



Modulation of the type I hypersensitivity late phase reaction to OVA by *Propionibacterium acnes*-soluble polysaccharide

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ABSTRACT

Late phase reaction (LPR) of immediate hypersensitivity is a Th2 response characterized by eosinophil recruitment and related to allergic asthma pathogenesis. Several strategies were developed trying to control the tissue damage observed in this reaction. Recently, we verified that killed *Propionibacterium acnes* (*P. acnes*), a Gram-positive bacillus, immunomodulated LPR in a murine model, potentiating or suppressing it depending on the treatment protocol used. However, the bacterium compounds responsible for this effect are not known, leading us to investigate if *P. acnes* purified soluble polysaccharide (PS) could be a major component involved on the modulation induced by the bacterium. Recently, we demonstrated that PS, like *P. acnes*, induces adjuvant effect on DNA vaccine, increases bone marrow dendritic cell precursors *in vivo* and its maturation *in vitro*, and modulates *in vitro* macrophage tumoricidal activity. Herein, we determined the chemical PS composition, which is mainly constituted by galactopyranose, ribopyranose, arabinopyranose, glucopyranose, ribofuranose and mannopyranose, and analyzed its capacity to modulate the immediate hypersensitivity in mice. Animals were subcutaneously implanted with coagulated hen's egg white (HEW) and 14 days later challenged with ovalbumin (OVA) in the footpad, developing a typical LPR after 24 h. Similarly to the whole bacterium, Th2 response to OVA was potentiated when PS was administered concomitantly to HEW implantation, by increase in footpad eosinophilia and IL-4-producing spleen cells, and decrease in anti-OVA IgG2a titers and IL-12- or IFN- γ -producing cells. On the other hand, the reaction was abrogated when HEW implantation was performed 1 week after PS-treatment, by decrease in footpad swelling, eosinophilia and anti-OVA IgG1 levels, and increase in IgG2a titers and IL-12-producing cells. These data suggest that PS seems to be the major *P. acnes* compound responsible for its effects on the modulation of immediate hypersensitivity reaction in mice.

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1. Introduction

The immediate hypersensitivity reaction is a Th2 mediated response followed by a late phase reaction (LPR), characterized by eosinophil infiltrate [1,2]. LPR underlies the pathogenesis of allergic asthma, in which the airway eosinophilia, the increased mucus production and structural remodeling lead to airway obstruction and bronchial hyperresponsiveness to inhaled allergen [3,4].

This reaction is mediated by specific immunoglobulin isotypes, such as IgE (humans and mice) and IgG1 (mice), induced by

Th2 cytokines, in particular IL-4, IL-13 and IL-5 [5,6]. IL-5 promotes eosinophils recruitment and differentiation, and these cells release pro-inflammatory cytokines that contribute to tissue damage [7,8].

It is expected that inhibition of Th2 cytokines may control over-expression of IgE and eosinophils [9], what have led to the development of several strategies to decrease Th2 responses to allergens through the Th1 pattern induction [10–12].

Propionibacterium acnes (*P. acnes*) is a Gram-positive bacillus which belongs to the normal human skin microflora [13,14], and it has been characterized as a typical Th1 response-inducing antigen [15–17]. In experimental models, killed *P. acnes* increases the endotoxic shock induced by LPS [18], activates the mononuclear phagocytic system [19], induces tumoricidal activity [20–22], acts

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as adjuvant for antibody response [23,24] and enhances resistance to infections [25–30]. The mechanisms implicated in these effects are related to a Th1 response, with increased production of IL-12, IL-18, IFN- γ , IL-1 α , IL-6 and TNF- α [31–35] and reduction of IL-4-producing CD4⁺ NKT cells [34].

The property of killed *P. acnes* to promote a Th1 response has led us, in a previous work, to study its effects on a murine model of type I hypersensitivity [36], described by Facincone et al. [37]. Accordingly, F₁ BALB/c $\times$ A/J mice were inoculated subcutaneously with coagulated hen's egg white (HEW) and 14 days later challenged with aggregated ovalbumin (OVA) in the footpad; a typical LPR of immediate hypersensitivity with intense eosinophilia was induced [37]. We demonstrated that when mice were pre-treated with killed *P. acnes* and sensitized with HEW after 1 week, the immediate hypersensitivity inflammation was abrogated, and mice developed a Th1 response after OVA challenge [36]. In contrast, when killed *P. acnes* was administered concomitantly to HEW implantation, the Th2 response to OVA was potentiated, with intense eosinophilia [36].

In this previous study, a commercially obtained phenol-killed *P. acnes* suspension was used [36], and it is known that polysaccharide components are preferentially exposed by phenol treatment. This led us to hypothesize that polysaccharide compounds could be implicated in the induction of Th1 or Th2 responses to OVA by the whole bacterium.

So we purified a soluble polysaccharide extracted from *P. acnes* cell wall (PS) and studied its biological effects. This polysaccharide, similarly to the whole bacterium, increased nitric oxide (NO) and TNF- α synthesis, enhanced phagocytic activity by peritoneal macrophages and promoted resistance to infection [38]. We also observed that PS was a major *P. acnes* component responsible for bacterium adjuvant effect to antibody response and IFN- γ production on a *Trypanosoma cruzi* DNA vaccine [24]. Besides, *P. acnes* and PS were able to modulate the number of different peritoneal cell populations and their functions, including macrophage tumoricidal activity [22]. We also demonstrated that PS was implicated in the *P. acnes* ability to induce increase in bone marrow CD34⁺ cells and their differentiation in dendritic cells (DCs) *in vivo* and *in vitro* [39]. This could be important in the immediate hypersensitivity modulation, since DCs are associated to Th1 or Th2 responses induction [40]. However, the fine structural characterization of this compound had not yet been performed.

Therefore, in the present work, we determined the carbohydrate composition of soluble PS and investigated if it is involved with the modulation of the Th2 immediate hypersensitivity reaction to OVA induced by *P. acnes*. Mice were treated with PS as described in our previous work for *P. acnes*-treatment [36] and submitted to the allergic model described by Facincone et al. [37]. Similarly to the whole bacterium [36], the Th2 response to OVA was abrogated when mice were sensitized with HEW 1 week after PS-treatment, but it was potentiated when PS was administered concomitantly to HEW implantation. These data suggest that PS is one of the major *P. acnes* compound responsible for its effects on Th1 or Th2 pattern modulation.

2. Materials and methods

2.1. Animals

Male or female 6-week-old F₁ hybrid mice (BALB/c $\times$ A/J) were used in all experiments. Experimental procedures were approved by the special University Ethics Committee for animal care and experimentation.

