Influence of immunomodulators of viral or bacterial origin and vaccine against Aujeszky's disease on the proportion of peripheral blood B cells in growing pigs

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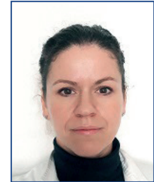

Abstract

The consequences of infection by Suid herpesvirus type 1 (SuHV-1) that causes Aujeszky's disease (AD) are well studied, however, the effects of immunomodulators (IMs) of microbial origin (viral and bacterial) when administered solely or in combination with the attenuated SuHV-1 vaccine are less known. The effects of parenteral administration of IMs, inactivated *Parapoxvirus ovis* (*P. ovis*) or a combination of inactivated *Propionibacterium granulosum* (*P. granulosum*) and detoxified *Escherichia coli* lipopolysaccharide (LPS) and attenuated SuHV-1, strain Bartha, on the proportion of peripheral blood $CD3^- CD21^+$ B cells were analysed in 30 crossbred, 3-month old pigs using flow cytometry (FCM). Specific antibodies for gE and gB of SuHV-1 were detected using the enzyme-linked immunosorbent assay (ELISA). Data were compared among six experimental groups: (1) pigs that separately received the vaccine, (2) IM of bacterial origin, (3) IM of viral origin, (4) simultaneous administration of the vaccine and bacterial IM, (5) simultaneous administration of the vaccine and viral IM, and (6) the control group of untreated pigs. Comparison of B

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cell proportions and the detection of specific antibodies in blood samples of vaccinated pigs on Day 11 of the experiment showed a transient decrease in B cell proportion, though this could not be assumed to be related since the control group showed a decrease in B cell content on the same day. The results showed that the use of IMs alone or in combination with the attenuated SuHV-1 vaccine did not have a significant impact on the proportion of peripheral blood B cells in growing pigs.

Key words: *B cells; pigs; flow cytometry; immunomodulators; Aujeszky disease*

Introduction

Numerous studies have confirmed that immunomodulators (IMs) of various origin (yeast derivatives, plant extracts and animal by-products) not only improve the production results in breeding and fattening of food animals, but may also stimulate a stronger non-specific immune response (Gallois and Oswald, 2008), especially in stress periods such as weaning of piglets (Li et al., 2005; Nofrarias et al., 2006; Zomborszky-Kovács et al., 2006; Nofrarias et al., 2007; Valpotić et al., 2016). Although, the effects of IMs administered together with vaccines against swine viral diseases have been investigated in numerous studies (Hiss et al., 2003; Biuk-Rudan et al., 2004; Terzić et al., 2004; Fraile et al., 2012; Šperanda et al., 2013), the mechanisms of their action are not yet fully clear.

IMs can be used alone or in combination with other substances with similar effects. For example, when *Propionibacterium granulosum* (*P. granulosum*) and *Escherichia coli* lipopolysaccharide (LPS) co-administered, they have a positive immunoregulatory effect on porcine innate immunity, *i.e.* NK cell activity and an early immune response which may contribute to protection against viral infections. Pigs that received PG/LPS had increased growth rate, and the PG/LPS combination proved to possess good adjuvant effects by enhancing the immune response to a vaccine prepared with the inactivated microorganism *Mycoplasma hyopneumoniae* and a vaccine with attenuated classical swine fever virus. It could be administered concomitantly in the field with vaccines, thus improving both non-specific and specific immune responses to enhance porcine resistance to infection (Lo et al., 2009).

Numerous studies of IMs of microbial origin have also been conducted with certain viruses. Biuk-Rudan et al. (2004) investigated the nonspecific immunoprophylactic effect in pigs when chemically inactivated *Parapoxvirus ovis* (*P. ovis*), strain D 1701 was administered ($10^{6.75}$ $TCID_{50}$/dose) and compared it with the results obtained in pigs vaccinated with gE^- vaccine against Aujeszky's disease (AD) and those obtained in control pigs after exposure to an experimental infection with a wild-type AD virus (Northern Ireland Aujeszky's-3, NIA-3). The IM did not affect the production of specific antibodies, making it less effective than vaccines in AD immunoprophylaxis. However, stimulating results were obtained as clinical signs of disease such as fever, spring frequency and weight loss were reduced or prevented.

