

Original article

Cat allergen peptide immunotherapy reduces CD4⁺ T cell responses to cat allergen but does not alter suppression by CD4⁺ CD25⁺ T cells: a double-blind placebo-controlled study

Background: We have previously described both modification of allergen immunotherapy using peptide fragments, and reduced regulation of allergen stimulated T cells by CD4⁺ CD25⁺ T cells from allergic donors when compared with nonallergic controls. It has been suggested that allergen immunotherapy induces regulatory T cell activity: we hypothesized that allergen peptide immunotherapy might increase suppressive activity of CD4⁺ CD25⁺ T cells.

Objective: To examine cat allergen-stimulated CD4 T cell responses and their suppression by CD4⁺ CD25⁺ T cells before and after cat allergen peptide immunotherapy in a double-blind placebo-controlled study.

Methods: Peripheral blood was obtained and stored before and after peptide immunotherapy or placebo treatment. CD4⁺ and CD4⁺ CD25⁺ were then isolated by immunomagnetic beads and cultured with allergen *in vitro*.

Results: Comparing cells from blood taken before with that after peptide immunotherapy there was a significant reduction in both proliferation and IL-13 production by allergen-stimulated CD4⁺ T cells, whereas no change was seen after placebo. CD4⁺ CD25⁺ T cells suppressed both proliferation and IL-13 production by CD4⁺ CD25⁺ T cells before and after therapy but peptide therapy was not associated with any change in suppressive activity of these cells.

Conclusion: Allergen peptide immunotherapy alters T cell response to allergen through mechanisms other than changes in CD4⁺ CD25⁺ T cell suppression.

T. R. F. Smith, C. Alexander, A. B. Kay, M. Larché, D. S. Robinson

Department of Allergy and Clinical Immunology, National Heart and Lung Institute and Leukocyte Biology Section, Biomedical Sciences Division, Faculty of Medicine, Imperial College London, UK

Key words: cat allergen; CD4⁺CD25⁺ T regulatory cells; peptide immunotherapy; T cells.

Trevor R. F. Smith
Department of Allergy and Clinical Immunology
National Heart and Lung Institute
and Leukocyte Biology Section
Biomedical Sciences Division
Faculty of Medicine
Imperial College London
UK

Accepted for publication 3 March 2004

Activation of Th2 type CD4⁺ T cells by allergen is thought to be a critical step in allergic inflammation in a variety of atopic allergic diseases including asthma, rhinitis and atopic dermatitis (1, 2). CD4⁺ CD25⁺ T cells have been described as naturally occurring regulatory or suppressive cells, initially characterized in mice as able to prevent activation of self-reactive T cells and thus prevent auto-immunity (3–6). We, and others, have recently described activity of such CD4⁺ CD25⁺ T cells from human peripheral blood in suppression of allergen-stimulated T cells from the same donors (7, 8). Indeed, when we compared activity of these cells from atopic and nonatopic donors the suppressive activity was greater in those without atopy than in those with atopic allergic disease (7), suggesting that inappropriate T cell responses to aeroallergens may normally be controlled by this regulatory subset.

Allergen injection immunotherapy was first described nearly 100 years ago and involves subcutaneous injection of allergen extract in an escalating dose regimen that results in clinical tolerance of allergen exposure (9). Controlled trials have shown such treatment to be effective in severe summer hayfever, cat allergic asthma,

and other allergies (10–12). Numerous studies have demonstrated alterations in T cell reactivity after allergen immunotherapy, with reduction in Th2 cytokine expression upon allergen stimulation often accompanied by increased expression of the Th1-associated cytokine interferon (IFN) γ (9, 13). More recently attention has focussed on IL-10, an immunodulatory cytokine which can down regulate production of both Th1 and Th2 cytokines (14). Several groups have suggested that allergen immunotherapy induces a CD25⁺ IL-10-producing regulatory T cell subset (15, 16).