2.2. Heat-killed *P. acnes* suspension

P. acnes (obtained from Instituto Adolfo Lutz, SP, Brazil) was cultured in anaerobic medium (Hemobac – Probac, SP, Brazil) for 3 days and washed three times by centrifugation. The bacterial pellet was re-suspended in 0.9% NaCl and autoclaved for 20 min at 120 °C. The protein concentration was determined by Bradford method [41].

2.3. *P. acnes*-soluble polysaccharide extraction

P. acnes-soluble polysaccharide was obtained as described [22,24,39]. Briefly, to 30 mL of heat-killed bacteria suspension (1 mg/mL), 30 mL of 90% phenol were added and the suspension was incubated for 10 min in a water bath at 70 °C, based on the Palmer and Gerlough protocol for polysaccharide extraction [42]. After centrifugation at 2000 $\times$ g under refrigeration, the water and intermediate (polysaccharide enriched) phases were collected. Water was added to phenol phase and this procedure was repeated three times. The aqueous and intermediate phases were pooled and the polysaccharide was precipitated with 3 volumes of ethanol. The suspension was kept at 4 °C overnight, and the polysaccharide was collected by centrifugation. The pellet was dried under reduced pressure, re-suspended in distilled water and designated soluble polysaccharide (PS). The carbohydrate concentration was determined by the Dubois method [43], and solutions were prepared based on total carbohydrate/mL. No protein was detected by Bradford method [41].

The apparent molecular weight of PS was estimated by high performance liquid chromatography using a TSG G2000 SW column, and mannan of 34–64 kDa (Sigma, MO, USA) was used as internal standard. The polysaccharide elution profile was determined by the Dubois method [42].

2.4. Sample preparation for polysaccharide characterization

Soluble polysaccharide from *P. acnes* (40 μ g) was heated at 85 °C overnight in 1 mL of 3 N methanolic hydrochloric acid. After cooling to room temperature, the mixture was extracted with 1 mL of hexane (Merck, Darmstadt, Germany), the lower phase was evaporated to dryness at 40 °C under nitrogen and further dried under vacuum in a desiccator (1 h). Samples were acetylated by heating in a mixture of 100 μ L pyridine (Sigma) and 100 μ L acetic anhydride (Sigma) at 60 °C for 1 h. The reaction mixture was evaporated with nitrogen (temperature below 40 °C), dissolved in 2 mL of dichloromethane (B&J, Muskegon, USA), and washed, first with a 0.05 M HCl solution (1 mL) and then twice with distilled water (1 mL). Samples were evaporated to dryness with nitrogen, dissolved in 200 μ L of hexane, and analyzed by GC/MS [44,45].

2.5. Gas chromatography–mass spectrometry (GC/MS)

Instrument used was a GC3800-MS1200 (Varian, Palo Alto, CA), operated in electron ionization (EI) mode. All component analyses were performed on a 30 m CP-Sil eight bonded phase fused silica capillary column (30 m 0.25 mm I.D., Chrompack, The Netherlands). Volumes of 1 μ L were injected in the splitless mode at an injector temperature of 290 °C; the helium head column pressure was 69 kPa. The initial column temperature, 40 °C, was increased to 300 °C (5 °C/min), where it was held for 5 min. The temperature of the interface (between GC and MS systems) was 290 °C. A re-N-acetylation step was used after methanolytic depolymerization to ensure that hexosamines, if present, would be detected as their acetamido derivatives. All derivatives were identified by comparison of retention times and

mass spectra compared to authentic standards and published data.

2.6. Amino acid analysis

Amino acid composition was determined by high performance liquid chromatography system (Shimadzu LC-10A/C R7A, Tokyo, Japan) with sodium citrate eluent and post-column derivatization with OPA (*O*-phthalaldehyde).

2.7. Heat-coagulated hen's egg white

Heat-coagulated hen's egg white (HEW) was prepared as previously described [37]. After solidification of egg white for 30 min in boiling water, it was separated, washed in distilled water and dehydrated in 100% ethanol for 24 h. Small fragments of 4 mm × 2 mm × 2 mm (approximately 40 mg) were obtained and re-hydrated in sterile PBS for 2 h at room temperature before implantation.

2.8. Heat-aggregated OVA

Grade II ovalbumin (Sigma) was diluted in saline (0.9% NaCl) at 20 mg/mL and heated in a dry bath for 5 min at 100 °C. After centrifugation at 3000 × g for 10 min, the supernatant was discarded and the pellet re-suspended in sterile saline at the same initial concentration.

2.9. Immediate hypersensitivity murine model

The immediate hypersensitivity murine model used in this work was developed by Facincone et al. [37]. Accordingly, when F₁ hybrid mice (BALB/c × A/J) are subcutaneously implanted with HEW (40 mg) and challenged, after 14 days, with 50 µL of heat-aggregated OVA (20 mg/mL) in the footpad, they develop a immediate hypersensitivity reaction, characterized by footpad swelling and eosinophil infiltration. Three peaks of footpad swelling can be observed, at 0.5, 6 and 24 h, the last corresponding to the type I immediate hypersensitivity late phase reaction [37].

2.10. *P. acnes*- or PS-treatment protocols

Based in our previous study, in which type I immediate hypersensitivity late phase reaction was differently modulated by two distinct treatment protocols with phenol-killed *P. acnes* [36], we used these two protocols to submit the animals (*n* = 6) to heat-killed *P. acnes*- or PS-treatment. In Protocol 1, mice were injected subcutaneously once a week with *P. acnes* (140 µg/350 µL/animal) or PS (25 µg/350 µL/animal) during 3 weeks, and HEW was implanted concomitantly to the last *P. acnes* or PS injection. In Protocol 2, HEW was implanted 1 week after the third dose of *P. acnes* or PS. For each protocol, the respective control group was treated with sterile saline at the same conditions. In both protocols, animals were challenged with aggregated OVA in the footpad, 14 days after HEW implant, as described above.

Animals treated with *P. acnes* were used for comparison purposes in anti-OVA IgG titers determination.

2.11. Analysis of footpad swelling

Footpad diameters were measured at 0.5, 3, 6, 9, 24 and 48 h after aggregated OVA challenge with a gauge caliper (Mitutoyo, Japan), in control and PS-treated groups. Swelling values were obtained by

subtracting the footpad diameter before challenge from the footpad diameters after different times of the challenge (Δ).