Studies have been performed of the effect of *P. ovis*, strain D 1701, alone and in combination with other viral vaccines. The results of a study with a subunit E2 vaccine against classical swine fever administered concomitantly with *P. ovis* (strain D1701) as an IM showed that its effect was evident on T lymphocytes but not on B lymphocytes in piglets (Terzić et al., 2004).

The adaptive immune and the innate immune systems are intimately bound

together to ensure that an effective immune response occurs in response to microbial invasion. While it is possible for some viruses and many pathogenic bacteria to be taken up by dendritic cells, other mechanisms have been developed to recognize pathogens that lack the appropriate markers for recognition. Lymphocytes overcome the constraints placed on the innate immune system by recognizing an almost infinite diversity of antigens, thus targeting each pathogen specifically (Thacker, 2004). B cells are cells that, like T lymphocytes, originate from the lymphoid lineage of bone marrow cells, but mature in secondary lymphatic organs such as bone marrow, lymph nodes, spleen, and Payer plates. B cells are an essential component of the adaptive immune system and provide antibody-mediated protection against infections. After stimulation of surface immunoglobulin receptors, and in some cases co-stimulatory T helper cell-derived signals, they respond with proliferation and affinity maturation of the antigen receptor, and finally differentiate into circulating memory B cells or antibody-producing plasma cells (Braun et al., 2017). B cells specifically recognize antigens using the B-Cell Receptor (BCR). If B cells have not previously been in contact with the antigen, they must also be activated first, though unlike T cells, B cells can respond to the antigen directly without being previously shown as part of the Major Histocompatibility Complex (MHC).

The marker of B lymphocyte lineage is not known as for T cells, but studies have shown that porcine B lymphocytes can express multiple molecules such as CD2, CD25, CD21, IgM, SLA-DR, CD45RC, CD5 (Šinkora et al., 1998; Šinkora et al., 2003; Wilson and Wilkie, 2007) and mAb-reactive molecules for human CD79α, CD20 and CD86 molecules (Jones et al., 1993; Faldyna et al., 2007). The expression of these molecules differs during the foetal and postnatal life of pigs, as well as during the maturation and differentiation of B lymphocytes.

In general, inactivated B lymphocytes are thought to express IgM, CD2 and CD21 molecules and to lose CD21 molecule expression upon activation or contact with a foreign antigen. The CD21 molecule with the CD19 and CD81 molecules forms a complex that together with the BCR enables the activation of B lymphocytes. It is also a type 2 complement receptor, i.e., it binds the degradation products of the C3 component of the complement system and promotes the antigen presentation on B lymphocytes (Zabel and Weis, 2001). Plasmablasts originating from activated B lymphocytes also lose the expression of the IgM molecule, which after the transition to plasma cells lose the expression of the CD2 molecule. Memory B lymphocytes also lose CD2 molecule expression but retain IgM molecule expression (Šinkora and Butler, 2009).

Although AD has been eradicated in domestic pigs in many countries, Suid herpesvirus type 1 (SuHV-1) is still present in wild boar populations, as seen by serological surveillance and virus isolation (Hahn et al., 2010; Müller et al., 2010; Boadella et al., 2012). The same authors concluded that infected wild boars pose a constant threat for the transmission of SuHV-1 to domestic pigs.

Marker vaccines against AD usually contain SuHV-1, which lacks one or more viral envelope glycoproteins or a viral enzyme, or genes encoding these glycoproteins/enzymes. The development of diagnostic tests that allow for the differentiation of serum antibodies induced by virulent strains of SuHV-1 and marker vaccines against AD has also made it possible to distinguish between infected and vaccinated pigs (Van Oirschot et al., 1986).

The purpose of this study was to gain insight into the effects of the studied IMs

of microbial origin and the vaccine on changes in the proportion of peripheral blood B cells, as this could be useful in future research on the use of IMs of microbial origin as adjuvants for vaccines against AD and, thus, could expand the current applications of IMs in pig farming.

