

# High Dose Allergen Stimulation of T Cells from House Dust Mite-Allergic Subjects Induces Expansion of IFN- $\gamma$ + T Cells, Apoptosis of CD4+IL-4+ T Cells and T Cell Anergy

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## Key Words

Allergy · Anergy · Apoptosis · Cytokines · Human · IFN- $\gamma$  · IL-4 · T cell

## Abstract

**Background:** During clinically effective allergen-specific immunotherapy a shift in cytokine dominance from IL-4, IL-5 predominant to IFN- $\gamma$  predominant has been observed. As antigen concentration influences Th cell priming, this study aimed to determine the effect of different allergen concentrations on human house dust mite (HDM)-specific T cell production of IL-4 and IFN- $\gamma$ , proliferation and apoptosis. **Methods:** HDM-allergic donor PBMC were cultured for 14 days with different concentrations of HDM extract (1, 10 and 100  $\mu$ g/ml). T cell intracellular IL-4 and IFN- $\gamma$ , division (CFSE labelling) and apoptosis (active caspase-3 staining) were analysed by flow cytometry. Proliferation was assessed by <sup>3</sup>H-thymidine incorporation. **Results:** Increased CD4+IFN- $\gamma$ + and CD8+IFN- $\gamma$ + T cell numbers were observed in high allergen concentration cultures compared with low concentration cultures whereas there were no differences in CD4+IL-4+ and CD8+IL-4+ T cell numbers. CFSE cell labelling revealed that high allergen concentration fa-

vours the expansion of IFN- $\gamma$ -producing CD4+ T cells. The proportion of apoptotic cells increased with allergen concentration and there was preferential apoptosis of CD4+IL-4+ T cells. HDM-induced proliferation was decreased in high allergen concentration cultures; this was reversible by IL-2 consistent with anergy. **Conclusion:** These results show that T cell division and apoptosis contribute to the cytokine skewing to predominant IFN- $\gamma$  production by T cells observed at high allergen concentration. Thus the use of hypoallergenic T cell reactive preparations which can be given safely at higher doses than natural extracts may enhance efficacy of allergen-specific immunotherapy.

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## Introduction

Allergic diseases affect up to 40% of the Western population. Allergic symptoms such as rhinitis and asthma are caused by the downstream effects of cytokines produced by Th2-type cells in response to allergen. IL-4 and IL-5 promote the maturation, migration and activation of mast cells, basophils and eosinophils [1]. IL-4 and IL-13 stimulate allergen-specific IgE production by B cells [2, 3].

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The cross-linking of membrane-bound IgE by allergen on mast cells and basophils results in the release of mediators including histamine, leukotrienes and prostaglandins that cause allergic inflammation. Allergen-specific immunotherapy (SIT), involving the repeated administration of allergen extract, is a current treatment for the symptoms of allergy in severely affected individuals [4]. This treatment modifies the natural course of allergic disease and may effect a cure; however, although efficacy rates for bee venom allergy are high, efficacy rates are lower for grass pollen and house dust mite (HDM) allergy [4]. Clinically successful SIT correlates strongly with decreased allergen-specific proliferative response and a shift from an IL-4, IL-5 dominant T cell cytokine profile to IFN- $\gamma$  dominance [5–12]. The mechanisms involved in this repolarisation are not well defined; however, deletion and/or anergy of allergen-specific Th2 cells or immune deviation have been proposed [13]. Changes in CD8+ T cell cytokine production during treatment are poorly studied.

Antigen concentration is a key factor that has been shown to influence Th cell differentiation. Several studies with non-allergen molecules have shown increased IFN- $\gamma$  production at higher antigen doses [14–18] but the effect of antigen concentration on IL-4 production seems to be more variable [15, 16, 18], presumably due to differences in duration of Th cell stimulation [18]. Cell division also plays a critical role in regulating cytokine production by Th cells [19] and this may be influenced by antigen concentration [20].

In the limited studies performed to date on the effect of antigen concentration on allergen-specific Th cell cytokine response, cytokines have been analysed as secreted product in culture supernatants [21, 22]. To more clearly dissect the mechanisms by which allergen concentration alters T cell functional phenotype, we have analysed cytokine responses at a single cell level by flow cytometry. We examined IL-4 and IFN- $\gamma$  production by allergen stimulated CD4+ and CD8+ T cells using intracellular cytokine staining. Culturing T cells with high concentration of allergen in comparison to low concentration resulted in the expansion of IFN- $\gamma$ -producing CD4+ T cells. We also report for the first time the effect of allergen concentration on programmed T cell death. Increased CD4+ IL-4+ T cell apoptosis was observed in high allergen concentration cultures. Elucidation of factors that alter cytokine profiles of allergen-specific Th cells in vitro could lead to the development of more rational SIT strategies for down-regulation of Th2-type responses in allergy.

## Methods

### Antigens

Freeze-dried HDM, *Dermatophagoides pteronyssinus*, kindly provided by Allergy Therapeutics Limited (UK), was prepared as an aqueous extract in PBS and sterilised. The concentration of the extract was determined by dry weight of freeze-dried HDM per volume of PBS. Analysis of the extract by SDS-PAGE and silver staining revealed a series of bands with a prominent band at 25 kD consistent with the molecular mass of Der p 1 (data not shown). Endotoxin (LPS) levels in HDM extract were below detectable levels ( $<3$  U/ml) as determined by the QCL-1000™ endotoxin detection kit (BioWhittaker, Norway). Glove extract (GE), as a source of latex allergens, was prepared by adding sterile PBS (1 ml/g glove) to the interior of latex gloves (Uniglove UG2A, Unimex trade agents P/L, Australia) as described previously [23].

### Study Population

Peripheral blood was taken from HDM-allergic donors attending the Alfred Hospital Allergy Clinic, Melbourne, Australia, with the approval of the Alfred Hospital Ethics Committee and written informed consent from each patient. Donors were selected on the basis of a history of clinical symptoms of perennial HDM allergy and HDM-specific IgE as determined by skin prick test (wheal  $>3$  mm) and/or EAST (Kallestad Allercoat Sanofi-Pasteur Diagnostics, score  $>2$ ).

### Cell Culture System

A cell culture system was designed to investigate the effects of repeated high concentration allergen stimulation on human T cell responses. PBMC ( $2.5 \times 10^6$ /well) were cultured in 24-well plates (Costar, USA) in complete medium (RPMI-1640 medium supplemented with 2 mmol/l L-glutamine, 100 IU/ml penicillin/streptomycin, Gibco BRL Life Technologies, USA, and 5% screened, heat-inactivated human AB+ serum, Sigma Chemical Company, USA) with HDM extract (1, 10 or 100  $\mu$ g/ml) in a final volume 2 ml/well for 7 days at 37°C in a 5% CO<sub>2</sub> humidified incubator. A 'no allergen' control could not be used in this study due to cell death after a few days of culture without antigen. On day 7 of culture, cells ( $1 \times 10^6$  cells/ml) were restimulated with HDM extract (1, 10 or 100  $\mu$ g/ml) in the presence of irradiated (3,000 rad) autologous PBMC ( $1 \times 10^6$ /ml) as a source of antigen presenting cells (APC). Recombinant human IL-2 (rIL-2; 25 U/ml; Cetus Corporation, USA) was added on day 8 of culture, and on day 11 of culture, 1 ml of culture medium was removed and replaced with fresh medium and rIL-2 (25 U/ml).

