GAPDH Rv	CGGCAGGTCAGGTCAACA		

2.4. Quantitative RT PCR

Table 1 shows the sequence of the oligonucleotides used to amplify regions of chicken cytokines, TLR21 and GAPDH control genes. Amplification and detection were carried out for each sample using equivalent amounts of RNA from each tissue. The standard curve of each gene was performed using ten-fold serial dilutions of plasmidic DNA, obtained from DH5α *Escherichia coli* transformed with recombinant pGEM-T Easy vector (Promega) carrying a short fragment (80–264 bp), coding for each cytokine and GAPDH (Carballeda et al., 2011). Quantification was carried out with the SYBR®Green Master Mix Kit (Applied Biosystems, Warrington, UK). Cycle threshold (CT) values were used to plot a standard curve in which the CT values decreases in linear proportion with the log of the template concentration. Sample CT values were extrapolated in the standard curve in order to determine the initial amount of each particular transcript.

2.5. Lymphocyte isolation

Single cell suspensions were obtained from pools of spleens by mechanical disruption in RPMI 1640, and mononuclear cells were isolated by centrifugation over histopaque density gradients (1.077 g/ml; Sigma, St. Louis, MO) at room temperature. Cells were isolated from the interface, washed, and live cells were counted using trypan blue exclusion.

Cecal tonsils were cut into very small pieces and mechanically disrupted by pressing with a syringe plunger, in RPMI 1640. Then, cellular suspensions were passed through a mesh (Cell Strainer, BD) and washed twice. Finally, cells were counted using trypan blue exclusion.

Intraepithelial lymphocytes were isolated from duodenum, as previously described by Göbel (2000) and once the cellular suspensions were obtained, viable cells were counted using trypan blue exclusion.

2.6. Flow cytometry analysis

Cells were resuspended in Staining Buffer (PBS $1\times$, 10% FBS, 0.1% Sodium Azide) and 1×10^6 cells per well were seeded on 96 well-plates (V-shape), and washed twice with the same buffer. Staining was performed by resuspending the cellular pellet of each well in 100 μl of staining buffer including different combinations of antibodies, or as single-color stainings for compensation. Cells were incubated at 4 °C for 30 min and washed twice with staining buffer by centrifugation at $290 \times g$ for 10 min.

Monoclonal antibodies (CD3-SPRD, CD4-PE, CD8α-FITC, CD8β-PE, KUL01-PE) were obtained from Southern Biotech (Birmingham, AL) and 28.4 mAb was kindly given by Dr Thomas Göbel (Ludwig-Maximilians-Universität (LMU) München) (Göbel et al., 2001). In this last case, a secondary antibody (goat anti-mouse-PE, Invitrogen) was also used. All antibodies were titrated in order to determine the optimal staining concentration for each one.

Positive cells were analyzed with a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA) and CellQuest software. Both, lymphocyte and monocyte gates were

Table 2
Relation between spleen and body weight of each animal.

	Animal weight (AW) (g ± SD)	Spleen weight (SW) (g ± SD)	SW/AW
Mock	245.73 ± 6.11	0.37 ± 0.04	0.0015
BV	239.50 ± 12.02	0.58 ± 0.11	0.0024[*]

Each value corresponds to the group mean ± the Standard Deviation.
[*] Statistically significant ($p < 0.005$).

defined by the forward/side scatter characteristics of the cells and 30,000 and 50,000 events were analyzed respectively for each sample.

2.7. Splenic nitrite production

Splenocytes were resuspended in RPMI medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 50 μM 2-mercaptoethanol and 10% FBS. Two million cells per well were seeded on 96 well-culture plates in the presence or absence of concanavalin A (ConA; 15 μg/μl) and incubated for 24 h at 41 °C in a 5% CO_2 atmosphere. Then, culture supernatants were collected and nitrite concentration was measured by Griess reaction (Griess, 1879). Briefly, 50 μl of culture media and two-serial fold dilutions of $NaNO_2$ (from 125 to 1 μM) used as a standard curve were displaced in a 96-well flat bottom microtiter plate (Nunc, Rochester, NY). Then 50 μl of each Griess Reagent (Sulfanilamide and naphthylethylenediamine) were added and the 530 nm absorbance of each well was measured with a multiscan reader (Thermo). NO_2 concentration was determined by extrapolation of the value obtained at 530 nm absorbance for each sample in the standard curve.