2.12. Histological analysis

Footpad lesions from control and PS-treated animals were excised 24 and 48 h after aggregated OVA-challenge, fixed in Bouin solution and appropriately prepared for HE staining. The number of eosinophils and other leukocytes infiltrating the lesions was evaluated by counting the number of cells in 10 different fields (~50 cells/field) from each histological preparation (two slide sections of each group).

2.13. Detection of specific OVA antibodies

Sera of control, *P. acnes*- or PS-treated mice were collected 24 and 48 h after OVA challenge, pooled and analyzed by ELISA for anti-OVA total IgG and its subclasses IgG1 and IgG2a. Briefly, 96-well plates were coated with soluble OVA (4 µg/100 µL/well) in PBS for 2 h at 37 °C, followed by overnight incubation at 4 °C. After blocking with PBS–1% BSA (100 µL/well) for 1 h at room temperature, samples were added (100 µL/well) diluted in PBS from 1:100–1:12,800 for total IgG detection and 1:25–1:12,800 for IgG1 and IgG2a, and incubated for 30 min at 37 °C. Reactions were detected using 100 µL/well of HRP-conjugated anti-mouse IgG 1:2,000 (Zymed, CA, USA) for 1 h at 37 °C, or biotinylated anti-mouse IgG1 or IgG2a 1:4000 (BD Pharmingen, CA, USA) for 1 h at 37 °C followed by 50 µL/well of HRP-conjugated streptavidin 1:4000 (BD Pharmingen) for 30 min at room temperature. Peroxidase activity was assessed with OPD-substrate and stopped with 4N H₂SO₄. Optical density (OD) was determined at 492 nm. The IgG1/IgG2a ratio was determined based on the mean of optical densities of each group.

2.14. Intracellular cytokines detection

In order to verify whether the cytokine pattern at the moment of challenge could be related to the modulation of Th2 response to OVA in PS-treated mice, spleen cells from control or PS-treated animals were obtained 14 days after HEW implantation, before aggregated OVA challenge. The number of IL-4-, IL-12- or IFN- γ -producing spleen cells was determined by intracellular staining and analyzed by flow cytometry. Briefly, cell suspension was counted with Trypan Blue to determine the cellular viability and concentration (total absolute number of cells/mL). Cells were incubated for 30 min at 4 °C with PBS–5% normal mouse serum to block Fc receptors, and permeabilized with 0.2% Triton-X for 6 min at room temperature. Intracellular staining was performed with PE-conjugated anti-mouse IL-4 or anti-mouse IL-12 and FITC-conjugated anti-mouse IFN- γ mAbs (BD Pharmingen). The percentage of IL-4⁺, IL-12⁺ or IFN- γ ⁺ cells was determined by flow cytometry (FACScan – Becton Dickinson, CA, USA) and converted to absolute number of cells based on the total cell concentration obtained by Trypan Blue counting.

2.15. Statistical analysis

Student's *t*-test was used to determine the statistical differences between control and PS-treated groups (Figs. 1 and 6, and Table 2) and ANOVA was used between control, *P. acnes*- and PS-treated groups (Figs. 3–5). Differences was taken as statistically significant when *p* < 0.05.

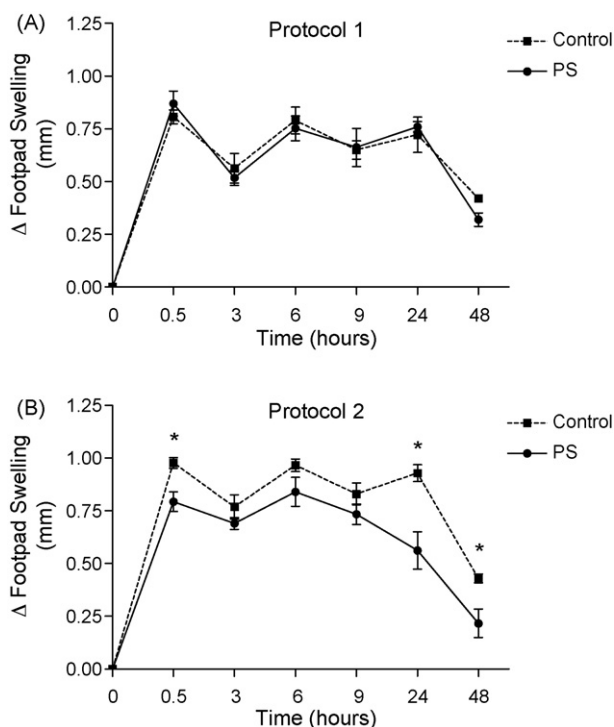


Fig. 1. Footpad swelling of control and PS-treated mice after OVA challenge. Footpad swelling of animals ($n=6$) treated with saline (control) or PS according to Protocols 1 (A) or 2 (B) was determined until 48 h after aggregated OVA challenge. These data are representative of two independent experiments with similar results. * $p < 0.05$ compared to the respective control group.

3. Results

3.1. *P. acnes*-soluble polysaccharide characterization

The apparent molecular weight of the soluble PS was estimated to be between 45 and 70 kDa by high performance liquid chromatography using TSG G2000 SW column.

Sugar analysis showed that PS is constituted by galactopyranose, ribopyranose, arabinopyranose, glucopyranose, ribofuranose, mannopyranose (60:16:13:8:7:1) and traces of xylopyranose and *N*-acetylglucosamine, as shown in Table 1. Amino acids represented less than 1% of the polysaccharide mass, confirming the purity of the soluble polysaccharide preparation. The main amino acids detected were glycine, alanine, glutamic acid and lysine, amino acids known to be components of cell wall peptidoglycan.

Table 1
Carbohydrate and amino acid composition of *Propionibacterium acnes*-soluble polysaccharide preparation.

Carbohydrates	t_R (min)	Amount ($\mu\text{mol/mg}$ of CH)	Amino acids	Amount ($\mu\text{mol/mg}$ of CH)
Xylopyranose	25.818	Trace	Asparagine/aspartic acid	0.0022
Arabinopyranose	27.455	0.654	Threonine	Trace
Ribopyranose	30.754	0.815	Serine	0.0057
Ribofuranose	31.362	0.365	Glutamine/glutamic acid	0.0072
Mannopyranose	33.241	0.055	Proline	Trace
Galactopyranose	33.742	2.969	Glycine	0.0231
Glucopyranose	35.152	0.422	Alanine	0.0175
<i>N</i> -Acetyl-glucosamine	36.968	Trace	Valine	Trace
			Leucine	Trace
			Histidine	0.0023
			Lysine	0.0067
			Arginine	Trace

Quantification of carbohydrates and amino acids obtained as described in Section 2. t_R = retention time in minutes; CH = carbohydrate.

