

ORIGINAL PAPER

Induction of interleukin-10 and suppressor of cytokine signalling-3 gene expression following peptide immunotherapy

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Clinical and Experimental Allergy

Summary

Background Allergen-derived (T cell epitope) peptides may be safer for immunotherapy than native allergen, as they do not cross-link immunoglobulin (Ig)E. However, HLA polymorphism results in multiple potential epitopes. Synthetic peptides of phospholipase (PL) A₂ were selected for a peptide vaccine, on the basis of binding affinity for commonly expressed HLA-DR molecules.

Objective To evaluate treatment with an HLA-DR-based PLA₂ peptide vaccine in subjects with mild honeybee allergy in an open, controlled study.

Methods Twelve volunteers with allergy to bee venom received nine intradermal injections of PLA₂ peptides, with six untreated subjects serving as controls. Outcome was assessed by the size of the late-phase cutaneous reaction to allergen, peripheral blood mononuclear cell (PBMC) proliferation, cytokine release, and expression of genes associated with immune regulation.

Results Subjects receiving peptides showed a decrease in the magnitude of the late-phase cutaneous reaction to bee venom compared with controls ($P=0.03$). The proliferation of venom-stimulated PBMCs decreased in treated subjects compared with controls ($P=0.01$).

Peptide treatment reduced the production of IL-13 by PLA₂-stimulated PBMCs ($P<0.01$) and IFN- γ ($P<0.01$), and increased the production of IL-10 ($P=0.02$). Transcription of the suppressor of cytokine signalling (Socs)3 gene was significantly increased following therapy. A transient, but modest, increase in allergen-specific IgG was also observed.

Conclusion HLA-DR-based T cell epitopes modify surrogate markers associated with successful immunotherapy and induction of immune regulation, supporting the concept that this form of treatment may be efficacious in human allergic disease.

Keywords allergy, cytokine, epitope, human, immunotherapy, T cells, tolerance/suppression/anergy, transcription factors

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Introduction

In contrast to other therapies for immunoglobulin (Ig)E-mediated disease, allergen-specific immunotherapy (SIT) is disease-modifying, reducing requirements for symptomatic medication, [1] and with effects lasting beyond the duration of treatment [2]. Randomized-controlled trials

have demonstrated the efficacy of SIT in hymenoptera venom allergy [3] and in allergic rhinitis and asthma triggered by aeroallergens [1, 4, 5]. However, the use of native allergen preparations is limited by unpredictable IgE-mediated systemic reactions. With increasing understanding of the central role of T cells in allergy, there has been considerable interest in the therapeutic use of short, allergen-derived T cell peptides as an alternative to conventional SIT [6]. Such preparations are envisaged to be safer, while retaining the immunomodulating effects of SIT through direct effects on T cells. However, HLA

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polymorphism theoretically confers huge diversity to T cell peptide epitopes, presenting potential limitations in the development of peptide-based desensitization vaccines.

Carballido *et al.* [7] used lymphocyte stimulation assays to select peptides of phospholipase A₂ (PLA₂, the major allergen of honeybee venom), which were later used to treat five highly allergic patients in an open-label study [8]. Two 27 amino acid peptides, from the cat allergen Fel d 1, were evaluated in a series of studies. Treatment was shown to modulate a number of parameters, including T cell function, resting lung function, and the ability of patients to tolerate allergen by inhaled challenge and in a cat exposure chamber [9–13]. In order to increase the utility of peptide-based vaccines, Oldfield *et al.* applied a different technique to peptide selection, preparing multiple overlapping peptides spanning most of the sequence (and thus T cell epitopes) of Fel d 1. In a double-blind placebo-controlled study, these peptides were shown to modulate several surrogate markers associated with successful immunotherapy, and importantly, to improve volunteers' subjective ability to tolerate cats [14]. Fellrath and colleagues devised three long overlapping peptides spanning the entire sequence of PLA₂. Treatment with this preparation modulated immunological parameters such as T cell proliferation to the peptides, cytokine production, and PLA₂-specific IgG₄ levels [15]. However, four of nine treated subjects became skin test positive to the peptides, two of whom developed mild generalized reactions a few hours following peptide injection.

A novel method for selecting candidate T cell epitopes for immunotherapy is by binding affinity for MHC molecules, with the aim of characterizing peptides that the majority of the target population are capable of restricting [16]. Using direct binding assays with overlapping peptides of PLA₂, 18mer T cell peptide epitopes have been characterized by affinity for commonly expressed HLA-DRB1 molecules that are abundantly expressed on antigen-presenting cells [17].

