



Expression of Th1, Th2, lymphocyte trafficking and activation markers on CD4+ T-cells of Hymenoptera allergic subjects and after venom immunotherapy



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ABSTRACT

Systemic reactions to Hymenoptera stings can be fatal and represent a reduction in the quality of life. The immune mechanisms involved in venom allergic subjects are barely known. Nevertheless, a shift towards a Th1-type response with an increase in IFN γ levels has been observed after venom immunotherapy (VIT). There is currently no information available about the expression of markers on CD4+ T-cells or their involvement in venom allergy, nor following VIT. For this, we have studied the expression of Th1 and Th2-cell markers, homing receptors and activation markers on CD4+ T-cells of subjects who presented systemic allergic reactions, mainly to *Polistes dominulus*, and after receiving a 4-month conventional VIT protocol. The markers studied were: CD26 (Th1), CD30 (Th2), CXCR4, CXCR3 (Th1), CCR4 (Th2), CD154 (CD40L), CD152 (CTLA-A), and ICOS. We also determined the IL-4 (Th2) and IFN γ (Th1) intracellular cytokine levels in T-cells and carried out a basophil activation test (BAT). Comparing venom allergic subjects with non-allergic healthy controls, we have found up-regulation of CD26, CXCR4, CXCR3, CD154 and ICOS. Conversely, a down-regulation of CD30, CD154 and CD152 occurred upon immune intervention, whereas the remaining markers were not affected. Equally, VIT has been shown to be effective, as evidenced by the decrease of basophil degranulation and increase of IFN γ levels in T-cells after the fourth month of treatment. These new findings highlight the possible application of these surface molecules as markers to distinguish between symptomatic and asymptomatic subjects sensitized to Hymenoptera venom, as well as revealing information about the immune changes associated with VIT.

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1. Introduction

Systemic reactions to Hymenoptera stings can produce life-threatening reactions in venom allergic patients. In the Mediterranean area of Spain it has been described a 2.3% of systemic reactions mainly to *Polistes* and *Vespula* (Fernandez et al., 1999). Venom allergy can be mediated by immunologic mechanisms IgE- or non-IgE-mediated, but also by non-immunologic (Johansson et al., 2001). However, in the greatest part of patients with venom allergy can be demonstrated an IgE-mediated reaction by allergic sensitization measured with positive specific-IgE (sIgE), and positive skin tests (Biló et al., 2005).

Venom immunotherapy (VIT) is the only intervention in patients with a history of systemic anaphylactic reactions that has proven effective in more than 80% of venom allergic patients (Golden, 2005). The immunologic mechanisms underlying VIT include: (a) a shift from Th2-type cytokines (IL-4) to Th1-type cytokines (IFN γ) (Schuerwegh et al., 2001); (b) an increase in the IL-10-producing regulatory T-cells (Treg) (Meiler et al., 2008); and (c) an increase in the number of peripheral Treg cells (Mamessier et al., 2006). Conversely, a decrease in the number of Treg cells with coexpression of lymph node homing receptors CCR7/CD62L was observed after VIT, indicating recirculation to secondary lymphoid organs (Kerstan et al., 2011).

However, to date, there is no information available regarding the expression of Th1- and Th2-cell markers, homing receptors and activation markers on CD4+ T-cells of venom allergic subjects, nor concerning their implication in the induction of tolerance after VIT. To assess the possible intervention of T-cell markers in

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Table 1

T-cells surface markers used in the study.

Surface marker	Immune response	Reference
CD26	Th1-cell marker	Annunziato et al. (1998), Krakauer et al. (2006)
CD30	Th2-cell marker	Romagnani et al. (1995), Oflazoglu et al. (2009)
CXCR4	Lymphocyte trafficking marker	Bai et al. (2009), Werner et al. (2013)
CXCR3	Th1-cell marker	Kim et al. (2001), Groom and Luster (2011)
CCR4	Th2-cell marker	Kim et al. (2001), Campbell and Hay Glass (2000)
CD154	Non-costimulatory activation marker	Kroczeck and Hamelmann (2005)
CD152	Costimulatory activation marker	Beier et al. (2007)
ICOS	Costimulatory activation marker	Beier et al. (2007)

Hymenoptera venom allergy and during VIT, we have investigated the surface expression of CD26, CD30, CXCR4, CXCR3, CCR4, CD154 (CD40L), CD152 (CTLA-4), and ICOS on CD4⁺ T-cells from patients with Hymenoptera venom allergy both before and after being treated with a VIT protocol (Table 1). These markers were selected in accordance with their involvement in the allergic pathology, CD26 and CXCR3 as Th1-cell markers (Schade et al., 2002; Campbell et al., 2001), CD30 and CCR4 as Th2-cell markers (Rojas-Ramos et al., 2007; Banfield et al., 2010), and the non-costimulatory (CD154) and costimulatory molecules (CD152 and ICOS) in the induction and regulation of immunoglobulin isotype switching (Kroczeck and Hamelmann, 2005). CXCR4 as marker of lymphocyte trafficking to areas of inflammation was also added in the study (Gonzalo et al., 2000). In addition, basophil activation tests (BAT) were performed and intracellular expression of IL-4 and IFN γ in T-cells was investigated to evaluate the efficacy of the VIT protocol.

