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Immunomodulatory properties of Beta-sitosterol in pig immune responses

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ABSTRACT

The ability to control an immune response for the benefit and production efficiency of animals is the objective of immunomodulation in food-producing animals; substances that exert this control are called immunomodulators. A Spanish product (Inmunicín MAYMO®), based on food plant phytosterols, is being commercialized as complementary feed. The main component of this product is Beta-sitosterol (BSS). BSS and its glycoside (BSSG) have been shown to exhibit anti-inflammatory, anti-neoplasic, anti-pyretic and immune-modulating activity demonstrated by *in vitro* and *in vivo* experiments. The objective of the present study was to characterize the effect of BSS on the pig immune system using *in vitro* cell cultures first and to elucidate whether BSS possesses any *in vivo* activity in fattener pigs after vaccination with porcine reproductive and respiratory syndrome virus (PRRSV) modified life vaccine (MLV). Firstly, our *in vitro* results showed that BSS increased viable peripheral blood mononuclear cell (PBMC) numbers and it activated swine dendritic cells (DCs) in culture. Secondly, pigs treated with phytosterols prior to vaccination with PRRSV-MLV vaccine exhibited some changes in immunological parameters at different times post-vaccination, such as the proliferation ability of PBMC after phytohemaglutinin stimulation and increased apolipoprotein A1 plasma concentration which may contribute to enhance PRRSV vaccine response. In conclusion, the data in this report show that BSS can be considered an immunomodulator in pigs.

1. Introduction

The use of immunomodulators could be a useful approach to enhance immune responses after vaccination or to overcome infectious diseases in swine [1]. Broad categories of immunomodulators include cytokines, pharmaceuticals, microbial products, nutraceuticals, and traditional medicinal plants. A nutraceutical is the part of the food that provides medical or health benefits, including prevention or treatment of disease [2]. Immunomodulators authorized in Europe for swine are usually administered alone or combined with vaccines by the parenteral route [3–5]. On the other hand, administration of products by the oral route is more suitable when the objective is to treat a large population of animals.

Inmunicin MAYMO®, based on food plant phytosterols, is being commercialized as complementary feed in Spain. The main component of this product is Beta-sitosterol (BSS). BSS and its glycoside (BSSG) have been shown to exhibit anti-inflammatory, anti-neoplasic, anti-pyretic and immune-modulating activities, both in *in vitro* and *in vivo* [6]. In humans, BSS seems to target specific T-helper (Th) lymphocytes, increasing Th1 activity and improving T-lymphocyte and natural killer (NK) cell activity [7,8]. At present, no information is available on its possible effects on the pig immune system.

Porcine reproductive and respiratory syndrome virus (PRRSV), a member of the *Arteriviridae* family, is widespread among swine populations, causing reproductive failure in sows and respiratory disorders in pigs [9]. The studies on PRRSV pathogenesis have shown a complex interaction between the virus and host's inflammatory and immune responses. In fact, contact with wild-type PRRSV, as well as with PRRS modified-live virus (MLV) vaccine, induces an unusual immunity response that probably plays a negative role, delaying virus elimination from the organism or avoiding the achievement of sterile immunity [9]. Therefore, this virus has been considered a suitable model to assess the *in vivo* effect of an immunomodulator. Our working hypothesis was that a substance that seems to target specific T-helper lymphocytes increasing Th1 activity, resulting in improved T-lymphocyte and natural killer cell activity [7] in humans and other animal species, would be potentially beneficial for pigs when PRRSV-MLV vaccinated.

Acute phase proteins (APPs) are produced by the liver upon stimulation by the pro-inflammatory cytokines such as interleukin-1β and -6 (IL-1β and IL-6) and tumor necrosis factor-α (TNF-α), in response to

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infection, inflammation or trauma [10] and the main APPs in pigs are haptoglobin, CRP (C-reactive protein) and Pig-MAP (Major Acute Phase protein). Moreover, alterations of lipoprotein concentrations have also been reported in several animal models after an acute injury [11,12]. High-density lipoprotein (HDL)-associated apolipoprotein (APOA1) is a negative acute-phase protein, *i.e.* a protein whose level is lowered by more than 25% during the acute phase reaction and this protein also exerts anti-inflammatory functions in both acute and chronic inflammation [13]. Thus, lipoprotein concentration after the administration of a substance with potential activity as an immunomodulator was evaluated.

