



TH1/TH2 balance in concomitant immediate and delayed-type hypersensitivity diseases

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ABSTRACT

In spite of the observation of mutual inhibitory properties of TH1 and TH2 CD4⁺ cells, a group of patients developed simultaneously immediate and delayed-type hypersensitivity reactions that are theoretically antagonistic. Patients presenting concomitant hypersensitivity reactions were evaluated for cytokine production.

PBMC from 45 patients and 13 non-atopic individuals were cultured with mite allergen and mitogen and the supernatants obtained were evaluated for cytokine production by ELISA.

The analysis of the cytokines levels revealed increased production of pro-inflammatory cytokine TNF- α in the non-atopic individuals after specific and mitogen stimulus. The IL-4 was largely observed on serum samples and IL-5 levels were higher in the double sensitized group (group DerpNi) after PHA stimulus. The IL-13 levels were increased in sensitized groups (Derp and DerpNi groups) after PHA stimuli. Atopic patients (Derp and DerpNi groups) presented lowest levels IFN- γ and the analysis of TGF- β production after rDER P I stimulation have shown increased levels among sensitized patients to *Dermatophagoides pteronyssinus* mite. IL-10 levels did not differ after antigen stimulation but basal production was higher on Derp and DerpNi groups. Furthermore, negative correlations were observed between IFN- γ levels and IL-4, IL-13 and IL-10.

This study has shown patients able to react, concomitantly, to the two types of antigens – rDER P I and NiSO₄, present distinct pattern of cytokine production. The increased levels of IL-13 in the sensitive individuals to mite antigen (rDER P I) and IFN- γ in NiSO₄ sensitized individuals confirm the role of the type TH2 response in the atopies and TH1 type in DCA.

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1. Introduction

The characterization of TH1 and TH2 lymphocytes subsets brought elucidative explanations regarding to pathogenic mechanisms of several diseases. Studies accomplished in the last years have confirmed that the differentiating process of these phenotypes is not a definitive event, but it is mutually excluding and presents crossed inhibiting effects [1].

In atopic diseases, experimental and theoretical data support the idea that activation of TH2 cells leads to production of IL-4, IL-5 and IL-13 that are cytokines involved in the synthesis of specific IgE and eosinophilic inflammation [2,3]. On the other hand, TH1 cells, that produce IFN- γ , are involved on delay type hypersensitivity [2]. In spite of this observation and of their mutual inhibitory properties, the TH1/TH2 differentiation does not explain some features such as increasing IFN- γ production by peripheral blood mononu-

clear cells (PBMC) stimulated by *Derf* antigens in atopic children [4] and the presence of activated TH1 cells in asthmatic patients with bronchial inflammation and hyper-responsiveness [5] and in some experimental models of asthma [6].

In the last 20 years it was observed an increase on the prevalence of atopic and autoimmune diseases all over the world, particularly in developed countries. Epidemiological studies suggest that the population socioeconomic improvement, better conditions of hygiene and health, allied to vaccination procedures would be associated to this phenomenon (Hygiene Hypothesis). The TH1/TH2 paradigm seems to support this hypothesis. The early exposition to microbial products, especially Gram-negative bacteria endotoxin, could perform a protecting effect against the development of allergic diseases, as it favors the development of a TH1 immune response [7]. In spite of the consistency of the role of endotoxin in protection against the development of atopic diseases, some studies suggest its participation in asthma exacerbation [8]. On the other hand, a TH2 type response induced by some parasites, especially helminthes seems to protect against atopic disease [9,10].

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The evaluation of this information suggests a larger complexity of immunological pathways involved on the induction of atopic disease. Moreover, considering that atopic IgE mediated allergic disease and contact dermatitis involve two distinct and mutual inhibitory mechanisms, the diagnosis of patients suffering simultaneously from these different conditions can not be easily explained in the point of view of TH1/TH2 paradigm.

In this study, patients presenting the two polar types of hypersensitivity – IgE- and cell-mediated hypersensitivities were analyzed for cytokine production by PBMC after mitogen and specific antigen stimulation.