2. Materials and methods

2.1. Subjects and controls

Ten patients with a history of systemic reactions to Hymenoptera stings from the Allergy Outpatient Clinic of the General University Hospital of Ciudad Real (Spain) were enrolled in the study. Two patients were female and eight were male, with a mean age of 49.3 ± 18.08 years (mean $\pm$ SD). Most patients had venom allergy to *Polistes dominulus* ($n=7$), another to *Vespula* spp. ($n=1$), *Apis mellifera* ($n=1$), and a final patient presented allergy to both *P. dominulus* and *Vespula* spp. ($n=1$). The sensitization state was determined through intradermal skin tests (IDT) and venom-specific IgE (sIgE) levels according to the diagnostic recommendations (Biló et al., 2005). The clinical characteristics of the venom allergic patients are summarized in Table 2. Moreover, 10 healthy volunteers, blood donors in the hospital stated above who were non-atopic and with no known allergies were included in the study. The mean age of the healthy controls was 44 ± 12 years (three females, seven males). All patients and controls gave their informed consent to participate in the study.

2.2. Venom immunotherapy protocol

Seven out of the ten venom allergic patients who, within the last 2 years, presented a history of anaphylaxis caused by Hymenoptera stings were eligible for venom immunotherapy (VIT) according to the criteria of the European Academy of Allergy and Clinical Immunology (Table 2) (Bonifazi et al., 2005). These patients were treated with a conventional VIT protocol of subcutaneous injections of the venom administered in the induction phase at doses increasing up to 100 μ g by the 30th day (Fig. 1). Maintenance injections of 100 μ g were then administered at monthly intervals. Blood samples of the active patients were collected at baseline time before VIT (T0), and after the fourth month of therapy (T4). The corresponding venom extracts were supplied by ALK-Abelló, S.A., (Madrid,

Spain). All patients gave their informed consent for the immune intervention.

2.3. Mononuclear cell isolation

Cells were isolated from heparinized venous blood by density gradient centrifugation (Ficoll-Hypaque, Sigma-Aldrich, St. Louis, MO, USA). Mononuclear cells were then washed twice in phosphate-buffered saline (PBS) and resuspended in 2 mL of RPMI-1640 cell culture medium (Gibco, Scotland, UK) supplemented with streptomycin (100 UI/mL) and penicillin (100 mg/mL). For surface staining conditions we used 1 mL of diluted lymphocytes obtained immediately after cell isolation. Cells from allergic patients were collected just before venom immunotherapy (T0) and after 4 months of treatment (T4).

2.4. Intracellular T-cell cytokine assay

Lymphocyte cells at a concentration of 1×10^6 mL⁻¹ (1 mL per tube) were stimulated for 16 h with 50 ng/mL of phorbol myristate acetate (PMA) (Sigma-Aldrich, Steinheim, Germany) and 1 μ M of ionomycin (Sigma, Steinheim, Germany) in 5% CO₂ at 37 °C. A BD GolgiPlugTM protein transport inhibitor (Becton Dickinson, San Jose, USA) was added for the last 5 h of the incubation period, for purposes of the intracellular cytokine staining protocol.

2.5. Antibodies used in flow cytometry

The following anti-human conjugated monoclonal antibodies were employed in the surface expression of CD4⁺ T-cells: fluorescein isothiocyanate (FITC) anti-CD4, phycoerythrin (PE) anti-CD26, PE anti-CD30, PE anti-CXCR4, PE anti-CXCR3, PE anti-CCR4, PE anti-CD154, PE anti-CD152, and PE anti-ICOS. For intracellular cytokine staining in CD3⁺ T-cells we used the monoclonal antibodies: PE anti-IL-4, PE anti-IFN γ , and PerCP-Cy5 anti-CD3. All the monoclonal antibodies were purchased from Becton Dickinson (BD, San Jose, USA). SimultestTM Control γ 1/ γ 1 (IgG1/IgG1) (BD) was used as a negative control to estimate the amount of non-specific staining.

2.6. Immunofluorescent staining and FACS analysis

In the surface staining for CD4⁺ T-cells, the cells were first incubated in the dark with the corresponding monoclonal antibody for

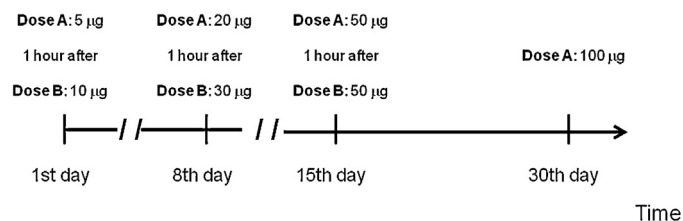


Fig. 1. Venom administration dosing schedule for patients treated with conventional VIT protocol.

