

Impaired secretion of interleukin-4 and interleukin-13 by allergen-specific T cells correlates with defective nuclear expression of NF-AT2 and jun B: relevance to immunotherapy

A. Faith*, D. F. Richards*, A. Verhoef†, J. R. Lamb‡, T. H. Lee* and C. M. Hawrylowicz*

*Department of Respiratory Medicine and Allergy, The Guy's, King's College and St Thomas' Hospitals School of Medicine, London, UK, †Department of Allergy and Clinical Immunology, National Heart and Lung Institute, Imperial College School of Medicine, London, UK, and ‡Respiratory Medicine Unit, Rayne Laboratory, University of Edinburgh Medical School, Edinburgh, UK

Summary

Background Allergen immunotherapy (IT) is a successful treatment associated with decreased Th2 cytokine production by allergen-specific T cells. We have previously demonstrated (Faith et al., J Immunol 1997; 159:53–57) that inhibition of Th2 cytokine production *in vitro* correlates with impaired tyrosine kinase activity through the TCR. The transcription factor complex, nuclear factor of activated T cells (NF-AT), which regulates Th2 cytokine production is controlled by the activity of tyrosine kinases.

Objective To address whether decreased Th2 cytokine production by allergen-specific CD4⁺ T cells following IT is correlated with altered translocation and nuclear expression of the NF-AT family member, NF-AT2, and the activator protein 1 (AP1) component of NF-AT, jun B.

Methods T cell lines specific for insect venom phospholipase A₂ (PLA) were derived from patients prior to and during conventional venom IT. Nuclear expressions of NF-AT and jun B were assessed following stimulation through the TCR. Th1 and Th2 cytokine and IL-10 production by insect venom-specific T cells were also determined. Results were compared with a well-established model system in which anergy was induced in cloned, allergen-specific Th2 cells.

Results Impaired translocation and decreased expression of NF-AT2 and jun B were detected in PLA-specific T cell lines derived from bee venom-allergic individuals following 16 weeks treatment compared to pre-treatment. These results correlated with significantly reduced production of IL-4 and IL-13 and significantly increased production of IFN- γ and IL-10 by PLA-specific T cells. Impaired IL-4 and IL-13 production also correlated with defective nuclear expression of NF-AT2/jun B in cloned, anergic allergen-specific Th2 cells.

Conclusion These results suggested that optimal production of IL-4 and IL-13 by allergen-specific T cells is dependent on the nuclear expression of NF-AT2 and jun B. Thus, specific inhibition of NF-AT2/jun B might be an option in novel and improved forms of allergen IT.

Keywords allergen, anergy, immunotherapy, NF-AT, Th2 cytokines

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Introduction

Skin and lung biopsies indicate that allergen-specific T cells secreting the Th2 cytokines, IL-4, IL-5 and IL-13, play a pivotal role in allergic inflammation reviewed in [1]. IL-4 and IL-13 regulate isotype switching of naïve B cells to IgE production [2], while IL-5 controls growth and survival of eosinophils [3]. A long-standing and efficacious treatment for allergic rhinitis, known as immunotherapy (IT), is the subcutaneous injection of allergen to which the patient is sensitized as reviewed in [4]. Successful IT has been associated

with decreased Th2 cytokine production by allergen-specific peripheral T cells [5, 6]. However, the immunological mechanisms responsible for abrogation of Th2 cytokine production remain unresolved.

We have previously reported that inhibition of Th2 cytokine production by cloned, anergic, allergen-specific T cells correlated with altered transmembrane signalling through the TCR, specifically ablation of p56lck and ZAP-70 tyrosine kinase activities and of intracellular calcium flux [7]. The calcium/calcineurin-dependent transcription factor, the nuclear factor of activated T cells (NF-AT), is regulated by p56lck and ZAP-70 reviewed in [8]. The NF-AT family comprises five transcription factors two of whose members, NF-AT1(NF-ATc2; NF-ATp) and NF-AT2 (NF-ATc1; NF-ATc) are predominant in T cells reviewed in [9]. Deficient nuclear translocation of both NF-AT1 and NF-AT2 was