The objective of the present study was to characterize the effect of phytosterols on the pig immune system using *in vitro* studies and to evaluate whether oral administration of these phytosterol mixture has an effect on the immune responses in fattener pigs after PRRS-MLV vaccination.

2. Materials and methods

2.1. Cells and viruses

Peripheral blood mononuclear cells (PBMCs) from healthy animals were separated by a density gradient centrifugation using Histopaque 1.077 (Sigma, Madrid, Spain) and were cultivated in RPMI-1640 (LONZA, Switzerland) supplemented with 5% fetal calf serum (FCS), 10 mM HEPES, 2 mM L-glutamine, 1% penicillin and streptomycin, and 0.05 mM 2-mercaptoethanol. Total PBMCs were cultured in the presence of variable concentrations of Inmunicin MAYMO®. After 16 h of culture, absolute numbers of PBMCs were estimated by counting viable cells after trypan-blue staining.

Bone marrow hematopoietic cells (BMHCs) were isolated from femurs of three healthy Large white × Landrace pigs of eight weeks of age, free from porcine reproductive and respiratory syndrome virus (PRRSV) and negative for type-2 porcine circovirus (PCV2) and influenza virus. BMHCs were cultured in RPMI medium, supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml polymyxin B (Sigma) and 1% penicillin/streptomycin. Bone marrow derived dendritic cells (BMDCs) were generated from BMHCs as previously described [14,15] with some modifications [16]. For cell stimulation, 0.8–1×10^6 BMDCs were cultured in flat-bottom 96-well plates at 37 °C and 5% CO_2. For studying BMDC responses, either 10 μg/ml (12 μM of BSS) or 100 μg/ml (123 μM of BSS) of Inmunicin MAYMO® was added to the cultures during overnight incubation. Photographs were taken using a NIKON ECLIPSE TS100 microscope at 200× magnification. D19, a well known oligodeoxyribonucleotide IFN-α inducer [17], was used as positive control. The vaccine strain of pseudorabies virus (PRV) was propagated in PK-15 cells. Cell culture supernatants from infected cells were titrated and inactivated by treatment with ultraviolet light (UV) for 30 min. This inactivated PRV was used for stimulation of DCs at a concentration equivalent to a multiplicity of infection (MOI) of 0.1. MOI was determined before inactivation. Finally, a PRRS-MLV European strain (Porcilis© PRRS, MSD Animal Health) vaccine was used for the *in vivo* studies.

All the *in vitro* experiments were performed in triplicates using at least three different animals. The cell culture supernatants from triplicates were aliquoted and stored at −80 °C for further cytokine analysis.

2.2. Cytokine detection

Anti-IFN-α monoclonal antibodies (K9 and K17) and IFN-α recombinant protein (PBL Biomedical laboratories, Piscataway, New Jersey) were used to quantify this cytokine using an ELISA assay as previously described [18]. IL-10 was tested by ELISA assay using an IL-10 detection kit following the manufacturer's instructions (DuoSet® Development System, R&D Systems Europe, UK). Our ELISA detection limit was 4 U/ml for IFN-α and 33 pg/ml for IL-10.