The suppressors of cytokine signalling (SOCS) family of intracellular proteins have been shown to regulate cytokine signalling pathways negatively [18]. Certain SOCS proteins have been implicated in T helper type 1 (Th1) and Th2 T cell polarization through their antagonistic actions on IL-4/STAT6 and IL-12/STAT4 signalling, respectively [19]. *In vitro* polarization of murine T cells results in enhanced expression of SOCS-1 in Th1 cells and substantially increased expression of SOCS-3 in Th2 cells. Expression of elevated levels of SOCS-3 has been correlated with disease severity and IgE levels in allergic diseases such as asthma and atopic dermatitis [20]. SOCS-3 has been considered to be a critical molecule in the determination of Th2 cell fate and thus, a prospective target for therapeutic intervention in allergic diseases. Foxp3 is a transcription factor that has been shown to be

up-regulated in CD4⁺ CD25⁺-regulatory T cell populations [21–23]. Up-regulation of this molecule following peptide therapy may indicate the induction of this class of regulatory cell.

In this study, we investigated the effect of repeated injections of HLA-DR-characterized PLA₂-derived T cell peptides on immunological parameters, including transcriptional modulation of genes encoding proteins associated with Th1/Th2 fate determination (SOCS family) and certain forms of T cell regulation (Foxp3). We hypothesized that peptide immunotherapy would be associated with reduced pro-inflammatory responses to allergen and modulation of expression of SOCS and Foxp3 genes.

Materials and methods

Volunteers

We recruited healthy volunteers (by open advertisement) with a recent history of mild systemic reaction following a honeybee sting (flushing, generalized urticaria, and/or angioedema), or a history of large local reaction only (defined as estimated size exceeding 4 cm and lasting for 2 or more days). All subjects were skin prick test positive to bee venom at a concentration of 100 µg/mL or less. Volunteers with a history of life-threatening anaphylaxis (airway or cardio-vascular compromise) were excluded and advised to seek conventional treatment. Subjects with possible asthma (as evidenced by any of the following: physician-diagnosed asthma at any age; current symptoms suggestive of asthma; obstructive spirometry; histamine PC₂₀ < 16) were specifically excluded. The study was approved by the Ethics Committee of the Royal Brompton and Harefield Hospitals NHS Trust, and the subjects provided informed, written consent to participate.

Peptides

Peptide-MHC binding characteristics were determined in an earlier study [17]. For clinical administration, peptides were synthesized by Fmoc chemistry, purified by high-performance liquid chromatography (HPLC), and supplied as lyophilized powder. Peptides were reconstituted with 10^{−4} M HCl and diluted in sterile, physiological saline solution. Peptide solutions were dispensed into sterile glass vials for single patient use by Nova Laboratories Ltd (Leicester, UK). Following dispensing, vials were tested for endotoxin and pyrogenicity.

Study protocol

This was an open-label, controlled study. Treated volunteers received nine incremental doses of peptide mixture at weekly intervals to a cumulative dose of 431.1 µg of

each peptide, administered by the intradermal route as follows: 0.1, 1, 5, 25, 50, 50, 100, 100 and 100 µg. At baseline and at 12 weeks, blood was taken for isolation of peripheral blood mononuclear cells (PBMCs). The late-phase cutaneous reaction was measured 6 h following the intradermal injection of 1.5 µg of bee venom (ALK-Abello, Horsholm, Denmark) (equivalent to about 1/30th of a bee sting) and following the intradermal injection of 1.5 µg of PLA₂ (Latoxan, Rosans, France). The early-phase reaction was measured 15 min following the intradermal injection of 50 µL of 0.1 µg/mL bee venom, and 50 µL of 0.1 µg/mL PLA₂. A 4 mm punch biopsy of the late-phase cutaneous reaction to bee venom was taken at 6 h. Control subjects attended the baseline and outcome visits at appropriate times only.

Peripheral blood mononuclear cell culture

Proliferation and cytokine secretion. PBMCs were isolated from heparinized blood by density centrifugation. Cells were cultured in medium supplemented with 5% human AB serum, 2 mM L-glutamine, and antibiotics (all Sigma Chemical Company, Poole, UK), for 6 days, in 96-well flat-bottomed plates at 2×10^6 cells/mL in the presence of medium alone, or previously determined optimal concentrations of PLA₂ (Latoxan), bee venom (ALK-Abello), individual peptides or purified protein derivative (PPD) of *M. tuberculosis* before the assessment of ³H-thymidine incorporation. To enable comparisons before and after treatment, stimulation indices were calculated as the ratio of counts in stimulated wells divided by those of unstimulated wells. For cytokine analysis, cells were cultured in the presence of medium or PLA₂ (Sigma, St Louis, Mo, USA) in 24-well flat-bottomed plates for 5 days before removal of supernatant to storage at -20°C and subsequent analysis for IL-13, IL-10, and IFN- γ concentration by ELISA (Pelikine, CLB, Amsterdam, Netherlands).