Correspondence: Alexander Faith, Department of Respiratory Medicine and Allergy, 5th Floor, Thomas Guy House, The Guy's, King's College and St Thomas' School of Medicine, St Thomas Street, London SE1 9RT, UK. E-mail: alex.fait@kcl.ac.uk

correlated with impaired production of IL-4, IL-5, IL-13 and the prototype Th1 cytokine, IFN- γ , in patients with SCID [10]. Nevertheless, evidence from NF-AT2-deficient mice indicates that this molecule specifically regulates differentiation of Th2 cells [11, 12]. In contrast, IL-13 and IL-5 were elevated in mice deficient in NF-AT1 after allergen challenge suggesting a capacity to down-regulate Th2 cytokine production [9]. There is, however, no evidence concerning regulation by NF-AT2 of Th2 cytokine production in allergen-specific human T cells.

Co-operative interaction between NF-AT and the transcription factor activator protein 1 (AP1), stabilizes and increases the affinity of the resulting complex [9]. AP1 selectively accumulated in murine Th2 compared to Th1 cells [13], and in a separate study the jun, but not the fos, component of AP1 was isolated from human Th2 cells [14]. Jun B was recently found to be selectively induced in murine Th2 cells and one of the factors regulating IL-4 expression [15].

We have, therefore, investigated changes in the expression of NF-AT2 and jun B in bee venom phospholipase A₂ (PLA)-specific T cell lines from venom-allergic patients undergoing IT and related this to alterations in Th2 cytokine production. The expression of NF-AT2 and jun B strongly correlated with IL-4 and IL-13 production, indicating cooperation between these two transcription factors to influence secretion of these cytokines positively by human allergen-specific T cells.

Materials and methods

T cell lines derived from insect venom-allergic patients

The study received prior approval from the Ethics Committee of Guy's Hospital. Allergic sensitivity to bee/wasp venom was confirmed by skin prick tests and RAST assay. Increasing doses (1–100 μ g) of wasp (patient 1) or bee (patients 2–4) venom extract (Alutard, ALK, Hungerford, UK) were administered subcutaneously. Maximum dosage was achieved after 8 weeks and patients then received maintenance injections at 4-weekly intervals. Venous blood was taken prior to treatment and at the indicated time-points. PBMC (2×10^6 /mL) were isolated by density gradient centrifugation (Lymphoprep) and stimulated with purified bee sting venom PLA (Boehringer Ingelheim, Vienna, Austria) (10 μ g/mL) or wasp venom extract (10 μ g/mL) for 7 days in complete medium, then cultured with IL-2 (5% Lymphocult T, Biotest Serum Institute, Frankfurt, Germany) for 4 days. T cells were harvested and restimulated for a further 7 days with PLA or wasp venom extract (10 μ g/mL) in the presence of irradiated, autologous PBMC. T cells were then stimulated with combinations of previously determined optimal concentrations of mAb to anti-CD3 (OKT3; purified in-house) and anti-CD28 (CLB-CD28; Netherlands Red Cross, Amsterdam, the Netherlands).

Cloned T cells

The derivation of the T cell clones, AC1 and MeT5.3A7, specific for the major group 2 antigen of house dust mite, Der p 2, and bee sting venom PLA, respectively, has been reported

[16, 17]. The cytokine profiles of AC1 and MeT5.3A7 indicate a Th2-Th0 and Th2 phenotype, respectively. The cloned T cells were resuspended in a complete medium, consisting of RPMI 1640 supplemented with 2 mM L-glutamine, 50 μ g/mL gentamycin and 10% heat-inactivated fetal calf serum (FCS).

Induction of anergy

MeT5.3A7 and AC1 were incubated for 16 h in the presence of a supraoptimal concentration (60 μ M) of the antigenic peptides, PLA_{45–62} and Der p 2_{28–40}, respectively, in 24- or 48-well culture plates [18]. The cells were then washed and incubated for an additional 48 h before restimulation with mAb to CD3/28.