Table 2
Characteristics of venom allergic patients.

Patient No.	Sex	Culprit insect	Systemic reaction ^a	IDT (μg/mL of venom) ^b	slgE (KU/L) ^c
1 ^{VIT}	F	<i>Polistes dominulus</i>	Grade IV	8.5 mm (0.01)	96.0
2 ^{VIT}	M	<i>Vespula</i> spp.	Grade I	11 mm (0.1)	11.6
3 ^{VIT}	M	<i>Polistes dominulus</i>	Grade IV	7 mm (1)	0.96
4 ^{VIT}	F	<i>Apis mellifera</i>	Grade III	6.5 mm (0.1)	39.6
5 ^{VIT}	M	<i>Polistes dominulus Vespula</i> spp.	Grade III	12.5 mm (0.1) 12.5 mm (0.01)	51.8 30.9
6 ^{VIT}	M	<i>Polistes dominulus</i>	Grade III	10.5 mm (0.01)	28.7
7 ^{VIT}	M	<i>Polistes dominulus</i>	Grade II	9.5 mm (1)	49.3
8	M	<i>Polistes dominulus</i>	Grade II	9 mm (0.1)	29.5
9	M	<i>Polistes dominulus</i>	Grade IV	7 mm (0.001)	20.8
10	M	<i>Polistes dominulus</i>	Grade II	7.5 mm (0.1)	3.82

Abbreviations: F, female; M, male; IDT, intradermal test; VIT, patients treated with venom immunotherapy.

^a According to the classification of systemic reactions published by Mueller (1966).

^b Venom extracts were supplied by ALK-Abelló S.A., Madrid, Spain.

^c Levels of slgE were measured by ImmunoCAP (Phadia AB, Uppsala, Sweden).

20 min. They were then washed, resuspended in PBS and analysed by flow cytometry. Cytokine intracellular staining of CD3+ T-cells was carried out according to the instructions of the BD intracellular staining kit (Cytofix/Cytoperm™ Plus Fixation/Permeabilization kit). Lymphocytes were acquired with a FACScan cytometer (Becton Dickinson) and analysed using CellQuest™ Pro software. At least 10,000 events were acquired and analysed in the lymphocyte gate using two-colour immunofluorescence.

2.7. Basophil activation test (BAT)

The basophil degranulation, expressed as the percentage of CD63+ basophils was determined using a BASOTEST kit (Glyco-type Biotechnology GmbH, Heidelberg, Germany). Heparinized whole blood samples were incubated with 0.1 or 1 μg/mL of the corresponding venom extracts (ALK-Abelló, S.A., Madrid, Spain) according to the kit's recommendations. All determinations were assayed in parallel with positive and negative controls (fMLP chemotactic peptide and wash solution, respectively). The cells were stained with PE anti-IgE and FITC anti-CD63 monoclonal antibodies, and a minimum of at least 1000 basophils were acquired for flow cytometry.

2.8. Statistical study

The non-parametric Mann–Whitney *U*-test was used to compare baseline data from allergic patients and controls. We used the non-parametric Wilcoxon matched-pairs signed-rank test to compare the paired venom allergic patients after intervention. Spearman's coefficient was used to analyse the correlation between quantitative variables. The results were analysed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). The differences were considered statistically significant at *p* values of less than 0.05.

3. Results

3.1. Population study

Venom allergic (VA) patients (*n* = 10) presented positive levels of sIgE with a median of 32.98 ± 26.74 KU/L (>0.35 KU/L) and positive IDT with a median of 9.23 ± 2.17 mm (wheal diameter of >5 mm). Each of the 10 VA patients presented differing grades of systemic reaction to Hymenoptera venom: (a) 1 with grade I; (b) 3 with grade II; (c) 3 with grade III; and (d) 3 with grade IV (Table 2).

At baseline time, T0, there were no significant differences ($p > 0.05$) between the percentage of CD3+ T-cells in VA patients ($65.41 \pm 5.80\%$) and controls ($69.62 \pm 7.24\%$). Equally, the percentage of CD4+ T-cells in VA patients compared to controls, also at T0, did not present significant differences ($p > 0.05$): $44.37 \pm 6.88\%$

vs. $40.89 \pm 4.72\%$, respectively. In the group of seven VA patients who were treated with the VIT protocol (Table 2), no significant differences were observed between the percentage of CD3+ and CD4+ T-cells at T0, nor after four months of therapy (T4) (data not shown).