### Intracellular Cytokine Staining

At day 14 of culture, cells were stimulated ( $2.5 \times 10^5$  cells/well) in a 96-well U bottom plate with immobilised anti-CD3 (OKT3; 10  $\mu$ g/ml) and rIL-2 (100 U/ml) in a final volume of 200  $\mu$ l/well for 6 h at 37°C in the presence of 10  $\mu$ g/ml Brefeldin A (Sigma Chemical Company, USA). Cells were then stained with anti-CD4-FITC and anti-CD8-CyChrome (Pharmingen, USA) or isotype control antibodies (mouse IgG<sub>1,k</sub>-FITC and IgG<sub>1,k</sub>-CyChrome). After washing, fixing for 20 min in 4% paraformaldehyde/PBS, and permeabilising for 20 min in FACS permeabilising solution (Becton Dickinson, USA) cells were double stained with anti-IL-4-PE and anti-IFN- $\gamma$ -APC (Pharmingen) or isotype control antibodies (mouse IgG<sub>1,k</sub>-PE and IgG<sub>1,k</sub>-APC; Pharmingen). Percentages of cytokine-positive CD4+ and CD8+ T cells were determined using a FACScaliber flow cytometer.

eter (Becton Dickinson) and Cellquest software. The specificity of cytokine staining was confirmed by pre-incubating anti-cytokine antibodies with the appropriate cytokine to block staining.

#### *Analysis of Cell Division*

After 7 days of PBMC culture with HDM extract (1, 10 and 100 µg/ml) cells were labelled with 1 µM 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Oregon) to analyse cell division. For this, harvested cells ( $1 \times 10^7$  cells/ml) were incubated with 1 µM CFSE in PBS for 10 min at 37 °C. To cease staining, cells were washed by the addition of a 10× volume of complete medium and centrifugation. Cells were washed a further two times to ensure there was no residual CFSE. CFSE-labelled cells were then cultured for 3 and 5 days with HDM extract (1, 10 and 100 µg/ml as for the first week of culture) and APC or with immobilised anti-CD3 (10 µg/ml) and rIL-2 (100 U/ml) as a positive control for cell division. After this time, cells were stimulated with immobilised anti-CD3 (10 µg/ml) and rIL-2 (100 U/ml) for 6 h at 37 °C in the presence of 10 µg/ml Brefeldin A, stained for intracellular cytokines and analysed by flow cytometry as before.

#### *Analysis of Apoptosis*

To analyse apoptotic cells and their cytokine profiles 14-day HDM extract-stimulated cultures were firstly harvested and stimulated with immobilised anti-CD3 (10 µg/ml) and rIL-2 (100 U/ml) for 6 h at 37 °C in the presence of 10 µg/ml Brefeldin A. Cells were then surface labelled with anti-CD4-CyChrome, fixed, permeabilised, and triple stained with anti-human active caspase-3-FITC (Pharmingen), anti-IL-4-PE and anti-IFN-γ-APC. The anti-active caspase-3 antibody binds the 17 kD active form of caspase-3 and not the proenzyme caspase-3. The percentages of active caspase-3 positive CD4+IL-4+ and CD4+IFN-γ+ T cells were determined by flow cytometry.

#### *Oligoclonal T-Cell Proliferation Assays*

T cells ( $5 \times 10^4$ /well) from 14-day cultures were incubated in 96-well round-bottom plates (Linbro, ICN Biomedicals, USA) in triplicate with equal numbers of APC in the presence of HDM extract at 10 µg/ml or IL-2 (100 U/ml) in a final volume of 200 µl/well. Cultures of T cells and APC in the absence of antigen, T cells alone, and APC alone were included as controls. After 72 h, cultures were pulsed with 1 µCi  $^3\text{H}$ -thymidine (Amersham Biosciences, UK) and harvested 16 h later. Proliferation as correlated with  $^3\text{H}$ -thymidine incorporation was measured by liquid scintillation spectroscopy and results expressed as Δ cpm (the mean of the triplicates with antigen minus the mean of the triplicates without antigen).

#### *Assessing HDM Extract for Mitogenicity, Toxicity and Non-Specific Induction of Cytokine Production*

HDM extract was tested for mitogenicity and toxicity using oligoclonal latex-reactive T cells which had been cultured in the presence of 25 µg/ml GE for 3 weeks. For the mitogenicity assay, latex-specific T cells ( $5 \times 10^4$ /well) were incubated in 96-well round-bottom plates (Linbro, ICN Biomedicals, USA) in triplicate with equal numbers of washed irradiated (3,000 rads) autologous PBMC and HDM extract at 1, 10 and 100 µg/ml (final volume 200 µl/well). For the toxicity assay the effect of addition of HDM extract at 1, 10 and 100 µg/ml to IL-2-stimulated (100 U/ml; volume 200 µl/well) latex-specific T cells ( $5 \times 10^4$ /well) was determined by proliferation assays. No APC were included in these toxicity assay cultures. For the mitogenicity and

toxicity assays, cells were incubated for 48 h at 37 °C in a 5% CO<sub>2</sub> humidified incubator, pulsed for the last 16 h with  $^3\text{H}$ -thymidine (1 µCi/well) and harvested onto printed glass fibre filters with a 96-well automatic cell harvester.  $^3\text{H}$ -thymidine incorporation was measured by liquid scintillation spectroscopy.

To test for non-specific induction of cytokine production by the HDM extract, latex-specific T cells ( $7.5 \times 10^5$ /well) were incubated in 48-well culture plates (Costar, USA) with equal numbers of washed irradiated autologous PBMC in the presence of 40 µg/ml GE with either 1 or 100 µg/ml HDM extract (final volume 1 ml/well) for 4 days at 37 °C in a 5% CO<sub>2</sub>. Cultures of T cells and APC with GE (40 µg/ml), or with IL-2 (100 U/ml), or with HDM extract (100 µg/ml), or without antigen were included as controls. After culture cells were harvested and analysed for intracellular IL-4 and IFN-γ.

#### *Statistics*

Data were found to be well approximated by a log normal distribution and was normalised via log-transformation prior to analysis. Statistical analyses were performed using Repeated Measures ANOVA with a Bonferroni adjustment for multiple comparisons. A two-sided p value of 0.05 was considered statistically significant. InStat 2.0 software was used for all statistical analyses.