2.8. Plasmatic IFN-γ concentration

Plasma samples of treated chickens were analyzed by ELISA using CytoSet Kit (Biosource, CA, USA) in 96-well plates, following manufacturer's instructions. Serial two-fold dilutions of recombinant IFN-γ were used to plot a standard curve, where OD values of each sample were extrapolated in order to calculate IFN-γ concentration in each sample.

2.9. Statistical analysis

Mann–Whitney test was used to determine significant differences between measures from mock-infected and BV treated chickens. A value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. BV inoculation induces the activation of a strong innate response

To evaluate the extent of the inflammatory response to BV inoculation, we measured first the spleen weight in relation to the total weight of the chicken. As can be seen in Table 2, the inoculation with BV induced a notable
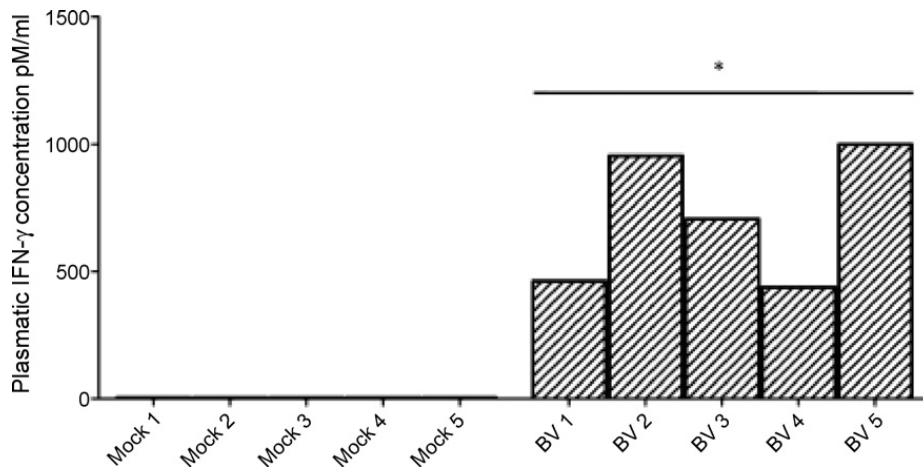
Fig. 1. IFN-γ concentration in plasma. Plasma samples of BV or Mock i.v. inoculated chickens were obtained at 3 hpi. Plasmatic concentrations of IFN-γ were individually determined by ELISA (CytoSet Kit, Biosource). $^*p < 0.05$.

inflammatory effect in chicken spleen, demonstrated by the significant increase ($p < 0.001$) of the spleen weight related to the body weight observed at 3 hpi. Then, we measured IFN-γ response in plasma by ELISA (Fig. 1). A significant increase in plasmatic IFN-γ concentration in all the animals inoculated with BV was found at 3 hpi ($p < 0.001$), reaching values near to 1000 pM (Fig. 1). Plasmatic concentration levels of IFN-γ in mock inoculated chickens were undetectable. Samples obtained at 16 hpi did not show differences in this measure between groups (data not shown).

Concomitantly, the transcriptional cytokine profile was established in spleen and duodenum. Fig. 2 shows that BV induced the expression of IFN-γ in spleen, together with the pro-inflammatory cytokine IL-6 and the LPS induced TNF-α factor (LITAF). Also, intravenous inoculation with BV induced a down-regulation in the expression of TGF-β. On the other hand, when cytokine expression was analyzed in duodenum, only IFN-γ showed to be over expressed in four out of the five animals (Fig. 2).

Chicken toll-like receptor (TLR) 21 acts as a functional homologue to mammalian TLR9 in the recognition of CpG motifs (Brownlie et al., 2009). As BV could be acting through this kind of motifs in the chicken, among other ways, the gene expression of TLR21 was analyzed. Fig. 2 shows an increase in the production of TLR21 mRNA in animals treated with BV.

Taken together, these data indicate that BV stimulates a strong innate immune response among chickens shortly after being administered, as the differences mentioned above were not detected at 16 hpi (data not shown).

