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Peripheral blood monocyte and T cell subsets in children with specific polysaccharide antibody deficiency (SPAD)

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ABSTRACT

Specific polysaccharide antibody deficiency (SPAD) is a well reported immunodeficiency characterized by a failure to produce antibodies against polyvalent polysaccharide antigens, expressed by encapsulated microorganisms. The clinical presentation of these patients involves recurrent bacterial infections, being the most frequent agent *Streptococcus* (*S.*) *pneumoniae*. In SPAD patients few reports refer to cells other than B cells. Since the immune response to *S. pneumoniae* and other encapsulated bacteria was historically considered restricted to B cells, the antibody deficiency seemed enough to justify the repetitive infections in SPAD patients. Our purpose is to determine if the B cell defects reported in SPAD patients are accompanied by defects in other leukocyte subpopulations necessary for the development of a proper adaptive immune response against *S. pneumoniae*. We here report that age related changes observed in healthy children involving increased percentages of classical monocytes (CD14++ CD16− cells) and decreased intermediate monocytes (CD14++ CD16+ cells), are absent in SPAD patients. Alterations can also be observed in T cells, supporting that the immune deficiency in SPAD patients is more complex than what has been described up to now.

1. Introduction

Primary immunodeficiencies (PIDs) represent the failure of one or more components of the immune system. In recent years more than 150 primary immunodeficiency syndromes have been described, and a number of these present an antibody defective production [1–3]. They comprise a heterogeneous group of medical conditions.

Specific polysaccharide antibody deficiency (SPAD) is an immune disorder of unknown, characterized by a failure to mount an antibody response to polyvalent polysaccharide antigens [2]. This defect may appear by itself or associated with other immunodeficiency syndromes [3,4]. The clinical presentation of these patients involves recurrent bacterial infections, mainly by Gram-positive agents, of which the most frequently found is *Streptococcus* (*S.*) *pneumoniae*, but have normal immunoglobulin serum levels.

Defects in antibody production are the main feature of humoral immunodeficiency patients, and in SPAD patients most reports refer to B cell defects [5–7]. Since the immune response to *S. pneumoniae* and other encapsulated bacteria was traditionally considered restricted to B cells, the antibody deficiency seemed enough to justify the repetitive infections with this kind of pathogens in SPAD patients. Nevertheless, in patients with other humoral PIDs, which also suffer this type of infections, alterations in other cell populations have been reported [4,8–10]. Selective deficits in blood dendritic cell subsets have been described in CVID and XLA but not in SPAD patients [11,12]. More recently, Jyonouchi et al. reported age-dependent changes in the numbers of dendritic cell subsets in healthy children and they also observed different patterns in the distribution of peripheral blood dendritic cell subsets in SPAD patients [13].

Three blood monocyte subsets have been described both in man and other mammals defined by the differential expression of CD14 and CD16 [14]. Classical monocytes strongly express CD14, the LPS receptor, and no CD16 on their surface (CD14++ CD16−), and represent the majority of circulating monocytes in humans. Two

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minor subpopulations are: intermediate monocytes (CD14++ CD16+) and non-classical monocytes that express low CD14 and CD16 (CD14+ CD16+) [14]. They are elevated in several conditions such as cancer, rheumatoid arthritis, asthma and sepsis [15,16]. The relationship between the three subpopulations has been described recently [17–20].

In the last decade several reports have been published referring to the T cell involvement in the immune response to *S. pneumoniae* [21–23]. In mice models T cell participation, in particular that of Th17 cells, in pneumococcal immunity has been reported [24–26] and the presence of memory T cells that recognize pneumococcal antigens has also been described [27]. A recent report in humans describes the involvement of Th1 and Th17 cells in the response to *S. pneumoniae* [26,28].

In order to evaluate if other peripheral blood cell populations differ in SPAD patients with respect to healthy controls, we undertook to study monocyte subpopulations and T cells in these patients. We decided to evaluate some activation and maturation T cell parameters, trying to determine if these cells also undergo age related changes. Our working hypothesis is that the B cell defects reported in SPAD patients may be accompanied by defects in other leukocyte subpopulations necessary for the development of a proper adaptive immune response against *S. pneumoniae*.

We here report that age related changes observed in healthy children involving increased percentages of classical monocytes (CD14++ CD16− cells) and decreased intermediate monocytes (CD14++ CD16+ cells), are absent in SPAD patients. Alterations can also be observed in T cells, supporting the idea that the immunodeficiency in SPAD patients is more complex than what has been described up to now.

2. Materials and methods

2.1. Study subjects

We studied 18 patients 7–19 years old; 10 boys and 8 girls that were diagnosed at a mean age of 5, which is the typical age of diagnosis [13]. These patients had a history of recurrent infections with encapsulated bacteria and presented symptoms of atopy. They were diagnosed for having low levels of protective anti-Spn antibodies after challenge with the Pneumo 23 vaccine (Aventis Pasteur) and normal immunoglobulin (Ig) levels [2]. The serum antibody concentration to 7 pneumococcal serotypes (1, 3, 5, 6b, 7, 14, 23F) was assessed in all patients 40 days after immunization with the vaccine. Impaired response to polysaccharide antigens was arbitrarily defined as titers fewer than 1.30 μg/mL in at least 4 of the 7 serotypes evaluated post immunization [10]. Patients with confirmed diagnosis of other PID were excluded from the study.