3.2. Expression of surface markers and intracellular cytokines in VA patients and controls

CD4+ T-cells from VA patients at T0 and controls were studied for surface expression of Th1-type markers (CD26 and CXCR3), Th2-type markers (CD30 and CCR4), the CXCR4-lymphocyte trafficking marker and activation markers (CD154, CD152 and ICOS) (Table 1). We found significant differences between VA patients and controls in the percentage of CD4+ CD26+ ($p = 0.041$), CD4+ CXCR3 ($p = 0.003$), CD4+ CXCR4+ ($p = 0.001$), CD4+ CD154+ ($p = 0.034$), and CD4+ ICOS+ ($p = 0.022$) (Table 3). However, for the remaining markers, CD30, CCR4 and CD152 no significant differences were observed with respect to the controls (Table 3) ($p > 0.05$).

The determination of intracellular cytokines was carried out on the population of CD3+ T-cells. There were no significant differences ($p > 0.05$) in the percentages of IL-4 and IFNγ producing-CD3+ T-cells of VA patients at T0 and controls; nor were there differences ($p > 0.05$) in ratio of IFNγ (Th1)/IL-4 (Th2) producing CD3+ T-cells (Table 4).

3.3. Study of surface markers in VA patients upon venom immunotherapy

Significant differences were observed between the matched-pairs of VA patients at T0 and after four months of VIT (T4) for the expression of CD30 (1.35 ± 0.98 vs. 0.44 ± 0.31 , for T0 and T4, respectively), CD154 (2.24 ± 2.67 vs. 0.45 ± 0.45) and CD152 (2.37 ± 2.40 vs. 0.45 ± 0.29) on CD4+ T-cells (Figs. 2B, F and G, respectively). However, for the markers (CD26, CXCR4, CXCR3 and ICOS) that were up-regulated at T0 we did not find significant differences ($p > 0.05$) with respect to normal controls (see Fig. 2A, C, D and H, respectively). No significant differences ($p > 0.05$) were recorded for expression of the CCR4 T-cell marker (Fig. 2E).

3.4. IL-4 and IFNγ-producing CD3+ T-cells after venom immunotherapy

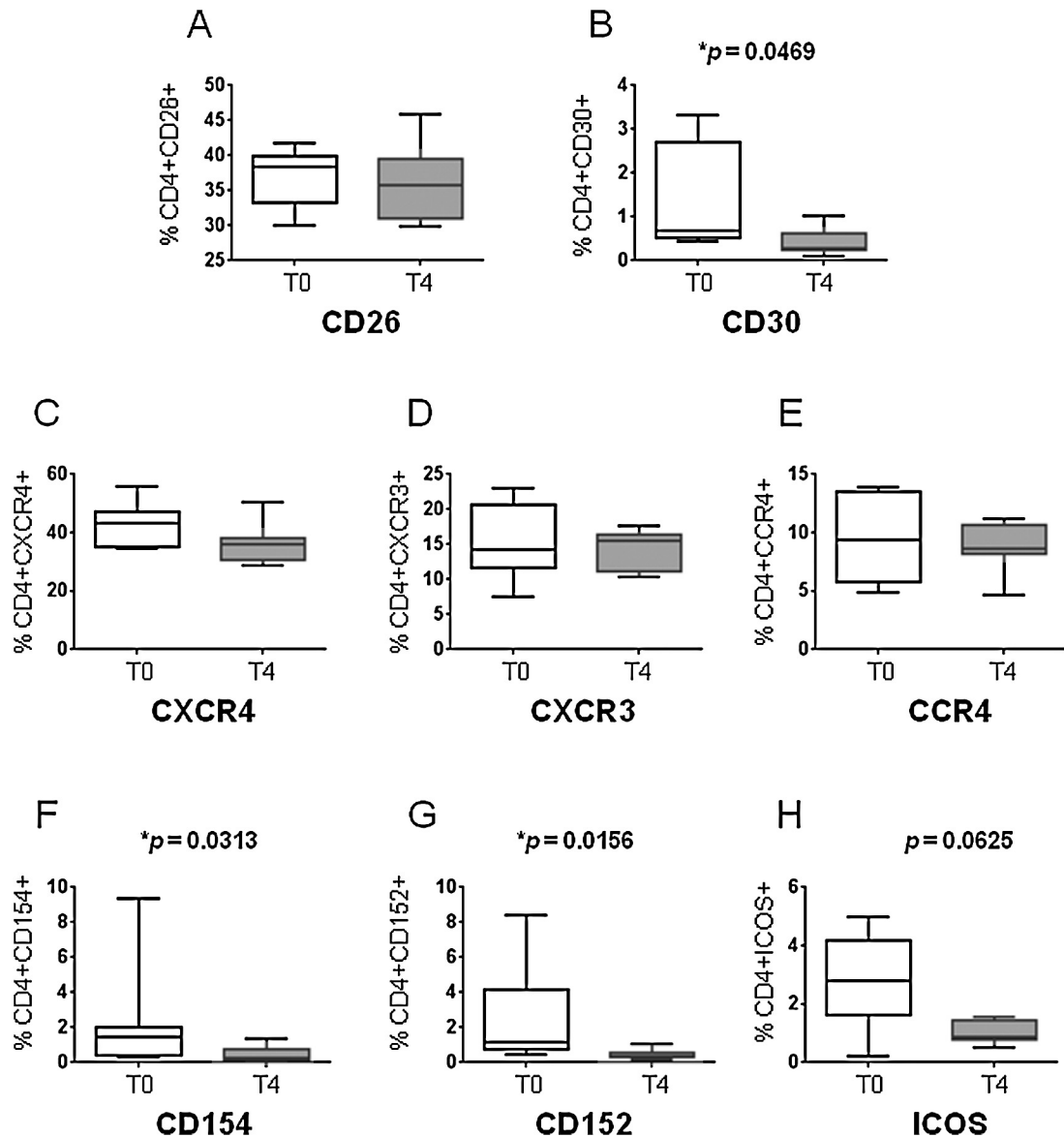
Upon intervention, five of the seven VA patients presented a decrease in the percentage of IL-4 producing T-cells; however the differences were not significant (Fig. 3A). In contrast, six of the seven VA patients experienced significant increases in IFNγ-producing T-cell expression levels after VIT treatment (Fig. 3B). The ratio of IFNγ (Th1)/IL-4 (Th2) producing T-cells presented a higher