3.2. Analysis of footpad inflammatory response in PS-treated mice submitted to the murine model of immediate hypersensitivity

Mice were treated with PS or saline (control) and submitted to HEW implantation and OVA challenge according to Protocols 1 and 2, as described in Section 2.

The analysis of footpad swelling after OVA challenge revealed that PS-treatment according to Protocol 1, in which HEW implantation was concomitant to the last PS injection, did not change the pattern of inflammatory response compared to the control group, including LPR, 24 h after challenge (Fig. 1A).

In contrast, when animals were submitted to Protocol 2, in which HEW implantation was performed 1 week after the last PS injection, there was a decrease in footpad swelling in PS-treated group, compared to the control. This was statistically significant at 0.5, 24 and 48 h after OVA challenge, and, interestingly, LPR (24 h) was totally abolished in PS-treated mice (Fig. 1B).

The footpad histological analysis 24 h after challenge showed an intense eosinophilia in lesions from control animals, as expected (Fig. 2A and C). Similar inflammatory reaction was observed at 48 h (data not shown).

Although PS did not alter the footpad swelling in mice treated according to Protocol 1 (Fig. 1A), it induced an increase in eosinophilia in the footpad of these animals 24 h after OVA challenge, compared to the control group (Fig. 2B), and this effect persisted after 48 h (data not shown). Determination of eosinophils and other leukocytes number by counting histological preparations 24 and 48 h after challenge revealed that the increase in eosinophil infiltrate, and consequent relative decrease of other leukocytes, in PS-treated group submitted to Protocol 1 was statistically significant (Table 2), indicating the potentiation of type I immediate hypersensitivity, including LPR.

On the other hand, a significant lower eosinophils number, and consequent relative enhancement of other leukocytes, was observed in the footpad infiltrate from PS-treated mice submitted to Protocol 2, compared to the control group (Fig. 2D, Table 2). In fact, this treatment protocol suppressed the type I hypersensitivity response, including decrease in footpad swelling (Fig. 1B).

3.3. Detection of specific anti-OVA total IgG in the sera of *P. acnes*- or PS-treated mice submitted to Protocols 1 or 2

Although we have already demonstrated the modulation of the type I hypersensitivity to OVA by the whole bacterium [36], the antibody response pattern to OVA in mice treated with *P. acnes* according to Protocols 1 or 2 had not yet been showed.

Therefore, the titers of anti-OVA total IgG in the sera of control, *P. acnes*- and PS-treated mice submitted to both protocols were determined 24 and 48 h after OVA challenge.

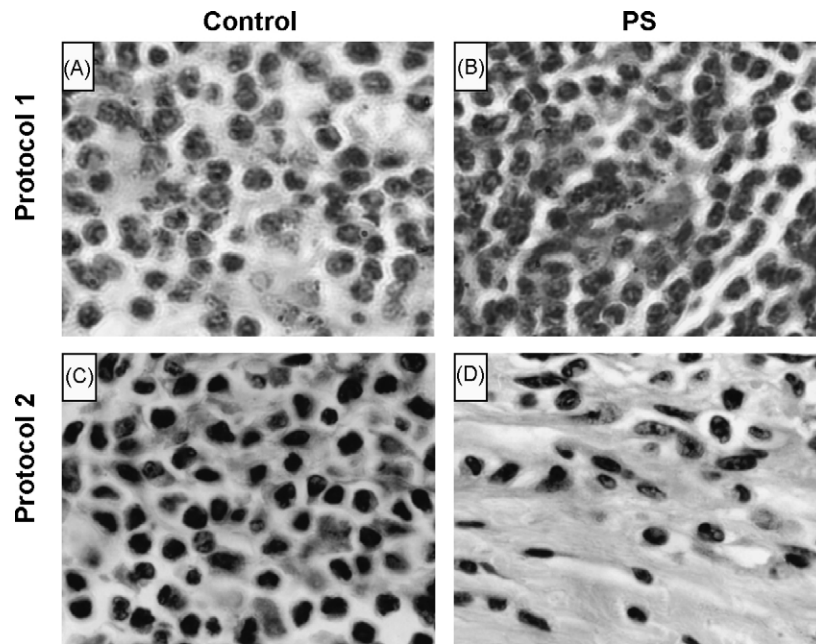


Fig. 2. Cellular infiltration in the footpad of control and PS-treated mice after OVA challenge. Footpads of control or PS-treated mice ($n=6$) submitted to Protocols 1 (A and B) or 2 (C and D) were excised 24 h after OVA challenge and appropriately prepared for HE staining. Magnification 1000 $\times$. Images are representative of two independent experiments with similar results.

We did not observe any difference in total IgG titers to OVA between control and *P. acnes*- or PS-treated groups submitted to Protocol 1, except a statistically significant decrease in IgG level in PS-treated animals at the dilution 1/800, 48 h after OVA challenge (Fig. 3A and B). In contrast, significant higher anti-OVA IgG titers were detected in the sera of PS-treated mice submitted to Protocol 2, 24 and 48 h after OVA challenge (Fig. 3C and D). Differently, in *P. acnes*-treated mice submitted to Protocol 2, lower levels of anti-OVA IgG titers were detected at 24 h, with statistical significance at the dilutions 1/200 and 1/800 (Fig. 3C), but significant higher levels were obtained at 48 h, compared to both control and PS-treated groups (Fig. 3D).

3.4. OVA-specific IgG subclasses in the sera of mice treated with *P. acnes* or PS according to Protocols 1 and 2

The subclasses of IgG to OVA were also evaluated in the sera of control and treated animals 24 and 48 h after OVA challenge.

Firstly, we detected higher levels of anti-OVA IgG1 than IgG2a in the sera of control animals at 24 and 48 h (Figs. 4 and 5). This is compatible to the Th2 pattern induced in this model of immediate hypersensitivity, once it is known that IgG1 is induced in a Th2 environment, while IgG2a is related to Th1 response. The IgG1/IgG2a ratio calculated for this group, 48 h after OVA challenge, confirmed the Th2 pattern, since the production of anti-OVA IgG1 by these

Table 3

IgG1/IgG2a ratio determination in sera from mice treated with saline (control), *P. acnes* or PS according to Protocols 1 or 2, 48 h after OVA challenge.