2.3. Animals

Twenty-four male 10-week-old pigs were included in the experiment. Animals were negative for porcine reproductive and respiratory syndrome virus (PRRSV) by RT-PCR as previously described [19]. All the animals were fed with standard feed according to the National Research Council recommendations [20] but twelve animals were treated with Inmunicin MAYMO® (identified as P) mixed with its feed and according to the product characteristics (2 kg of Inmunicin MAYMO® per ton of final feed) for eight weeks (from 10 to 18 weeks of age) whereas 12 animals did not receive Inmunicin MAYMO® (identified as NP). At 13 weeks of age, 12 animals were vaccinated with a PRRS-MLV European strain (Porcilis© PRRS, MSD Animal Health) vaccine (identified as V) and 12 animals received 2 ml of saline physiologic serum (identified as NV). This experimental design included four experimental groups: NP/NV, NP/V, P/NV and P/V. Pigs were housed on a production farm where the same air space was shared between them but without direct contact (nose to nose) between pigs. Blood samples were collected at 0, 1, 2 days post-administration of vaccine or physiological serum.

2.4. Plasma lipids

Total cholesterol concentration in plasma was measured in a microtiter assay, using Infinity™ commercial kits (Thermo Scientific, Madrid, Spain). Plasma HDL cholesterol was quantified in the supernatant by a fluorescent enzymatic method (Amplex Red, Molecular Probes, USA), after precipitation of apolipoprotein B particles with phosphotungstic acid-$MnCl_2$ (Roche, Barcelona, Spain). Apolipoprotein A1 (APOA1) was quantified by ELISA using specific polyclonal antibodies (Biodesign, Saco, ME) as previously described [21].

2.5. Flow cytometry and cell proliferation

Flow cytometry was performed using an indirect labeling for all the surface markers using hybridoma supernatants and direct labeling for FITC-CD172a (clone BL1H7, AbD Serotec). All hybridoma supernatants were kindly donated by Dr. J. Domínguez (INIA, Spain). The secondary antibody was R-Phycoerythrin anti-mouse IgG (Jackson ImmunoResearch, Suffolk, UK). Different subpopulations of PBMCs were determined by flow cytometry with the following antibodies: anti-CD8 (clone 76-2-11), anti-CD4 (clone 74-12-4), anti-CD45 (clone 2A5), anti-SLA-II (clone 2E9/13), anti-SLA-I (clone 4B7/8) and anti-CD21 (clone BB6-11C9.6). Briefly, 2.5×10^5 cells/50 μl/well were labeled for 1 h at 4 °C for each CD marker. After incubation, they were washed with cold PBS with 2% FCS by centrifugation at 450 g, 4 °C for 10 min. Then, the secondary antibody R-Phycoerythrin diluted 1:200 was added when required and cells were incubated for 1 h at 4 °C. Afterwards, they were washed as before and resuspended in PBS with 2% FCS. Stained cells were acquired on Coulter® EPICS XL-MCL cytometer and analyzed by EXPO 32 ADC v.1.2 program.

PBMCs were seeded at a density of 0.5×10^6 cells/well and were stimulated with phytohemaglutinin (PHA) at different concentrations. Unstimulated cells were used as negative controls (Mock). Proliferation was detected using the colorimetric cell proliferation BIOTRAK Kit from Amersham following the manufacturer's instructions and results were expressed as optical density at 450 nm.

2.6. Statistical analysis

Experimental groups were compared through ANOVA followed by Tukey-kramer post hoc test for multiple comparisons of unpaired observations. In the particular case of the proliferation study, the area

under the curve (AUC) of proliferation *versus* PHA at different concentrations was previously calculated by the trapezoidal rule with the NCSS 2004 and PASS 2005 software (Kavysville, Utah, USA).

The significance level was established at 0.05 and all the analyses were carried out with the NCSS 2004 and PASS 2005 (Kavysville, Utah, USA).

3. Results

3.1. Inmunicin MAYMO® effects on cell cultures

Total PBMCs from three healthy pigs were cultured in the presence of variable concentration of Inmunicin MAYMO® as shown in Fig. 1. After 16 h of culture, absolute numbers of PBMCs were higher when Inmunicin MAYMO® was present at a dose of 10 μg/ml (12 μM of BSS) or 100 μg/ml (123 μM of BSS) compared with untreated cells although only a statistical tendency ($p = 0.07$) was observed.