2. Casuistic and methods

2.1. Patients

Forty-five patients selected from the Allergy and Immunology section of the Universidade Federal do Triângulo Mineiro (UFTM) were enrolled in this study. Every subject has referred signs and symptoms of allergic rhinitis, asthma and/or contact dermatitis, and has presented immediate-type and/or delayed-type sensitization identified through *in vivo* tests performed with standardized allergenic extracts. *Dermatophagoides pteronyssinus* (58.500 UBE/ml), *Dermatophagoides farinae* (20.175 UBE/ml) e *Blomia tropicalis* (37.650 UBE/ml) allergens (IPI-ASAC Brasil–São Paulo–SP) were used on the skin prick test (SPT) and a set of standard chemical products, including the nickel sulfate, were employed on the patch test (PT), as previously described [11,12]. The patients were arranged into four groups according to the observed skin reactions to these allergens. *Derp Group* was composed by individuals with exclusive positive skin reaction to *Derp*, while those with positive reaction only to nickel sulfate on the PT constitute the *Ni Group*. Those patients with simultaneously reaction to *Derp* and nickel sulfate established the *DerpNi Group*. Thirteen individuals without complaints of allergic diseases and no reaction to stimulating antigens (*negative prick and patch tests*) were considered *negative controls*.

All patients with suspect or confirmed immunological compromising and those with established diagnosis of any other allergic diseases such as atopic dermatitis and urticaria were excluded from this study. Usage of drugs 15 days before and presence of infectious diseases in the previous 7 days, were criteria to postpone the “*in vitro*” assays.

Mononuclear cells (PBMC) and serum of each patient was obtained from circulating blood through peripheral vein puncture. PBMC were used in supernatants production procedures.

This study was approved by the Ethical Committee of the UFTM and started after signed free consent informs was obtained.

2.2. Cell culture

Peripheral mononuclear cells (PBMC) were isolated from heparinized blood by centrifugation (400 × g for 20 min, in room temperature) on Ficoll-Hypaque (PHARMACIA-Upsala, Sweden). The cells were collected, washed three times RPMI medium (GIBCO-Grand Island, NY, USA) and suspended in RPMI supplemented with 2 mM L-glutamine (GIBCO), 40 µl/ml gentamicin (ARISTON-Brazil), 2 mM HEPES (GIBCO), 50 mM 2-mercaptoetanol (GIBCO) and 5% fetal calf serum (complete RPMI) [13].

Twenty four cavities plates (FALCON-Lincoln Park, NJ, USA) were used to cultivate 2.0×10^6 cells per milliliter per well in presence of RPMI medium, 5 µg/ml recombinant *D. pteronyssinus* antigen (rDER P I) (INDOOR Biotechnologies, Charlottesville, VA, USA), 5 µg/ml nickel sulfate (NiSO₄) (SIGMA-St Louis, Mo, USA), 5 µg/ml LPS (SIGMA) and PHA (5 µg/ml) (SIGMA) for supernatants production.

The cells were incubated at 37 °C in a 5% CO₂ atmosphere. At 48 h and 120 h the supernatants were collected and stored at –70 °C for analysis of the cytokines production. The procedures were achieved in sterile conditions.

2.3. Cytokines quantification

For cytokines analysis, microplates (NUNC – Roskilde, Denmark) were sensitized overnight with anti-TNF-α monoclonal antibody (Mab) (PHARMINGEN-San Diego, CA, USA); anti-IL-4 Mab (MabTech-Nacka, Sweden); anti-IL-5 Mab (PHARMINGEN); anti-IL-10 Mab (PHARMINGEN); anti-IL-13 Mab (MabTech); anti-TGF-β Mab (R&D System-Minneapolis, MN, USA) and anti-IFN-γ Mab (MabTech). Nonspecific binding were prevented by incubating the plates with 2% BSA (SIGMA) in PBS. Plates were incubated overnight with 100 µl of 1:2 dilution supernatants or serum in 2% BSA-PBS and 100 µl of standard cytokines (PHARMINGEN and R&D). The plates were washed 4 times in PBS and 0, 05% Tween and incubated with biotinilated antibodies (Ac-B anti-IL-4, IL-5, IL-10, IL-13, TNF-α, TGF-β and IFN-γ at 1 µg/ml) for 4 h. Plates were then washed and incubated with alkaline phosphatase conjugated with streptavidin (MabTech). Finally, the plates were washed again and the enzymatic activity was developed by incubating the plates with p-nitrophenil phosphate (SIGMA). The absorbance was read at 405 nm (Abs405) in microplates reader set (BIORAD, Hercules, CA, USA). The sensitivity of the tests ranged from 5 to 30 pg/ml.

2.4. Statistical analysis

According to the nature of the studied variables, non-parametric tests were used: Kruskal–Wallis, Mann–Whitney and Spearman correlation test. The level of rejection of the null hypothesis was lower than 5% ($p < 0,05$). Statview 4.0 and Epi-Info 6.04 were employed for database storage and analysis.