Table 3Expression of surface markers on CD4⁺ T-cells in normal controls (C) (*n* = 10) and venom allergic (VA) patients (*n* = 10) at baseline time (T0). Data are given as percentages.

Cellular marker type	Surface marker	C (mean ± SD)		VA (mean ± SD)	<i>p</i> Value
Th1	CD4 + CD26+	33.66 ± 4.97	vs.	39.36 ± 5.51	0.041 [*]
Th1	CD4 + CXCR3+	6.72 ± 3.95	vs.	15.05 ± 5.12	0.003 ^{**}
Th2	CD4 + CD30+	0.72 ± 0.34	vs.	1.35 ± 0.98	0.19
Th2	CD4 + CCR4+	11.17 ± 1.72	vs.	9.72 ± 3.32	0.414
Homing	CD4 + CXCR4+	25.37 ± 5.72	vs.	42.57 ± 7.28	0.001 ^{**}
Activation	CD4 + CD154+	0.58 ± 0.22	vs.	2.24 ± 2.67	0.034 [*]
Activation	CD4 + CD152+	1.33 ± 0.63	vs.	2.37 ± 2.4	0.462
Activation	CD4 + ICOS+	1.01 ± 0.16	vs.	2.74 ± 1.51	0.022 [*]

^{*} *p* < 0.05.^{**} *p* < 0.01.**Table 4**Percentage of interleukin producing-CD3⁺ T cells in non-allergy controls (C) and venom allergic (VA) patients at baseline time. Data are given as mean ± SD.

Intracellular cytokine	C (<i>n</i> = 10)		VA (<i>n</i> = 10)	<i>p</i> Value
CD3 + IL4+	1.43 ± 0.56	vs.	1.29 ± 0.62	0.514
CD3 + IFN γ +	5.02 ± 4.03	vs.	4.08 ± 3.90	0.369
Ratio IFN γ /IL-4	4.40 ± 4.43	vs.	3.90 ± 4.88	0.624

**Fig. 2.** Percentage of CD26 (A), CD30 (B), chemokine receptors ((C)–(E)), and activation markers ((F)–(H)) expressed on CD4⁺ T-cells from VA patients at T0 and upon venom immunotherapy (T4). Data are presented in box and whisker plots showing the median and range. * *p* < 0.05.

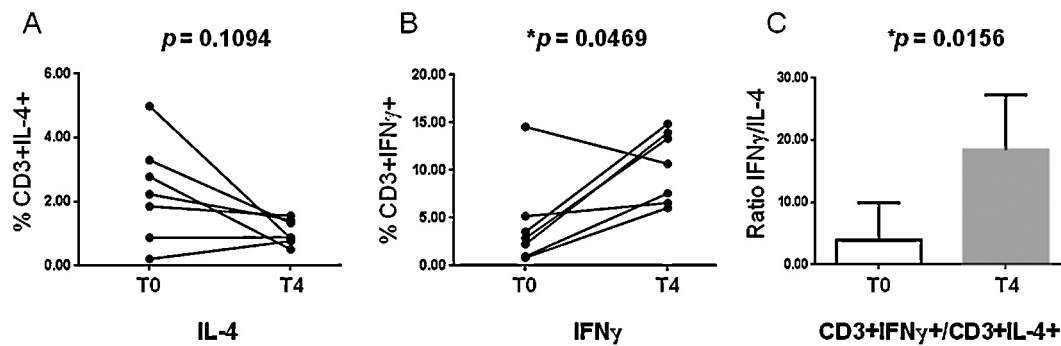


Fig. 3. Percentage of IL-4 (A) and IFN γ (B) producing-CD3+ T-cells of venom allergic patients at T0 and after 4 months of venom immunotherapy (T4). Histogram (C) shows the ratio of IFN γ (Th1)/IL-4 (Th2) producing CD3+ T-cells at T0 and T4. Data are shown as mean $\pm$ SD. * $p < 0.05$.