3.2. BV inoculation induces modifications in the immune cells pattern

In order to evaluate if the inoculation of BV was able to induce modifications in the frequency of immune cells, mononuclear cells isolated from spleen, cecal tonsils and duodenum were stained with different combinations of monoclonal antibodies and evaluated by flow cytometry (Table 3). In spleen, an increase of CD3$^+$CD4$^+$ and CD3$^+$CD8αα$^+$ (but not CD3$^+$CD8αβ$^+$) lymphocytes was observed, together with an important increase in the

percentage of monocytes/macrophages population. A fall in the frequency of NK cells was evidenced by the decrease of CD3$^-$CD8α$^+$28.4$^+$ and CD3$^-$CD8αα$^+$ cells. Other organs, such as cecal tonsils and duodenum, were also evaluated. In cecal tonsils, a drop in the frequency of CD3$^+$CD4$^+$ T-lymphocytes was recorded. Another feature observed in cecal tonsils was the increase of NK cells, revealed by the increase in the percentage of CD3$^-$CD8α$^+$28.4$^+$ cells. On the other hand, the evaluation of duodenum samples did not show differences between BV treated animals and the corresponding control (data not shown).

3.3. BV inoculation activates NO response in spleen

Also, the splenic nitrite production, as a measure of macrophage activation, was determined in ConA or mock stimulated splenocytes from BV or mock inoculated chickens. A significant increase ($p < 0.05$) in NO production in both ConA and mock stimulated splenocytes from BV inoculated chickens was observed (Fig. 3). Thus, intravenous inoculation with BV affects chicken spleen promoting the activation of immune functions.

4. Discussion

One of the most relevant advances in immunology is the understanding that the innate immune response is of crucial and controlling importance for fighting against a pathogen, as well as for the development of the adaptive immune response. It is now recognized that the innate immune system provides an important initial response to pathogens, which can limit or prevent infection. Thus, the action of the innate immune response favoring the preservation of health before a vaccine could provide a protective status against a pathogen, would be of great help to support control and prevention sanitarian campaigns. In this study, we evaluate the effect of the in vivo administration of baculovirus on the innate immune response of chickens. As soon as 3 hpi, we found an up regulation of the gene expression of IFN-γ, IL-6 and LITAF in the spleen of baculovirus treated chickens, together with a decrease of the TGF-β gene expression, indicating a strong pro-inflammatory
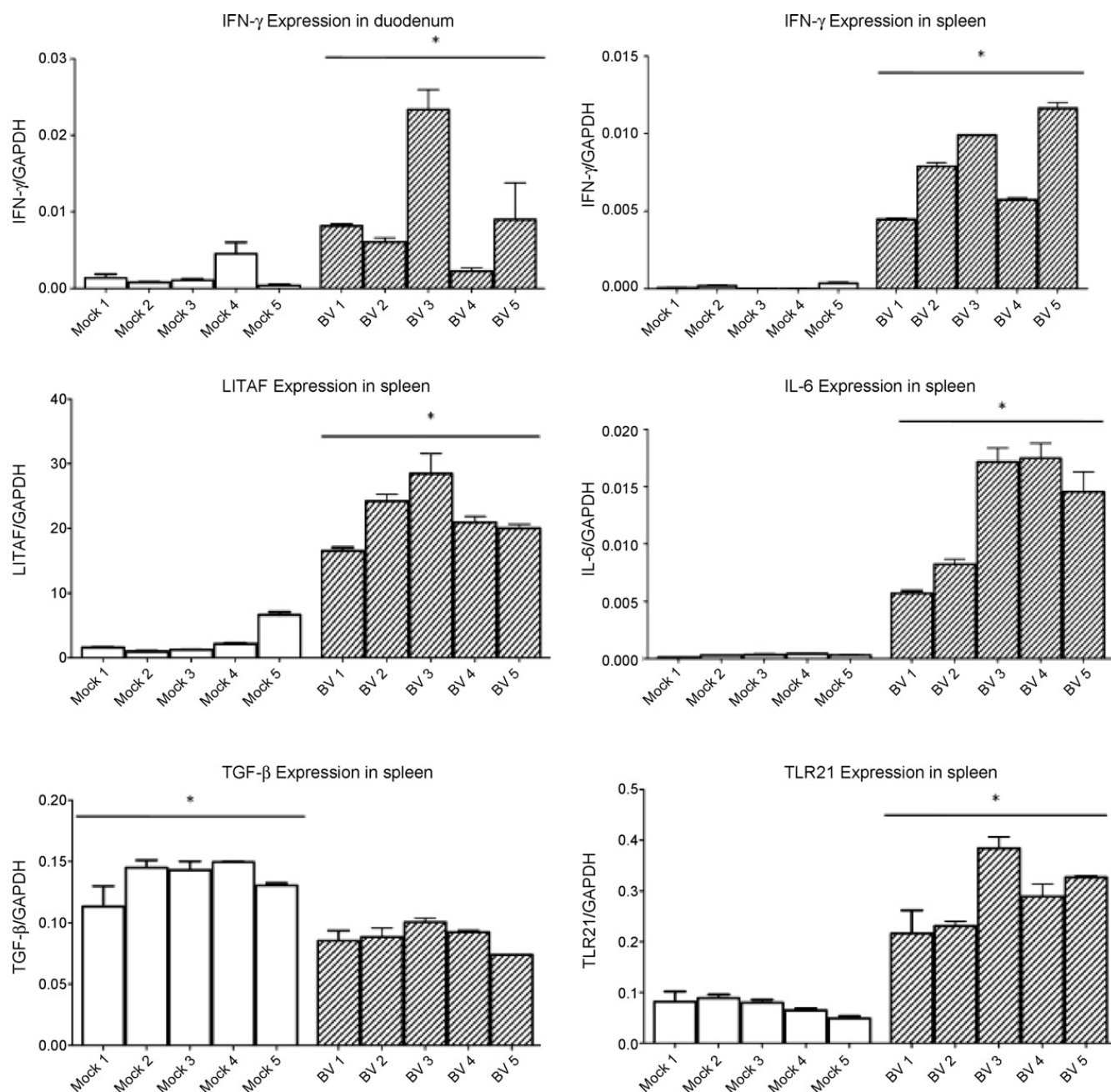
Fig. 2. Transcriptional pattern of cytokine and TLR21 genes. Total RNA was extracted from spleen and duodenum of BV or Mock i.v. inoculated chickens at 3 hpi and cDNA was synthesized. mRNA levels were determined by quantitative real time PCR using specific primers and SYBR®Green method. The expression level of each mRNA was calculated in relation to the expression level of GAPDH. Each bar represents mean ± SEM of 2 replicates of each sample obtained from 5 BV or Mock inoculated chickens at 3 hpi. *$p < 0.05$.