3. Results

The group evaluated was composed by 58 patients with 35 (± 12) years old average, prevailing the female individuals (Table 1). The response to *Der p* and nickel sulfate allergens in cutaneous tests accomplished previously [14] allowed to group the participants into groups (Table 1) and identified the *D. pteronyssinus* antigen (*Derp* I) and nickel sulfate (NiSO₄) as relevant antigens that justified their usage as specific stimulus on cell culture. Briefly, on this previous study a group of patients who developed simultaneously types I and IV hypersensitivity reactions were identified. Ninety two patients ($n = 92$) were submitted to clinical evaluation and SPT with aeroallergens and PT with a set of standardized chemical substances. Positive PT to at least one substance was observed in 74% patients and the analysis of the SPT demonstrated that 64% patients had a positive reaction to at least one aeroallergen. Forty-six patients

Table 1
General characteristics of the evaluated subjects.

	Characteristic	N	%
Age	35 years (±12)		
Gender	Female	46	79
Groups	Skin prick test* Patch test**		
Derp group	+	15	26
Derp Ni group	+	15	26
Ni group	–	15	26
Negative group	–	13	22

* Skin prick test – used several aeroallergens. *D. pteronyssinus* was considered the relevant antigen to the evaluated individuals.

** Patch test – used standard set of chemical substances. Nickel sulfate was considered the relevant substance to the evaluated individuals.

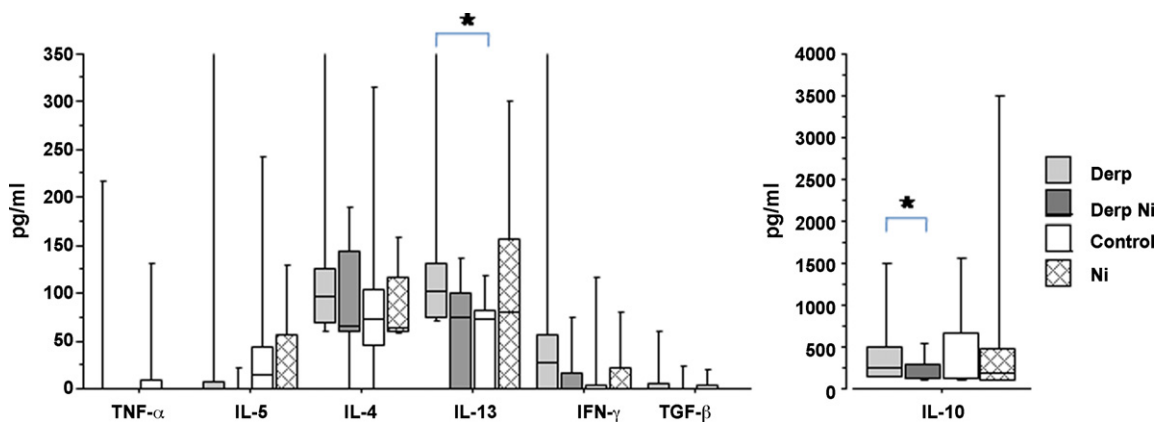


Fig. 1. TNF- α , IL-5, IL-4, IL-13, IFN- γ , TGF- β and IL-10 levels in serum samples (pg/ml) according to the skin tests response (*Derp* group: patients with positive reaction to *D. pteronyssinus* at the skin prick test; *Ni* group: patients with positive reaction to nickel sulfate at the patch test; *DerpNi* group: patients with simultaneous positive reaction to *D. pteronyssinus* and nickel sulfate at the skin prick test and patch test, respectively; *Control* group: asymptomatic and non-sensitive individuals). Significant differences between groups are indicate * (Mann–Whitney, $p < 0.05$).

(50%) demonstrated positive reaction simultaneously to SPT and PT (double sensitized group) suggesting a great deal of complexity in immunoregulatory mechanisms that govern the pathogenesis of these theoretically antagonistic group of hypersensitivity, the IgE- and cell-mediated diseases.

To investigate the immune response pattern of these double sensitized patients the “*in vitro*” cytokines production after antigens and mitogens stimuli were performed. The cytokines TNF- α and IL-4 were quantified in serum and in the 24 h culture supernatants and IL-5, IL-13, IFN- γ , TGF- β and IL-10 were evaluated in serum and in 120 h culture supernatants.

The levels of the cytokines in serum are shown in the Fig. 1, show that IL-13 was significantly higher in *Derp* group patients.