This result led us to investigate whether porcine dendritic cells (DCs) were modulated by Inmunicin MAYMO® *in vitro*. Dendritic cells, a heterogeneous population of hematopoietic cells, are the most potent antigen presenting cell in the body [22]. They play a versatile role in orchestrating immune responses against an array of invading pathogens, including viral infections. If DCs were activated by BSS, they might induce lymphocyte proliferation or prevent lymphocytes from cell death. To check this hypothesis, BMDCs from 3 healthy pigs were incubated with variable concentrations of the studied product. As shown in Fig. 2A, an increased DC aggregation after overnight treatment was observed. Homotypic aggregation of DCs has been related to DC activation mediated by MHC-II [23] in other systems. Therefore, we investigated surface markers such as SLA-II or SLA-I by flow cytometry. Levels of both molecules were significantly upregulated compared with untreated controls ($p<0.05$) in BMDC cultures after Inmunicin MAYMO® treatment using the highest dose (Fig. 2B).

Also, activation of DCs may involve IFN-α secretion after encountering any stimuli, particularly if a viral infection is present. The IFN-α concentration in DC cultures was 4 and 5 times higher than untreated control ($p<0.05$) when Inmunicin MAYMO® was added *in vitro* at a dose of 10 μg/ml (12 μM of BSS) or 100 μg/ml (123 μM of BSS) respectively (Fig. 3). No IL-10 production was detected in culture supernatants in these *in vitro* conditions.

In summary, BSS, the main component of Inmunicin MAYMO® has a detectable *in vitro* effect on cells from the porcine immune system by increasing the viability of PBMCs and by modulating DC activity.

3.2. Clinical examination and response to vaccination

In order to elucidate whether phytosterols possess *in vivo* activity, fattener pigs were treated as described in the Materials and methods section. None of the groups involved in the *in vivo* study (NP/NV, NP/V, P/NV and P/V) had any relevant clinical signs at any time point during Inmunicin MAYMO® oral treatment or during the vaccination period.

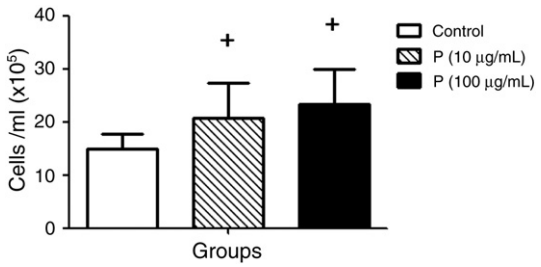

Fig. 1. Average viable PBMCs (number ± SD) after 16 h of culture in the absence (control) or presence of variable amounts of Inmunicin MAYMO® (P). Viable cells were estimated using trypan-blue staining. + indicates statistical tendency ($0.05<p<0.10$).

Treatment with Inmunicin MAYMO® did not cause any adverse reactions during the trial.

Firstly, no variation in cholesterol and HDL cholesterol concentration was observed among all the studied groups at 0, 1 and 2 days post vaccination. However, APOA1 plasma concentration was significantly higher in the groups that received Inmunicin MAYMO® (P/NV and P/V) in the diet than in the ones that did not receive this product (NP/NV and NP/V) at days 1 and 2 of the trial. Interestingly, the APOA1 concentration was very similar among the animals that received Inmunicin MAYMO® independently of whether they were vaccinated or not (Table 1).