Peripheral blood mononuclear cell culture for RNA extraction. Frozen PBMC obtained before and after peptide immunotherapy were thawed, washed and cultured as follows: PBMC were stimulated at 4×10^6 cells in 1 mL medium supplemented with 10% human serum, 2 mM L-glutamine, and antibiotics, in the presence or absence of 5 µg/mL PLA₂ (Sigma) in 24-well flat-bottomed plates. On day 5, 20 ng/mL recombinant IL-2 (R&D Systems, Abingdon, UK) was added to all wells. After 12 days, cells were recovered and restimulated, either with solid-phase $\alpha\text{CD3}-\alpha\text{CD28}$ (BD Pharmingen, Oxford, UK; 10–1 µg/mL respectively), or left unstimulated (both at 2×10^6 cells/mL). After 4 h of restimulation, cells were washed, centrifuged, and resuspended in 0.5 mL 'RNA-later' (Qiagen, Crawley, UK) and stored for subsequent RNA extraction.

Real-time reverse transcriptase-polymerase chain reaction mRNA expression analysis

Total RNA from T cells was extracted using the RNeasy kit (Qiagen, Basel, Switzerland) according to the manufacturer's instructions. During this procedure, a DNase digestion with the RNase-free DNase set, for use with RNeasy/QIAamp columns (Qiagen), was used. RNA was reverse-transcribed using TaqMan Reverse Transcription Reagents, including Random Hexamers (Applied Biosystems, Foster City, CA, USA). The resulting cDNA was analysed by real-time PCR (ABI PRISM 7000 Sequence Detection System; Applied Biosystems). The probe for SOCS1 was hSOCS1-686T, 5'-acc tga act cgc acc tcc tac ctc ttc atg. The primers for SOCS1 were hSOCS1-646F (forward) 5'-ccc tgg ttg ttg tag cag ctt, and hSOCS1-754R (reverse) 5'-caa ccc ctg gtt tgt gca a. The probe for CIS1 was hCIS1-252T, 5'-cca ata cca gcc aga ttc ccg aag gta. The primers for CIS1 were hCIS1-203F (forward) 5'-tgt gca tag cca aga cct tct c, and hCIS1-272R (reverse) 5'-tcg ctg gcc gta atg gaa. The probe for SOCS3 was hSOCS3-588T, 5'-cgt caa cgg cca cct gga ctc cta t. The primers for SOCS3 were hSOCS3-565F (forward) 5'-ctt cag cat ctc tgt cgg aag a, and hSOCS3-668R (reverse), 5'-gca tcg tac tgg tcc agg aac t. The probe for SOCS5 was hSOCS5-1329T 5'-tcg acg ccc atg acc cgt gtg tat. The primers for SOCS5 were hSOCS5-1298F (forward), 5'-cga att gag cag tgg aat cac a and hSOCS5-1413R (reverse), 5'-aac atg cac gaa ctg gga tct t. Foxp3 forward primer was 5'-cac ccg cac aaa gca ctt g; Foxp3 reverse primer was 5'-ccc ggc ctt cca cag aa. As endogenous control, eukaryotic 18S rRNA (VIC/TAMRA Probe, Primer Limited) from Applied Biosystems was used. For all SOCS genes and the 18S rRNA endogenous control gene amplification, the TaqMan Universal PCR Master Mix (Applied Biosystems) was used according to the manufacturer's instructions. For Foxp3 gene expression, the SYBR Green PCR Master Mix (Applied Biosystems) was used.

All PCRs were performed in duplicate. Mean values were used for the calculation. The relative gene expression, the was calculated according to the comparative threshold cycle number (C_t) method as described previously (Applied Biosystems). Briefly, the formula for each sample is $2E^{-(C_{t\text{target}} - C_{t\text{standard}})}$. This yields a quantification of the target PCR products relative to the PCR products for the internal calibration (18S rRNA) and unstimulated control cells.

Immunohistochemistry

Skin biopsies were fixed in 4% paraformaldehyde for 4 h and then transferred to 15% sucrose overnight, before mounting in cryomatrix using isopentane pre-cooled to -80°C . Six micrometre sections were cut on a cryostat and transferred to glass slides. Sections were stained for

the following markers (DAKO-Envision): CD3, CD4, CD8, CD68, CD25, neutrophil elastase, tryptase and congo red. Slides were washed with phosphate-buffered saline, incubated for 30 min with secondary antibody and then washed again before development with fast red, counterstaining with haematoxylin and mounting in glycerol. An individual blinded to the status of the participants counted positive cells.

Phospholipase A₂-specific and bee venom-specific immunoglobulin G

Allergen-specific IgG was measured by an antigen-binding test, essentially according to the procedure described previously [24]. Briefly, serum (1.25 µL/test) was pre-incubated with the solid phase, which was either cyanogen bromide-activated Sepharose-coupled protein A (0.5 mg/test; Pharmacia, Uppsala, Sweden for measuring PLA₂-specific total IgG), or Sepharose-coupled monoclonal anti-IgG4 (CLB Sanquin, Amsterdam, the Netherlands) for measuring PLA₂-specific IgG₄. Non-bound serum components were removed by washing before ~1 ng ¹²⁵I-labelled PLA₂ (Sigma) was added. After overnight incubation and washing, Sepharose-bound radioactivity was measured. High samples were retested at appropriate dilutions. Test results were converted into arbitrary units per millilitre (AU/mL) by comparison with dilutions of a reference serum.