All the studied patients required 400–600 mg/kg/day intravenous gamma globulin (ivGG) treatment, due to recurrent infections. Blood samples were drawn before ivGG infusion at least one month after the previous one. Since it has been reported that healthy children, but not SPAD patients, present age-associated changes in the development and maturation of dendritic cells [13], we separated our study subjects in two age groups: 7 –13 ($n = 11$) and 14–19 years ($n = 7$) for some determinations.

The control group consisted of age matched healthy children, undergoing laboratory tests prior to programmed surgery (“Healthy individuals”). They were also separated into two age groups: 7–13 ($n = 7$) and 14–19 years ($n = 15$).

All children had received BCG vaccination early in life according to standard immunization protocols from our country. None of the children had been immunized with a conjugated *S. pneumoniae* vaccine at the time the blood samples were drawn. Informed consent was obtained for sample extraction as well as for inclusion in the study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, and was approved by the Ethics Committee of the Ricardo Gutiérrez Children Hospital and the Ethics Committee of the Institutes of the Academia Nacional de Medicina.

2.2. Antigens

ATCC 49619 *S. pneumoniae* (Spn) as well as strain 14 (Spn14) and strain 3 (Spn3) of the same microorganism, cultured, washed and heat inactivated were kindly provided by the Central Bacteriology Laboratory of the National Institute of Infectious Diseases ANLIS Dr. Malbrán. H37-RV *Mycobacterium tuberculosis* (Mtb), cultured, washed and heat inactivated was kindly provided by the Mycobacteria Service of the same Institution, and used as a control. Proper antigen concentration to be used was determined at the beginning of the study by a dose response curve.

2.3. Cell purification and culture

Heparinized blood was collected and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque gradient centrifugation. Cells were collected from the interphase and resuspended in RPMI 1640 tissue culture medium (Gibco Lab, NY, USA) containing gentamycin (85 μg/ml) and 15% heat inactivated fetal calf serum (Gibco Lab) (complete medium).

PBMC were cultured at 1×10^6 cells/ml in 1 ml of complete medium in Falcon 2003 tubes, at 37 °C in humidified 5% CO_2 atmosphere, in the presence or absence of 0.1 UDO/ml of either Spn (2×10^6 bacteria/ml) or Mtb (3×10^6 bacteria/ml) for different time periods. Due to small sample size, the same culture was used both for activation markers expression and intracellular cytokine production evaluation by flow cytometry. The 48 h time point ($t = 48$) was chosen as a compromise to obtain peak levels of cytokines and measurable levels of activation markers.

2.4. Cell populations and subpopulations

CD3, CD4 or CD8 T cell populations that express CD25 or CD69 were evaluated in PBMC by flow cytometry on the day of sample collection ($t = 0$), using anti-CD3 PE-Cy5 (BD Pharmingen), anti-CD4 PE (BD Pharmingen), anti-CD8 FITC (Caltag Laboratories), anti-CD25 FITC (BD Pharmingen) and anti-CD69 PE (BD Pharmingen).

We also determined Monocyte and T cell subpopulations in PBMC at $t = 0$. We evaluated three different subpopulations of monocytes, according to the expression of CD14 and CD16 markers, by flow cytometry. We used anti-CD14 FITC (Biolegend) and anti-CD16 PE (BD Pharmingen) antibodies. To assess effector and memory T cells, we determined the expression of CD27 and CD45RA or CD45RO in CD3+ cells, using anti-CD3 PE-Cy5 (BD Pharmingen), anti-CD27 PE (Biolegend), anti-CD45RA FITC (Biolegend) and anti-CD45RO FITC (Biolegend). Foxp3 T regulatory cells were assessed with the Foxp3 Staining Buffer Set (eBioscience, CA, USA), using anti-CD4PE-Cy5 (eBioscience), anti-CD25-PE (eBioscience) and anti-Foxp3-FITC (eBioscience) antibodies for flow cytometric analysis.

2.5. Cytokine evaluation by flow cytometry

Intracellular expression of TNF-α in monocytes was evaluated in whole blood from patients and healthy individuals. Cells were cultured in the presence of GolgiPlug (BD Biosciences, USA), stimulated with either Spn, or with LPS (from Escherichia coli 011:84, Sigma Chemicals Co, Saint Louis, USA) as a positive control, or

without stimulus for 4 h ($t = 4$). Then they were labeled with anti-CD14 PE-Cy5 (Biolegend) and anti-CD16 FITC (Biolegend) monoclonal antibodies. After fixing and permeabilizing cells (Fix and Perm kit, Caltag), TNF-α production ability was evaluated with anti-TNFα PE (Biolegend) by flow cytometry.

Intracellular T cell cytokines were evaluated by flow cytometry in whole blood cells from patients and healthy individuals. Cells were stimulated with phorbol myristic acetate (PMA) (50 ng/ml)/Ionomycin (1 μM) in the presence of GolgiPlug (BD Biosciences, USA) for 5 h and then stained with membrane anti-CD3PECy5 and anti-CD8FITC, to indirectly assess CD4 T cells as previously described [29]. After fixation and permeabilization using the Fix & Perm kit (Caltag Laboratories, USA), cells were stained with intracellular PE conjugated antibodies for several cytokines: TNF-α (Caltag Laboratories), IL-4 (BD FastImmune), IL-5 (BD Pharmingen), IL-13 (BD FastImmune), IFN-γ (Caltag Laboratories), IL-10 (BD Pharmingen) and IL-9 (Biolegend).