Protocol	Treatment	Mean of optical densities		IgG1/IgG2a ratio
		IgG1	IgG2a	
1	Control	1.274	0.128	10.0
	<i>P. acnes</i>	1.419	0.016	88.7
	PS	1.223	0.029	42.2
2	Control	1.504	0.128	11.8
	<i>P. acnes</i>	1.334	0.391	3.4
	PS	1.365	0.232	5.9

Sera from control, *P. acnes*- or PS-treated mice were collected 48 h after OVA challenge and analyzed by ELISA to determine the anti-OVA IgG1 and IgG2a titers. Sera were diluted from 1/25 to 1/12,800. The means of optical densities (ODs), considering all the dilutions, were obtained to determine the IgG1/IgG2a ratio for each group.

animals was 10.0–11.8-fold higher than anti-OVA IgG2a (Table 3).

When mice were treated with PS according to Protocol 1, there was a significant decrease in anti-OVA IgG1 levels in the first two dilutions (1/25 and 1/50) at 24 and 48 h, compared to the control group (Fig. 4A and B). However, this seemed to be due to saturation of ELISA reaction, since we noted that optical densities (ODs) were very similar between these dilutions. In the other serum dilutions analyzed, no differences were observed between control and

Table 2

Percentage of eosinophils and other leukocytes in footpad inflammatory reaction of control and PS-treated mice according to Protocols 1 or 2.

Protocol	Time after OVA challenge	Eosinophils (%)			Other leukocytes (%)		
		Control	PS		Control	PS	
1	24 h	32.7 $\pm$ 1.8	46.3 $\pm$ 1.3	$p < 0.05$	67.3 $\pm$ 1.8	53.7 $\pm$ 1.3	$p < 0.05$
	48 h	28.5 $\pm$ 0.3	42.7 $\pm$ 0.4	$p < 0.05$	71.5 $\pm$ 0.3	57.3 $\pm$ 0.4	$p < 0.05$
2	24 h	35.0 $\pm$ 1.0	16.0 $\pm$ 3.0	$p < 0.05$	65.0 $\pm$ 3.5	84.0 $\pm$ 3.0	$p < 0.05$
	48 h	32.5 $\pm$ 0.5	11.0 $\pm$ 1.0	$p < 0.01$	67.5 $\pm$ 0.5	89.0 $\pm$ 1.0	$p < 0.01$

Footpads of control or PS-treated mice ($n=6$) submitted to Protocols 1 or 2 were excised 24 and 48 h after OVA challenge and stained by HE. Data represent means $\pm$ S.E.M. of cells counted in 10 different fields of each histological preparation, obtained in two reproducible experiments.

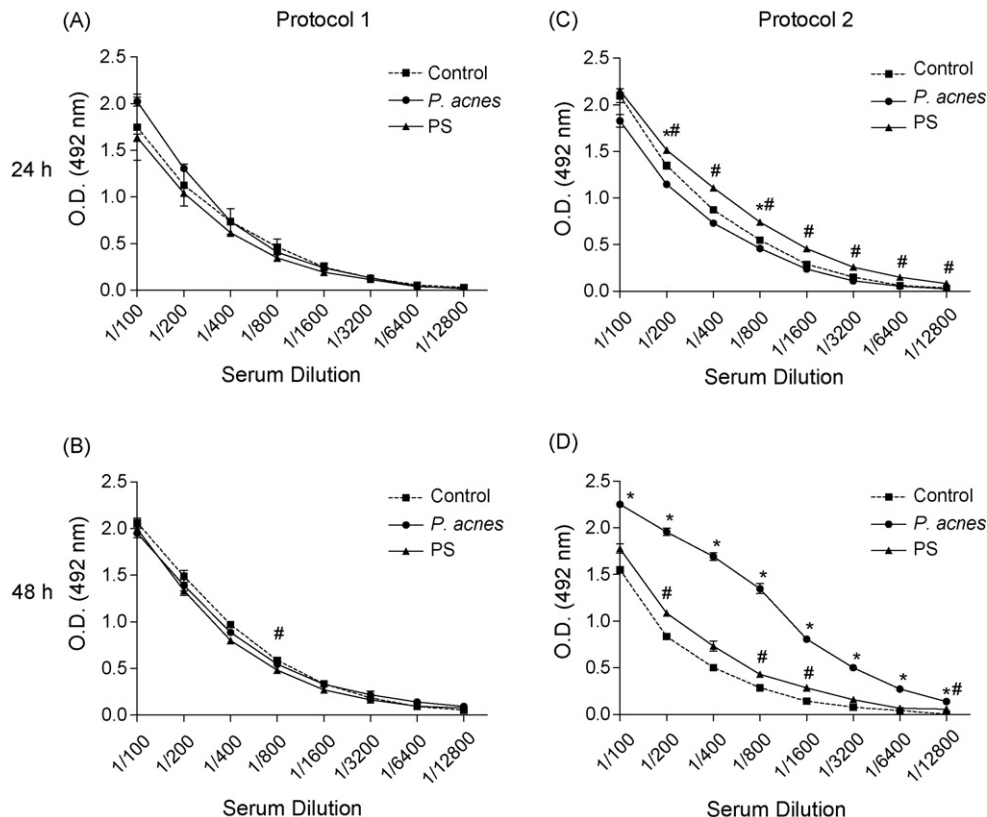


Fig. 3. Anti-OVA total IgG titers in the sera of control, *P. acnes*- or PS-treated mice submitted to both protocols. The sera of mice ($n=6$) treated with saline (control), *P. acnes* or PS according to Protocols 1 (A and B) or 2 (C and D) were collected 24 h (A and C) and 48 h (B and D) after aggregated OVA challenge, and analyzed by ELISA to determine the titers of anti-OVA total IgG. These data are representative of two independent experiments with similar results. * $p < 0.05$ between *P. acnes* and saline groups. # $p < 0.05$ between PS and saline groups.

treated groups (Fig. 4A and B), indicating that PS-treatment according to Protocol 1 did not change anti-OVA IgG1 levels. Besides, lower titers of IgG2a to OVA were detected in this group, compared to the control, with significant differences in various dilutions until 1/200, mainly at 48 h (Fig. 5A and B). These results indicate the potentiation of Th2 response in PS-treated animals submitted to Protocol 1. However, this potentiation was not as intense as in *P. acnes*-treated mice submitted to the same protocol, in which not only the anti-OVA IgG2a titers were decreased (Fig. 5A and B), but also the anti-OVA IgG1 titers were concomitantly increased, mainly at 48 h (Fig. 4A and B). This was confirmed by the IgG1/IgG2a ratio obtained for *P. acnes*- or PS-treated animals submitted to Protocol 2 (88.7 and 42.2, respectively), despite the ratios for both groups have been much higher than control group (10.0), 48 h after challenge (Table 3).