Intracellular staining

All reagents for intracellular staining were obtained from Pharmingen (Abingdon, UK). A previously published method [19] with modifications was used to detect intracellular cytokine production. Monensin was added to cultures for the final 4 h to allow accumulation of cytokines within the cell, and PMA (0.25 ng/mL) (Sigma, Poole, UK) and ionomycin (25 ng/mL) (Calbiochem, Beeston, UK) were then added during the final 1–2 h of culture. Cytofix/Cytoperm Plus was then added and the cells were permeabilized and stained for expression of IL-13 or IFN- γ with specific mAb conjugated to FITC (Pharmingen). Fluorescence analysis was performed on a FACSCalibur flow cytometer (Becton Dickinson, Abingdon, UK).

ELISA

Cytokines were measured using commercially available matched antibody pairs. All antibody pairs were purchased from Pharmingen. Cytokine concentrations were determined by reference to standards purchased from R & D Systems (Abingdon, UK). The lower limits of detection of the ELISA to IFN- γ was 50 pg/mL; IL-4 50 pg/mL; IL-5 200 pg/mL; IL-10 50 pg/mL; IL-13 100 pg/mL.

Preparation of nuclear extracts

T cells (1×10^7) were washed in ice-cold PBS and lysed in cytosolic buffer containing 0.2% Nonidet P-40, 10 mM KCl, 1 mM DTT, 10 mM Hepes (pH 7.9), 2 mM MgCl₂, 10 mM NaF, 0.5 mM sodium orthovanadate, 10 μ g/mL leupeptin and aprotinin, 5 mM EDTA and 5 mM iodoacetamide and left for 15 min on ice. Nuclei were obtained by centrifugation at 13 000 g. for 30 s in an Eppendorf microcentrifuge. The nuclei were washed once in ice-cold cytosolic buffer, pelleted and lysed into a hypertonic buffer, containing 400 mM NaCl, 1 mM DTT, 20 mM Hepes, pH 7.9, 1 mM MgCl₂, 5 mM EDTA, 0.5 mM sodium orthovanadate, 10 mM NaF, 10 μ g/mL leupeptin and aprotinin and 5 mM iodoacetamide, and gently shaken for 15 min at 4°C. The lysate was then centrifuged at 13 000 g. for 3 min and the insoluble material was discarded. Protein concentrations of the nuclear extract were determined using the Folin–Ciocolteu reagent (Sigma).

Immunoblotting

Extracts were diluted 1:2 in Laemmli sample buffer and boiled before electrophoresis. Lysates were electrophoresed on 8–16% SDS-PAGE gels (Novex, San Diego, CA, USA). Proteins were transferred onto nitrocellulose blots and probed with mAb to NF-AT2, jun B, (Santa Cruz Biotechnology, Santa Cruz, CA, USA; Affinity Bioreagents, Golden, CO, USA) and GAPDH (Chemicon, Temecula, CA, USA). Blots were detected by enhanced chemiluminescence (Amersham, Chalfont St. Giles, UK). Densitometry was performed using Image Quant software (Amersham).

Statistics

The paired *t*-test was used to compare cytokine production and transcription factor expression pre- and post-IT.

Results

Bee/wasp sting venom immunotherapy induces abrogation of IL-4 and IL-13 cytokine production by allergen-specific T cells

The effect of immunotherapy on cytokine production by T cells was determined in short-term bee-venom PLA-specific T cell lines derived from three patients and wasp venom-specific T cell lines derived from a fourth patient. Lines were established prior to and 8 and 16 weeks following the start of treatment. These time-points were chosen as a previous study had reported diminished IL-4 production by PBMC from bee venom allergics following 8 weeks of conventional venom IT [20]. T cells were restimulated with anti-CD3/28 mAb to avoid contamination of nuclear extracts with NF-AT

derived from APC [9]. The number of recovered T cells was $1.6 - 2.5 \times 10^6/\text{mL}$ from a starting concentration of $2 \times 10^6/\text{mL}$ and was independent of the time-point studied. The four patients studied demonstrated a significant decrease in IL-4 and IL-13 production following 8 and 16 weeks treatment (Fig. 1). In contrast to IL-13, production of IL-10 and IFN- γ , secreted at low levels prior to treatment, increased significantly after treatment. Although there was a trend to decreased levels of IL-5 after 16 weeks treatment, this did not reach significance. Intracellular cytokine staining also demonstrated significantly decreased IL-13 and significantly increased IFN- γ production following 8 weeks IT (Table 1 and Fig. 2). The effect of IT on IFN- γ secreting cells was particularly marked in patient 2. Follow-up investigation showed decreased skin prick test weals (7×5 mm, 6×5 mm and 6×5 mm prior to and 2×3 , 3×3 and 5×4 mm 18 months after commencing IT for patients 2, 3 and 4 respectively). Patient 1 was unavailable for testing.