Secondly, changes in PBMCs subpopulations during the first two days after PRRSV-MLV vaccination were analyzed. Total lymphocytes ($CD45^+$ cells), B cells ($CD21^+$ cells) and T lymphocytes subpopulations such as $CD4^+CD8^-$, $CD4^-CD8^+$and $CD4^+CD8^+$ in PBMCs did not exhibit any alteration in any group at this time point (data not shown). However, a general increase ($p<0.05$) was observed in the subpopulation phenotypically described as $CD172a^+$ $CD4^+$cells, namely plasmacytoid dendritic cells (pDCs) or natural interferon producing cells (NIPCs). All animals in group NP/V exhibited an increase in pDC at day 2 post-vaccination (Fig. 4). It is conceivable to think that this effect was produced by PRRSV-MLV vaccination. However, this pDC increase was completely abolished in all animals in group P/V, showing a pattern similar to the animals in groups P/NV and NP/NV. Interestingly, vaccinated animals treated with Inmunicin MAYMO® (P/V) showed the same profile as unvaccinated animals (NP/NV), which may suggest that treatment with Inmunicin MAYMO® would prevent some of the alterations in the immune system caused by viral infections such as the ones when applying the PRRS-MLV vaccine. In the NP/NV group, there were two animals with higher pDC numbers than controls at day 2 which could be explained by individual variations between pigs. IFN-α levels observed in serum were below the detection limit in all the experimental groups.

Finally, lymphocyte function measured as the ability to proliferate in the presence of different concentrations of PHA in total PBMCs at 0, 1 and 2 days post-vaccination was analyzed. PRRS-MLV vaccination induced a decrease in PHA proliferation responses in the untreated group of animals (NP/V) (4 animals out of 6) during the first two days after vaccination. In contrast, PBMCs from Inmunicin MAYMO® treated animals showed a better proliferation ability than their untreated counterparts although only a statistical tendency ($p = 0.1$) was observed (Fig. 5).

4. Discussion

In this study, our first goal was to characterize the effect of phytosterols on the swine immune system using *in vitro* cell cultures. Secondly, we tested whether these substances could also have an effect *in vivo* using PRRSV-MLV vaccination as a tool to decipher its effect on the immune system.

First of all, our results showed that Inmunicin MAYMO® had a detectable effect on the immune system by increasing PBMCs viability *in vitro* and by increasing the proliferation ability of PBMCs after PHA stimulation in vaccinated animals. Recently, Desai et al. [24] also described that BSS has an effect on proliferation of PBMCs in humans. This result could have multiple interpretations that are far from the scope of this paper, but the importance of decreasing cell death and/or inducing a certain degree of responsiveness might have a major impact on any immune response against an encountered pathogen.

The range of concentrations for *in vitro* studies was selected according to the information available in adult human serum collected after standard diets [25] where the observed BSS serum concentration ranged between 1.7 and 29 μg/ml. Thus, the *in vitro* immune effects of this phytosterol mixture using concentrations between 10 μg/ml (12 μM BSS) or 100 μg/ml (123 μM BSS) were considered reasonable