PBMC from healthy donors or SPAD patients were cultured with Spn, Mtb or no stimulus for 48 h, adding GolgiPlug (BD Biosciences, USA) 6 h before the end of the culture. Cells were then washed, fixed, permeabilized and labeled with the corresponding monoclonal antibodies. We determined CD4 T cells capacity to produce IL-17, IFN-γ or IL-9, after bacterial antigen stimulation. We used the following antibodies for flow cytometric evaluation: anti-CD4 PE-Cy5 (eBioscience), anti-IL-17 PE (eBioscience), anti-IFN-γ FITC (eBioscience), anti-CD3 PE-Cy5 (BD Pharmingen), anti-CD8 FITC (Caltag Laboratories) and anti-IL-9 PE (Biolegend).

2.6. Statistical analysis

GraphPad Prism 5.0 was used for statistical analysis. Statistical significance between parameters measured in Healthy individuals and SPAD patients, at the time of the extraction of the sample (0 h), was determined by the Mann–Whitney test. To compare the response to the same stimulus (Spn or Mtb) in Healthy individuals and SPAD patients, we used the Mann–Whitney test. To compare the different culture conditions within a specific group (at 48 h), statistical significance was determined by a Repeated Measures one-way ANOVA, followed by a *post hoc* Tukey's Multiple Comparison Test.

3. Results

3.1. Monocytes

In our evaluation of PBMC obtained from healthy individuals, we observed a higher percentage of classical (CD14++ CD16−) monocytes, as well as a lower percentage of intermediate (CD14++ CD16+) monocytes in children from 14 to 19 years old when compared to children from 7 to 13 years old (Table 1 and Fig. 1A). Our results show that in healthy individuals there is a difference in the absolute number of intermediate monocytes, while no significant differences were found for the classical and non-classical (CD14+ CD16+) monocytes (Table 1). We did not observe these differences in the monocyte distribution of SPAD patients (Table 1). As shown in Fig. 1B, SPAD patients have a different ratio of CD14++ CD16+ over total CD14++ monocytes when compared to healthy individuals of the same age group. We hypothesized that the altered ratios of monocyte subsets in SPAD patients may compromise the capacity of these cells to produce cytokines and to prime T cells during bacterial infections. Hence we decided to evaluate, both in SPAD patients and healthy individuals, the capacity of monocytes to respond to bacterial antigens. It is important to mention that functional studies that required drawing a second blood sample were performed in children from the 14 to 19 year old group.

We evaluated the spontaneous variation of CD16 expressing monocytes in non-stimulated whole blood cultures of healthy individuals, confirming previous reports that describe the loss of CD16 expression in culture [17,30]. Fig. 2A depicts in a healthy donor sample how the percentage of the three subpopulations can spontaneously change with time. We also confirmed that the percentages of monocytes from both healthy and SPAD individuals observed in whole blood samples corresponded to the data obtained from PBMC (Fig. 2B vs. Table 1). Taking this information into account we chose the 4 h time point to evaluate the capacity of the three monocyte subpopulations to produce TNF-α in the presence of heat inactivated *S. pneumoniae* reference strain (Spn), both in SPAD patients and in healthy individuals. We observed an increase in TNF-α production in the three monocyte subpopulations that was slightly higher in SPAD patients than in healthy individuals, even though differences were non-significant (Fig. 2C and D). CD14++ CD16− cells were the main TNF-α producers after antigenic stimulation both in healthy individuals and SPAD patients, although there was a lower percentage of these cells in the latter.

3.2. T cells

We studied the age-dependent frequency of both naïve and memory T cells in SPAD patients and in healthy individuals. Results are presented in Fig. 3. We observed a decrease in the percentage of naïve (CD27+ CD45RA+) T cells and an increased percentage of memory (CD27+ CD45RO+) T cells in healthy individuals that was not observed in SPAD patients (Fig. 3A and B), but we found no significant differences in the frequencies of effector CD27- T cells when SPAD patients were compared to healthy individuals (data not shown). Considering the absolute number of cells (Fig. 3C and D), our results show a decrease in the number of memory T cells that seems to be related to age in healthy individuals, while no significant differences were found for naïve T cells.

We wondered if the alterations we observed in SPAD patients could impact on their capacity to produce a sufficient T cell memory response towards encapsulated bacteria, so we decided to

Table 1
Changes in monocyte subpopulations in Healthy and SPAD children. Number of cells/μl and percentage of CD14++ CD16−, CD14++ CD16+ and CD14+ CD16+ monocytes in healthy and SPAD children from 7 to 13 ($n = 7$ and $n = 11$) and from 14 to 19 years old ($n = 15$ and $n = 7$) analyzed ex vivo ($t = 0$). Results are expressed as ẋ ± SEM. Significance: $^{*}p < 0.05$ (7–13 vs. 14–19 years); $^{\#}p < 0.05$, $^{\#\#}p < 0.001$, $^{\#\#\#}p < 0.0001$ (Healthy vs. SPAD) by Mann–Whitney test.