A clinical drawback of allergen immunotherapy is that whole allergen extracts cross link IgE and can thus trigger anaphylaxis. This has limited the use of allergen immunotherapy, particularly in the UK (17). Our group has described a modification of immunotherapy to use peptide fragments of cat allergen, which do not cross link IgE but do interact with T cells in an MHC restricted fashion (18, 19). Previous studies have shown that this peptide therapy can reduce allergen-induced cutaneous responses (both late phase and early phase) (18). In addition cat peptide therapy reduced blood CD4⁺ T cell reactivity to whole allergen, and this was accompanied by reduced Th2

cytokine production but increased production of IL-10 (18, 19). We hypothesized that allergen peptide immunotherapy might act to increase suppression by CD4⁺ CD25⁺ T cells. In this study we have examined the effect of peptide immunotherapy on peripheral blood CD4⁺ responses and CD4⁺ CD25⁺ suppression of allergen-stimulated cultures in a double-blind placebo-controlled trial.

Methods

Subjects and immunotherapy study design

Cat-allergic asthmatics were recruited from the allergy clinic of the Royal Brompton Hospital, Imperial College London staff or by advertisement. All studies were approved by the Ethics Committee of the Royal Brompton and Harefield Hospitals NHS Trust, and written informed consent was obtained.

Inclusion criteria for Fel d 1 peptide immunotherapy: volunteers, age 18–55 years, had a history of cat-allergic rhinitis and asthma. Subjects gave a positive skin prick test (wheal diameter > 5 mm) to cat dander (all ALK), in the presence of a positive histamine and negative vehicle control. Volunteers had withheld oral and inhaled corticosteroids for the 2 months prior to the trial.

The study was randomized, placebo-controlled and double-blinded. It involved 16 cat allergic asthmatics, eight received a mixture of 12 Fel d 1 derived peptides and eight placebo. The 12 peptides spanning chain 1 and 2 of the major cat allergen protein Fel d 1, were synthesized and validated as previously described (19).

The subjects that passed the inclusion criteria returned for a further 12 visits over a period of 30 weeks. Before and after peptide/placebo therapy patients' cutaneous, bronchial and nasal responses to allergen challenge were measured and recorded (C. Alexander unpublished data).

Peptide/placebo therapy began on visit 5. Peptides starting at 1 µg were injected intradermally, followed by escalating doses (5, 10, 25, 50, 100, 100 µg) on subsequent visits. Pre- and post-peptide/placebo therapy 2 × 150 ml peripheral blood was taken into heparinized syringes and peripheral blood mononuclear cells (PBMCs) were separated by density centrifugation on Histopaque (Sigma Chemicals, Poole, UK). Extracted PBMCs were cryogenically stored in foetal calf serum containing 15% DMSO (Sigma).

Cell separation and culture

The cell culture system with standardized cat allergen extract (kindly supplied by Leti, Barcelona, Spain) was as previously described (7). Briefly, CD4⁺ T cells were isolated by negative immunomagnetic selection, followed by positive selection for CD25⁺ T cells using MACS (Miltenyi, Bisley, UK). CD4⁺ cells were enriched to a median of 95% pure, range 91–99%, and CD25⁺ a median of 78%, range 50–90%, as determined by flow cytometric analysis. Cells were cultured in 96 well flat-bottomed plates in triplicate at a density of 2 × 10⁶ cells/ml in RPMI 1640 medium with 5% human AB serum, penicillin, streptomycin and L-glutamine (Sigma) for 6 days with allergen extract added at 100 µg/ml. Autologous irradiated mononuclear cells were added as antigen presenting cells to all cultures at a density of 2 × 10⁶ cells/ml. In all cases triplicate medium control wells (no allergen) were included as a negative control. Cultures were of un-fractionated CD4⁺, CD4⁺ CD25⁺ cells alone, CD4⁺ CD25[−] cells alone, or mixed CD4⁺ CD25[−] and CD4⁺ CD25⁺ T cells at

a ratio of 2 : 1, or CD4⁺ CD25[−] at 3 × 10⁶ cells per ml to control for increased cell density of the CD25⁺/CD25[−] cultures. At day 6, 100 µl of supernatant was collected for cytokine analysis using enzyme-linked immunosorbent assay (IL-13; Pelikine, CLB, Amsterdam, The Netherlands) or Luminex Bead Array (IL-5, IL-10 and IFNγ; Luminex, Riverside, CA). Tritiated thymidine (Amersham, Chalfont St Giles, UK) was then added for the final 6 h of culture after which cells were harvested and incorporated radioactivity counted as index of proliferation. Data are expressed as counts per minute, or stimulation indices (used to allow pre-post therapy comparisons) which are calculated by comparing Ag-containing wells with those containing medium alone.