significant difference with respect to IFN γ as marker of response to venom immunotherapy (Fig. 3C).

3.5. Degranulation of basophils in the induction of tolerance after VIT

CD63 expression on basophils was determined at T0 and T4 in the VA subjects treated with immunotherapy using 0.1 and 1.0 μ g/mL of venom extracts (submaximal and maximal concentrations, respectively) (Eržen et al., 2012). We found significant differences in the percentage of CD63+ basophils both at 0.1 μ g/mL ($27.48 \pm 25.49\%$ vs. $8.87 \pm 8.58\%$, at T0 and T4, respectively) (Fig. 4A) and at 1 μ g/mL of venom extract ($60.65 \pm 31.13\%$ vs. $32.98 \pm 30.1\%$) (Fig. 4B).

3.6. Correlation study in VA subjects at baseline time and after VIT

The surface markers (CD26, CD30, CXCR4, CXCR3, CCR4, CD154, CD152, and ICOS) expressed on CD4+ T-cells and the intracellular cytokines IL-4 and IFN γ in T-cells were analysed using a two-tailed Spearman's correlation test. At T0, the VA subjects ($n = 10$) demonstrated positive correlations among the surface markers CD26/ICOS (Fig. 5A) and CD154/ICOS (Fig. 5B). After the immune intervention in the active subjects ($n = 7$) we found a positive correlation between CD154 and CD152 (Fig. 5C). Nevertheless, the VA subjects presented no further correlations ($p > 0.05$) in the remaining markers at either T0 or T4, after VIT (data not shown).

4. Discussion

Systemic allergic reactions after Hymenoptera stings are due to hypersensitivity that is mainly mediated by venom specific-IgE. The affected subjects have skin, gastrointestinal, respiratory and cardiovascular manifestations representing an impaired quality of life and

sometimes with fatal results (Biló and Bonifazi, 2009). Neither the symptomatic or asymptomatic sensitization nor the immunological mechanisms involved in venom allergy are well known (Golden, 2005). Nevertheless, it is apparent that immune changes occur following immunotherapy, with a shift towards a Th1-type response (Ozdemir et al., 2011). Therefore, we have investigated the surface expression of Th1- and Th2-type markers, CXCR4-lymphocyte trafficking marker and activation markers on CD4+ T-cells of subjects with systemic reactions, mainly to *P. dominulus*, and the role of these markers after venom immunotherapy.

At baseline time in venom allergic patients we have presented novel findings with regard to up-regulation of CD26, CXCR3, CXCR4, CD154 and ICOS on CD4+ T-cells. CD26 (dipeptidyl peptidase IV, DPPIV) in peripheral blood is preferentially expressed on CD4+ memory T-cells and is up-regulated after T-cell activation, it also acts as a costimulatory signal (Ohnuma et al., 2008). Further to this, it is expressed in a variety of tissues including endothelial and epithelial cells (Mentlein, 1999). Despite the substantial evidence regarding the involvement of CD26 in Th1-type diseases, such as multiple sclerosis (Ofazoglu et al., 2009), rheumatoid arthritis (Hatchi et al., 2003) and Graves' disease (Eguchi et al., 1989), the expression of CD26 is also associated with Th2-dependent allergic inflammation (Ohnuma et al., 2005). This is supported in work by Lun et al. (2007), who have described an up-regulation of CD26 on CD4+ T-cells from patients with allergic asthma. Therefore, CD26 appears to have a role in the inflammatory process. In addition, it has been reported that T-cells which migrate through monolayers of endothelial cells express high *in vitro* levels of CD26 (Masuyama et al., 1992), this is despite its function as a peptidase of different cytokines and IFN γ -induced chemokines (IP-10 and CXCL9) (Proost et al., 2001). Accordingly, it has been found that the levels of these IFN γ -induced chemokines are reduced in patients with allergic asthma (Lun et al., 2006).