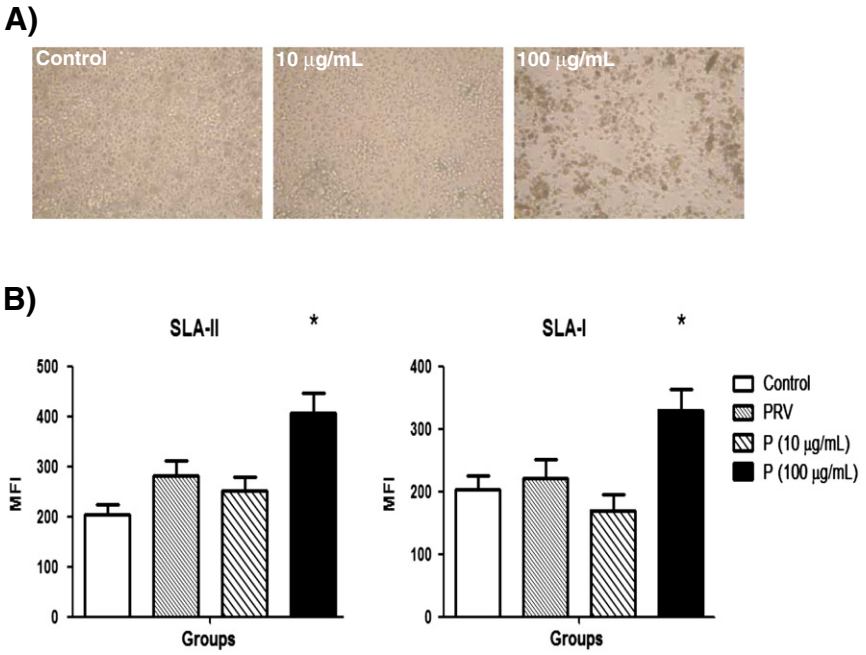

Fig. 2. BMDC activation in the presence of BSS. BMDCs were incubated with 0 (Control), 10 (12 μM of beta-sitosterol) or 100 μg/ml (123 μM of beta-sitosterol) Inmunicin MAYMO® (P). A) Photographs were taken with a NIKON ECLIPSE TS100 camera at 200× magnification. Homotypic aggregation was detected at 100 μg/ml. B) Mean fluorescence intensity (MFI) of BMDCs stained for SLA-I, and SLA-II after treatment. Herpesvirus porcine type I (PRV) was used as positive control. Representative results from three animals are represented.

taking into account that information on BSS serum concentration in swine is not presently available.

Given the pivotal importance of DCs in triggering immune responses, myeloid DCs were chosen to test the Inmunicin MAYMO® immunomodulatory effect *in vitro*. We used BMDCs because they are phenotypically and functionally well characterized [16,26]. Also, BMDCs have been extensively used as an *in vitro* model to test immune modulators before carrying out experimental studies [27,28]. BMDCs tend to aggregate in response to stimuli by upregulating surface stimulation molecules and the same pattern was observed when Inmunicin MAYMO® was present in BMDC cultures; indeed, SLA-II or SLA-I were upregulated at the cell surface. These changes were similar to those observed in DCs after interaction with a pathogen and they are generally associated with an efficient presentation of endocytosed and processed antigen [26].

Similar to other species, it has been shown that swine DCs can modulate the type of T-cell response induced [26] since the Th1/Th2 profile will be influenced by the nature of each antigen presented as well as by the cytokine environment. In our *in vitro* conditions, stimulation of BMDCs with Inmunicin MAYMO® seems to drive the BMDC response towards a Th1 pattern with IFN-α secretion and absence of IL-10. These results agree with data published by Yuk et al. [29] suggesting that beta-sitosterol (the main component of Inmunicin MAYMO®) could be a potential therapeutic molecule in asthma [30] due to its ability to drive the immune response towards a Th1 response. Moreover, Lee et al. [31] showed that daucosterol, a beta-sitosterol glycoside, had an immunomodulating activity that mediated induction of Th1-dominant cytokine production from $CD4^+$ T cells and this profile is involved in protection of mice against disseminated candidiasis. In this disease, driving Th1 or Th2 responses correlates with the severity of the fungal infection and Th1-type dominance can reduce severity. Finally, Brull et al. [32] demonstrated that sitosterol is able to shift immunity towards a Th1 dominant response in humans, at least *ex vivo*, and this effect is a plant sterol specific one and not a sterol effect in general. Moreover, they clearly demonstrated that TLR2 activation was essential to induce this Th1 shift in human PBMCs by plant stanols and plant sterols. These authors hypothesized that the consumption of these substances in the diet by subjects suffering from a disease caused by a Th2 dominant immune response may have beneficial health effects. Indeed, the data presented in this paper fully agree with published data in other systems, this being the first in pigs. Until now, the mechanisms of action

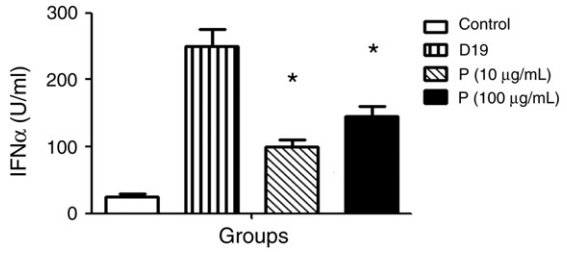

Fig. 3. IFN-α production in BMDCs treated with Inmunicin MAYMO® (P) at 0 (control), 10 (12 μM of BSS,) or 100 μg/ml (123 μM of BSS) or in the presence of D19 as positive control. Asterisk indicates statistically significant differences ($p<0.05$) *versus* control.