## **Results**

### *High Allergen Concentration Increases the Proportion and Number of CD4+IFN-γ+ T Cells and CD8+IFN-γ+ T Cells, but Decreases the Proportion of CD4+IL-4+ T Cells*

To determine the effect of repeated allergen stimulation at different concentrations on CD4+ and CD8+ T cell cytokine production, intracellular IL-4 and IFN-γ staining was performed on 14-day HDM extract-stimulated cultures. Results for one HDM-allergic donor are shown in figure 1a. Repeating this experiment on PBMC from another 9 donors (fig. 1b) revealed significantly higher CD4+IFN-γ+ T cell proportions in cultures stimulated with 100 µg/ml HDM extract in comparison to 1 µg/ml ( $p < 0.01$ ) and 10 µg/ml ( $p < 0.05$ ). In addition significantly lower CD4+IL-4+ T cell proportions were observed in the 10 µg/ml ( $p < 0.05$ ) and 100 µg/ml ( $p < 0.01$ ) HDM extract-stimulated cultures compared to the 1 µg/ml HDM extract-stimulated cultures (fig. 1b). Very few CD4+IFN-γ+IL-4+ T cells were observed. CD8+IFN-γ+ T cell proportions were significantly higher in cultures stimulated with 100 µg/ml HDM extract in comparison to 1 µg/ml ( $p < 0.01$ ; fig. 1c). At each HDM extract concentration tested only a very small percentage of CD8+ T cells stained positive for IL-4. When data were expressed as cell numbers per culture, CD4+IFN-γ+ and CD8+IFN-γ+ T cells were significantly higher in cultures stimulated with 100 µg/ml HDM extract (median values  $10 \times 10^5$

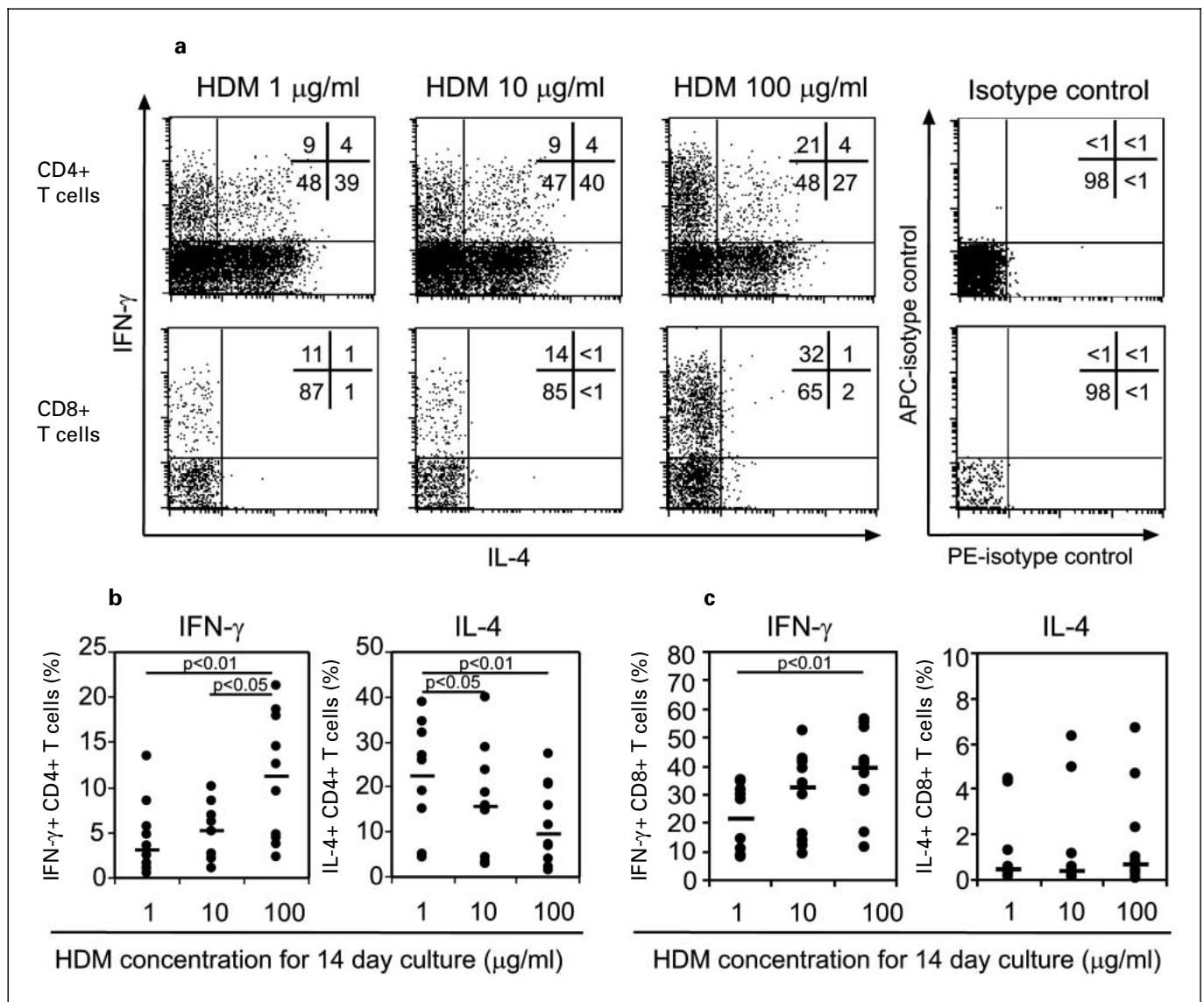


**Fig. 1.** The effect of allergen concentration on CD4+ and CD8+ T cell cytokine profiles. PBMC from 10 HDM-allergic donors were cultured with HDM extract (1, 10 and 100 µg/ml) for 14 days, stimulated with anti-CD3/IL-2 in the presence of Brefeldin A for 6 h and analysed by flow cytometry following labelling with anti-CD4, anti-CD8, anti-IL-4 and anti-IFN-γ. **a** CD4+ and CD8+ T cell cytokine profiles for one representative HDM-allergic donor. Isotype controls were used to set quadrant markers and quadrant percentages are shown. **b** Percentages of IFN-γ positive CD4+ T cells were significantly greater at 100 µg/ml HDM extract in comparison to 1 µg/ml ( $p < 0.01$ ) and 10 µg/ml ( $p < 0.05$ ) HDM extract. The percentages of IL-4+ CD4+ T cells were significantly lower in cultures stimulated with 10 µg/ml ( $p < 0.05$ ) and 100 µg/ml ( $p < 0.01$ ) HDM extract in comparison to 1 µg/ml HDM extract. **c** Percentages of IFN-γ positive CD8+ T cells were significantly greater in cultures stimulated with 100 µg/ml HDM extract in comparison to 1 µg/ml ( $p < 0.01$ ). There was no change in percentages of IL-4+ CD8+ T cells. Each symbol in the graphs in **b**, **c** represents one HDM-allergic donor and the bars represent the medians.

and  $8.4 \times 10^5$  respectively) in comparison to 1  $\mu\text{g/ml}$  ( $p < 0.05$ ) (median values  $2.7 \times 10^5$  and  $3.3 \times 10^5$ , respectively). CD4+IL-4+ and CD8+IL-4+ T cell numbers did not differ significantly between cultures (range of median values for all concentrations was  $11\text{--}16 \times 10^5$  for CD4+IL-4+ and  $0.07\text{--}0.1 \times 10^5$  for CD8+IL-4+). No dose-dependent changes were found for total CD4+ and CD8+ T cell numbers or cytokine negative CD4+ T cell number (data not shown).

To exclude the possibility that HDM extract could non-specifically activate T cells, as a mitogen, latex-specific T cells were cultured in the presence of 1, 10 and 100  $\mu\text{g/ml}$  HDM extract for 4 days. Latex-specific T cells did not proliferate in the presence of 1, 10 or 100  $\mu\text{g/ml}$  HDM extract, demonstrating that the extract did not have mitogenic properties (fig. 2a). Furthermore, to exclude that the HDM extract could non-specifically activate T cells to produce cytokines, IL-4 and IFN- $\gamma$  production by latex-specific T cells was assessed after culture for 4 days in the presence of GE with HDM extract. HDM extract did not enhance the production of IL-4 or IFN- $\gamma$  by GE-stimulated CD4+ T cells (fig. 2b). In addition, the proliferative response of latex-specific T cells to IL-2 was not affected by coculture with HDM extract at 1, 10 or 100  $\mu\text{g/ml}$  indicating that the HDM extract was not toxic (fig. 2c).