On the other hand, *P. acnes*- or PS-treatment according to Protocol 2 clearly induced suppression of Th2 pattern, because there was a significant decrease in IgG1 levels at 48 h (Fig. 4D), concomitantly to a significant increase in IgG2a titers, at 24 and 48 h (Fig. 5C and D), with consequent diminishment of the IgG1/IgG2a ratio at 48 h in both groups (3.4 and 5.9, respectively), compared to the control (11.8) (Table 3). However, the response suppression seemed to be more intense in *P. acnes* group, in which the increase in anti-OVA IgG2a titers was more pronounced, mainly at 48 h (Fig. 4D), leading to a lower IgG1/IgG2a ratio than in PS-treated group (Table 3). Importantly, although there was an obvious Th2 suppression in both treated groups, the response did not seem to be converted to Th1, because the anti-OVA IgG1 levels remained higher than IgG2a (Figs. 4C and D, 5C and D) and the IgG1/IgG2a ratios did not reach values lower than 1.0 (Table 3).

3.5. Absolute number of cytokines-producing spleen cells in PS-treated animals according to Protocols 1 and 2 before aggregated OVA challenge

In order to verify whether the cytokine pattern at the moment of challenge could influence the exacerbation or suppression of the Th2 response to OVA in PS-treated mice, spleen cells from control and PS-treated groups were obtained 14 days after HEW implantation, before OVA challenge, and the number of IL-4- or IFN- γ -producing cells was determined by intracellular staining.

Firstly, we confirmed the Th2 pattern in control mice, once the number of IL-4-producing cells was higher than IL-12 $^{+}$ or IFN- γ^{+} cells in these animals (Fig. 6A and B).

When mice were treated with PS according to Protocol 1, in which we observed potentiation of the immediate hypersensitivity reaction, there was a significant increase in the number of IL-4-producing spleen cells before OVA challenge, simultaneously to a decrease in IL-12- or IFN- γ -producing cells, compared to the control group (Fig. 6A), confirming the Th2 response enhancement.

In PS-treated mice submitted to Protocol 2, in which there was suppression of the immediate hypersensitivity, we also detected higher number of IL-4-producing cells before OVA challenge, compared to the control group (Fig. 6B), but this increase was not as evident as in PS-treated animals submitted to Protocol 1 (Fig. 6A). Besides, differently from Protocol 1, there was a significant increase in IL-12-producing cells and an apparent enhancement of IFN- γ^{+} cells in mice treated with PS according to Protocol 2, compared to the control (Fig. 6B). These results suggest the suppression of Th2 response, but not the conversion to Th1, because the number of IL-

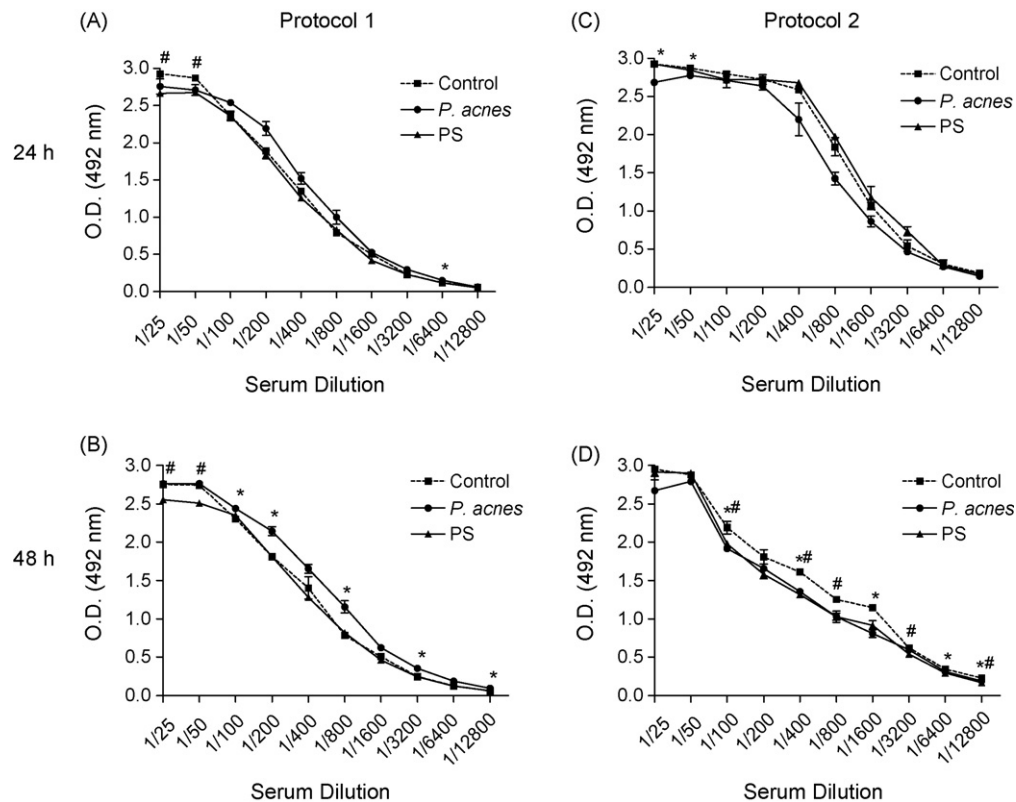


Fig. 4. Anti-OVA IgG1 titers in the sera of control, *P. acnes*- and PS-treated animals submitted to both protocols. The sera of control, *P. acnes*- and PS-treated mice ($n=6$) submitted to Protocols 1 (A and B) or 2 (C and D) were collected 24 h (A and C) and 48 h (B and D) after aggregated OVA challenge, and analyzed by ELISA to determine the titers of anti-OVA IgG1. These data are representative of two independent experiments with similar results. * $p < 0.05$ between *P. acnes* and saline groups. # $p < 0.05$ between PS and saline groups.

4⁺ cells in this group remained higher than IL-12⁺ and IFN- γ ⁺ cells (Fig. 6B).

4. Discussion

Among *P. acnes* immunomodulatory effects, a most relevant one is its capacity to potentiate or suppress the Th2 response to OVA in a murine model of type I hypersensitivity, depending on the treatment protocol used, as we previously described [36]. However, the bacterium compounds responsible for this effect are not known, leading us to investigate if the *P. acnes* soluble polysaccharide (PS) could be a major component involved in the Th2 response modulation induced by the whole bacterium.