Statistical analysis

The significance of placebo/active intra-group pre- and post-therapy changes were determined using a nonparametric Wilcoxon matched pairs test.

The significance of placebo and therapy inter-group pre- and post-therapy changes were determined using a nonparametric Mann–Whitney test.

Results

CD4⁺ CD25⁺ T cells suppress cat allergen-induced proliferation of and cytokine production by CD4⁺ CD25[−] T cells.

First, we sought to confirm that human peripheral blood CD4⁺ CD25⁺ T cells from atopic allergic donors could suppress cat allergen-induced proliferation and cytokine production by CD25[−] T cells. When cultured with cat allergen for 6 days proliferation of CD4⁺ CD25[−] T cells, obtained from study subjects before treatment, was significantly greater than that seen for the whole CD4⁺ population, whereas CD4⁺ CD25⁺ T cells showed minimal proliferative response to allergen stimulation. Addition of CD4⁺ CD25⁺ T cells to CD4⁺ CD25[−] T cells from the same donor resulted in significant suppression of proliferation (Fig. 1A). Furthermore, CD4⁺ CD25⁺ T cells also significantly suppressed cat allergen-stimulated IL-13 production by CD4⁺ CD25[−] T cells (Fig. 1B).

Peptide immunotherapy reduces CD4⁺ T cell responses to allergen

Peptide immunotherapy was well tolerated and resulted in significant reduction in the late asthmatic response to inhaled challenge to whole cat allergen (clinical data from this study are presented elsewhere, C. Alexander et al. unpublished data). In accordance with our previous findings, in this double-blind placebo-controlled study there was a significant reduction in cat allergen-induced CD4⁺ T cell proliferation and IL-13 production comparing data before and after treatment in those patients randomized to active treatment but not in placebo-treated patients. The effect of treatment was also significant between groups for proliferation (Fig. 2). There was no significant effect of treatment on production of IL-5, IL-10 or IFNγ (data not shown).

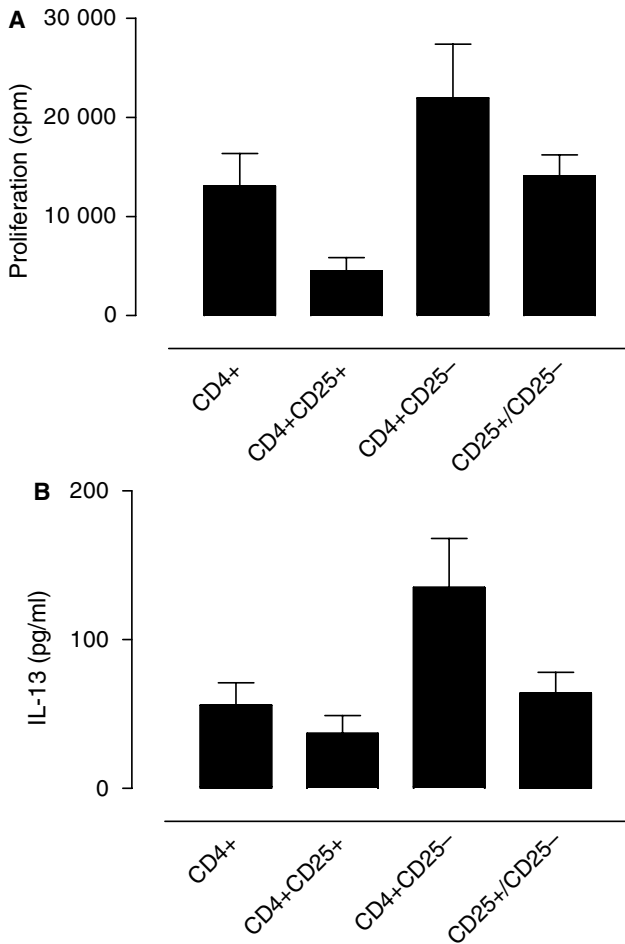


Figure 1. CD4⁺ CD25⁺ T cells suppress allergen stimulated T cell proliferation and cytokine production. Proliferation (A) and interleukin (IL)-13 production (B) by cat allergen-stimulated peripheral blood cells: CD4⁺ T cells, CD4⁺ CD25⁺ T cells, CD4⁺ CD25⁻ T cells, or mixed CD4⁺ CD25⁺ and CD4⁺ CD25⁻ T cells at a ratio of 1 : 2. Data are from 17 individual donors and triplicate cultures for each, and show results before treatment. Suppression by CD4⁺ CD25⁺ T cells (comparing CD4⁺ CD25⁻ alone with CD4⁺ CD25⁻ plus CD4⁺ CD25⁺) is significant at $P < 0.05$ for both proliferation and IL-13 production. CPMs is counts per minute for tritiated thymidine incorporation.