	Healthy		SPAD	
Cells/μl (%)	7–13	14–19	7–13	14–19
CD14++ CD16−	179.90 ± 44.56 (52.64 ± 9.01)	155.00 ± 17.57 (89.79 ± 1.34*)	165.00 ± 33.92 (76.30 ± 4.30#)	313.40 ± 63.64 (80.02 ± 5.25#)
CD14++ CD16+	122.20 ± 36.06 (39.83 ± 9.69)	9.10 ± 1.43* (6.97 ± 1.35*)	29.12 ± 6.99# (15.32 ± 3.67#)	75.74 ± 24.93### (19.19 ± 4.58##)
CD14+ CD16+	13.52 ± 3.37 (3.96 ± 0.61)	5.66 ± 0.79 (3.11 ± 0.33)	13.08 ± 2.57 (6.52 ± 1.23)	25.16 ± 8.36 (5.65 ± 1.07)

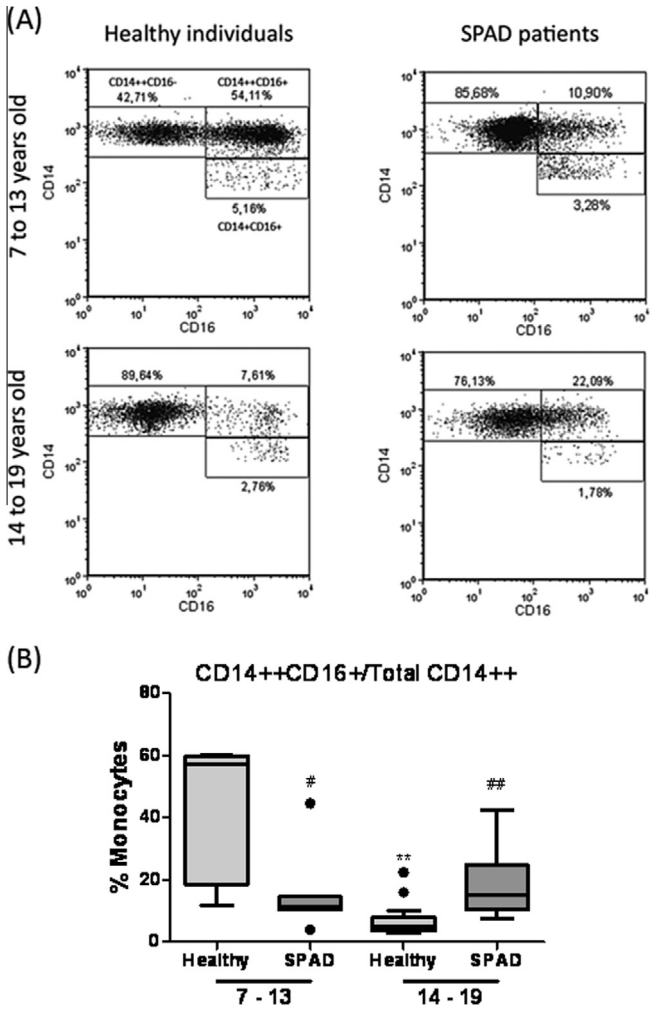

Fig. 1. Monocyte subpopulations in Healthy and SPAD children. (A) Dot plots depicting the changes in the percentages of classical (CD14++ CD16−), intermediate (CD14++ CD16+) and non-classical (CD14+ CD16+) monocytes analyzed ex vivo (at $t = 0$) in healthy and SPAD children. (B) Percentage of CD14++ CD16+ monocytes over total CD14++ monocytes in healthy and SPAD children from 7 to 13 ($n = 7$ and $n = 11$) and 14 to 19 ($n = 15$ and $n = 7$) years old. Significance: $^{**}p < 0.001$ (7–13 vs. 14–19 years); $^{\#}p < 0.05$, $^{\#\#}p < 0.001$ (Healthy vs. SPAD) by Mann–Whitney test.

study the *in vitro* T cell response to Spn from healthy and SPAD children, analyzing the expression of activation surface markers and the production of cytokines.

To determine the basal percentages of activated T cells we studied the expression of CD69 and CD25 at $t = 0$. As shown in Table 2, healthy individuals from 14 to 19 years old have a higher absolute number of CD8+ CD69+ T cells. SPAD patients 7–13 years old showed a significantly higher percentage of CD8+ CD69+ cells when compared to healthy individuals of the same age. When considering CD25 expression in CD4 and CD8 T cells from healthy individuals, we observed that the percentage of the former decreases and the percentage of the latter increases with age. These changes were not observed in SPAD patients, so we decided to study the absolute number and percentage of T regulatory (Treg) cells, the basal cytokine production of CD4 T cells and the CD25 expression and cytokine production of T cells cultured in the presence or absence of bacterial antigens in these children.

Overall, SPAD patients presented a lower number (0.34 ± 0.07 vs. 0.70 ± 0.09, $p < 0.05$; results expressed as mean ± SEM) and percentage (4.63 ± 1.04 vs. 12.58 ± 2.79, $p < 0.05$; results expressed as mean ± SEM) of CD4+ CD25+ Foxp3+ cells when compared to Healthy individuals. We didn't find any differences in the expression of TNF-α, IFN-γ, IL-10, IL-4, IL-13 and IL-9 from CD4 T cells when Healthy and SPAD children were compared, but we did find that the percentage of CD4+ IL-5+ cells was significantly higher in SPAD patients (Table 3). This value was still less than half of the expected value in atopic asthmatic patients [6].

We cultured PBMCs from both healthy individuals and SPAD patients for 48 h in the presence of heat inactivated Spn, or either one of two of the most frequently isolated strains of this bacterium in Argentina: strain 14 (Spn14) and strain 3 (Spn3) [31] or of the H37-RV strain of *M. tuberculosis* (Mtb) as a control. As shown in Fig. 4, CD25 expression seems to be higher in CD4 T cells from SPAD patients when stimulated with any of the three pneumococcus strains and it is significantly different for Spn3 (Fig. 4C). No changes in the percentage of CD4+ CD25+ T cells were observed in response to Mtb (Fig. 4D). We also observed a significant decrease in the percentage of CD8+ CD25+ T cells after stimulation with Spn in SPAD patients, while no differences were found with the other bacterial antigens (data not shown).