Table 3
Evaluation of mononuclear cell populations by flow cytometry.

Source of cells	$CD3^+CD4^+$	$CD8\alpha^+\beta^+$	% Positive cells in the gate		$CD8\alpha^+$ ($CD3^-28.4^+$)	$KULO1^+$
			$CD8\alpha^+\alpha^+$	$CD3^-\ CD8\alpha^+$		
Spleen/Mock	41	41	15	3	4	6
Spleen/Bv	52	35	28	2	2	11
CT/Mock	27	31	3	13	12	ND
CT/Bv	19	31	5	14	19	ND

Chicken leukocytes were isolated from pools of spleens and cecal tonsils of chickens intravenously inoculated with BV or culture supernatant of Sf9 mock infected cells and sacrificed at 3 hpi. Cells were stained with different combinations of antibodies and analyzed by flow cytometry. For $CD3^+$, $CD4^+$, $CD8\alpha^+$ and $CD8\beta^+$ the gating strategy was location of the lymphocytes in a forward/side scatter-defined gate. The identification of $CD8\alpha^+\beta^+$ and $CD8\alpha^+\alpha^+$ cells was done in the $CD3^+$ gate. For 28.4 mAb, following the identification of the lymphocytes, $CD8\alpha^+$ cells were gated and the presence of $CD3^-$ 28.4^+ cells was analyzed. $KULO1^+$ cells were studied in the monocytes/macrophages gate defined in a forward/side scatter graph. Results were expressed as the percentage of stained cells in the gate. ND: non determined.
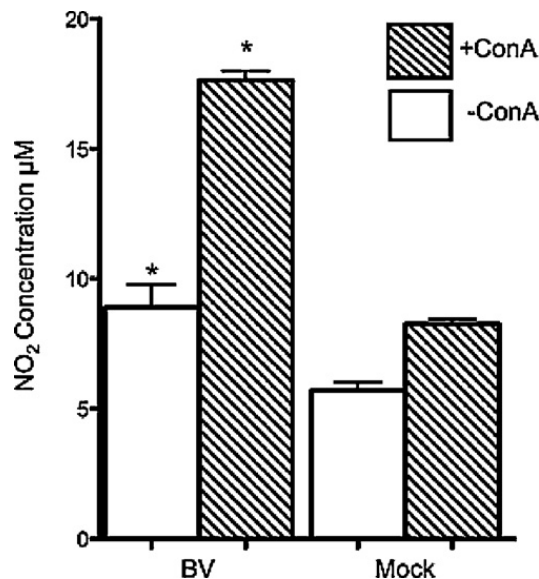
Fig. 3. Nitrite concentration (mean ± SEM) in splenocyte cultures were measured in BV or Mock i.v. inoculated chickens. Splenocytes were isolated from disaggregated spleen tissues at 3 hpi. Mononuclear cells were plated at 2×10^6 viable cells/well by triplicate in the presence (or absence) of ConA. Supernatant fluid was collected at 24 h and nitrite concentrations were determined using Griess reagent for each well. Each bar represents mean ± SEM of 3 replicates of each pool, constituted by samples obtained from 5 BV or Mock inoculated chickens at 3 hpi. $^*p < 0.05$.