We focused our interest on this component because the *P. acnes* suspension used by us to modulate the immediate hypersensitivity reaction was phenol-killed [36], and it is known that polysaccharide components are preferentially preserved and exposed by phenol-treatment. In our laboratory, we purified a soluble polysaccharide phenol-extracted from *P. acnes* cell wall which induces similar effects to the whole bacterium [22,24,38,39]. However, the fine structural characterization of this compound had not yet been performed.

By high performance liquid chromatography it was determined that soluble PS presents apparent molecular weight between 45 and 70 kDa and contains galactopyranose, ribopyranose, arabinopyranose, glucopyranose, ribofuranose, mannopyranose in proportion of 60:16:13:8:7:1.

After the molecule characterization, the PS capacity to modulate the immediate hypersensitivity reaction in mice was studied.

For the development of immediate hypersensitivity, we used a murine model described by Facincone et al. [37], in which a typical

LPR is evoked 24 h after aggregated OVA challenge in the footpad. This model fits our purpose since adjuvant is not required and it was the model used in our previous work demonstrating the modulation of immediate hypersensitivity by *P. acnes* [36].

Herein, mice were treated with PS and submitted to this same immediate hypersensitivity model. The PS dose and the two protocols used in our experiments were based in previous works of our laboratory [22,24,36,38,39]. Besides, animals treated with heat-killed *P. acnes* were also used to compare the effects of *P. acnes*- and PS-treatments concerning the synthesis of anti-OVA IgG. The *P. acnes* dose was also based in previous works [22,24,36,39].

PS-treatment according to Protocol 1, in which HEW was implanted concomitantly to the last PS injection, did not change mice footpad swelling after OVA challenge, but induced an increase in eosinophils infiltration at 24 and 48 h, similarly to the whole bacterium [36]. These results suggest that the mechanism by which PS potentiated the immediate hypersensitivity could be related to eosinophils differentiation and/or recruitment, but not edema formation. The capacity of *P. acnes* and PS to induce differentiation and/or recruitment of other leukocytes has already been demonstrated [22,39,46,47]. These data also indicate that PS is an important component responsible for bacterium effects on eosinophils enhancement.

The increase in eosinophils number in this PS-treated group demonstrates the enhancement of immediate hypersensitivity, suggesting there was an augmentation in Th2 response to OVA. This was corroborated by the decrease in anti-OVA IgG2a titers at 24 and 48 h, since this antibody subclass is induced by Th1 cytokines, and the increase in IgG1/IgG2a ratio at 48 h, similarly to what was observed in *P. acnes*-treated group. Besides, we detected a more

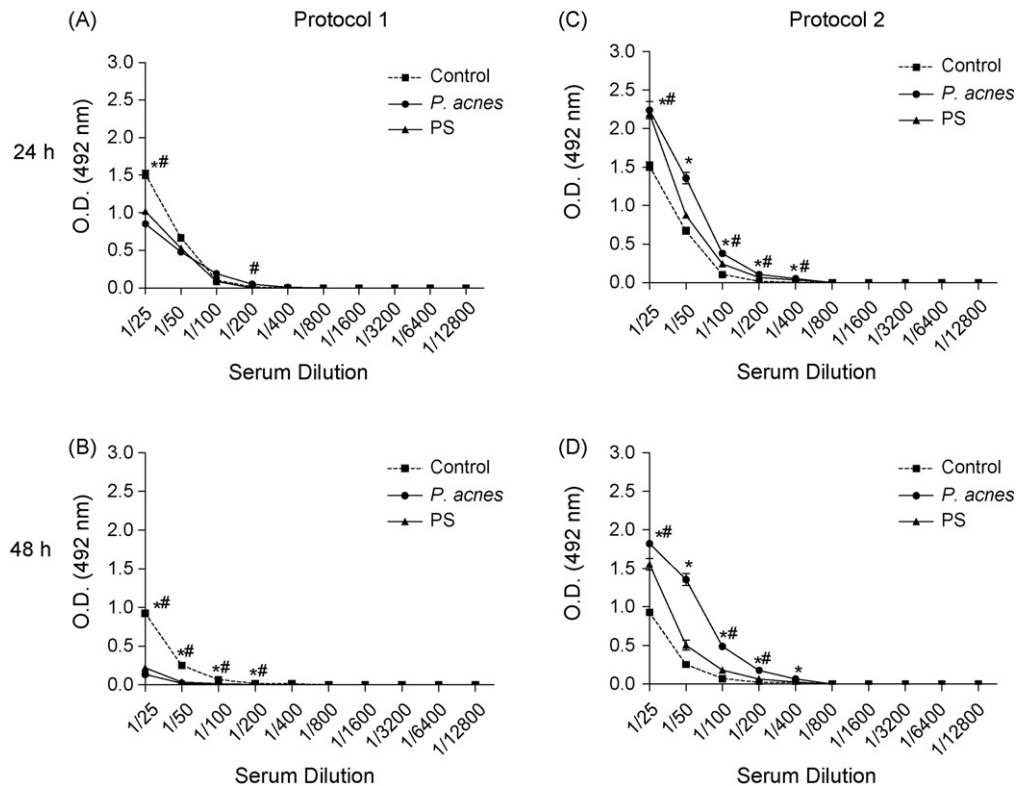


Fig. 5. Anti-OVA IgG2a titers in the sera of control, *P. acnes*- and PS-treated groups submitted to both protocols. The sera of mice ($n=6$) treated with saline (control), *P. acnes* or PS and submitted to Protocols 1 (A and B) or 2 (C and D) were collected 24 h (A and C) and 48 h (B and D) after OVA challenge, and analyzed by ELISA to determine the titers of anti-OVA IgG2a. These data are representative of two independent experiments with similar results. * $p < 0.05$ between *P. acnes* and saline groups. # $p < 0.05$ between PS and saline groups.

intense Th2 environment in PS-treated group than in the control before OVA challenge, as demonstrated by higher number of IL-4-producing spleen cells and lower number of IL-12⁺ or IFN- γ ⁺ cells. We believe that this enhanced Th2 pattern at the moment of OVA challenge contributes to the potentiation of immediate hypersensitivity reaction, mainly the increase in eosinophils number during LPR, once it has been demonstrated that these cells are recruited and activated in response to Th2 cytokines, such as IL-4 and IL-5 [48].

Therefore, it seems that when PS is administered concomitantly to another antigen, it potentiates the cellular immune response to it. Since OVA is a Th2-inducing antigen, in the present work, the concomitant administration of PS and HEW implantation has led to Th2 enhancement, and consequent potentiation of the immediate hypersensitivity reaction to OVA. We have already showed that, when mice were simultaneously treated with *P. acnes* or PS and a Th1-inducer *T. cruzi* DNA vaccine, this concomitant administration also potentiated the Th1 pattern [24].