The analysis of the cytokines levels in culture supernatants revealed that the IFN- γ production after PHA stimulation was significantly higher among non-sensitized (negative group) and nickel sulfate individuals (*Ni* group) when compared to patients sensitized to *Derp* alone or *Derp* and nickel (*Derp* and *DerpNi* groups) ($p < 0.05$) (Fig. 2a). This result suggests that subjects sensitized to *Derp* have the ability to down modulate the production of IFN- γ .

On its turn, TNF- α levels were significantly higher in the non-sensitive individuals (negative group) after stimulus

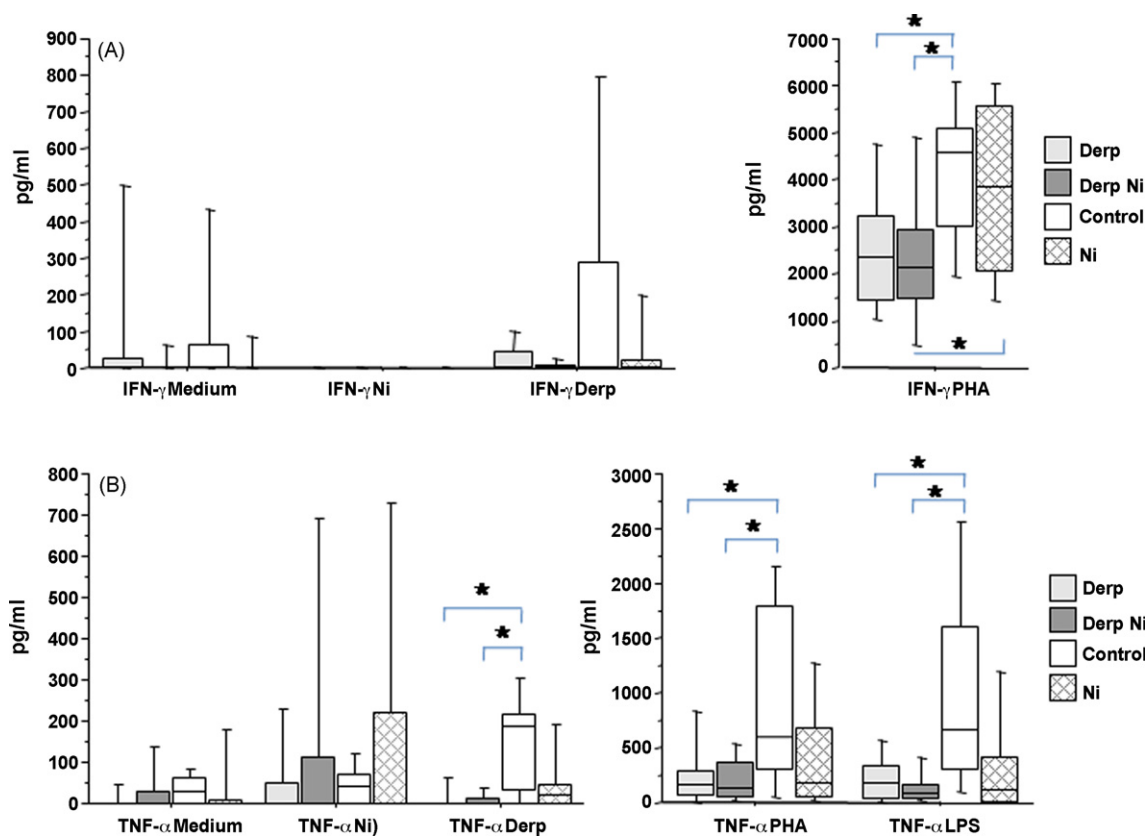


Fig. 2. IFN- γ (A) and TNF- α (B) production by peripheral blood mononuclear cells (PBMC) cultured with different stimuli considering the four groups. (*Derp* group: patients with positive reaction to *D. pteronyssinus* at the skin prick test; *Ni* group: patients with positive reaction to nickel sulfate at the patch test; *DerpNi* group: patients with simultaneous positive reaction to *D. pteronyssinus* and nickel sulfate at the skin prick test and patch test, respectively; *Control* group: asymptomatic and non-sensitive individuals). Significant differences between groups are indicate * (Mann–Whitney, $p < 0.05$).