Materials and methods

Animals

The study was performed on 30 commercial crossbred pigs without antibodies for the AD virus. At the beginning of the study, pigs were about three months old, of both sexes and with an average bodyweight (bw) of 24 kg. All pigs were marked with ear tags and randomly assigned into six separate groups of five pigs each, followed by a seven-day period of acclimatization. They were fed *ad libitum* with commercial feed for fattening pigs and clinically monitored daily. Immune status, i.e., the presence of antibodies for glycoproteins B (gB) and E (gE) of AD virus was checked by the enzyme-linked immunosorbent assay (ELISA).

Vaccine and immunomodulators

All veterinary-medicinal products (VMPs) used during the experiment, i.e., two commercial VMPs of microbial origin with immunomodulatory effect and the vaccine, have been authorized for use in pigs in the Republic of Croatia. Both IMs and the vaccine were injected into the neck muscles according to the protocol listed in Table 1.

Vaccination was performed with a vaccine against AD (V_{AD}, Genera d.d., Croatia) that contains attenuated AD virus (SuHV-1, Bartha strain K/61 gE^-) and which has been in use in Croatia for decades. The reconstituted vaccine contains at least $10^{4.5}$ $TCID_{50}$ per 1 mL.

The immunomodulator of viral origin (IM_V, Zoetis SA, Belgium) contains inactivated *P. ovis*, strain D1701, and 1 mL contains the amount of virus that *in vitro* stimulates the formation of at least 230 interferon units.

The immunomodulator of bacterial origin (IM_B, Laboratorios Calier, Spain) contains LPS of *Escherichia coli* (0.02 mg/mL) and inactivated *P. granulosum* (0.25 mg/mL).

The study was conducted in accordance with ethical principles and approved by the institutional ethics committee and ethics committee of the Croatian Ministry of Agriculture (records No.: UP/I-322-01/14-01/86, file No.: 525-10/0255-14-2).

Table 1. Administration of the IMs (IM_V or IM_B) and V_{AD} with blood sampling

Applied IM_V* and IM_B**, V_{AD}*** and blood sampling (✓) according to the days (D) of the experiment					
Group	D1	D4	D7	D11	D18
1.	IM_B ✓	IM_B, V_{AD}	✓	✓	✓
2.	IM_B ✓	IM_B	✓	✓	✓
3.	IM_V ✓	IM_V, V_{AD}	✓	✓	✓
4.	IM_V ✓	IM_V	✓	✓	✓
5.	✓	V_{AD}	✓	✓	✓
6.	✓	-	✓	✓	✓

* 2 mL/animal; ** 1 mL/10 kg bw; *** 1 mL/animal

Blood sampling and sample preparation

Blood samples were collected by venepuncture from the anterior vena cava on Days 1, 7, 11 and 18 of the experiment.

Blood (10 mL) for the detection of serum antibodies against gE and gB of SuHV-1 was collected into sterile tubes without an anticoagulant. After blood clotting in tubes without an anticoagulant, sera were further separated by centrifugation at 2500 rpm for 10 minutes (Rotina 420, Hettich, Germany) and stored in new tubes, after which they were ready for analysis.

Blood samples (6 mL) for isolation of peripheral blood mononuclear cells (PBMCs) were collected in sterile tubes containing the anticoagulant lithium heparin. Isolation of PBMCs from peripheral blood samples was performed according to Andrišić et al. (2020).

Detection of serum antibodies for gE and gB SuHV-1

Commercial ELISA tests HerdChek Anti-PRV gpI (IDEXX Laboratories, USA) and HerdChek Anti-PRV gB (IDEXX Laboratories, USA) were used to detect serum antibodies to gE (former name gI) and gB of SuHV-1. Samples were analysed according to the manufacturer's instructions, and the absorbance was measured at 650 nm using a spectrophotometer (Tecan Sunrise Basic, Austria). Results were expressed as sample/negative (S/N) value and the test results were interpreted as follows: serum sample contains antibodies to gE and gB of SuHV-1 if S/N is less than or equal to 0.60; a sample is suspect if S/N is less than or equal to 0.70 and greater than 0.60; and serum sample does not contain antibodies for gE and gB of SuHV-1 if S/N is greater than 0.70.