Peptide immunotherapy does not alter suppressive activity of CD4⁺CD25⁺ T cells for cat allergen stimulated proliferation or IL-13 production by CD4⁺ CD25⁻ T cells

In contrast to the effect on whole CD4⁺ T cells, the suppressive effect of CD4⁺ CD25⁺ on cat allergen-stimulated CD4⁺ CD25⁻ T cells was not different comparing patients before and after peptide allergen immunotherapy (Fig. 3). Nor was there any effect of peptide therapy on suppression of allergen-induced IL-13 production (Fig. 3), or production of IL-5, IL-10 or IFN γ (data not shown).

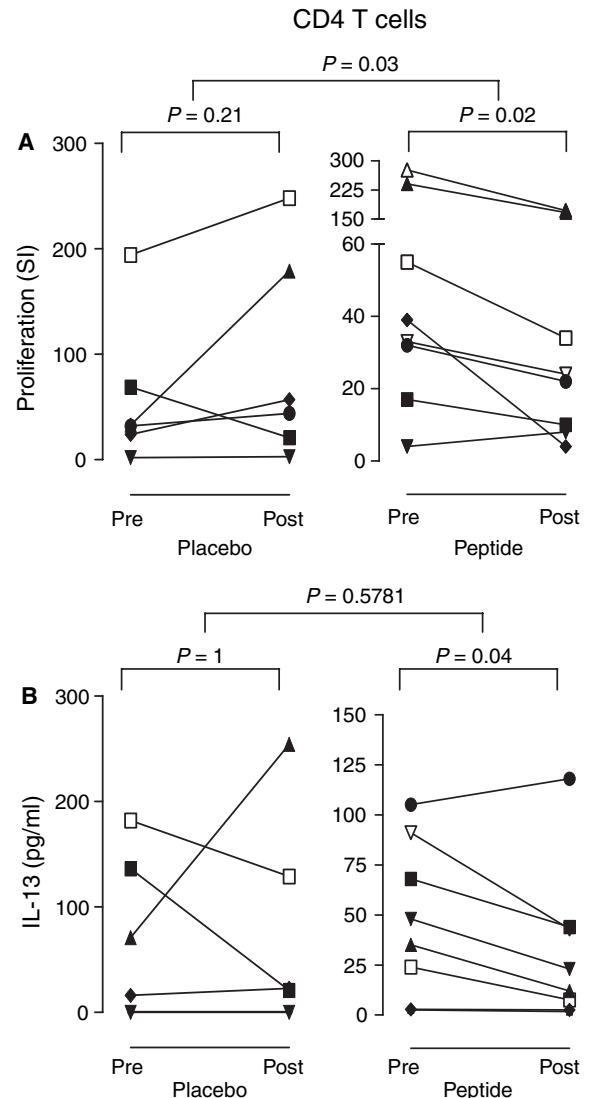


Figure 2. Peptide immunotherapy results in a reduction in cat allergen-induced proliferation and interleukin (IL)-13 production by CD4⁺ T cells. Proliferation of CD4⁺ T cells is shown as stimulation index (ratio of counts per minute for stimulated to unstimulated cells) before and after therapy for placebo-treated and peptide-treated patients (A). The IL-13 production is shown as pg/ml before and after therapy for placebo-treated ($n = 6$) and peptide-treated ($n = 8$) patients (B).

Discussion

We show here, in a clinically effective double-blind, placebo-controlled study, that although CD4⁺ T cell responses to cat allergen were significantly reduced by peptide therapy, this had no effect on suppressive activity of CD4⁺ CD25⁺ T cells in allergen-stimulated cultures. We suggest that peptide immunotherapy may induce T cell tolerance by mechanisms other than changes in CD4⁺ CD25⁺ suppressive function.