IgG₄ against whole bee venom was tested in a RAST-type assay, using Sepharose-immobilized bee venom (Sigma), 1.25 µL serum per test and radiolabelled anti-IgG₄, as described previously [25]. Results are in AU/mL.

Statistical analysis

Values are expressed as medians. Within-group changes were compared by Wilcoxon's rank sum test. Changes between groups were compared using the Mann-Whitney *U*-test. Measurements of serum IgG levels were compared by a paired *t*-test. A *P*-value of less than or equal to 0.05 was considered to be significant.

Results

We recruited 12 volunteers in to the active treatment group and six in to the control group. Volunteers were sequentially assigned to the active treatment group and control group. The baseline characteristics of the study volunteers are shown in Table 1. Most participants had developed mild systemic reactions to bee stings, rather than isolated large local reactions, and the groups were well matched for age, sex, skin reactivity to bee venom, and specific IgE levels. All subjects completed the study as per the protocol. No early or late adverse events were observed during this study.

Table 1. Volunteer characteristics (median, range)

	Control	Treated
Age	22 (19–36)	25 (20–34)
Sex	Four males, two females	Eight males, 4 females
Phenotype	Three isolated large local reactions Three mild systemic reactions	Four isolated large local reactions Four systemic reactions
EPC* (µs/mL)	10 (1–100)	10 (1–100)
RAST (IU/mL)	3.98 (0.34–8.17)	4.04 (0.34–12.50)

*EPC, end-point concentration on skin prick testing; lowest concentration of bee venom producing a wheal size of 3 mm with erythema.

Decreased cutaneous late-phase reactions following peptide therapy

The late-phase reaction to PLA₂ decreased significantly in the peptide-treated group (*P* = 0.04), but not in the control group; the decrease was modest in the treated group and failed to reach significance between groups. The late-phase cutaneous reaction to bee venom (Fig. 1) was significantly smaller at week 12 (2 weeks after completion of dosing) in the treated group (*P* = 0.02), and differed significantly from the control group at outcome (*P* = 0.03). Responses in the treated group were variable, with a minority of subjects showing increased reaction size. Baseline values did not differ statistically between treated and control subjects. No changes in the size of the early-phase reaction to bee venom or PLA₂ were observed in either control or peptide-treated subjects (data not shown).

Reduced proliferative responses to bee venom, phospholipase A₂ and peptides following therapy

In the peptide-treated group, proliferation to each of the four peptides was reduced by week 12, although this only reached significance for peptides 2 and 3 due to the small and variable proliferative responses to peptides conferred by heterogenous expression of restriction elements (Fig. 2). There were no significant changes in proliferation to peptides in the control group. Proliferation to PLA₂ and to bee venom did not differ between weeks 0 and 12 in the control group (Fig. 3), but decreased significantly in the peptide-treated group (*P* < 0.01) and when compared with untreated controls (*P* = 0.01). Proliferation to the recall antigen PPD did not differ between baseline and outcome in either group (data not shown).

The production of IL-13 and IFN-γ by PLA₂-stimulated PBMCs (Fig. 4) decreased significantly in peptide-treated subjects (*P* < 0.01), while the production of IL-10 increased between weeks 0 and 12 (*P* = 0.02). Cytokine analysis was not performed on control subjects. Cytokine data for treated individuals are shown in Table 2.

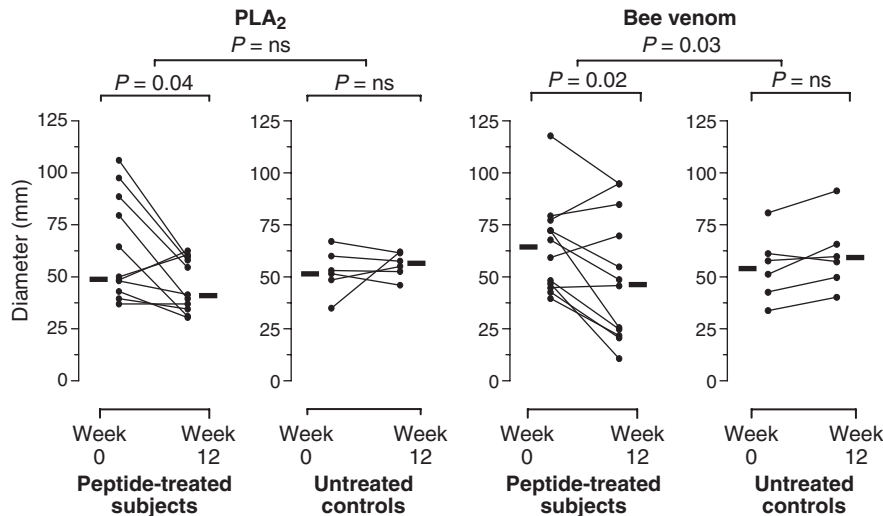


Fig. 1. Peptide immunotherapy reduces the magnitude of the cutaneous late-phase reaction to allergen challenge. Peptide-treated ($n = 12$) and untreated controls ($n = 6$) were challenged intradermally (volar aspect of the forearm), with $1.5 \mu\text{g}$ of bee venom and $1.5 \mu\text{g}$ of phospholipase A_2 , at the initiation of the study (week 0) and at week 12.