#### *High Allergen Concentration Increases CD4+ T Cell Division and Alters the Relationship between IFN- $\gamma$ Production and Cell Division Number*

We next investigated the possible mechanisms involved in skewing cytokine production of CD4+ T cells towards IFN- $\gamma$  predominance after repeated stimulation with high HDM extract concentration over 14 days. Cell division has been shown to play an important role in regulating cytokine production by murine T cells [19]. Hence we analysed T cell division in our 14-day cultures by CFSE cell labelling and flow cytometry. By staining with CFSE at day 7 and analysing cultures 3 and 5 days later in parallel with intracellular cytokine staining we could assess whether the relationship between cell division and cytokine production could be altered by different allergen concentrations. Division intervals were determined from CFSE profiles of anti-CD3/IL-2-stimulated CD4+ T cells because defined peaks were not observed for HDM extract-stimulated cultures. CFSE profiles of HDM extract-stimulated cultures at day 10 and 12 are shown for one HDM-allergic donor (fig. 3a, b). Repeating this experiment on another 5 donors revealed increased numbers of CD4+ T cells undergoing division in higher HDM con-

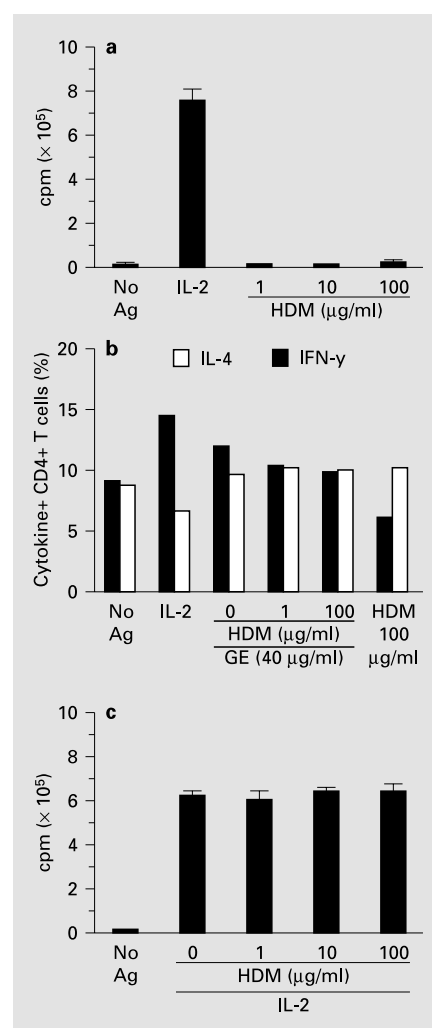


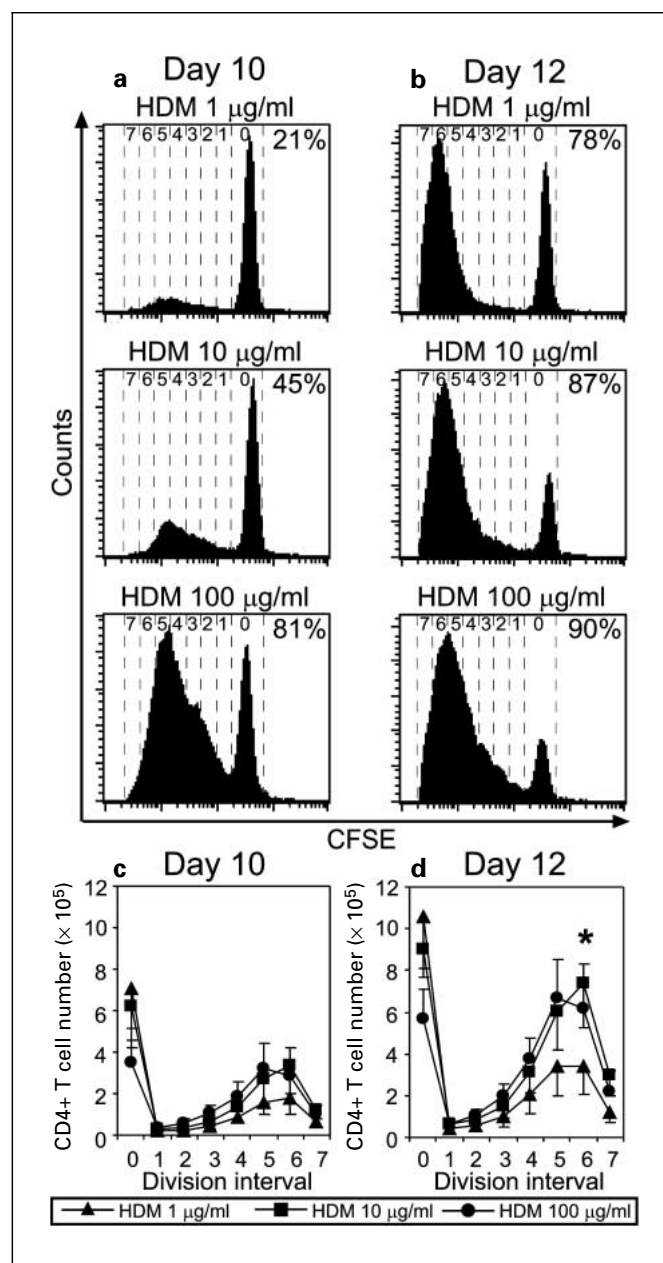
**Fig. 2.** Assessing HDM extract for toxicity and mitogenicity. Latex-specific T cells were cultured for three weeks in the presence of GE and irradiated PBMC as APC. **a** Mitogenicity of HDM extract was determined by culturing latex-specific T cells with APC and HDM extract at 1, 10 and 100  $\mu\text{g/ml}$ . After 72 h, proliferation as correlated with  $^3\text{H}$ -thymidine incorporation (cpm) was determined. **b** The ability of HDM extract to nonspecifically induce IL-4 and IFN- $\gamma$  production was determined by culturing latex-specific T cells with equal numbers of washed irradiated autologous PBMC in the presence of 40  $\mu\text{g/ml}$  GE with either 1 or 100  $\mu\text{g/ml}$  HDM extract (final volume 1 ml/well) for 4 days at 37°C in a 5% CO<sub>2</sub>. Cultures of T cells and APC with GE (40  $\mu\text{g/ml}$ ), or with IL-2 (100 U/ml), or with HDM extract (100  $\mu\text{g/ml}$ ), or without antigen were included as controls. After 4 days, cells were stimulated for 6 h with anti-CD3/IL-2 and analysed by flow cytometry following staining with anti-CD4, anti-IL-4 and anti-IFN- $\gamma$ . **c** Toxicity of the HDM extract was determined by culturing latex-specific T cells with APC, IL-2 and HDM extract at 1, 10 and 100  $\mu\text{g/ml}$ . After 72 h, proliferation as correlated with  $^3\text{H}$ -thymidine incorporation (cpm) was determined.

centration cultures (fig. 3c, d;  $p < 0.05$  for division 6 in 100  $\mu\text{g/ml}$  HDM extract-stimulated cultures in comparison to 1  $\mu\text{g/ml}$  HDM extract-stimulated cultures on day 12).