First we confirmed in this group of patients with cat allergy, that CD4⁺ CD25⁺ T cells had suppressive

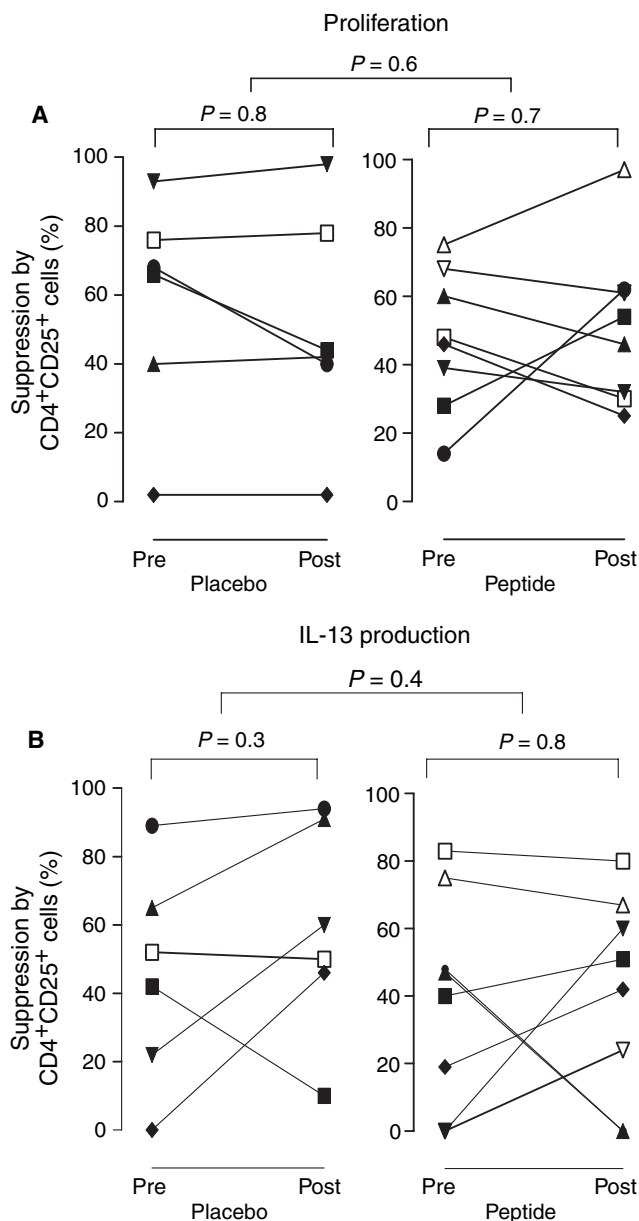


Figure 3. Peptide immunotherapy does not alter suppression of allergen-stimulated T cells by CD4⁺ CD25⁺ T cells. Percentage suppression of allergen stimulated proliferation (A) and interleukin-13 production (B) by CD4⁺ CD25⁺ T cells by CD4⁺ CD25⁺ T cells is shown before and after therapy for placebo ($n = 6$) and peptide ($n = 8$) treated patients.

activity for both proliferation and IL-13 production against cat allergen stimulated CD4⁺ CD25⁺ T cells. It is of note that this suppression was quite variable from one patient to another, but this was not related to cat-specific serum IgE or clinical symptom scores (data not shown).

Cat peptide immunotherapy was associated with a reduction in CD4⁺ T cells reactivity to cat allergen, in agreement with our previous studies (18, 19). However there was no effect on CD4⁺ CD25⁺ T cell suppression. Here we have studied peripheral blood responses, and it is

possible that allergen-specific regulatory T cells are recruited to sites of disease such as the airway. Nonetheless we were able to detect changes in blood CD4⁺ T cell reactivity, which were not accompanied by changes in suppression by blood CD4⁺ CD25⁺ T cells.

The numbers of patients in each group from whom blood was available before and after treatment was relatively small. It is therefore possible that a larger study might detect differences in suppression by CD4⁺ CD25⁺ T cells before and after peptide therapy. However, given the lack of any trend towards difference in either patient group or between groups it seems unlikely that our failure to find differences after peptide therapy simply reflects type II error, particularly since we did see significant changes for CD4⁺ T cell responses.