immune response. On the other hand, flow cytometry assays also demonstrated that BV induced modifications in the mononuclear cells pattern of different organs. In spleen, an increase of $CD3^+CD4^+$ and $CD3^+CD8\alpha\alpha^+$ lymphocytes was observed, together with an increase in the percentage of monocyte/macrophage population.

One of the many interesting profits that the use of baculovirus for stimulating innate immunity offers is the limit of the duration of the BV-induced response, avoiding the potential damage a strong inflammatory immune response on an extended time period could produce. In this way we analyzed the immune response at 16 hpi and we did not find neither up-regulation of inflammatory cytokines expression nor increase in spleen NO production, indicating a time frame for the immune response elicited by BV.

Recently, chicken TLR21 was identified as having mammalian TLR9-like homologue function in CpG-induced innate immune response (Brownlie et al., 2009; Keestra et al., 2010). On the other hand, unmethylated CpG motifs are present at high frequency within the AcMNPV genome (Ayres et al., 1994). Han and coworkers reported the identification of the TLR by which BV induced activation in a macrophage-like cell line. They showed that stimulation of HD11 cells (chicken macrophage-like cell line) by BV resulted in a strong up-regulation of TLR21 expression, while other TLRs were either unaffected or down-regulated. The authors suggested that TLR21 plays a key role in BV-induced innate immunity of chicken (Han et al., 2010). In the present work, we found an increase in mRNA expression level of TLR21 in the spleen of BV-treated chickens. Nevertheless, the spleen cell population responsible for our observations, possibly macrophages, remains to be firmly identified.

Moreover, an indirect effect on monocytes/macrophages could be presumed since baculovirus inoculation induced an increase of IFN-γ at the mRNA and the protein levels, and a priming effect of NO response in splenocytes was observed. NO is a multi-functional mediator with diverse physiological and pathological roles in host defense against infectious agents (MacMicking et al., 1997) and the production of NO by activated monocytes/macrophages is an important innate immune response sign of cellular antiviral and bactericidal activity.

We report here the effect of BV *in vivo* and even though further studies in order to understand the mechanisms by which BV affect chicken innate immune response *in vivo* are needed, results obtained in the present work clearly agree with the results reported by Niu et al. and demonstrate the ability of BV to stimulate chicken innate immunity modifying the expression pattern of related genes and the profile of immune cells. Our results encourage future studies aimed at defining the mechanisms by which BV render such innate immune response in chickens.

The results presented here suggest that BV could be a valuable tool for providing a protective status to susceptible chickens during the "window of susceptibility" that conventional vaccines indefectibly render.

References

Abe, T., Takahashi, H., Hamazaki, H., Miyano-Kurosaki, N., Matsuura, Y., Takaku, H., 2003. Baculovirus induces an innate immune response and confers protection from lethal influenza virus infection in mice. J. Immunol. 171, 1133–1139.

Ayres, M.D., Howard, S.C., Kuzio, J., Lopez-Ferber, M., Possee, R.D., 1994. The complete DNA sequence of *Autographa californica* nuclear polyhedrosis virus. Virology 202, 586–605.

Bai, B., Lu, X., Meng, J., Hu, Q., Mao, P., Lu, B., Chen, Z., Yuan, Z., Wang, H., 2008. Vaccination of mice with recombinant baculovirus expressing spike or nucleocapsid protein of SARS-like coronavirus generates humoral and cellular immune responses. Mol. Immunol. 45, 868–875.

Brownlie, R., Zhu, J., Allan, B., Mutwiri, G.K., Babiuk, L.A., Potter, A., Griebel, P., 2009. Chicken TLR21 acts as a functional homologue to mammalian TLR9 in the recognition of CpG oligodeoxynucleotides. Mol. Immunol. 46, 3163–3170.

Carballeda, J.M., Chimeno Zoth, S., Gómez, E., Gravisaco, J.M., Berinstein, A., 2011. Activation of the immune response against Infectious Bursal Disease Virus after intramuscular inoculation of an intermediate strain. Immunobiology 216, 1028–1033.