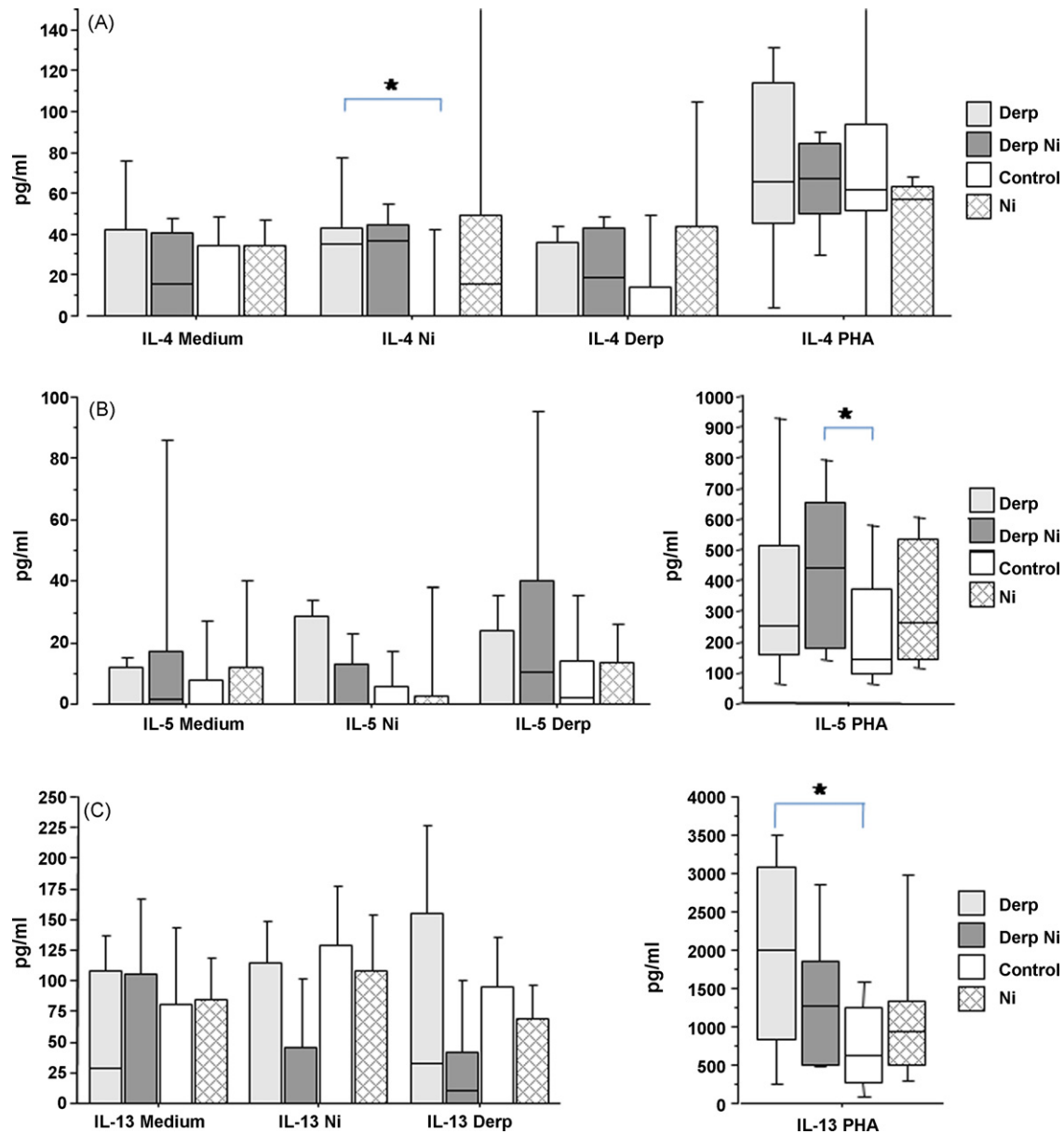


Fig. 3. IL-4 (A), IL-5 (B) and IL-13 (C) production by PBMC cultured with different stimuli considering the four groups. (Derp group: patients with positive reaction to *D. pteronyssinus* at the skin prick test; Ni group: patients with positive reaction to nickel sulfate at the patch test and patch test, respectively; Control group: asymptomatic and non-sensitive individuals). Significant differences between groups are indicate * (Mann–Whitney, $p < 0.05$).

with LPS ($p = 0.017$), PHA ($p = 0.02$) and rDER P I ($p = 0.001$) (Fig. 2b).