We detected significant suppression of IL-13 by CD4⁺ CD25⁺ T cells prior to therapy, and reduced allergen-induced IL-13 production by CD4⁺ T cells in those patients treated with peptide, but saw no significant changes in other cytokines measured. This may reflect the sensitivity of assay systems used, the lack of consumption of IL-13 in the cultures or other factors, but these data are in accord with previous findings of allergen-cultured human peripheral blood T cells (20). Certainly IL-13 is implicated as an important effector cytokine in allergic disease, particularly airway hyper-responsiveness (21–23), which was the clinical endpoint showing significant change with treatment in this study.

We, and others have previously shown increased production of IL-10 after both peptide and whole allergen immunotherapy (15, 16, 18, 19). Peptide given intravenously or orally induced a CD4⁺ CD25⁺ regulatory population in an animal model (24). In addition, IL-10 regulatory T cells have also been induced in animal models by peptide therapy (25). It may be that allergen-peptide or indeed whole allergen immunotherapy induces such IL-10 regulatory T cells, which may be an inducible regulatory subset that act in concert with naturally occurring CD4⁺ CD25⁺ T cells. However, in this study we saw no significant change in IL-10 production, and the doses used in animal models and human peptide tolerance regimens are markedly different. Whilst it has been suggested that IL-10 may be produced by CD4⁺ CD25⁺ T cells (16), we and others have previously shown no effect of blocking IL-10 on suppression by these cells (7, 26). Separate regulatory T cell populations characterized by high IL-10 production have been described in a variety of settings (25, 27, 28). It thus remains to be confirmed whether peptide immunotherapy induces changes in T cell responsiveness by induction of other regulatory T cell subsets, or by other mechanisms such as a deletion or redistribution of allergen-reactive T cells. One possibility is production of IL-10 by non-T cells (Verhoef et al. unpublished data). Further studies will be required to compare these subsets.

A better understanding of the activity of immunotherapy in altering the balance between suppressors and effector memory cells in allergic disease may allow use of

adjuvants, such as immunosuppressive drugs, to optimize and simplify the immunotherapy treatment regimen.

Acknowledgments

These studies were funded by the Medical Research Council, UK. TRFS is an MRC PhD student, DSR is supported by a Wellcome

Trust research Leave Award for Clinical Academics, ML is a National Asthma Campaign Senior Fellow and CA was supported by a TV James Fellowship from the British Medical Association. The authors thank Justine Arbery RGN for her expert help in the clinical study.