The presence of antibodies to gE and gB indicates exposure to the field strains of SuHV-1 and/or vaccine containing gE antigen.

Immunophenotyping of PBMCs

Isolated PBMCs were defrosted in a water bath (38 °C), washed twice in RPMI 1640 medium and counted in each sample using a Bürker-Türk haemocytometer. The samples of PBMCs were then washed in phosphate-buffered saline (PBS) buffer solution and dead cells were stained with Zombie Aqua™ Fixable Viability Dye (BioLegend, USA) according to the manufacturer's instructions. After washing in PBS, the samples were adjusted to 2.5×10^6 PBMC/mL and 180 μL of each PBMC sample was transferred into tubes for fluorescence-activated cell sorting (FACS). Then, PBMC were mixed with mouse serum IgG (Invitrogen, USA) to block unspecific staining, and monoclonal antibodies (mAbs) were added. These mAbs were commercially available and were specific for porcine CD3 (epitope ε) and CD21 were directly stained with fluorochromes (mAb for CD3 with Alexa Fluor® 405; Novus Biologicals, USA and mAb for CD21 R-phycoerythrin; Southern Biotech, USA). After 30 minutes of incubation at 4 °C, the labelled PBMCs were washed in PBS and fixed with 2% (w/v) formaldehyde in PBS (pH=7.4).

Flow cytometry analysis (FCM)

The FCM analysis of labelled PBMCs was performed using a BD LSR II flow cytometer (BD Biosciences, USA) equipped with 405 nm, 488 nm and 640 nm lasers. Live individual lymphocytes (1×10^4 per sample) were acquired using FACSDiva Software Version 5.0.3 (BD Biosciences, USA). After that, the proportion of B cells ($CD3^-CD21^+$) within lymphocytes was analysed. Raw data were analysed by Flow Jo Software Version 7.6.5 (FlowJo, LCC, USA), and the results are shown in Figure 1.

Statistical analysis

Statistical analysis was performed using Stata version 13.1 (STATA Corp.,

USA). Using ANOVA, the relative values of the proportion of B cells were compared among all groups of animals at each sampling (*post-hoc* Tukey's test). The observed differences in relative values of B cells between time points within the same group were verified by repeated measures ANOVA (*post-hoc* paired t-test). Statistical analysis of results that did not follow normal distribution was performed using Kruskal-Wallis test. The results of statistical comparison, i.e., P values less than 0.05 were considered statistically significant.

Results and Discussion

When the immune status was checked in the serum samples of the pigs selected for the experiment, antibodies for gB and gE of SuHV-1 (R > 0.70) were not detected at the beginning of the study (Day 1) or after the second blood collection (Day 7). In the current study, the absence of specific antibodies for SuHV-1 at the beginning of the experiment served only to confirm that the pigs had not been exposed to the AD virus from the field or that they originated from vaccinated sows. After the third blood collection (Day 11), antibodies for gB of SuHV-1 (S/N < 0.60) and no antibodies for gE of SuHV-1 (S/N > 0.70) were detected in the serum of vaccinated pigs (pigs in the 1st, 3rd, and 5th groups), whereas antibodies for neither gB of SuHV-1 nor gE of SuHV-1 (S/N > 0.70) were present in the sera of the pigs in the 2nd, 4th, and 6th groups. After the fourth blood collection (Day 18), the finding of SuHV-1-specific antibodies was similar to that after Day 11.