Finally, we studied the capacity of CD4+ T cells to produce inflammatory cytokines in the presence of bacterial antigens. We found no significant differences in the percentage of IL-17+ or IFN-γ+ CD4 T cells, cultured either with Spn or Mtb when healthy individuals were compared to SPAD patients (Fig. 5A and B). Nevertheless a marked tendency to a lower percentage of IFN-γ+ CD4 T cells was observed in SPAD patients for both antigens (Fig. 5A and B). Remarkably, we observed a significantly higher percentage of IL-9+ CD4 T cells in SPAD patients in response to Spn (Fig. 5B).

4. Discussion

Young children have an immature immune system and different aspects develop at different ages. Production of IgG2 subclass antibody, which contains most of the antibody response to polysaccharide antigens, appears at 2–5 years of age or later [32]. In SPAD patients defects in B cells have long been known: defective B cell switching [5,10] as well as an increase in CD5 positive B cells [7] have been reported. This seemed to agree with the traditional concept of the immune response to encapsulated bacteria, since response to *S. pneumoniae* was considered T cell independent. Nevertheless, recent reports have demonstrated that other cell populations are involved in this immune response, including cells from the innate immunity and T cells [28,33]. Snapper proposed that during infections with polysaccharide-encapsulated extracellular bacteria, polysaccharide-specific IgG responses are largely CD4 T cell dependent [34]. His group reported the need of inflammatory monocytes to induce a polysaccharide-specific antibody response to intact *S. pneumoniae* in a mouse model [35].

Differences in cells other than B cells had not been described until recently in SPAD patients, when age dependent changes in dendritic cell subsets observed in normal children, were reported absent in them [13]. As we have shown, SPAD patients have a different ratio of CD14++ CD16+/CD16− (intermediate/classical) monocytes than healthy individuals of the same age group (Table 1 and Fig. 1), suggesting that there might be an alteration in the correct development of these cells.

To determine if the imbalance we observed in monocyte subpopulations had an impact on their capacity to produce inflammatory cytokines, we evaluated their response to bacterial antigens. In our experimental system, classical monocytes appear to be the major TNF-α producers after antigenic stimulation of the three monocyte subpopulations, both in healthy individuals and SPAD patients, even though there was a lower percentage of these cells in the latter (Fig. 2). Patients with tuberculosis have a high ratio

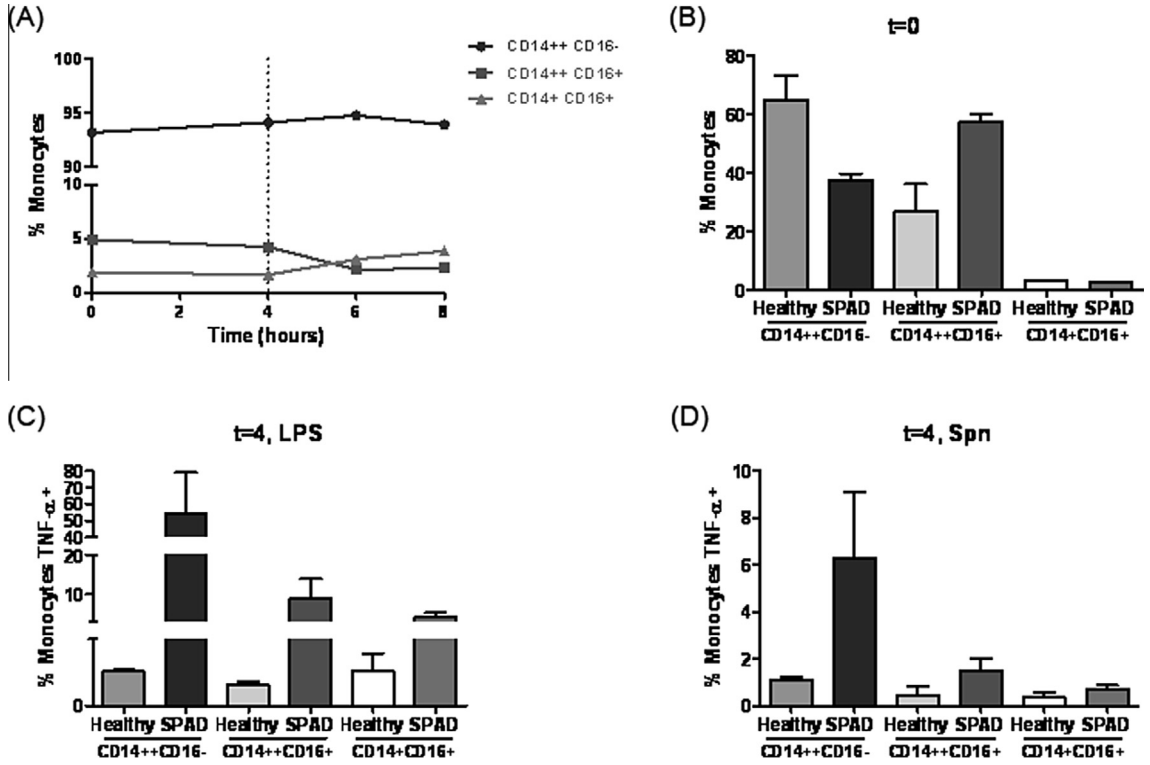

Fig. 2. Cytokine expression induced by bacterial antigens in monocyte subpopulations from Healthy and SPAD children. (A) Spontaneous variation of monocyte subsets from a healthy donor in a non-stimulated culture, at different time points. (B) Percentage of monocytes in whole blood at $t = 0$ ($n = 5$). Percentage of monocytes expressing TNF-α after 4 h stimulation in culture with (C) LPS or (D) Spn ($n = 5$). No significant differences by Mann–Whitney test.