Immunohistochemistry

Eosinophils were recruited to sites of cutaneous allergen challenge both before ($P < 0.01$; diluent vs. allergen) and after ($P < 0.01$; diluent vs. allergen) peptide treatment. There was a trend towards a reduction in the recruitment of eosinophils to the late-phase cutaneous reaction (measured at 6 h) within the peptide-treated group ($P = 0.09$) and when compared with the untreated control group ($P = 0.09$). None of the other cellular markers changed significantly in either the control or peptide-treated groups (data not shown).

Expression of Socs gene family members and Foxp3 in allergen-stimulated T cells

Modulation of the level of transcription of *Socs1*, *3*, *5*, *Cis* and *Foxp3* was evaluated in total cellular mRNA extracted

from allergen-stimulated PBMC cultures derived from peripheral blood taken before and after the peptide treatment period. Levels of transcription were heterogeneous for all molecules analysed. The levels of transcription of *Socs3* were significantly enhanced following peptide treatment (Fig. 5). No other transcript was reliably modulated during this period, although transcription of *Socs1* and *Foxp3* also appeared to increase.

Transient induction of allergen-specific immunoglobulin G and immunoglobulin G_4 during peptide therapy

Allergen-specific IgG levels (total IgG to PLA_2 , IgG $_4$ to PLA_2 , and IgG $_4$ to whole bee venom) were measured before, during (mid-point), and after (week 12) peptide therapy. Modest transient increases in IgG antibodies were observed during the course of peptide therapy. At the

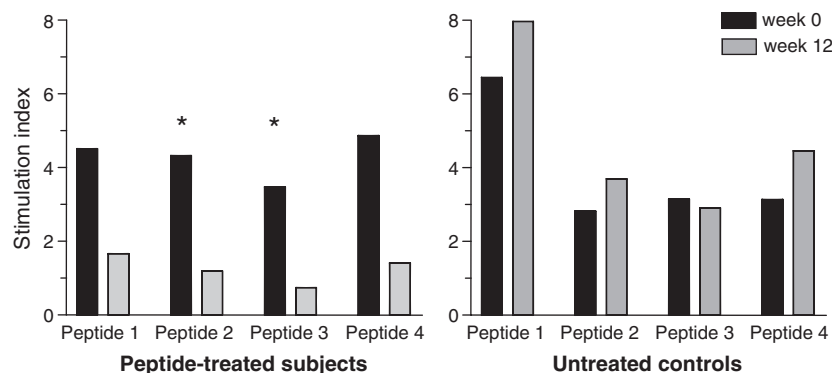


Fig. 2. Peptide treatment reduces peripheral blood mononuclear cell (PBMC) proliferative responses to phospholipase (PLA_2) peptides. Proliferative responses of PBMC were evaluated before (week 0) and after (week 12) peptide treatment and at the same time points in untreated controls. Cells were cultured with individual PLA_2 peptides (see Fig. 1) for 6 days.

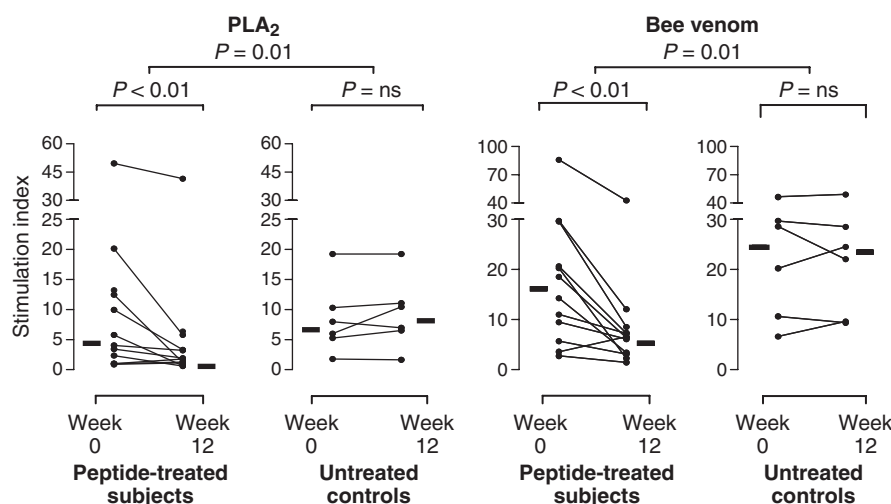


Fig. 3. Peptide treatment reduces peripheral blood mononuclear cell (PBMC) proliferative responses to phospholipase (PLA)₂ and bee venom. Proliferative responses of PBMC were evaluated before (week 0) and after (week 12) peptide treatment and at the same time points in untreated controls. Cells were cultured with PLA₂ or purified bee venom for 6 days.