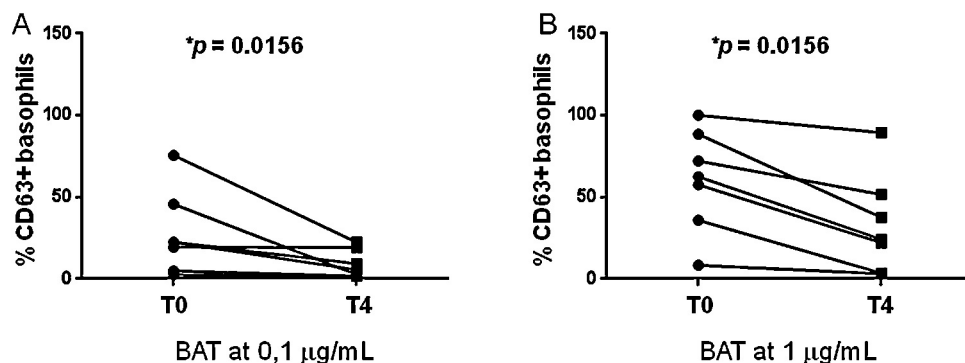


Fig. 4. Basophil response performed in the active VA patients at T0 and after 4 months of therapy (T4).

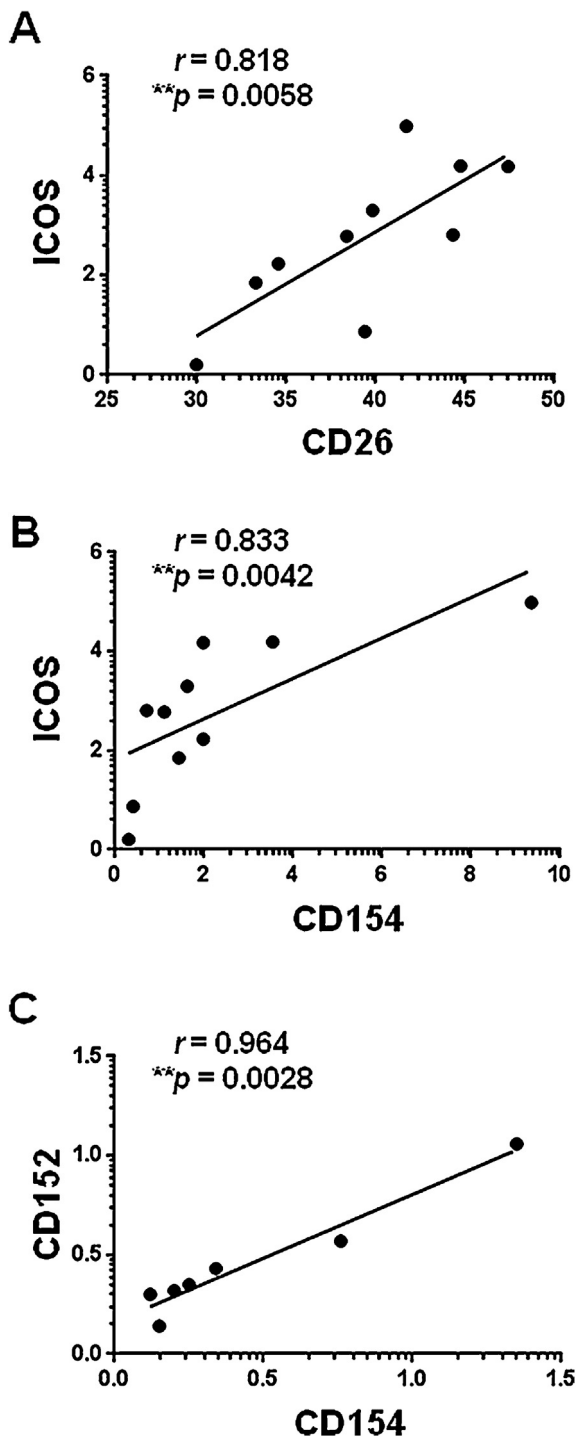


Fig. 5. ((A) and (B)) Relationships among surface markers in VA patients at T0 ($n = 10$). (C) Significant correlation found in the seven VA patients treated with VIT at the fourth month. Spearman's rho analysis and p values were calculated by two-tailed test (* $p < 0.05$, ** $p < 0.01$). Correlation coefficients (r).

CXCR3 is a chemokine receptor which is highly expressed on Th1-type cells, however Th2-type cells also express this receptor but in lower percentages (Kim et al., 2001). Similarly, the CCR4 Th2-cell marker is barely expressed on Th1-type cells (Kim et al., 2001). CXCR3 has a role in the migration of T-cells to the peripheral tissues and lymphoid nodes during inflammation, thus facilitating the interaction with antigen presenting cells (APCs) (Groom and Luster, 2011). It is up-regulated in peripheral blood T-cells collected from patients with different autoimmune diseases, such as multiple