Impaired translocation and expression of NF-AT2/jun B in T cell lines from patients receiving bee sting venom IT

We next determined whether inhibition of IL-4 and IL-13 production following bee sting venom IT correlated with altered expression and translocation of NF-AT2 and jun B. There was strong induction of NF-AT2 and weaker induction of jun B in nuclear extracts from PLA-specific T cell lines from the three patients prior to treatment. Results are shown for patients 2 and 4 (Fig. 3). The expression of both factors was maximal after stimulation for 3 h with anti-CD3/28. The NF-AT2 band seen after 3 h stimulation represents dephosphorylated NF-AT proteins that are believed to be essential for optimal binding to DNA [9]. Following 16 weeks IT, there were alterations in both the time course and level of

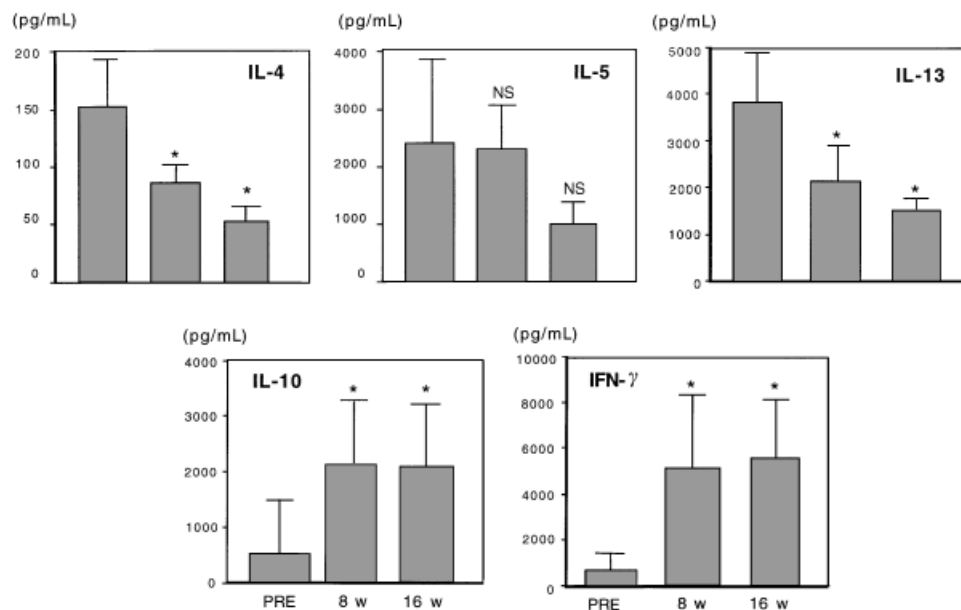


Fig. 1. Inhibition of IL-4 and IL-13 production by PLA-specific T cells following allergen IT. PBMC were obtained from four insect venom allergic patients prior to treatment, and 8 and 16 weeks after the start of IT. PBMC were stimulated with purified bee venom PLA or wasp venom extract for 7 days, and T cells expanded in IL-2. T cells were then restimulated with PLA or wasp venom extract for a further 7 days, then challenged with anti-CD3/28 mAb. Supernatants were harvested after 48 h and cytokine concentrations were determined by ELISA. *represents significant inhibition ($P < 0.05$) of IL-4 and IL-13 and significant enhancement ($P < 0.05$) of IL-10 and IFN- γ production after 8 and 16 weeks treatment. ns = not significant.