mid-point measurement, significant increases were observed in PLA₂ IgG ($P=0.03$), PLA₂ IgG₄ ($P=0.04$), and bee venom IgG₄ ($P=0.04$). However, at the end of the treatment, no significant increases (above baseline) were observed (trend towards an increase in PLA₂ IgG; $P=0.08$). Individual measurements at baseline and after therapy for subjects undergoing peptide treatment are shown in Table 2.

Discussion

This is the first description on the use of peptides, selected on the basis of binding affinity for MHC molecules, in the treatment of human disease. We have demonstrated the

modulation of immunological parameters known to be associated with clinically successful venom immunotherapy in subjects with honeybee allergy. Furthermore, down-regulation of Th1 and Th2 responses to allergen, together with up-regulation of IL-10 protein production and elevated transcription of the *Socs3* gene, may provide a partial mechanistic basis for the effects of peptide therapy in this and other studies. PLA₂ peptides were not associated with any adverse events in these volunteers with a mild disease phenotype.

The use of direct binding assays to characterize T cell epitopes is a means of identifying small numbers of peptides that bind to multiple HLA molecules represented at high frequency in the target population, enabling the rational design of a desensitizing vaccine. Direct binding assays offer advantages over determination of T cell epitopes through the induction of proliferative responses in PBMC, as the latter is generally characterized by low levels of proliferation. Furthermore, induction of immunoregulatory cytokines by certain peptides may further suppress proliferative responses. In this study, each of the selected peptides was able to elicit a proliferative and cytokine response in PBMC from a varying number of subjects. Treatment of allergic volunteers with the peptide mixture induced immune modulation to individual peptides, as well as to whole allergen. Modulation of the response was indicated by a modest reduction in the magnitude of the late-phase cutaneous reaction to whole allergen, reduced allergen-stimulated lymphocyte proliferation, and reduced pro-inflammatory cytokine production. Reductions in pro-inflammatory outcomes were associated with an increase in production of the regulatory cytokine IL-10 and expression of the *Socs3* gene.

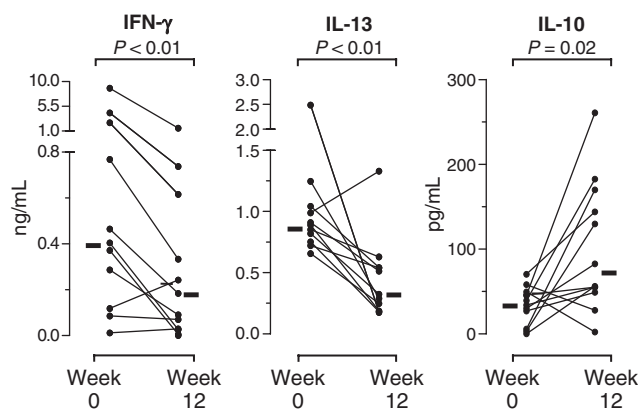


Fig. 4. Peptide treatment reduces Th1 and Th2 cytokine production increases IL-10. Peripheral blood mononuclear cell were isolated before and after peptide treatment and were cultured with phospholipase A₂ for 5 days. Cytokines were measured by ELISA in culture supernatants.

Table 2. Individual data for peptide-treated subjects

Subject	LPR		Proliferation PLA (SI)		Proliferation PLA (SI)		IL-10 (pg/mL)		IL-13 (ng/mL)		IFN (ng/mL)		IgG4 PLA		IgG total		IgG4 WBV	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
T1	79.5	85.0	13.2	6.4	29.9	8.5	70.5	144.0	853.8	630.3	4328.1	737.8	< 1	< 1	6	7	8	7
T2	47.0	11.0	49.6	34.3	86.1	42.0	58.1	27.8	722.9	540.3	2501.5	615.4	170	230	236	317	159	227
T3	40.0	22.0	2.3	0.6	9.4	5.9	3.2	129.9	888.6	186.8	464.8	183.9	1	5	15	34	5	10
T4	48.5	28.5	5.8	0.7	20.3	2.1	5.6	169.9	819.9	323.1	12.1	29.3	5	3	7	6	9	2
T5	59.5	65.0	20.1	5.8	30.0	12.0	39.3	182.4	752.6	181.5	404.3	25.7	18	22*	39	38*	59	79*
T6	68.0	49.0	10.0	3.3	20.7	7.2	45.8	55.0	2489.1	176.5	85.2	70.2	21	19	21	19	25	23
T7	43.0	21.0	0.9	1.1	3.4	6.4	27.0	49.0	656.8	248.9	372.3	5.4	3	8	16	41	6	19
T8	72.5	55.0	3.4	1.9	5.5	2.9	30.0	82.5	910.4	512.0	286.4	91.2	12	59*	20	75*	20	75*
T9	45.0	46.0	1.0	1.8	10.9	7.1	34.0	55.0	989.2	1330.7	768.7	333.8	9	39	32	75	9	24
T10	77.5	95.0	1.0	1.3	2.5	1.2	45.4	260.8	1246.3	289.1	8825.7	1522.8	8	7	9	9	24	25
T11	118.0	95.0	12.4	1.2	18.6	6.3	5.9	56.1	652.9	182.8	169.9	123.6	6	22*	7	22*	16	36*
T12	72.5	26.0	4.0	0.9	14.3	3.2	49.4	2.3	1043.3	542.7	118.9	242.3	2	4	3	4	16	21

