Molecular Immunology 62 (2014) 178–185

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

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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ARTICLE INFO

Article history:
Received 13 May 2014
Received in revised form 13 June 2014
Accepted 16 June 2014

Keywords:
Hymenoptera allergy
Venom immunotherapy
T-cell marker
Homing-receptor
Non-costimulatory activation marker
Costimulatory activation marker

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 IFNγ 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 IFNγ (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 IFNγ 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.

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 (IFNγ) (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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http://dx.doi.org/10.1016/j.molimm.2014.06.023
0161-5890/

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	Kroczek 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 (Kroczek 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 IFNγ 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 ± 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 μg by the 30th day (Fig. 1). Maintenance injections of 100 μg 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 μM of ionomycin (Sigma, Steinheim, Germany) in 5% CO_2 at 37 °C. A BD GolgiPlug™ 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-IFNγ, and PerCP-Cy5 anti-CD3. All the monoclonal antibodies were purchased from Becton Dickinson (BD, San Jose, USA). Simultest™ Control γ1/γ1 (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

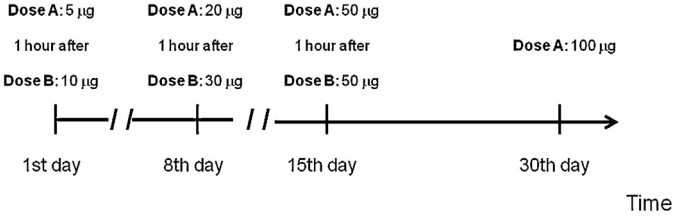

Fig. 1. Venom administration dosing schedule for patients treated with conventional VIT protocol.