Table 1. Intracellular cytokine production pre- and post-immunotherapy

Patient	Pre-immunotherapy		Post-immunotherapy	
	IL-13	IFN- γ	IL-13	IFN- γ
1	16*	4	6	19
2	13	9	1	30
3	13	4	3	14
4	18	5	7	10
Mean (SD)	15 (2.5)	6 (2.1)	4 (2.5)	18 (8.6)
P-value†			0.001	0.03

*Percentage of IL-13 or IFN- γ -producing cells. †Pre-immunotherapy vs. post-immunotherapy.

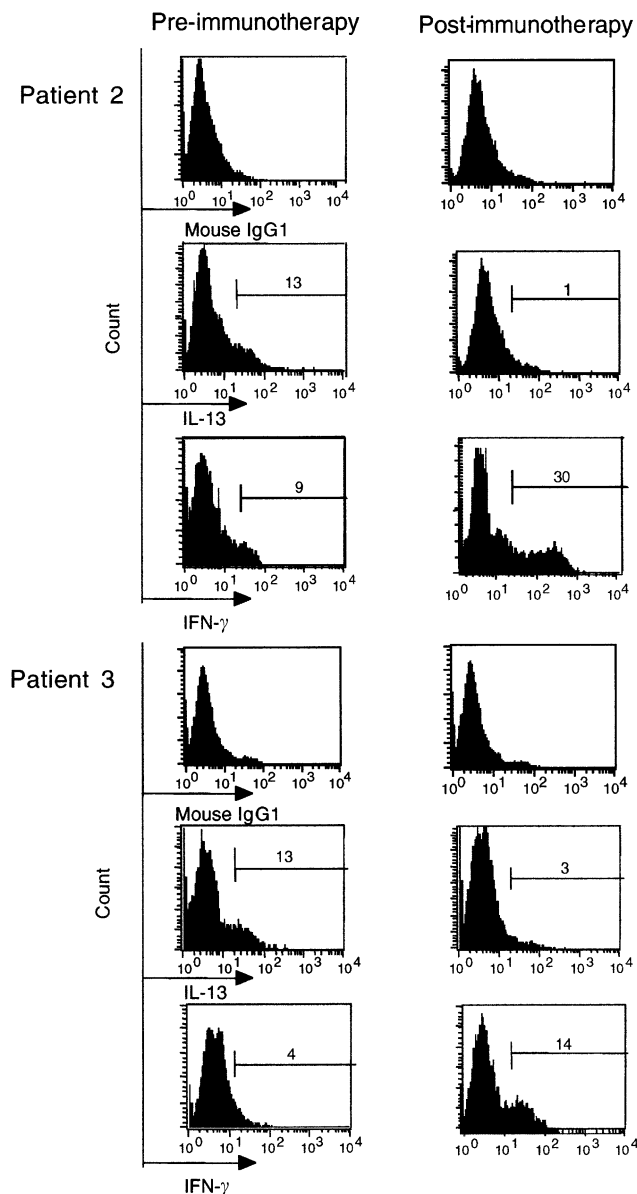


Fig. 2. Inverse effect on percentage of IL-13 and IFN- γ secreting cells during IT. PLA-specific T cell lines were established (see Fig. 1) from two bee venom allergic patients prior to treatment and 8 weeks after commencing IT. Intracellular cytokine staining for IL-13 and IFN- γ was performed following stimulation with anti-CD3/28 for 48 h. Figures represent percentages of positive cells.

expression of NF-AT2. NF-AT2 was ablated at the 3 h time-point. Following stimulation for 6 h, expression increased but was still 3–6-fold less compared to pre-IT. Post-IT, jun B was almost undetectable in nuclear extracts from T cell lines. The combined results for the three bee venom allergic patients, presented graphically (Fig. 3), demonstrate significant decreases post-IT in nuclear expression of NF-AT2 and jun B ($P < 0.01$) in T cells restimulated for 3 h.