Chimeno Zoth, S., Gómez, E., Carballeda, J.M., Carrillo, E., Berinstein, A., 2011. Expression of a secreted version of the hemagglutinin–neuraminidase glycoprotein of *Newcastle disease virus*: its evaluation as a diagnostic reagent. J. Vet. Diag. Invest. 23, 519–523.

Chimeno Zoth, S., Gómez, E., Carballeda, J.M., Taboga, O., Carrillo, E., Berinstein, A., 2009. Potential use of the hemagglutinin–neuraminidase glycoprotein of Newcastle disease virus expressed in Rachiplusia nu larvae as an immunogen for chickens. Clin. Vaccine Immunol. 16, 775–778.

Facciabene, A., Aurisicchio, L., La Monica, N., 2004. Baculovirus vectors elicit antigen-specific immune responses in mice. J. Virol. 78, 8663–8672.

Göbel, T.W.F., 2000. Isolation and analysis of natural killer cells in chickens. Method. Mol. Biol. 121, 337–345.

Göbel, T.W.F., Kaspers, B., Strangassinger, M., 2001. NK and T cells constitute two major, functionally distinct intestinal epithelial lymphocyte subsets in the chicken. Int. Immunol. 13, 757–762.

Griess, P., 1879. Bemerkungen zu der abhandlung der H.H. Weselsky und Benedikt "Ueber einige azoverbindungen". Chem. Ver. 12, 426–428.

Gronowski, A.M., Hilbert, D.M., Sheehan, K.C., Garotta, G., Schreiber, R.D., 1999. Baculovirus stimulates antiviral effects in mammalian cells. J. Virol. 73, 9944–9951.

Han, Y., Niu, M., An, L., Li, W., 2010. Involvement of TLR21 in baculovirus-induced interleukin-12 gene expression in avian macrophage-like cell line HD11. Vet. Microbiol. 144, 75–81.

Hervas-Stubbs, S., Rueda, P., Lopez, L., Leclerc, C., 2007. Insect baculoviruses strongly potentiate adaptive immune responses by inducing type I IFN. J. Immunol. 178, 2361–2369.

Keestra, A.M., de Zoete, M.R., Bouwman, L.I., van Putten, J.P., 2010. Chicken TLR21 is an innate CpG DNA receptor distinct from mammalian TLR9. J. Immunol. 185, 460–467.

Lu, L., Yu, L., Kwang, J., 2007. Baculovirus surface-displayed hemagglutinin of H5N1 influenza virus sustains its authentic cleavage, hemagglutination activity, and antigenicity. Biochem. Biophys. Res. Commun. 358, 404–409.

MacMicking, J., Xie, Q.W., Nathan, C., 1997. Nitric oxide and macrophage function. Annu. Rev. Immunol. 15, 323–350.

Niu, M., Han, Y., Li, W., 2008. Baculovirus up-regulates antiviral systems and induces protection against infectious bronchitis virus challenge in neonatal chicken. Int. Immunopharmacol. 8, 1609–1615.

O'Reilly, D.R., Miller, L.K., Luckow, V.A., 1994. Titering virus stocks. In: O'Reilly, D.R., Miller, L.K., Luckow, V.A. (Eds.), Baculovirus Expression Vectors, A Laboratory Manual. W.H. Freeman, New York, NY, pp. 130–132.

Strauss, R., Hüser, A., Ni, S., Tuve, S., Kiviat, N., Salif Sow, P., Hofmann, C., Lieber, A., 2007. Baculovirus-based vaccination vectors allow for efficient induction of immune responses against *Plasmodium falciparum* circumsporozoite protein. Mol. Ther. 15, 193–202.

Thiem, S.M., 1997. Prospects for altering host range for baculovirus bioinsecticides. Curr. Opin. Biotechnol. 8, 317–322.

Via, S.T., Zu Altenschildesche, G.M., Doerfler, W., 1983. Autographa californica nuclear polyhedrosis virus (AcNPV) DNA does not persist in mass cultures of mammalian cells. Virology 125, 107–117.

Wu, Q., Fang, L., Wu, X., Li, B., Luo, R., Yu, Z., Jin, M., Chen, H., Xiao, S., 2009. A pseudotype baculovirus-mediated vaccine confers protective immunity against lethal challenge with H5N1 avian influenza virus in mice and chickens. Mol. Immunol. 46, 2210–2217.