Intracellular cytokine staining of CFSE labelled cultures was also performed on day 12 to analyse the relationship between cytokine production and cell division. Representative profiles are shown in figure 4a, b and a summary of results for 6 HDM allergic donors is shown in figure 4c, d. Culturing PBMC with 100  $\mu\text{g/ml}$  HDM extract altered the relationship between CD4+IFN- $\gamma$ + T cell proportion and cell division interval in cultures (fig. 4c). CD4+IFN- $\gamma$ + T cell proportions in cultures stimulated with 100  $\mu\text{g/ml}$  HDM extract increased progressively from division interval 4 to 7 whereas in the lower concentration cultures the proportions were similar between division intervals 1 to 6 and then decreased at division interval 7. The overall profile for the proportions of IFN- $\gamma$  positive CD4+ T cells at all division intervals was significantly different ( $p < 0.05$ ) for the 100  $\mu\text{g/ml}$  HDM extract-stimulated cultures in comparison to the lower concentration cultures. In contrast HDM concentration did not alter the relationship between CD4+IL-4+ T cell proportion and cell division number (fig. 4d). CD4+IL-4+ T cell proportions peaked at division intervals 4 or 5 for all concentrations tested.

### CD8+ T Cells Divide and Produce IFN- $\gamma$ in HDM-Stimulated Cultures

To determine whether the relationship between CD8+ T cell division and IFN- $\gamma$  production was also altered at high allergen concentration, 7-day HDM extract-stimulated cultures for 4 HDM-allergic donors were labelled with CFSE and analysed for cell division and intracellular cytokines by flow cytometry 3 and 5 days later. CFSE pro-



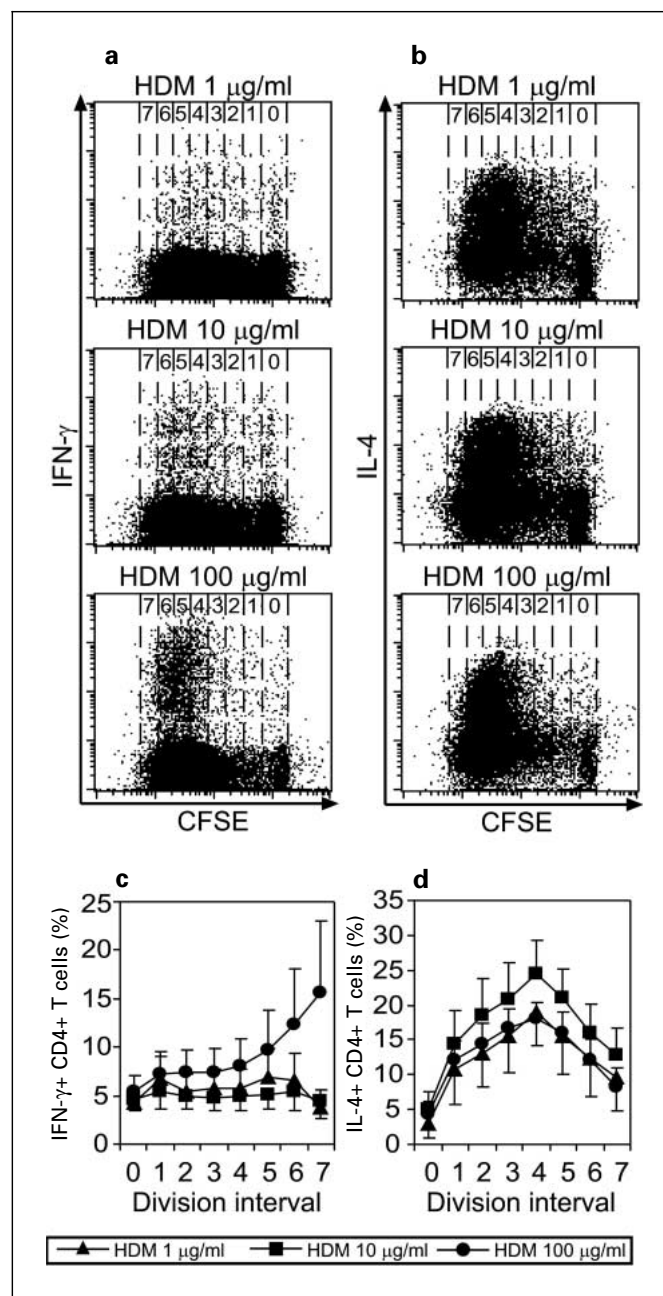
files of CD8+ T cells in HDM-stimulated cultures on day 12 for one HDM-allergic donor are shown in figure 5a. Repeating this experiment on another 3 donors revealed, that as for CD4+ T cells, numbers of CD8+ T cells undergoing division increased from day 10 to day 12 in HDM extract-stimulated cultures (data not shown), and there was a trend towards increased CD8+ T cell division in cultures stimulated with 10 and 100 µg/ml HDM extract (fig. 5c). As CD8+ T cells produced minimal IL-4 at any HDM concentration, shown previously in figure 1, dividing cells were only analysed for IFN-γ production (fig. 5b, d). The relationship between cell division and IFN-γ production was not altered by allergen concentration. In general the proportions of dividing CD8+ T cells producing IFN-γ were greater than those for the CD4+ T cells.

#### Increased Th2 Cell Apoptosis at High Allergen Concentration

To investigate the underlying mechanism for the observed increase in CD4+ T cell division in high allergen concentration cultures (fig. 3) without an overall increase in CD4+ T cell number, apoptosis of a subset of CD4+ T cells was considered. To investigate this, 14-day HDM extract-stimulated cultures from 8 donors were stained for intracellular active caspase-3 and cytokines. The results for one HDM-allergic donor shown in figure 6a, b

**Fig. 4.** High allergen concentration alters the relationship between cell division and IFN-γ production but not IL-4 production. Seven day HDM extract-stimulated (1, 10 and 100 µg/ml) cultures were labelled with CFSE and restimulated with HDM extract and APC. On day 12, CFSE labelled cultures were stimulated with anti-CD3/IL-2 in the presence of Brefeldin A for 6 h, labelled with anti-CD4, anti-IL-4 and anti-IFN-γ and analysed by flow cytometry. Representative profiles of CFSE fluorescence versus IFN-γ (a), and versus IL-4 (b) generated by gating on CD4+ T cells in HDM-stimulated cultures for one HDM-allergic donor are shown. The dotted lines in the plots represent division intervals determined from the anti-CD3 (10 µg/ml) and rIL-2 (100 U/ml) positive control. The mean percentages of CD4+IFN-γ+ T cells (c) and CD4+IL-4+ T cells (d) and standards errors at each division interval for six HDM-allergic donors are shown. The overall profile for the proportions of IFN-γ positive CD4+ T cells at all division intervals was significantly different ( $p < 0.05$ ) for the 100 µg/ml HDM extract-stimulated cultures in comparison to the 1 and 10 µg/ml cultures.

demonstrate increased proportions of active caspase-3 positive CD4+IL-4+ cells but not CD4+IFN-γ+ T cells with increasing HDM extract concentration. There was a significant increase ( $p < 0.05$ ) in the proportion of active caspase-3 positive CD4+IL-4+ T cells in cultures stimulated at 100 µg/ml HDM extract in comparison to those stimulated at 1 µg/ml (fig. 6c). Allergen concentration



did not alter proportions of active caspase-3 positive CD4+IFN- $\gamma$ + T cells which were low at all concentrations (fig. 6d). The percentage of active caspase-3 positive CD8+IFN- $\gamma$ + T cells was increased in cultures stimulated with HDM extract at 10  $\mu$ g/ml (median value 10.4 %;  $p < 0.05$ ) and 100  $\mu$ g/ml (median value 9.9 %; not significant) in comparison to 1  $\mu$ g/ml (median value 6.4%).