References

- KAY AB. Allergy and allergic diseases. *N Engl J Med* 2001;**344**:30–37.
- ROBINSON DS. Th-2 cytokines in allergic disease. *Br Med Bull* 2000;**56**:956–968.
- SAKAGUCHI S, SAKAGUCHI N, ASANO M, ITOH M, TODA M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 1995;**155**:1151–1164.
- SAKAGUCHI S. Regulatory T cells: key controllers of immunologic self-tolerance. *Cell* 2000;**101**:455–458.
- McHUGH RS, SHEVACH EM. The role of suppressor T cells in regulation of immune responses. *J Allergy Clin Immunol* 2002;**110**:693–702.
- MALLOY KJ, POWRIE F. Regulatory T cells in the control of immune pathology. *Nat Immunol* 2001;**2**:816–822.
- LING EM, SMITH T, NGUYEN XD, PRIDGEON C, DALLMAN MJ, ARBERY J et al. Relation of CD4+CD25+ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. *Lancet* 2004;**363**:608–615.
- BELLINGHAUSEN I, KLOSTERMANN B, KNOP J, SALOGA J. Human CD4+CD25+ T cells derived from the majority of atopic donors are able to suppress Th1 and Th2 cytokine production. *J Allergy Clin Immunol* 2003;**111**:862–868.
- DURHAM SR, TILL SJ. Immunologic changes associated with allergen immunotherapy. *J Allergy Clin Immunol* 1998;**102**:157–164.
- VARNEY VA, GAGA M, FREW AJ, ABER VR, KAY AB, DURHAM SR. Usefulness of immunotherapy in patients with severe summer hay fever uncontrolled by anti-allergic drugs. *BMJ* 1991;**302**:265–299.
- BOUSQUET J, CALVAYRAC P, GUERIN B, HEJJAOUI A, DHIVERT H, HEWITT B et al. Immunotherapy with a standardized *Dermatophagoides pteronyssinus* extract. I. In vivo and in vitro parameters after a short course of treatment. *J Allergy Clin Immunol* 1985;**76**:734–744.
- VARNEY VA, EDWARDS J, TABBAH K, BREWSTER H, MAVROLEON G, FREW AJ. Clinical efficacy of specific immunotherapy to cat dander: a double-blind placebo-controlled trial. *Clin Exp Allergy* 1997;**27**:860–867.
- DURHAM SR, WALKER SM, VARGA EM, JACOBSON MR, O'BRIEN F, NOBLE W et al. Long-term clinical efficacy of grass-pollen immunotherapy. *N Engl J Med* 1999;**341**:468–475.
- MOORE KW, DE WAAL MALEFYT R, COFFMAN RL, O'GARRA A. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 2001;**19**:683–765.
- JUTEL M, AKDIS M, BUDAK F, AEBISCHER-CASAULTA C, WRYSZCZ M, BLASER K et al. IL-10 and TGF- β cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. *Eur J Immunol* 2003;**33**:1205–1214.
- FRANCIS JN, TILL SJ, DURHAM SR. Induction of IL-10⁺ CD4⁺ CD25⁺ T cells by grass pollen immunotherapy. *J Allergy Clin Immunol* 2003;**111**:1255–1261.
- Committee on Safety of Medicines/Medicines Control Agency. Desensitising vaccines. *BMJ* 1986;**293**:948.
- OLDFIELD WL, LARCHE M, KAY AB. Effect of T-cell peptides derived from Fel d 1 on allergic reactions and cytokine production in patients sensitive to cats: a randomised controlled trial. *Lancet* 2002;**360**:47–53.
- OLDFIELD WL, KAY AB, LARCHE M. Allergen-derived T cell peptide-induced late asthmatic reactions precede the induction of antigen-specific hyperresponsiveness in atopic allergic asthmatic subjects. *J Immunol* 2001;**167**:1734–1739.
- TILL SJ, DURHAM SR, DICKASON R, HUSTON D, BUNGRE J, WALKER S et al. IL-13 production by allergen-stimulated T cells is increased in allergic disease and associated with IL-5 but not IFN- γ expression. *Immunology* 1997;**91**:53–57.
- WILLS-KARP M, LUYIMBAZI J, XU X, SCHOFIELD B, NEBEN TY, KARP CL et al. Interleukin-13: central mediator of allergic asthma. *Science* 1998;**282**:2258–2261.
- GRUNIG G, WARNOCK M, WAKIL AE, VENKAYYA R, BROMBACHER F, RENNICK DM et al. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 1998;**282**:2261–2263.
- HUMBERT M, DURHAM SR, KIMMITT P, POWELL N, ASSOULI B, PFISTER R et al. Elevated expression of messenger ribonucleic acid encoding IL-13 in the bronchial mucosa of atopic and nonatopic subjects with asthma. *J Allergy Clin Immunol* 1997;**99**:657–665.
- THORSTENSON KM, KHORUTS A. Generation of anergic and potentially immunoregulatory CD25+CD4 T cells in vivo after induction of peripheral tolerance with intravenous or oral antigen. *J Immunol* 2001;**167**:188–195.
- SUNDSTEDT A, O'NEILL EJ, NICOLSON KS, WRAITH DC. Role for IL-10 in suppression mediated by peptide-induced regulatory T cells in vivo. *J Immunol* 2003;**170**:1240–1248.
- DIECKMANN D, PLOTTNER H, BERCHTOLD S, BERGER T, SCHULER G. Ex vivo isolation and characterization of CD4(+)CD25(+) T cells with regulatory properties from human blood. *J Exp Med* 2001;**193**:1303–1310.
- GROUX H, O'GARRA A, BIGLER M, ROULEAU M, ANTONENKO S, DE VRIES JE et al. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 1997;**389**:737–742.
- BARRAT FJ, CUA DJ, BOONSTRA A, RICHARDS DF, CRAIN C, SVELKOUK HF et al. In vitro generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. *J Exp Med* 2002;**195**:603–616.