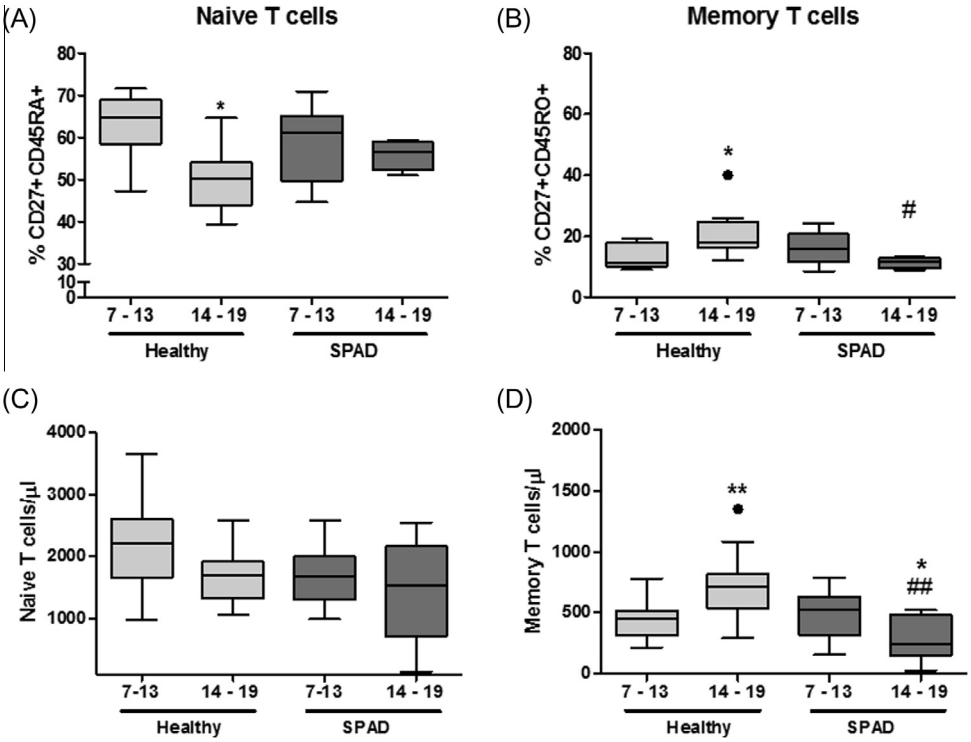

Fig. 3. Changes in Naïve and Memory T cells in Healthy and SPAD children. CD27+ CD45RA+ and CD27+ CD45RO+ T cell subsets in healthy and SPAD children from 7 to 13 ($n = 7$ and $n = 11$) and from 14 to 19 years old ($n = 15$ and $n = 7$) analyzed ex vivo ($t = 0$). Data expressed in percentages (A and B) and in cells/µl (C and D). Significance: $^{*}p < 0.05$ and $^{**}p < 0.001$ (7–13 vs. 14–19 years); $^{\#}p < 0.05$ and $^{\#\#}p < 0.001$ (Healthy vs. SPAD) by Mann–Whitney test.

Table 2
Changes in T cell subsets in Healthy and SPAD children. Number of cells/μl and percentage of activated CD4 and CD8 T cell subsets in healthy and SPAD children from 7 to 13 ($n = 7$ and $n = 11$) and from 14 to 19 years old ($n = 15$ and $n = 7$) analyzed ex vivo ($t = 0$). Data expressed as $\dot{x} \pm$ SEM. Significance: $^{*}p < 0.05$, $^{**}p < 0.001$ (7–13 vs. 14–19 years); $^{\#}p < 0.05$, $^{\#\#}p < 0.001$ (Healthy vs. SPAD) by Mann–Whitney test.

	Healthy		SPAD	
Cells/μl (%)	7–13	14–19	7–13	14–19
CD4+ CD69+	8.79 ± 1.52 (0.26 ± 0.05)	13.02 ± 4.29 (0.35 ± 0.10)	21.65 ± 5.57 (0.65 ± 0.20)	17.52 ± 3.97 (0.50 ± 0.12)
CD8+ CD69+	2.38 ± 0.61 (0.07 ± 0.02)	10.59 ± 5.35* (0.43 ± 0.21)	13.17 ± 5.77 (0.72 ± 0.30$^{\#\#}$)	6.11 ± 1.82 (0.48 ± 0.25)
CD4+ CD25+	32.08 ± 6.42 (3.94 ± 1.17)	24.88 ± 4.34 (0.72 ± 0.11**)	26.63 ± 7.92 (0.94 ± 0.22$^{\#}$)	36.10 ± 5.62 (1.33 ± 0.40)
CD8+ CD25+	7.55 ± 0.84 (0.66 ± 0.22)	6.11 ± 1.03 (0.18 ± 0.03*)	5.90 ± 0.83 (0.18 ± 0.03)	4.92 ± 1.26 (0.38 ± 0.18)

Table 3
Percentage of CD4 T cells expressing: TNF-α, IFN-γ, IL-10, IL-4, IL-5, IL-13 or IL-9; after stimulation of whole blood cells with PMA/ionomycin for 5 h, from both healthy ($n = 13$) and SPAD ($n = 10$) children. Significance: $^{\#}p < 0.05$; by Mann–Whitney test.