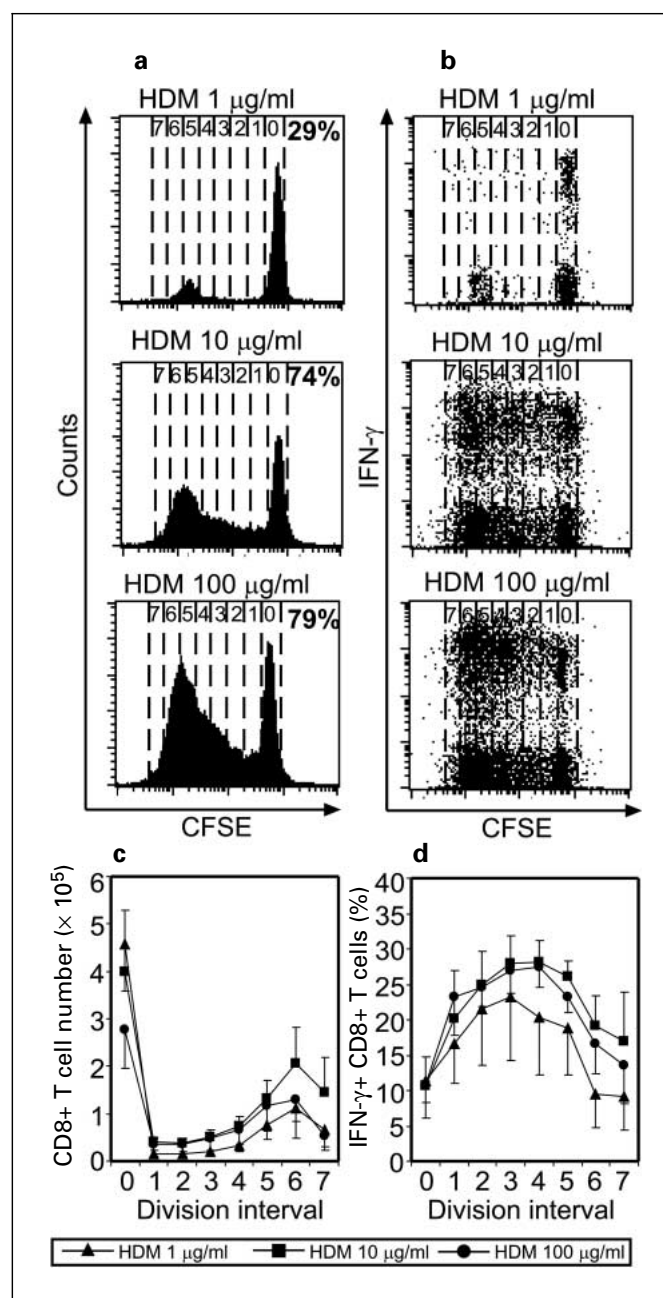
#### *Decreased Proliferative Responses to HDM by T Cells Previously Cultured with High Allergen Concentration*

As altered T cell cytokine production can accompany high dose antigen-induced anergy of allergen-specific cloned T cells [24], we determined whether anergy could be induced by stimulation with high concentrations of HDM extract over the 14-day culture period. After 14 days culture of 10 HDM-allergic donor PBMC with 1, 10 or 100  $\mu$ g/ml HDM extract, cells were restimulated with an immunogenic concentration of HDM extract (10  $\mu$ g/ml) or IL-2 (100 U/ml) (fig. 7). Pretreatment with 100  $\mu$ g/ml HDM extract resulted in significantly lower proliferative response to 10  $\mu$ g/ml HDM extract compared to pretreatment with 1  $\mu$ g/ml ( $p < 0.01$ ) or 10  $\mu$ g/ml ( $p < 0.05$ ) HDM extract. Cells pretreated with 1, 10 and 100  $\mu$ g/ml HDM extract showed similar proliferative responses to IL-2.

**Fig. 5.** CD8+ T cells divide and produce IFN- $\gamma$  in HDM-stimulated cultures. PBMC, cultured with HDM extract (1, 10 and 100  $\mu$ g/ml) for 7 days, were harvested, labelled with CFSE and restimulated with HDM extract (1, 10 and 100  $\mu$ g/ml) and APC. **a** Representative histograms of CFSE fluorescence gated on CD8+ T cells from HDM-stimulated cultures on day 12 are shown with the percentage of cells that have undergone 1 or more divisions in the top right hand corner of each plot. **b** Representative profiles of CFSE fluorescence versus IFN- $\gamma$  were generated by gating on CD8+ T cells from cultured cells labelled with anti-CD8 and anti-IFN- $\gamma$  at day 12 after stimulation with anti-CD3/IL-2 in the presence of Brefeldin A for 6 h. The dotted lines in the plots represent division intervals determined from the anti-CD3 (10  $\mu$ g/ml) and rIL-2 (100 U/ml) positive control. **c** Performing this experiment on 4 HDM-allergic donors revealed a trend towards increased numbers of CD8+ T cells undergoing division in the 10 and 100  $\mu$ g/ml HDM-stimulated cultures in comparison to 1  $\mu$ g/ml at day 12. The mean number and standard error of CD8+ T cells at each division interval are shown. **d** The mean percentage and standard error of IFN- $\gamma$  positive CD8+ T cells at each division interval in HDM-stimulated cultures for the 4 donors are graphed.

## Discussion

Forty percent of the Western population is atopic and therefore at risk for the development of allergic diseases. Allergic responses are a result of the downstream effects of Th2-type cytokines produced by Th cells in response to allergen. SIT, a current treatment for allergic disease, modifies the natural course of allergy and can potentially