Table 1
Cholesterol, HDL-cholesterol and APOA1 concentration determined in pig plasma after applying a PRRSV-MLV European strain vaccine (V) or placebo (NV) in pigs that were receiving a standard diet (NP) or a standard diet supplemented with Inmunicin MAYMO® (P). Vaccine or placebo was administered at day 0. See Materials and methods section for further details.

		NP-NV	NP-V	P-NV	P-V
Cholesterol (mmol/l)	Day 0	95 ± 13	86 ± 12	99 ± 11	95 ± 13
	Day 1	110 ± 20	110 ± 15	118 ± 19	113 ± 11
	Day 2	87 ± 10	81 ± 16	96 ± 12	92 ± 13
HDL-cholesterol (mmol/l)	Day 0	25 ± 4	26 ± 4	28 ± 4	27 ± 4
	Day 1	28 ± 6	29 ± 3	29 ± 4	30 ± 5
	Day 2	23 ± 4	26 ± 9	27 ± 4	29 ± 4
APOA1 (mg/ml)	Day 0	3.7 ± 0.4	3.5 ± 0.4	4.2 ± 1.0	4.2 ± 1.0
	Day 1	3.0 ± 0.3	2.9 ± 0.5	3.7 ± 0.6*	3.6 ± 0.8
	Day 2	3.7 ± 0.7	4.0 ± 1.4	5 ± 1.2*	5.2 ± 1.6*

* $p \leq 0.05$ any group *versus* NP-NV.

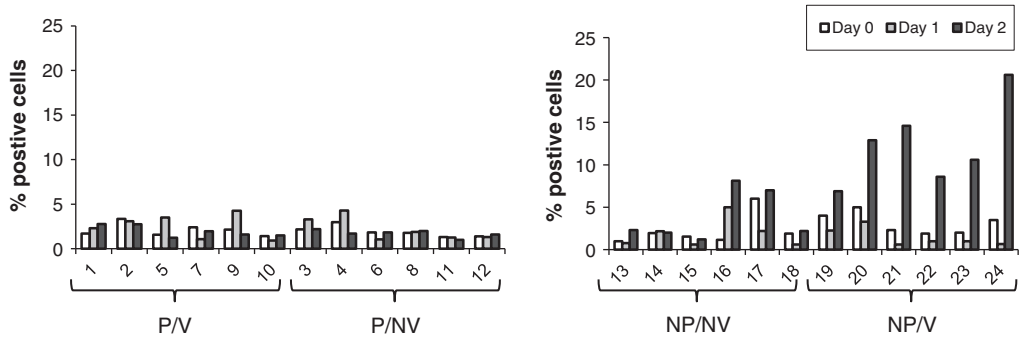

Fig. 4. Percentage CD172a$^+$ CD4$^+$ PBMC subpopulation analyzed by flow cytometry. PBMCs were obtained from animals fed with Inmunicin MAYMO® (P) in the diet or not (NP) or vaccinated with PRRSV-MLV vaccine (V) or not (NV). Blood samples were collected at day 0 (white bars), 1 (gray bars) and 2 (black bars) post-vaccination. Stained cells were acquired using a Coulter® EPICS XL-MCL cytometer and analyzed by EXPO 32 ADC v.1.2 program. See the Materials and methods section for details. Data from all animals are shown.

of plant sterols in pigs have not been described but it is reasonable to believe that TLR2 activation, at least in DCs, might be involved because TLR2 is a highly conserved molecule between species.