% T cells	Healthy ($n = 13$)	SPAD ($n = 11$)
CD4+ TNF-α+	36.34 ± 5.38	29.13 ± 4.66
CD4+ IFN-γ+	13.90 ± 2.01	10.39 ± 2.05
CD4+ IL-10+	0.32 ± 0.09	0.45 ± 0.10
CD4+ IL-4+	0.45 ± 0.12	0.86 ± 0.19
CD4+ IL-5+	0.27 ± 0.03	0.67 ± 0.14$^{\#}$
CD4+ IL-13+	0.97 ± 0.39	0.74 ± 0.19
CD4+ IL-9+	0.12 ± 0.02	0.26 ± 0.05

of CD16+/CD16− monocytes during chronic infections, and their CD16+ monocytes have an altered capacity of differentiation towards dendritic cells [30]. In a recent report a lower spontaneous monocyte production of IL-10 was observed in SPAD children compared to healthy controls [36]. Overall, alterations both in CD16 expression and in the production of inflammatory and anti-inflammatory cytokines, may affect the ability of these monocytes to become competent antigen presenting cells.

An optimal adaptive immune response requires immunological memory generation [37]. The variation of naïve/memory T cell subsets in healthy children was previously described using the classical phenotypic analysis of CD45RA and CD45RO expression in T lymphocytes [38], but this analysis has been expanded to include CD27 as a relevant marker [37,39,40]. When we studied the expression of the different isoforms of CD45 in CD27+ T cells of SPAD patients, we did not observe the age related increase in memory T cells present in healthy individuals (Fig. 3). It is of note that in our experiments in SPAD patients there was a tendency to a reduced response against Mtb by two subpopulations of effector T cells (CD4+ IL-17+ and CD4+ IFN-γ+) when compared to healthy individuals, even when they all have been vaccinated with BCG (Fig. 5). This may imply that SPAD patients do not respond in the same way as healthy individuals do against some protein antigens. We observed an increased percentage of CD4+ IL-5+ T cells in SPAD patients after stimulation of whole blood cells with PMA (Table 3); Th2 cytokines are well known for inhibiting Th1 cytokine production [41]. Furthermore, it has recently been described that, in contrast to IL-2 and TNF-α, IFN-γ production by CD4 memory T cells is characteristic of T cells at later stages of differentiation [40]. Altogether these data highlight the importance of studying further the differentiation of the T memory cell compartment in response to different bacterial antigens in SPAD patients.

In humans, it has been described that CD4+ T memory cells may be driven towards the Th9 profile [42]. IL-9 plays a role as a regulator of pathogenic vs. protective mechanisms of immune responses [43]. It was recently reported that human T cells can release IFN-γ and IL-17 when stimulated with Spn [28] and IL-9 has been associated to the Th17 effector response [43,44]. We observed a high percentage of CD4+ IL-9+ cells when stimulating

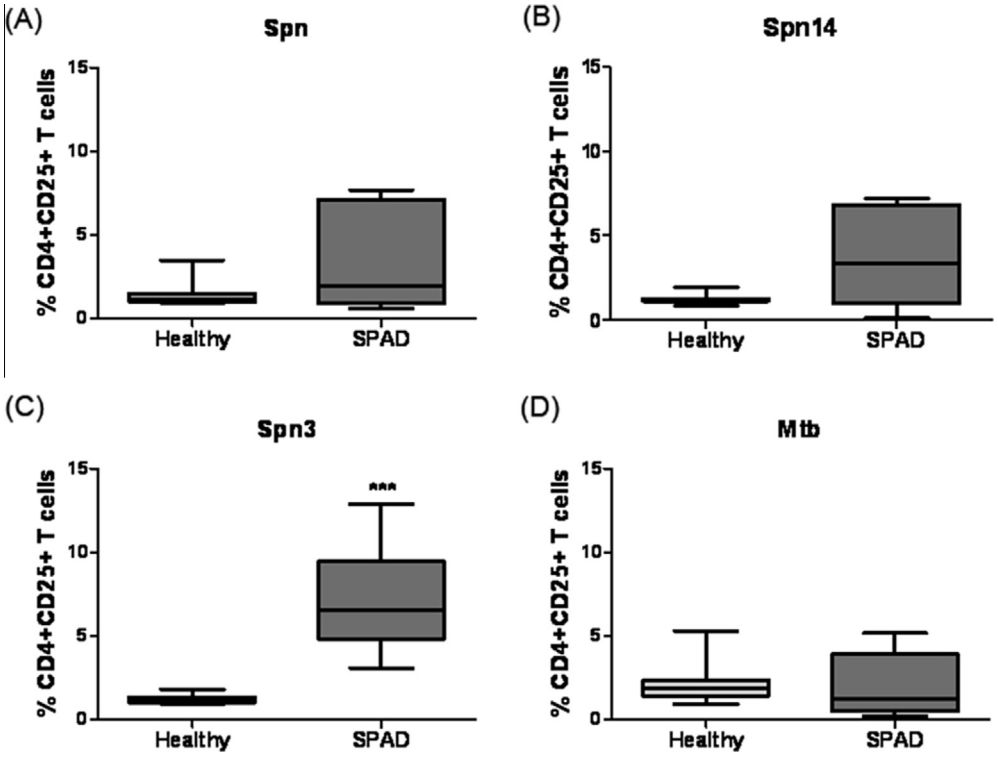

Fig. 4. CD25 expression induced by bacterial antigens in CD4 T cells from Healthy and SPAD children. Percentage of CD4+ CD25+ T cells in healthy ($n = 11$) and SPAD ($n = 13$) children after 48 h stimulation in culture with (A) Spn, (B) Spn14, (C) Spn3 or (D) Mtb. Significance: $^{***}p < 0.0001$; by Mann–Whitney test.