Since the marker for pig B lymphocytes is not well established, they were immunophenotypically defined in this experiment as lymphocytes that do not express the T cell marker (CD3)

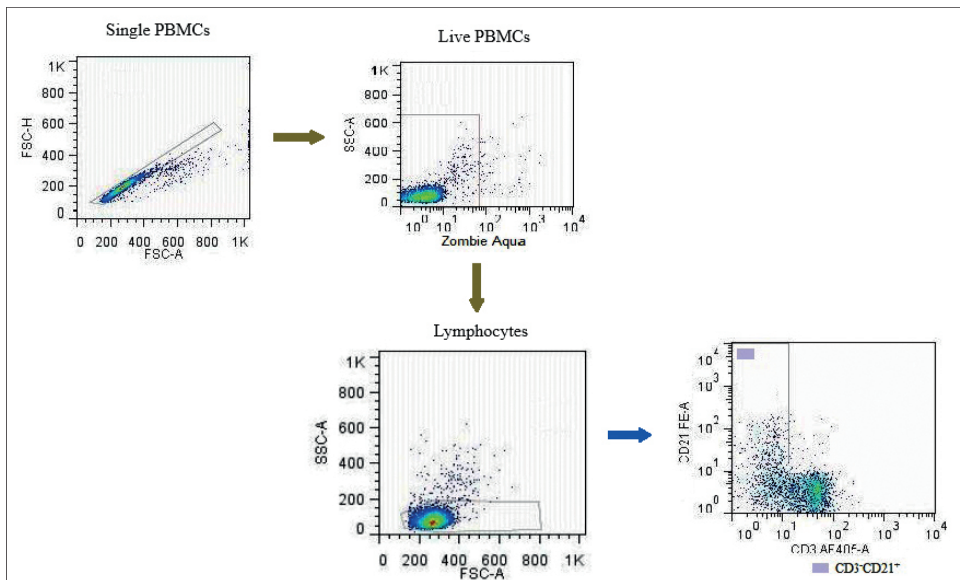

Fig. 1. Gating strategy for FCA: gating was first applied to (A) single PBMC based on FSC-H (Forward Scatter-Hight) and FSC-A (Forward Scatter-Area), then to (B) live PBMC based on labelling with Zombie Aqua™ and then to (C) lymphocytes based on SSC-A (Side Scatter - Area) and FSC-A. (D) Lymphocytes were then analysed for expression of CD3 (CD3 AF 405 - Alexa Fluor® 405) and CD21 (CD21 FE - phycoerythrin) cell surface differentiation antigens

but express the CD21 molecule ($CD3^{-}CD21^{+}$). Although the CD21 molecule is not specific only for B lymphocytes, it can be assumed with high probability that pigs PBMC in the lymphocyte Gate expressing the CD21 molecule are B lymphocytes, since this molecule in pigs is also expressed on follicular dendritic cells that can be mostly found in lymph nodes (Piriou-Guzylack and Salmon, 2008).

At the beginning of this experiment, the mean value of the proportion of B cells within total peripheral blood lymphocytes (PBL) for all groups was similar to that for crossbred pigs of a similar age (6.9%), as reported by Terzić et al. (2002).

The mean value of the proportion of B cells within PBL (Table 2) for all groups of pigs at the first day of the experiment was 5.93%. The dynamics of the mean value of the B cells content in all groups was similar throughout the experiment, i.e., an increase between Day 1 and Day 7, a decrease between Day 7 and Day 11, and an increase between Day 11 and Day 18. Only group 4 showed a decrease in the mean value of the proportion of B lymphocytes between Day 11 and Day 18. However, no statistically significant differences were found in the mean value of the B cell content between groups during the period of blood sampling, nor between individual days of blood sampling in the same groups (Table 2).

On Day 11, a transient decrease was recorded in the mean value of the B cells proportion in all groups receiving the vaccine, and on Day 11, serum antibodies for gB of SuHV-1 were detected for the first time in those same groups. These findings could indicate higher activation of B cells and transformation to plasmablasts. According to Šinkora and Butler (2009), after contact with the antigen, B cells transform into plasmablasts that lose the expression of the CD21 molecule. However, this finding could not be confirmed in our study because the control group also recorded a decrease of B cell proportion on Day 11. These results were obtained according to the predicted experimental protocol; however, earlier or later and more frequent blood sampling for cytometric analysis after the application of the investigated IMs, could give more precise answers about their effect on B cell kinetics in growing pigs.