*Mid-treatment value, post-treatment serum not available.

SI, stimulation index; PLA, phospholipase A2; WBV, whole bee venom.

Severe reactions to honeybee stings are life-threatening and often recur. Therefore, it was considered inappropriate to enrol patients who satisfied criteria for conventional immunotherapy. We elected to study subjects with mild systemic reactions and/or large local reactions, with the latter forming a minority of our study group. No differences in baseline characteristics were observed between patients with systemic reactions and large local reactions with respect to skin reactivity, specific IgE levels, T cell proliferation and cytokine production, and the outcomes following peptide treatment were similar. Therefore, we feel that such volunteers represent a useful model for studies with immunological outcomes, while appreciating that direct inferences of clinical efficacy cannot be drawn.

Outcome was assessed in this study by measuring of a variety of parameters, and not every subject responded in every parameter. Peptide responses were particularly variable, with the reduction in proliferation only reaching statistical significance for peptides 2 and 4. In two of 12 treated subjects, the late-phase cutaneous reaction to PLA₂ increased. Similarly, four individuals showed slight increases in the late-phase reaction to bee venom. These variations probably reflect variability in a biological system. The MHC-restricted nature of peptide recognition meant that not all subjects would be expected to recognize each peptide. Every volunteer responded in at least two modalities. Furthermore, HLA typing (data not shown) revealed that, in theory at least, all subjects were able to present at least one of the four peptides. Because of the promiscuous nature of MHC binding among the chosen peptides [17], no clear associations between HLA haplotype and biological response were observed.

The early-phase reaction to allergen was not altered shortly after peptide treatment, in keeping with the observation that prolonged treatment with conventional immunotherapy is required to affect IgE levels [26]. In a recent study, using peptides from the cat allergen Fel d 1, we demonstrated that a period of 3–9 months was required in order to observe significant reductions in this parameter [14]. The reduction in the late-phase reaction to PLA₂ was smaller than that observed with bee venom. However, purified PLA₂ may also contain other proteins (hyaluronidase and mellitin) that contribute to the magnitude of the reaction. Relatively high doses of PLA₂ were required to elicit consistent late-phase reactions, which may have masked the effect of therapy to some degree.

The immunological mechanisms underlying immunotherapy, both with whole allergens and with T cell peptides (which may be similar), remain an area of controversy. Using a peptide-based vaccine, we have observed outcomes similar to numerous studies of conventional immunotherapy: reduced allergen-specific T cell proliferation and cytokine production; reduced late-phase cutaneous reaction to allergen; and altered late-phase reaction cellularity. The modulation of T cell responses by peptides may be related to the presentation of cognate ligand to resting T cells in the absence of activation signals to the APC. Furthermore, increased IL-10 production by PBMCs following peptide therapy concurs with the results of several recent studies with conventional SIT [27–29] and is supportive of a role for regulatory T cells in the mechanism of allergen desensitization.

In order to address the molecular mechanisms associated with peptide immunotherapy, we measured expression of a number of genes that have previously been