Inhibition of IL-4 and IL-13 production in cloned, anergic Th cells correlates with defective nuclear translocation of NF-AT2 and jun B

Induction of anergy in cloned, human, allergen-specific Th cells represents a model system in which molecular changes associated with abrogation of Th2 cytokines can be investigated [7]. Binding of transcription factors to an NF-AT consensus sequence was attenuated in cloned, anergic influenza-specific Th cells [21]. Anergy induction in the bee sting venom PLA-specific Th2 clone, Met5.3A7, by exposure to a supraoptimal concentration of p45–62 abrogated IL-13 (Fig. 4a) and IL-4 (data not shown) production. When cloned Th cells were stimulated with anti-CD3/28, increased expression of NF-AT2 and the appearance of a strong band of slightly lower molecular weight than in unstimulated cells were observed (Fig. 4c). In contrast, anergic Th cells showed no induction of NF-AT2 on restimulation. We next examined the jun B component of NF-AT. Induction of jun B was observed in normal Th cells stimulated with anti-CD3/28. There was no evidence of expression of jun B in nuclear extracts from anergic Th cells either before or after stimulation. Similar results were obtained for the house dust mite-specific Th clone, AC1 (Fig. 4b).

Discussion

The results from this study indicate that changes in TCR signalling are associated with IT for bee sting allergy. Impaired translocation and expression of NF-AT2 and jun B were detected in PLA-specific T cell lines derived from bee venom-allergic individuals during IT and correlated with significantly decreased IL-4 and IL-13 production.

Several studies of rush IT for bee/wasp venom allergy have demonstrated decreased Th2 cytokine production by allergen-specific T cells [20, 22–24], while two studies of conventional IT have reported reductions in IL-4 secretion by venom-specific T cells [20, 25]. We decided to investigate the effect of conventional venom IT on IL-13 and IL-5 production, which has not been reported previously. Following treatment there was (a) significant reduction in IL-4 and IL-13 production by PLA-specific T cell lines and (b) lower levels of IL-5 production, although this was not statistically significant.

Our results, decreased Th2 cytokine and augmented IFN- γ production, indicated a role for immune deviation in conventional venom IT, as suggested by previous studies [20, 25]. The maximum concentration of bee venom injected, 100 μ g, is equivalent to approximately two stings and this dose was repeated as maintenance therapy. We have reported that the intensity of TCR ligation determines the pattern of cytokine production by CD4⁺ T cells [26]. Thus, TCR

ligation by high concentrations of allergen skews towards IFN- γ production. However, it is necessary to enter several caveats. Expansion of IFN- γ producing allergen non-specific

T cells could contribute to the result, although this was not observed in cultures of T cells prior to immunotherapy. Another possibility is the emergence of a regulatory T cell population during IT [27]. Such T cells, described as Tr1, secrete IL-10 and IFN- γ and have recently been reported to inhibit IgE production in a murine model of allergic asthma [28]. We detected increased production of the immunoregulatory cytokine, IL-10, during IT. The immunoregulatory effects of IL-10 [29, 30] may constrain potentially damaging inflammatory reactions due to concomitant secretion of IFN- γ .

Inhibition of IL-4 and IL-13 production correlated with defective translocation and nuclear expression of NF-AT2 in PLA-specific T cell lines, providing evidence for positive regulation of IL-4 and IL-13 by NF-AT2. This result was reinforced by the observation that cloned, anergic PLA-specific Th2 cells also showed defective induction of NF-AT2. The duration of NF-AT in the nucleus has been suggested to determine cytokine production by T cells [10]. NF-AT was not detectable in the nucleus 16 h following stimulation of PLA-specific T cells (data not shown) and the delayed time course for appearance of NF-AT2 during IT may, therefore, contribute to diminished IL-4 and IL-13 production.

In contrast to the effect on Th2 cytokine production, IT augmented IFN- γ production in PLA-specific T cell lines concomitant with impaired expression of NF-AT2. Moreover, defective induction of NF-AT2 in cloned, anergic Th cells had no effect on IFN- γ production. Stimulation with anti-CD3/28 mAb in the presence of cyclosporin A (CsA), which impedes nuclear import of NF-AT by inhibiting the phosphatase, calcineurin, has also been shown to increase IFN- γ secretion [31], suggesting a causal relationship. However, mRNA for IFN- γ is deficient in SCID patients with defective expression of both NF-AT1 and NF-AT2 [10]. Therefore, an alternative possibility is that NF-AT1 and NF-AT2 have the potential to regulate individual cytokines differentially [32].