Our observations and the presented results could be useful to complement the existing knowledge and to plan the future research of adjuvant effects of IMs in pigs vaccinated with the attenuated virus. Experimental infection would give more accurate results. However, as this was only a pilot study, additional research

Table 2. Kinetic of proportions of B cells ($CD3^{-}CD21^{+}$) within PBL in the experimental groups of pigs at days of blood sampling

Mean value and standard deviation of the mean value (% ± SD)/day of sampling (D)				
Group	D1	D7	D11	D18
1. (IM_B+V_{AD})	5.66 ± 4.23	9.74 ± 4.57	5.10 ± 2.37	6.54 ± 1.22
2. (IM_B)	5.75 ± 2.92	7.82 ± 2.30	4.79 ± 3.15	4.57 ± 1.47
3. (IM_V+V_{AD})	6.29 ± 3.37	7.43 ± 1.21	4.59 ± 0.89	7.28 ± 3.44
4. (IM_V)	5.24 ± 1.71	5.40 ± 2.39	6.13 ± 2.11	4.65 ± 1.76
5. (V_{AD})	6.56 ± 3.65	10.53 ± 5.70	8.66 ± 5.63	10.01 ± 5.75
6. (CG*)	6.11 ± 3.02	9.87 ± 3.29	4.96 ± 1.84	6.13 ± 2.78

* Control group

and measurement tools are required to establish correlations between B cells and antibody levels in the peripheral blood of pigs.

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Utjecaj imunomodulatora virusnog ili bakterijskog podrijetla i cjepiva protiv bolesti Aujeszkoga na udio B limfocita periferne krvi u tovljene prasadi

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Posljedice infekcije herpes virusom-1 svinja (SuHV-1) uzročnikom bolesti Aujeszkoga (BA) dobro su istražene, ali manje je poznat učinak imunomodulatora (IM) mikrobnog podrijetla (virusnog ili bakterijskog) primijenjenih u kombinaciji s atenuiranim cjepivom protiv BA. Učinak parenteralne primjene IM-a, inaktiviranog virusa *Parapoxvirus ovis* ili kombinacije inaktivirane bakterije *Propionibacterium granulosum* i lipopolisaharida bakterije *Escherichia coli* i cjepiva koje sadržava atenuirani virus BA (SuHV-1, soj Bartha) na udio $CD3^-CD21^+$ B limfocita periferne krvi analiziran je protočnom citometrijom u krvi 30 komercijalnih križanaca svinja u dobi od tri mjeseca bez protutijela za virus BA. Prisustvo je protutijela za glikoproteine B (gB) i E (gE) virusa BA provjereno imunoenzimnim testom (engl. *Enzyme Linked Immunosorbant Assay*, ELISA). Dobiveni su rezultati uspoređeni između 6 pokusnih skupina tj. između svinja koje su primile: cjepivo (1. skupina), IM bakterijskog podrijetla (2. skupina), IM virusnog podrijetla (3. skupina), svinja koje su istovremeno primile cjepivo i IM bakterijskog podrijetla (4. skupina) ili cjepivo i IM viruisnog podrijetla (5. skupina) te netretirane, kontrolne skupine svinja (6. skupina). Usporedba udjela B limfocita u uzorcima krvi cijepljenih svinja 11. dan pokusa pokazali su prolazno smanjenje srednje vrijednosti udjela B limfocita za koje se nije moglo pretpostaviti da je povezano s pojavom specifičnih protutijela, jer je smanjenje srednje vrijednosti udjela B limfocita bilo i u kontrolnoj skupini istoga dana. Naši su rezultati pokazali da primjena samo IM-a, kao i njihovih kombinacija s atenuiranim cjepivom protiv BA, nisu imali značajan utjecaj na udio B limfocita periferne krvi u tovljene prasadi.

Ključne riječi: *B limfociti, svinje, protočna citometrija, imunomodulatori, bolest Aujeszkoga*