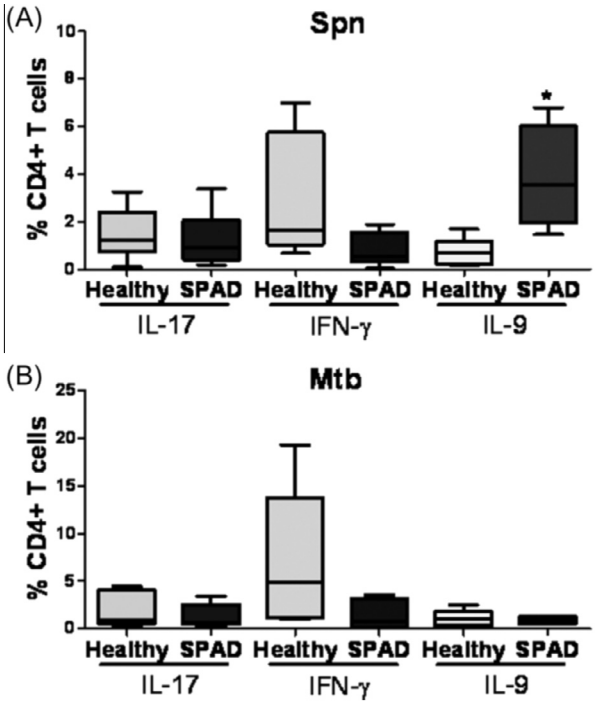

Fig. 5. Cytokine expression induced by bacterial antigens in CD4 T cells from Healthy and SPAD children. Percentage of CD4+ T cells expressing: IL-17, IFN-γ, or IL-9 in healthy ($n = 5$) and SPAD ($n = 5$) children after 48 h stimulation in culture with (A) Spn or (B) Mtb. Significance: $^{*}p < 0.05$; by Mann–Whitney test.

with Mtb, but not with Spn (Fig. 5). In *in vitro* experiments CD4+ T cells can differentiate into Th9 cells in the presence of TGF-β and IL-4 [45], while IFN-γ may suppress the TGF-β dependent Th9 differentiation [46]. Altogether, these data suggest that the Th1 response may be slightly altered in SPAD patients. It may be helpful to perform experiments with live *S. pneumoniae* in the future to characterize it.

We also observed alterations in the percentage of activated T cells at 0hs. When we evaluated CD25 expression, we were surprised by the fact that SPAD patients presented a high percentage of CD4+ CD25+ T cells in response to all the bacterial stimuli studied when compared with healthy individuals, which was significantly higher when Spn3 was the stimulus (Fig. 4C). This may be related to the altered mucoid capsular phenotype, present in this serotype, associated to its virulence [47]. Although we could not measure the percentage of Treg cells in 48 h cultures due to insufficient amount of sample, we did find that SPAD patients had a basal lower percentage of Foxp3 Treg cells when compared to Healthy individuals. The concept of Th cell plasticity explains immunopathogenesis phenomena [48] and *in vitro*, the conversion of Th17 cells into Treg cells and vice versa has been extensively studied [49–51]. Moreover, in a mouse model it has been described that IL-4 (a Th2 cytokine, elevated in atopic diseases) may inhibit the generation of TGF-β induced Foxp3+ Treg cells; instead, these cells differentiate into Th cells that can produce IL-9 and IL-10, but lack regulatory properties [52]. Since we did not observe an increase in the Th17/Th1 effector response in SPAD patients, which is the expected response to Spn or Mtb stimulation in humans [28,53], we believe that these CD25+ T cells could be differentiating to IL-9/IL-10 producing T cells. Further evaluation of CD4 T cells functional response to bacterial antigens might clarify a possible connection to the atopic manifestations these children often present.

It has been reported that the Th cytokine response to pneumococci is mainly derived from CD45RO+ memory T cells [28]. Furthermore, it has been described that antigen presenting cells that were previously activated via Toll-like receptors (TLRs) may reactivate memory Th17 and Th1 cells [54–56]. A compromise in NFκB pathway proteins, like TLR-2 and MyD88, was previously associated to selective polysaccharide antibody deficiency [3]. It still remains to be determined whether the abnormalities reported in SPAD patients B cells, dendritic cells, T cells and monocytes have a common molecular origin or if a defect in a signaling pathway affecting one of the cell lineages may have a negative impact on the development of the other immune system cells. Two candidate pathways to consider could be TLR-9 and NOD-like Receptor 2 which, as TLR-2, are important in the recognition of *S. pneumoniae* [28,57].

In conclusion, we have observed several differences in monocytes and T cell populations in SPAD patients that had not been previously described, but a larger number of patients should be studied to determine the extent of the age-related alterations in these cell types. We consider that the immunodeficiency in SPAD patients is much more complex than what was believed up to date. Further studies on their immune response against *S. pneumoniae* should provide a tool to better understand the basis and phenotypic expression of this complexity. These studies may eventually help develop better diagnostic tools or even alternative treatment options.

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