The jun component of NF-AT has been shown to confer CD28 responsiveness at the IL-4 promoter in transformed human T cells [33]. We observed impaired expression of jun B in PLA-specific T cell lines during venom IT and in cloned Th cells following anergy induction. These results correlated with diminished secretion of IL-4 and IL-13. Co-operation between NF-AT and jun B is required for IL-4 and IL-13 production by transformed human T cells [34] and experiments in murine Th1 clones indicate that in the absence of cooperation between NF-AT and jun components, anergy results [35].

Impaired expression of jun B in T cell lines following immunotherapy correlated with significantly increased levels of IFN- γ . Induction of anergy in murine Th1 clones had no effect on IFN- γ production reviewed in [36]. Nevertheless, the expression of jun B was ablated on restimulation of the

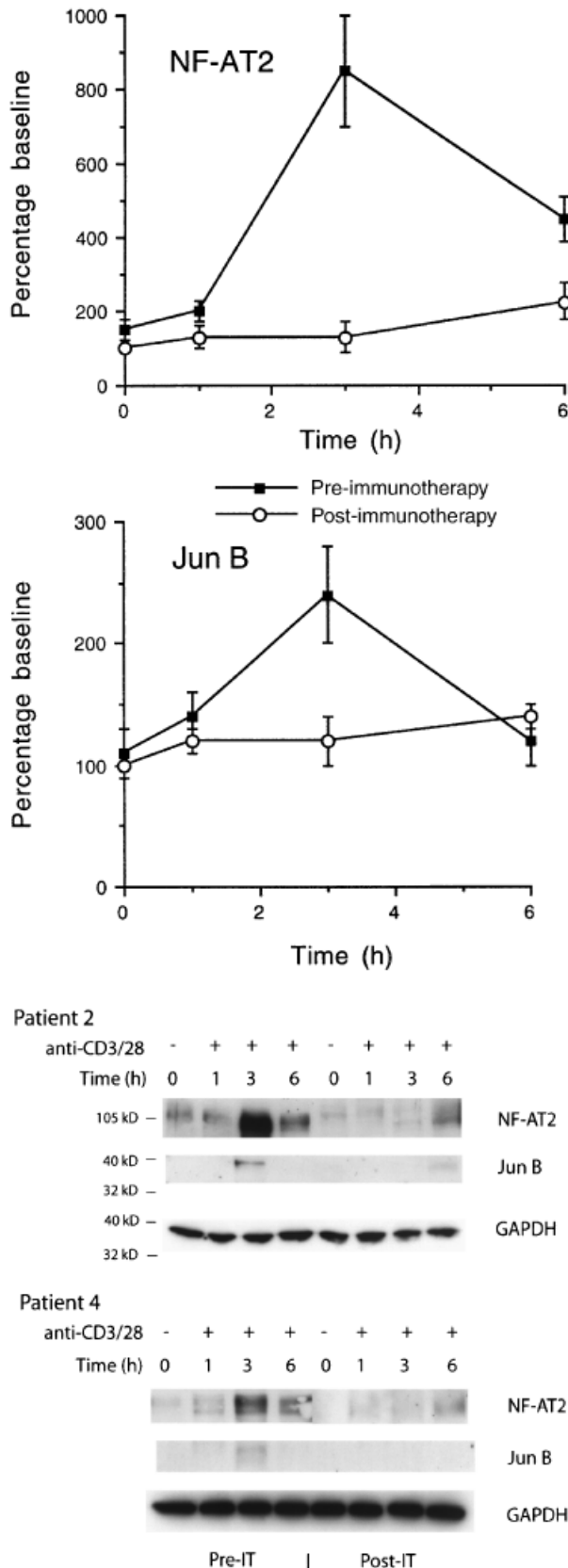


Fig. 3. Defective nuclear expression of NF-AT2 and jun B in PLA-specific T cells derived from bee venom-allergic patients during the course of IT. PLA-specific T cell lines were established (see Fig. 1) prior to treatment and 16 weeks after commencing IT. Nuclear lysates were prepared at the indicated time-points following stimulation with anti-CD3/28, resolved on 8–16% gradient gels, and immunoblotted with mAb to NF-AT2. Blots were then stripped and reprobed with mAb to jun B and GAPDH. The expression of NF-AT2 and jun B from patients 2–4 was determined by densitometry and the mean levels represented graphically as a percentage of the baseline (0 time-point) measurement.