Our results demonstrated that oral administration of this phytosterols mixture has an effect on the innate immune response in fattener pigs after PRRS-MLV vaccination. Thus, a significant increase was observed in pDCs of untreated vaccinated group (NP/V) when compared with animals treated with Inmunicin MAYMO® (P/V) at 2 days post-vaccination. Moreover, the P/V group showed the same profile as unvaccinated animals (NP/NV). It has been reported that the phenomena occurring in the initial phases after infection or vaccination with PRRSV could explain the unusual development of the cell-mediated response [9,33]. Interestingly, our results suggest that the effect induced by the virus at early stages of infection is overcome by the administration of the immunomodulator. It is well-known that, as occurs with other viral infections, PRRSV is highly susceptible to the action of IFN-α [34] which is mainly secreted by pDCs. The fact that pDCs are a subpopulation of cells whose main function is to secrete IFN-α upon viral challenge [35] led us to investigate whether IFN-α levels were altered in the serum of those animals. Attempts to detect IFN-α in serum were unsuccessful but this may not reflect local levels of this family of cytokines in target tissues.

Finally, it has been described in the literature that the lowering of HDL concentration upon acute inflammation might generate particular conditions favoring the development of chronic inflammation [12]. Following this line of thought, the increase in APOA1 elicited by Inmunicin MAYMO® could provide protection against maintenance of inflammation in a particular target tissue as well as decreasing the production of inflammatory cytokines at the systemic level [13]. This is a very interesting insight, considering the role of this protein not only in innate immunity [36] but also in the acute phase response [37]. Further studies are required to elucidate whether the immunomodulatory effect observed on the innate immune response after applying a PRRSV-MLV vaccine in this system may reflect a broadly improved viral response in pigs. This last point is especially important taking into account that very scarce information is available on the essential components of the immune system that are effective in protection against PRRSV infection [38].

Conflict of interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

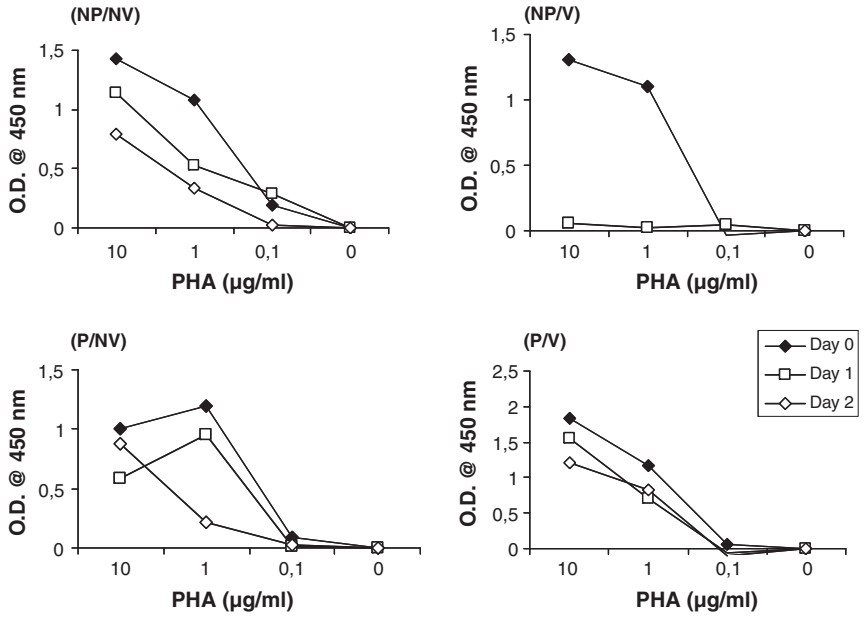

Fig. 5. Proliferation of PBMCs after stimulation with different concentrations of PHA or with mock. PBMCs were obtained from animals fed with Inmunicin MAYMO® (P) in the diet or not (NP) or vaccinated with PRRSV MLV vaccine (V) or not (NV). Blood samples were collected at day 0 (◆), 1 (□) and 2 (◇) post-vaccination. Data from one representative animal per group is shown and results are expressed as optical density at 450 nm.

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