sclerosis, SLE and type I diabetes mellitus, amongst others (Groom and Luster, 2011), as well as in the lungs of patients with allergic asthma (Campbell et al., 2001). Equally, the CXCR4-lymphocyte trafficking receptor has a function in the recruitment of T-cells in the areas of inflammation for autoimmune diseases such as rheumatoid arthritis (Nanki et al., 2000) and in the lungs of patients with allergic airway diseases (Gonzalo et al., 2000; Lukacs et al., 2002). The up-regulation of CD26, CXCR3 and CXCR4 could play a part in the fast inflammatory response in periphery tissues following Hymenoptera stings. Actually, the symptoms usually start within minutes of a sting (Biló and Bonifazi, 2009). Therefore, despite of the ratio $\text{IFN}\gamma$ (Th1)/IL-4 (Th2) do not reveal differences at baseline time between VA patients and controls, and cannot be established a predominant Th2-type response in these patients. It is noteworthy that both Th1-type markers, CD26 and CXCR3 also act in Th2-allergic inflammation as it has been argued previously, and similarly could contribute in the induction of systemic reactions after Hymenoptera stings in these subjects.

CD154 or CD40L is a non-costimulatory activation molecule, which expressed on Th2-type cells interacts with CD40 on B-cells, and together with IL-4 and IL-13 signal the immunoglobulin isotype switching to IgE. Various costimulatory molecules, for example ICOS, are involved in isotype switching and activation of B-cells following Th2 interaction (Beier et al., 2007). In fact, it has been shown that ICOS has a predominant involvement in Th2-type responses. In mouse models of allergic airway inflammation, the blockade of ICOS reduces airway inflammation and decreases production of IgE and Th2-type cytokines (Gonzalo et al., 2001). Similarly, CD154 blocking in murine models reveals a decrease in IgE production (Hogan et al., 1997). To date, in venom allergic subjects, there are only reports of an increase of CD154 on the surface of blood basophils, with respect to normal controls (Siegmond et al., 2000). However, in this study we have described that both activation markers CD154 and ICOS are up-regulated in venom allergic subjects compared with non-allergic healthy controls. Moreover, we have found a positive correlation between these two markers (CD154 and ICOS) and between CD26 and ICOS. These results reveal that CD4+ T-cells in venom allergic subjects have a higher activation state compared with non-allergic persons.

We have observed a down-regulation of the activation markers CD154 and CD152 (CTLA-4), a decrease in the surface expression of the Th2-type marker CD30, as well as a shift to Th1-type cytokines ($\text{IFN}\gamma$), in seven VA patients after being treated with venom immunotherapy (VIT) for four months. Likewise, the significant decrease of CD63 expression both at submaximal and maximal concentration of venom extract has proven the induction of tolerance to Hymenoptera venom during the VIT. The basophil activation test (BAT) measured by CD63 expression on basophils, is an *in vitro* sensible and reliable test which avoid the risks of anaphylaxis of the skin challenge test in the determination of tolerance after VIT (Eržen et al., 2012). In addition to being painful, the sting challenge test is complex and it requires proper equipment and supervision in an intensive care unit.

The low expression of CD30 (Th2) on CD4+ T-cells after VIT is consistent with the previous findings reported by Foschi et al. (2008). They recorded decreased levels of soluble CD30 (CD30s) after three months of venom immunotherapy. Furthermore, long-term reductions in levels of soluble CTLA-4 (sCTLA-4) have been described in venom allergic patients after venom immunotherapy (Riccio et al., 2012), in agreement with the low expression of CD152 (CTLA-4) on CD4+ T-cells observed in the present study. The down-regulation of CD154 (CD40L) on CD4+ T-cells in pollen allergic patients has recently been reported by Urra et al. (2014) during long-term allergen-specific immunotherapy (SIT). We found similar results in venom allergic patients, with a decrease of CD154 expression by the fourth month of therapy. Moreover, our results

agree with previous data for CD154 (CD40L) expression on PBMCs, in which an earlier decrease was observed after just the first week of venom immunotherapy (Bellinghausen et al., 1997). Our study has also revealed a positive correlation between both CD152 and CD154 markers following venom immunotherapy.

5. Conclusions

An important issue in Hymenoptera venom allergy which has not yet been clarified is the ability to differentiate asymptomatic sensitized subjects from those who will likely suffer a systemic reaction to Hymenoptera stings. However, despite the prevalence of asymptomatic sensitization being as high as nearly 30% of the adult population (Biló and Bonifazi, 2009), no marker has yet been described which can predict who is susceptible to a future reaction and whether or not it will be systemic. In this study we have presented important data about the up-regulation of different markers expressed on CD4+ T-cells involved in lymphocyte trafficking and activation state of subjects with systemic reactions to Hymenoptera stings. Further studies will be necessary to clarify whether these markers can be employed to differentiate between symptomatic and asymptomatic sensitized subjects. In addition, the down-regulation of activation markers could be used in future studies that monitor the efficacy of the venom immunotherapy.

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