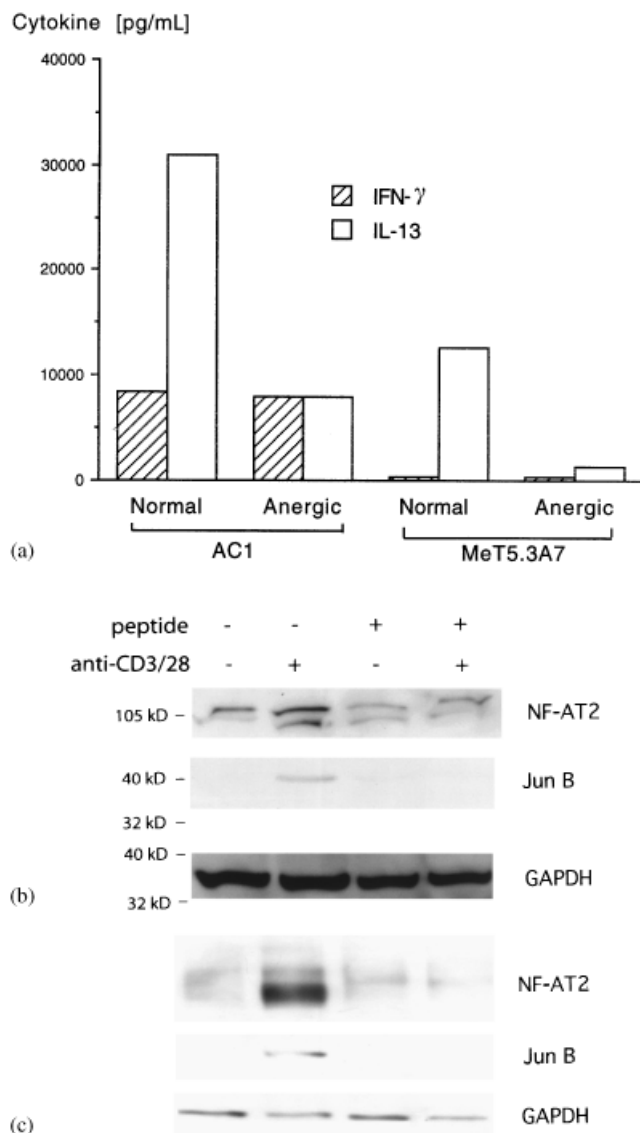


Fig. 4. Impaired IL-13 production in cloned, anergic Th cells correlates with defective nuclear expression of NF-AT2 and jun B. Cloned T cells AC1 and MeT5.3A7 were untreated or exposed for 16 h to a supraoptimal concentration of their respective antigenic peptides in the absence of APC. Cloned T cells were then restimulated for 48 h with mAb to CD3/28 and (a) IL-13 and IFN- γ production determined. Nuclear lysates were prepared from (b) MeT5.3A7 and (c) AC1 prior to and following 3 h stimulation with anti-CD3/28, resolved on 8–16% gradient gels and immunoblotted with mAb to NF-AT2. Blots were stripped and reprobed with mAb to jun B and GAPDH.

anergic T cells [37]. Moreover, introduction of a jun B transgene slightly inhibited IFN- γ production by murine T cells on restimulation with nominal antigen [15]. The consensus from these results indicates that IFN- γ production is not dependent on jun B. Therefore, the presence and activity of jun B may be associated with selective promotion of IL-13 and IL-4.

In conclusion, these results indicate that inhibition of IL-4 and IL-13 production correlates with defective nuclear expression of NF-AT2 and jun B. NF-AT has recently been reported to bind differentially to a proximal region of the IL-4 promoter in Th1 and Th2 cells [32], thus opening the

possibility of modulating this interaction to inhibit Th2 cytokine production specifically.

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