Regarding to the TH2 cytokines, IL-4 was largely observed on serum samples from all subjects groups and IL-13 serum levels was significantly higher in Derp group compared to Negative group ($p = 0.03$) (Fig. 3). Interestingly, IL-4 levels, in culture supernatants, were significantly higher on Derp group, compared to Negative group after Ni stimulation (Fig. 3a). IL-5 levels were higher in the double sensitized group (DerpNi) compared to Negative group after PHA stimulus ($p = 0.022$) (Fig. 3b). The IL-13, other important TH2 cytokine was increased in supernatants stimulated with PHA in Derp and DerpNi groups compared to Negative and Nickel groups ($p = 0.017$) (Fig. 3c). Although no differences were observed after antigen stimulation, this result suggests that subjects sensitized to Derp have higher ability to produce IL-13 independent of their reaction to nickel in delay hypersensitivity test.

The analysis of regulatory cytokines revealed higher levels of TGF- β after specific stimulation with rDER P I or PHA among the patients sensitized to *D. pteronyssinus* – Derp and DerpNi groups when compared to non-sensitized individuals and nickel sensitized group ($p < 0.05$) (Fig. 4a).

The IL-10 was also investigated and there were no significant differences in its level, after antigens or PHA stimulation, when the four groups were compared. On the other hand, basal production (non stimulated cultures) of IL-10 reveal that subjects sensitized to nickel alone produced significantly lower IL-10 levels than patients of Derp and DerpNi groups ($p = 0.04$) (Fig. 4b). Higher levels of IL-10 were observed in serum samples of group Derp individuals compared to DerpNi ($p = 0.05$) (Fig. 1).

When all patients were considering together, a negative correlation was observed between IFN- γ levels in PHA stimulated cultures, and TH2 cytokines, IL-4 and IL-13 and also between IFN- γ levels and IL-10 (Fig. 5).

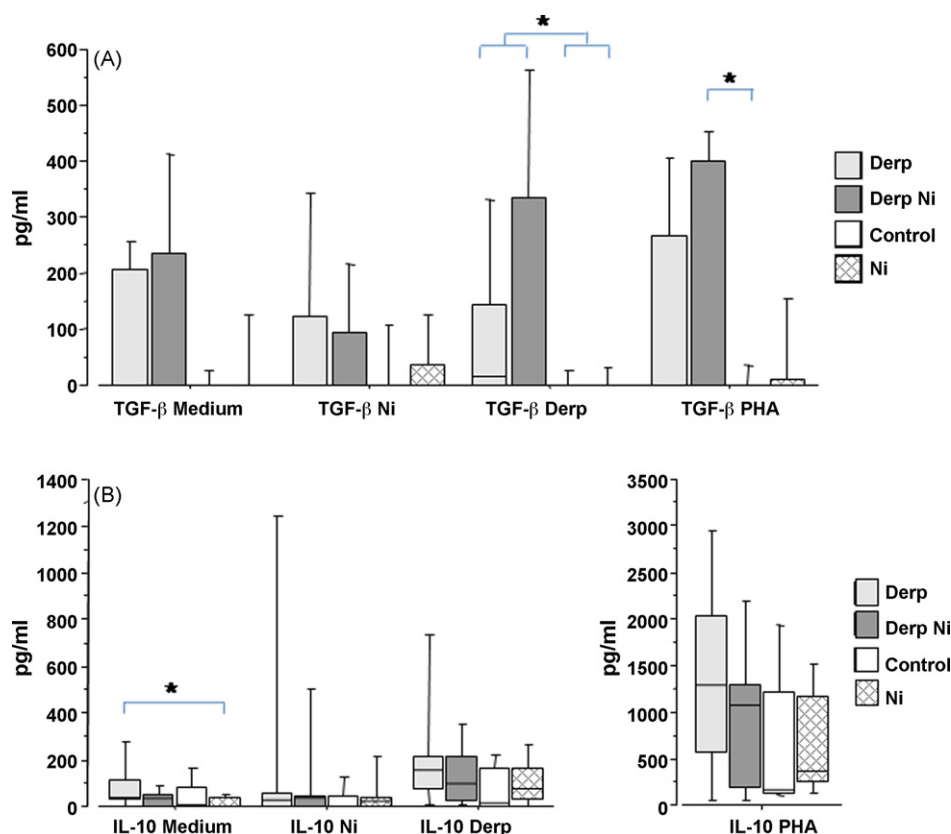


Fig. 4. TGF- β (A) and IL-10 (B) production by PBMC cultured with different stimuli considering the four groups. (Derp group: patients with positive reaction to *D. pteronyssinus* at the skin prick test; Ni group: patients with positive reaction to nickel sulfate at the patch test; DerpNi group: patients with simultaneous positive reaction to *D. pteronyssinus* and nickel sulfate at the skin prick test and patch test, respectively; Control group: asymptomatic and non-sensitive individuals). Significant differences between groups are indicate * (Mann–Whitney, $p < 0.05$).