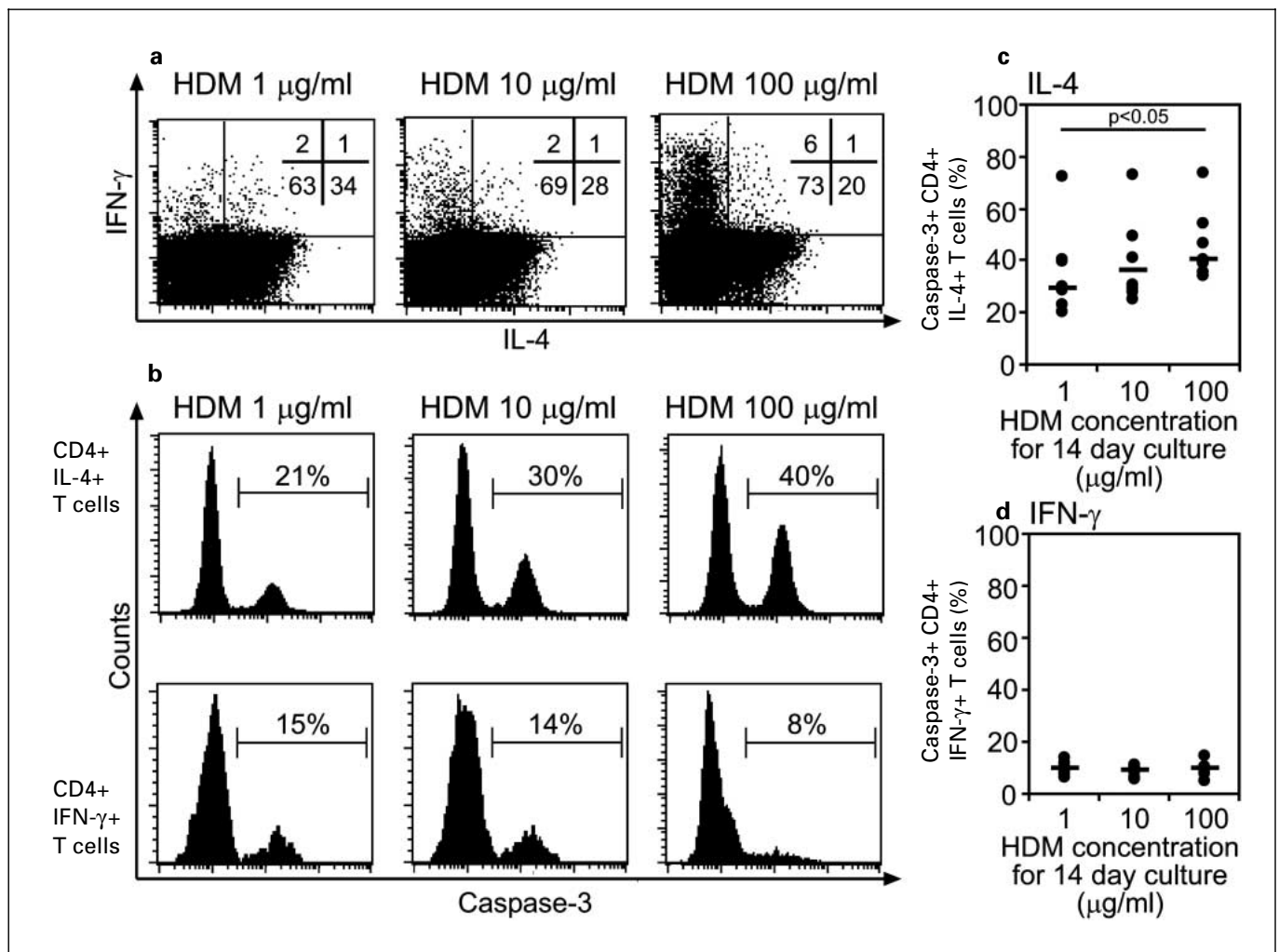


**Fig. 6.** The effect of allergen concentration on apoptosis of cytokine-positive CD4+ T cells. PBMC from 8 HDM-allergic donors were cultured with HDM extract (1, 10 and 100  $\mu\text{g/ml}$ ) for 14 days. Cultured cells were stimulated with anti-CD3/IL-2 in the presence of Brefeldin A for 6 h and analysed by flow cytometry following labelling with anti-CD4, anti-active caspase-3, anti-IL-4 and anti-IFN- $\gamma$ . **a** Cytokine profiles of CD4+ T cells for one representative HDM-allergic donor. Percentages in each quadrant are shown. **b** Histograms showing the percentages of CD4+IL-4+ T cells and CD4+IFN- $\gamma$ + T cells positive for active caspase-3 staining for one HDM-allergic donor. **c** The percentages of active caspase-3-positive CD4+IL-4+ T cells increased significantly at 100  $\mu\text{g/ml}$  HDM extract in comparison to 1  $\mu\text{g/ml}$  HDM extract ( $p < 0.05$ ). **d** The percentages of active caspase-3 positive CD4+IFN- $\gamma$ + T cells were not altered by allergen concentration. **c, d** each symbol represents one HDM-allergic donor and the bars represent the median values for the 8 donors.

effect a cure. However efficacy of SIT varies depending on the allergen and there are associated side effects. By determining factors that can down-regulate allergen-specific Th2-type responses in vitro, more rational strategies for SIT can be devised. As high antigen concentration promotes such changes for T cell responses to other antigens and SIT is associated with the administration of consider-

ably higher doses of allergen than are encountered naturally [25, 26], we investigated the effect of allergen concentration on cytokine production, division and apoptosis of allergen-specific T cells in vitro. Using intracellular cytokine staining and flow cytometry we analysed changes at the single cell level. Culturing cells in the presence of high allergen concentration in comparison to lower

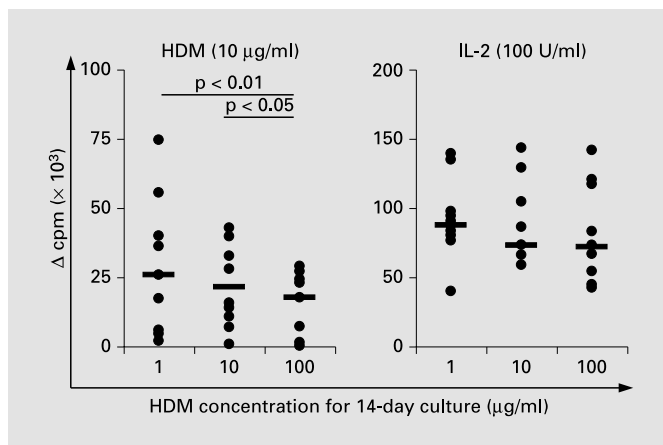


**Fig. 7.** Decreased proliferation of T cells pretreated with high allergen concentration upon restimulation with an immunogenic allergen concentration. PBMC from 10 HDM-allergic donors were cultured with 1, 10 and 100  $\mu\text{g/ml}$  HDM extract for 14 days and restimulated in the presence of either 10  $\mu\text{g/ml}$  HDM extract or IL-2 (100 U/ml). After 72 h, proliferation as correlated with  $^3\text{H}$ -thymidine incorporation ( $\Delta$  cpm) was determined. Cells pretreated with 100  $\mu\text{g/ml}$  HDM extract had significantly decreased proliferation upon restimulation with an immunogenic concentration of allergen in comparison to cells pretreated with 1  $\mu\text{g/ml}$  ( $p < 0.01$ ) and 10  $\mu\text{g/ml}$  ( $p < 0.05$ ) HDM extract. There was no difference in proliferative response to IL-2 for cells pretreated with different allergen concentrations. Each symbol in the graphs represents one HDM-allergic donor and the bars represent the medians.

concentrations resulted in increased CD4+IFN- $\gamma$ + and CD8+IFN- $\gamma$ + T cell numbers with no difference in CD4+IL-4+ and CD8+IL-4+ T cell numbers although CD4+IL-4+ T cell proportions were decreased. CFSE cell labelling demonstrated an association between increased IFN- $\gamma$  production and highly dividing CD4+ T cells at high allergen concentration. High allergen concentration also induced increased apoptosis of IL-4-producing Th cells.