On the other hand, when mice were treated with PS according to Protocol 2, in which HEW implantation was performed 1 week after the last PS injection, we observed a decrease in footpad swelling after aggregated OVA challenge. Interestingly, LPR (24 h) was abolished in this group, differently to what had been observed with *P. acnes*, which had also decreased footpad swelling, but had not abrogated LPR [36]. These data suggest a better effect of PS in suppressing the immediate hypersensitivity reaction, maybe because the *P. acnes* dose used in our previous work could not be sufficient to induce the same effect obtained with the purified PS dose. Besides, *P. acnes* contains several other compounds, what could explain why the modulation induced by the whole bacterium is not exactly the same obtained with purified PS.

PS-treatment according to Protocol 2 also induced a decrease in eosinophils recruitment to the lesions, similarly to *P. acnes* [36], confirming the immediate hypersensitivity suppression.

One possible explanation for this effect is that PS could induce a Th1 environment before HEW implantation, contributing to the suppression of Th2 response to OVA at the moment of implant. Lower production of Th2 cytokines could then induce diminished recruitment and/or activation of eosinophils after OVA challenge. In fact, the capacity of *P. acnes* to induce a Th1 pattern has already been demonstrated [31–35]. We have showed that PS also promotes a Th1 response when administered in the absence of other antigens [38], corroborating our theory that a Th1 environment has been induced before HEW implantation in PS-treated animals submitted to Protocol 2.

In accordance to our hypothesis, we detected lower anti-OVA IgG1 titers in the sera of PS-treated animals 48 h after OVA challenge and higher levels of IgG2a at 24 and 48 h, indicating Th2 suppression, what was confirmed by the decrease in the IgG1/IgG2a ratio. It is important to note that, despite this effect, the cellular immune response to OVA was not converted to Th1, since IgG1 levels remained higher than IgG2a, and IgG1/IgG2a ratio did not reach values lower than 1.0.

Similar results were obtained for *P. acnes*-treated animals submitted to Protocol 2, but, in this case, the Th2 response suppression seemed to be more pronounced, as indicated by the lower IgG1/IgG2a ratio, compared to both control and PS-treated groups. Although this ratio have not reached values lower than 1.0, we have already demonstrated that *P. acnes* can induce a Th1 response to OVA using this model of type I hypersensitivity reaction, as indicated by higher levels of IFN- γ than IL-4 in the sera of treated mice [36]. However, despite the conversion to a Th1 response, the levels

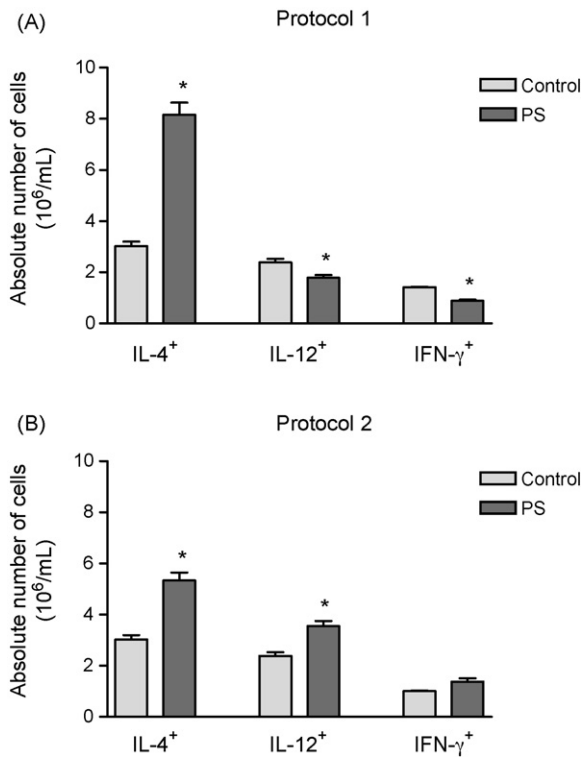


Fig. 6. Absolute number of cytokines-producing spleen cells from control and PS-treated mice before OVA challenge. Spleen cells from mice ($n = 6$) treated with saline (control) or PS according to Protocols 1 (A) or 2 (B) were obtained 14 days after HEW implantation, before aggregated OVA challenge. The percentage of IL-4⁺, IL-12⁺ or IFN-γ⁺ spleen cells was determined by intracellular staining and flow cytometry analysis, and converted to absolute number of cells based on the total cell concentration obtained by Trypan Blue counting. These data are representative of two independent experiments with similar results. * $p < 0.05$ compared to the respective control group.

of anti-OVA IgG1 remained higher than IgG2a in this group, maybe due to the persistence of the Th2-inducing antigen (OVA) implanted in high dose in the subcutaneous tissue.

The analysis of cytokines-producing spleen cells before OVA challenge showed a higher number of IL-4⁺ cells in PS-treated mice submitted to Protocol 2, compared to the control, but the increase induced in this group was not as intense as the observed in PS-treated animals submitted to Protocol 1. Besides, differently from Protocol 1, there was a significant increase in the number of IL-12-producing cells in Protocol 2, and an apparent augmentation of IFN-γ⁺ cells. These data confirm there was a suppression of Th2 response to OVA in this group, but not a conversion to Th1, because the number of IL-4⁺ cells remained higher than IL-12- or IFN-γ-producing cells, in accordance to what we have observed concerning anti-OVA IgG subclasses.

So, we believe that PS could induce a Th1 environment before HEW implantation, what would decrease Th2 response to OVA at the moment of implant, but not completely abrogate it. Then, after OVA challenge, the Th2 response would be suppressed, leading to diminished edema and eosinophils recruitment, but not inverting the IgG subclasses pattern.

Interestingly, this was different to what we had observed with *P. acnes*-treatment, in which the cellular immune response to OVA was converted to Th1 in mice submitted to Protocol 2 [36], but also not inverting the IgG subclasses pattern. These results suggest that PS is an important bacterium component involved on the immediate hypersensitivity suppression, but probably other *P. acnes* fractions also participate of this effect.

In conclusion, our results demonstrate that PS is an important *P. acnes* compound responsible for potentiation or suppression of immediate hypersensitivity, although other bacterium fractions can also participate of these effects. The molecular mechanisms by which the Th1/Th2 balance is modulated probably involves antigen presenting cells, which have an important role in the Th1/Th2 induction, what is under investigation.

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