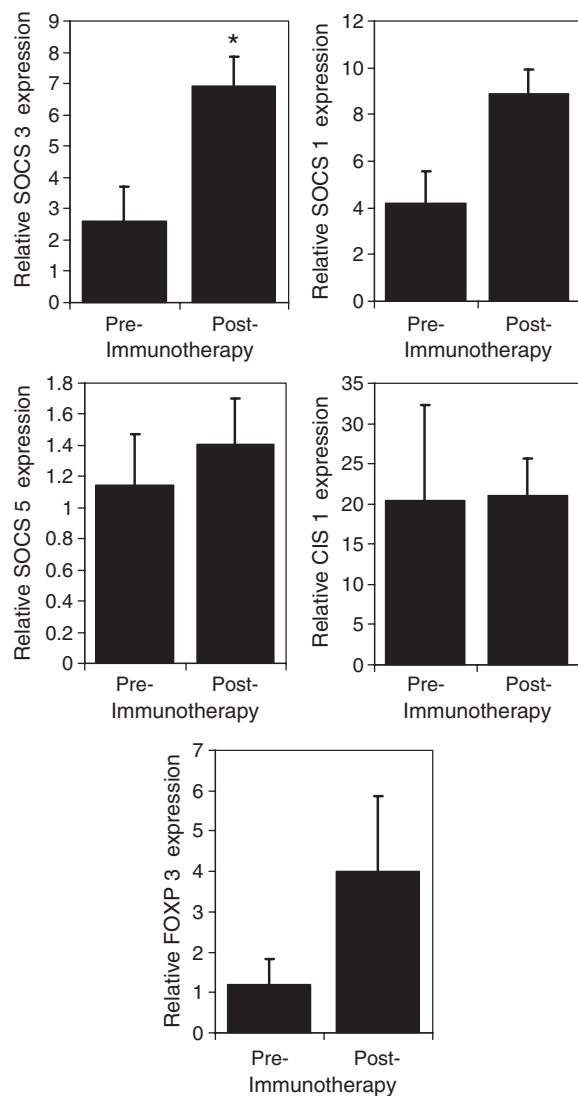


Fig. 5. Peptide treatment induces the expression of *Socs3* mRNA. Quantitative RT-PCR was performed on mRNA isolated from peripheral blood mononuclear cell stimulated with allergen, both before and after the treatment period.

associated with polarization to Th1/Th2 and with certain types of regulatory T cells. We demonstrate modest up-regulation of the *Socs3* mRNA, with no significant change in levels of expression of *Socs1*, *Socs5*, *Cis1*, or *Foxp3*. However, only a small number of individuals were analysed and data were heterogeneous. *Foxp3* expression was equivocal in the present study. The lack of statistically significant change in *Foxp3* expression may support other recent observations from our laboratory that failed to implicate naturally occurring (thymus-derived) CD4⁺CD25⁺-regulatory T cells in the antigen-specific hyporesponsiveness observed following peptide therapy [30]. However, studies on larger numbers of individuals will be required to determine an association of this and other factor(s) with peptide immunomodulation.

SOCS proteins have been shown to be pleiotropic and to exert effects on a number of cytokine signalling pathways. SOCS-3 is structurally closely related to SOCS-1 and CIS1 and is rapidly induced by a wide range of cytokines including IFN γ , IL-2, and IL-10. SOCS-3 has been shown to be preferentially expressed in Th2 cells in the mouse [19] and has been associated with allergic disease and disease severity. SOCS-3 down-regulates Th1 cytokine signalling, at least in part, by repression of IL-12/STAT4 signalling, leading to development and maintenance of Th2 phenotype [19]. Recent data suggest that this effect is mediated through direct association of the SOCS-3 SH2 domain and a phosphorylated tyrosine in the human IL-12 receptor β 2 subunit (IL-12R β 2) [31]. The correlation of SOCS-3 expression and Th2 development/maintenance has identified this molecule as a potential target for therapeutic intervention in allergic disease. It has been suggested that a reduction in SOCS-3 levels may down-regulate allergic responses [20].

Peptide therapy was associated with reduced antigen-specific proliferative responses and a reduction in pro-inflammatory cytokines such as IL-4, IL-13, and IFN γ . Concomitant increases in IL-10 production have also been observed in a number of peptide therapy studies [14, 29]. IL-10 signalling has been shown to induce expression of SOCS-3 in various cell types [32, 33], and may be responsible for the increases observed here. Here, the induction of SOCS-3 following peptide therapy is not associated with enhanced Th2 responses to allergen, but rather with the induction of antigen-specific hyporesponsiveness or 'tolerance'. Thus, during peptide immunotherapy, Th1 responses may be down-regulated through mechanisms that include the induction of SOCS-3 expression, with alternative mechanisms responsible for the suppression of Th2 cytokines. In light of this, a review of the role of SOCS-3 solely as a promoter of Th2 development may be appropriate.

In contrast to immunotherapy with intact allergens, peptide therapy was only associated with a transient and marginal increase in serum levels of allergen-specific IgG. This observation may reflect the specific targeting of T cells rather than B cells. Although the peptides used may include B cell epitopes, the monomeric nature of the peptides makes it unlikely that B cell receptor cross-linking would be achieved during therapy. Failure to activate allergen-specific B cells in an IL-10-rich environment may result in relatively static levels of allergen-specific IgG isotypes. The requirement for whole allergen in the induction of allergen-specific IgG₄ has been reported elsewhere [8]. However, Wachholz et al. [34] recently reported dissociation between levels of IgG₄ induced by conventional immunotherapy and blocking activity. These results support the notion that only relatively modest concentrations of high-affinity IgG₄ contribute to blocking of IgE-allergen binding. Thus, in the

current system, a small increase in IgG/G4 may have a biological effect. Clearly, further studies evaluating antibody affinity will be required to address this issue.

To conclude, this is the first description of the use of HLA-DR-characterized T cell peptides in allergic human volunteers and the first to evaluate a potential role for SOCS-3 in the induction of immunological tolerance in humans.

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