Increased production of IFN- $\gamma$  by allergen-specific T cells could lead to a reduction in allergen-induced pathology. In human B cells, IFN- $\gamma$  inhibits IL-4-induced expression of the germline epsilon transcript and consequently prevents IgE production [27]. Via this mechanism IFN- $\gamma$ , induced by high dose allergen administration for SIT, could reduce inflammatory cell mediator release and thus decrease clinical symptoms upon allergen exposure. In a murine model of allergic asthma, Huang and colleagues showed that IFN- $\gamma$  reversed bronchial hyper-responsiveness and bronchoalveolar lavage eosinophilia [28]. Increased IFN- $\gamma$  positive T cell numbers at sites of

allergen challenge have been demonstrated following clinically successful immunotherapy [29]. Thus high allergen administration during SIT may enhance this outcome thereby increasing the effectiveness of SIT at down-regulating allergen-specific Th2-type responses in the tissues.

Mechanisms for immune deviation elicited by changes in antigen concentration are not fully understood. However, ligand density may play a role in determining the activation threshold of T cells and subsequent signal transduction for cytokine gene transcription. It has been suggested that with high antigen dose, the number of T cell receptors engaged is increased, resulting in strong interactions and sustained patterns of signal transduction leading to IFN- $\gamma$  production [30]. In contrast IL-4 production results from low antigen doses inducing weak interactions and transient signalling patterns. Antigen concentration has also been shown to influence the relationship between cytokine profile and Th cell division number [20]. In agreement, we observed IFN- $\gamma$  production by more highly dividing CD4+ T cells at high allergen concentration than for lower allergen concentrations. In addition, with increased antigen concentration, there may be increased amounts of polyclonal activators present in the HDM extract, which could alter T cell cytokine production. These polyclonal activators, such as bacterial LPS, peptidoglycan and other specific pathogen-associated microbial products, can bind Toll-like receptors (TLR) expressed by human monocytes, dendritic cells, B cells and T cells [31, 32]. Binding of these ligands to TLRs on APC results in the production of inflammatory cytokines such as IL-12, TNF- $\alpha$  and IL-6 [31, 32], thus altering the cytokine milieu of antigen-stimulated T cells and influencing Th differentiation. We believe polyclonal activators may only be playing a minor role, if any, in our cell culture system as LPS levels in the HDM extract were shown to be undetectable, and IL-4 and IFN- $\gamma$  production by latex-specific T cells was not enhanced by culture with glove extract in combination with HDM extract. Furthermore we have observed similar skewing of T cell cytokine production at high concentrations of other allergens including grass pollens and latex glove extract.

In addition to changes in T cell cytokine production, many studies demonstrate decreased allergen-stimulated lymphoproliferative responses following SIT [13]. The induction of T cell anergy has been suggested as a possible mechanism. [13, 33]. Anergy, defined by diminution of antigen-specific proliferative response with reversal by IL-2 [34], has been induced in allergen-specific cloned T cells by high dose peptide treatment and can result in altered T cell cytokine production [24]. In our study, 14-

day pretreatment of HDM-allergic donor PBMC with high allergen concentration resulted in reduced proliferative responses to stimulation with an immunogenic dose of allergen with retained responsiveness to IL-2 consistent with allergen-specific Th cell anergy. The precise mechanism for the induction of anergy is not clear but induction by IL-10 is one such mechanism [35]. IL-10 positive cells in skin at sites of allergen challenge and in blood are increased after venom SIT [36]. Whether these cells represent the CD4+ IL-10-producing regulatory T cell population described in other models is yet undefined.

As apoptosis can follow anergy induction after high dose antigen treatment [37], deletion of allergen-specific Th cells could also lead to in part the reduced proliferative responses seen in our cultures stimulated with high HDM extract concentration. Furthermore, preferential deletion of IL-4- and IL-5-producing T cells could contribute to the shift towards dominant T cell IFN- $\gamma$  cytokine production. In support of this proposed mechanism, we observed increased apoptosis of IL-4-producing CD4+ T cells at high allergen concentration with no change in the number of apoptotic IFN- $\gamma$ -producing CD4+ T cells. Our results differ from a previous study which suggested that Th1 cells had increased susceptibility to apoptosis [38]. In Janssen and colleagues' study, *in vitro* polarised Th1 and Th2 cell lines were stimulated for several hours with anti-CD3, and activation-induced cell death sensitivity was shown to be correlated with up-regulation of Fas ligand (FasL) resulting in Fas-dependent apoptosis [38]. They suggested mechanisms for activation-induced cell death may differ depending on the stimulus and time. Analysis of FasL expression by allergen-specific T cells in our culture system could address this issue. Nevertheless, the results that we observed are consistent with a recent report showing that Th2-type cells from SIT-treated atopic patients cultured with specific allergen *ex vivo* undergo increased apoptosis [39]. Thus designing SIT preparations that induce apoptosis of allergen-specific effector Th2-type cells could lead to increased efficacy of this treatment.

CD8+ T cell IFN- $\gamma$  production was also enhanced at high allergen concentration. Recently the first human MHC class I-restricted T cell epitopes of the allergen Der p 1 were identified using MHC class I tetramers [40], providing evidence for involvement of MHC class I peptide presentation to CD8+ T cells in immune responses to allergens. In addition, CD8+ T cells have been implicated in the modulation of allergic disease. In animal models of allergy CD8+ T cells transferred from allergen sensitised mice could reduce IgE production and prevent airway

hyperresponsiveness in sensitised recipients [41] and IFN- $\gamma$  produced by CD8+ T cells transferred into sensitised rats inhibited allergen-induced eosinophilic inflammation [42]. Of note, we observed IFN- $\gamma$  production by CD8+ T cells after fewer cell divisions in comparison to CD4+ T cells suggesting that the CD8+ T cell IFN- $\gamma$  production induced by high allergen concentration may have preceded and promoted the cytokine shift observed for the CD4+ T cell population. In murine studies IFN- $\gamma$  production by CD8+ T cells was shown to occur after TCR stimulation independently of cell division indicating that there are different thresholds for IFN- $\gamma$  production and cell division in CD8+ T cells [43]. This could explain our failure to observe a change in the relationship between CD8+ T cell division and IFN- $\gamma$  production at high allergen concentration as seen for the CD4+ T cells. Although CD8+ T cell IFN- $\gamma$  production has been shown in some studies to be decreased or unchanged in individuals receiving SIT [44, 45], the results from this current study suggest that this may be antigen dose related. The induction of regulatory CD8+ T cells producing IFN- $\gamma$  may be possible through the use of higher allergen concentrations in SIT preparations. Concomitant IL-10 production by T cells during SIT as mentioned earlier would down-regulate deleterious effects of IFN- $\gamma$  in addition to Th2 mediated inflammation [46].

In conclusion our studies indicate that the administration of higher doses of allergen in SIT would be more effective at down-regulating allergen-specific Th2-type responses. The use of hypoallergenic preparations such as peptides based on dominant T cell epitopes [33, 47] or mutant allergens [48, 49] should permit further refinement of efficacy and safety of this potentially curative treatment. Laboratory analysis of allergen-specific T cell responses will also provide valuable assays for monitoring SIT efficacy.

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