4. Discussion

The aim of this study was to evaluate cellular immune response of patients with concomitant IgE- and cell-mediated hypersensitivity. So, mononuclear cells were cultured in presence of mitogens and specific antigens. The supernatants produced were evaluated for the presence of TH1, TH2 and regulatory cytokines.

Although high production of TNF- α has not been associated with atopic diseases [15], it is secreted mainly by activated mononuclear cells and were quantified with the purpose of evaluating the presence of inflammatory activity in these individuals. Reduced production of TNF- α by cells, specifically and nonspecifically stimulated, was observed among the allergic sensitized individuals (Derp, DerpNi and Ni groups) when compared to those of Negative group, suggesting an attempt of controlling the inflammatory response in these groups.

The observation of higher levels of IFN- γ in Ni group and lower levels of IFN- γ associated to higher levels of IL-13 in Derp group confirmed the predominance of TH1 response pattern among individuals presenting the delayed-type hypersensitivity/DCA and the TH2 pattern among the immediate type hypersensitivity/atopies as observed by others [15,16]. The identification of a double sensitized group (DerpNi), presenting clinical signs and symptoms of both polar types of hypersensitivity suggest that the regulatory mechanisms by mutual inhibition of CD4 $^{+}$ T cells phenotypes are not operating. The analysis of cytokines production in order to identify the predominant pattern of response on DerpNi group was the initial step for the comprehension of the apparent controversy related to TH1/TH2 balance on hypersensitivity diseases.

Atopy (allergic rhinitis and asthma) and contact dermatitis are inflammatory diseases broke out by antigens of distinct bio-

chemical nature that are not handle similarly by the immune system inducing inflammatory damage to different target organs by different effectors mechanisms [17,18]. This could favor the compartmentalization and dichotomy of immunological response such as described in some infectious diseases [19]. If so, perhaps the addressing of effectors cells commanded by “homing” receptors could favor the differentiation of polar and antagonistic subtypes of cells T (TH1/TH2) in different organs where the reciprocal inhibitory control would be hiding. This hypothesis may be supported by the observation of negative correlation between IFN- γ levels in PHA stimulated cultures and IL-4, IL-13 and IL-10.

The analysis of cytokines production in DerpNi group revealed lower levels of IFN- γ than those found in Ni group and even lower than in Derp group. This data is pointing to an *in vitro* TH2 response inhibitory mechanism over the TH1 response that does not reflect on clinical manifestation, *in vivo*, probably due to the compartmentalization of immune response. Perhaps this would be greater evidence that mutual inhibition mechanism does occur.

The set of clinical manifestations presented by patients with double sensitized group may suggest impaired mechanisms of reciprocal control, so that mechanisms of cellular regulation acting after phenotypical differentiation could be necessary to modulate the inflammatory response at the target organ. The presence of mutations in the gene *Foxp3* in X-Linked Autoimmunity-Allergic Disregulation Syndrome [20] and the decreased inflammation in airway and bronchial hyperresponsiveness in experimental models by the regulatory cytokines TGF- β and IL-10 [21,22] indicate the participation of regulatory cells in negative modulation of bronchial inflammation in asthma and allergic diseases.

Some authors have demonstrated that repeated antigenic presentation can lead to the development of regulatory T cells favoring

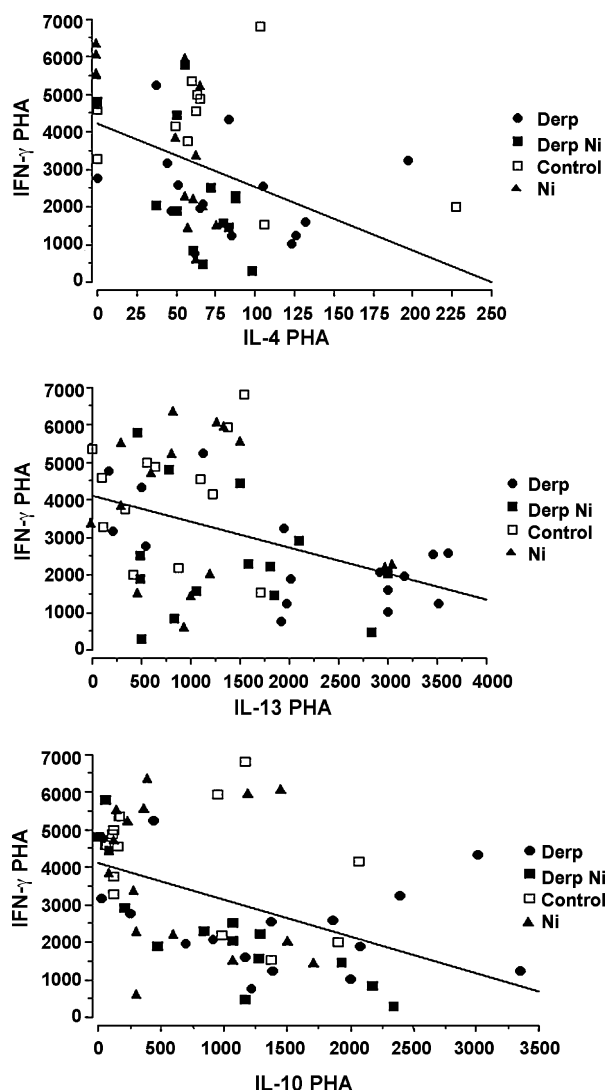


Fig. 5. Correlation between levels of IFN- γ and IL-4, IL-13 and IL-10 in PBMC cultures stimulated with PHA. (Derp group: patients with positive reaction to *D. pteronyssinus* at the skin prick test; Ni group: patients with positive reaction to nickel sulfate at the patch test; DerpNi group: patients with simultaneous positive reaction to *D. pteronyssinus* and nickel sulfate at the skin prick test and patch test, respectively; Control group: asymptomatic and non-sensitive individuals). Significant negative correlation was observed when all groups were considered together (Spearman $p < 0.05$).

the immunological tolerance and regulation of activated cells. When the production of such cytokines is insufficient or the production of TH2 or TH1 cytokines is excessive, the consequence is tissue lesion and disease [23]. Regulatory mechanisms of active immune response were evaluated by quantification of inhibitory cytokines (IL-10 and TGF- β). It was possible to detect the presence of IL-10, cytokine produced by cells TH2, TH1, macrophages and dendritic cells, in serum and in supernatant of cells of atopic individuals cultured without any stimuli (Derp and DerpNi groups), suggesting a spontaneous production of IL-10.

Regarding to TGF- β , patients with immediate type hypersensitivity (Derp and DerpNi group) were able to produce the highest levels of this cytokine after specific (rDER P I) and nonspecific stimulation (PHA). The presence of TGF- β , a cytokine that perform different functions associated to the presence of the IL-10 in these groups could indicate an attempt of negatively modulate the immunological mechanisms. This ineffectiveness of TGF- β to control immune response may be explained by the recent find-

ing that STAT6 binding to *FoxP3* promoter, inhibiting its expression [24,25].

The exact meaning of the presence of high levels of regulatory cytokines observed on these individuals with immediate type hypersensitivity is not clear. The set of data presented here suggests the attempt of controlling the exaggerated cellular activation by antigens, the following inflammation and their consequences. These regulatory mechanisms probably initiated by cellular activation seem also not to be well succeeded because they all manifest their diseases clinically. In spite of this, it is in DerpNi group that probably these regulatory mechanisms were less efficient since they are double-sensitized individuals.

The data related to the non-sensitized individuals (Negative group) seem to bring important information. They were the higher producers of TNF- α and presented considerable levels of IFN- γ to the different stimulus, probably indicating that the response in non allergic individuals may be predominantly of TH1 type. Besides, the negligible levels of IL-10 and the absence of TGF- β in this group could suggest the participation of some other mechanism of tolerance and/or regulation of immunological response to guarantee the individuals condition of non-allergic.

5. Conclusion

At the end of this study, the presence of patients able to react, concomitantly, to the two types of antigens – rDER P I and NiSO₄, developing antagonistic hypersensitivity mechanisms is documented. The increased levels of IL-13 in the sensitive individuals to mite antigen (rDER P I) and IFN- γ in NiSO₄ sensitized individuals confirm the role of the type TH2 response in the atopies and TH1 type in DCA. The increased levels of regulatory cytokines IL-10 and TGF- β in atopic patients suggest attempts to control the inflammatory response. The presence of detectable response observed in a non-sensitized group (Negative group) demonstrates the requirement of studies regarding to regulatory and tolerance immunological mechanisms to non-self antigens in allergic and asymptomatic individuals.

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