

Original article

Increased allergen concentration enhances IFN- γ production by allergic donor T cells expressing a peripheral tissue trafficking phenotype

Background: Clinically effective allergen-specific immunotherapy correlates with decreased circulating allergen-specific IL-4⁺ T cells but increased IFN- γ ⁺ cells at sites of allergen challenge. Whether immunotherapy promotes trafficking of IFN- γ ⁺ T cells to peripheral tissues is unknown. As aeroallergen is administered at higher concentrations during immunotherapy than those encountered naturally, the effect of allergen concentration on adhesion molecule (CD62L and CD49d) and chemokine receptor (CCR3 and CCR5) expression by peripheral-blood T cells was analysed in parallel with cytokine production.

Methods: House dust mite-allergic donor peripheral blood mononuclear cells were cultured for 14 days with different allergen concentrations. Cytokine profiles of were analysed by flow cytometry.

Results: Cultures stimulated with 100 μ g/ml house dust mite extract compared with 1 μ g/ml had increased proportions and numbers of CD62L^{lo}, CD49d^{hi} or CCR5⁺ T cells expressing IFN- γ . CCR3-positive CD4⁺ and CD8⁺ T cell numbers were very low and did not differ between cultures. In contrast the proportions of 'peripheral tissue trafficking' CD4⁺ T cells expressing IL-4 were decreased in cultures stimulated with high in comparison with low allergen concentration.

Conclusion: These results indicate the importance of achieving high allergen doses during immunotherapy to promote IFN- γ production and expression of a 'peripheral tissue trafficking' phenotype by allergen-specific CD4⁺ and CD8⁺ T cells. The net change in cytokine milieu at sites of allergen encounter would then down-regulate clinical manifestations of allergic disease.

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Key words: adhesion molecule; allergy; chemokine receptor; immune regulation; T cell.

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Accepted for publication 2 May 2004

Conventional aeroallergen specific immunotherapy (SIT) involves incremental administration of allergen extract to induce clinical tolerance and modify the natural course of the disease (1). During clinically successful SIT, T cell responses are modified, with deviation from IL-4, IL-5 cytokine production to IFN- γ predominant (2–4). The mechanisms for these cytokine changes include immune deviation (2–4), T cell anergy (2–4), induction of T regulatory cells (5, 6), and enhanced allergen-induced apoptosis (7). There are also increased IFN- γ ⁺ cell numbers at sites of allergen challenge (8–10), but it is not clear whether SIT promotes trafficking of circulating IFN- γ ⁺ T cells to peripheral tissues.

Trafficking of T cells from the circulation into tissues is dependent on interactions between T cell-expressed adhesion molecules and ligands on endothelial cells. Naïve T cells gain access to lymph nodes by L-selectin (CD62L) binding to sulphated carbohydrates on CD34, a vascular addressin expressed by high endothelial

venules. Upon entry to the lymph node and after antigen activation, T cells shed CD62L and upregulate expression of lymphocyte function-associated antigen-1 (LFA-1) and very late activation antigen-4 (CD49d) (11). CD49d and LFA-1 bind to vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1), respectively, on activated peripheral vascular endothelium. This change in adhesion molecule expression is associated with reduced recirculation through lymphoid tissue and increased movement into peripheral tissues (11). Differences in chemokine receptor expression by Th1- and Th2-type cells may result in differential recruitment of these subsets to sites of inflammation (12). A subset of Th2-type cells expresses CCR3 which binds eotaxin, MCP-3,4 and RANTES (13, 14), whereas expression of CCR5, which binds MIP-1 α , MIP-1 β and RANTES, is largely on Th1-type cells (15), although expression by Th2-type cells has been reported (13).

Aeroallergen SIT is associated with administration of considerably higher allergen concentrations than encountered naturally (16, 17). To further elucidate mechanisms of SIT, we previously analysed the effect of allergen concentration on T cell responses by flow cytometry and found preferential expansion of IFN- γ -producing T cells at high allergen concentration in comparison with low (18). This observation is consistent with the well-recognized influence of antigen concentration on Th cell differentiation (19, 20). The current study determined whether repeated stimulation of peripheral blood mononuclear cells (PBMC) with high allergen concentration could differentially alter expression of surface molecules that influence trafficking to peripheral tissues by allergen-specific Th1- and Th2-type cells. As T cells expressing low levels of CD62L or high levels of CD49d would represent peripheral tissue trafficking T cells, the cytokine profile of allergen-activated T cells expressing these markers was analysed. Additionally expression of CCR3 and CCR5 was examined, as ligands for these receptors (eotaxin, RANTES and MIP-1 α) are reportedly increased in bronchoalveolar lavage fluid from atopic asthmatics after allergen challenge (21).

Methods

Antigen

Freeze-dried house dust mite (HDM), *Dermatophagoides pteronyssinus*, kindly provided by Allergy Therapeutics Limited (Worthing, West Sussex, UK), was prepared as a sterilized aqueous extract in PBS (1 mg HDM/1 ml PBS). Endotoxin levels in the extract were undetectable (<3 U/ml; QCL-1000TM endotoxin detection kit, BioWhittaker, Walkerville, MD). Mitogenicity of the extract was excluded by culturing with oligoclonal latex-reactive T cells, and toxicity was excluded by culture with latex-reactive T cells and IL-2 (data not shown). The HDM extract did not induce increased IFN- γ production by latex-reactive T cells (data not shown).

Study population

Peripheral blood was taken from 10 HDM-allergic donors (six females, four males; mean age 36 years) attending the Alfred Hospital Allergy Clinic, Melbourne, Australia with Alfred Hospital Ethics Committee approval and written informed consent obtained from each patient. Donors were selected on the basis of a history of clinical symptoms of HDM allergy and positive HDM-specific IgE as determined by skin prick test (wheal >6 mm) and/or Kallestad Allercoast EAST (Sanofi-Pasteur Diagnostics, Chaska, MN; score >2).

Allergen-stimulated PBMC cultures

PBMC separated by density gradient centrifugation on Ficoll Paque (Amersham Pharmacia, Uppsala, Sweden) were cultured in 24-well plates (2.5×10^6 /well; Greiner, Frickenhausen, Germany) in complete medium (RPMI 1640 medium with 2 mmol/l L-glutamine, 100 IU/ml penicillin/streptomycin, Gibco Invitrogen, Auckland, NZ, and 5% screened, heat-inactivated human AB+ serum, Sigma, St Louis, MO) with HDM extract (1, 10 or 100 μ g/ml) in a final volume of 2 ml/well for 7 days at 37°C, 5% CO₂. A 'no allergen' control was not used due to cell death after several days of

culture without antigen stimulation. On day 7 of culture, cells (1×10^6 cells/ml) were restimulated with HDM extract (1, 10 or 100 μ g/ml) and irradiated (3000 rads) autologous PBMC (1×10^6 /ml) as a source of antigen presenting cells in a final volume of 2 ml/well. Recombinant human IL-2 (rIL-2; 25 U/ml; Cetus Corporation, Emeryville, CA, 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). The viability of cultures at 14 days was >90%. For cultures from two donors, insufficient cells were generated to perform all analyses.

Monoclonal antibodies and cell staining

All antibodies for flow cytometry were purchased from Pharmingen (San Diego, CA). T cell subset staining was performed using anti-human CD4-Allophycocyanin (APC) or anti-human CD4-Cy-Chrome and anti-human CD8-Fluorescein (FITC) or anti-human CD8-Cy-Chrome. IgG_{1, κ} -FITC, IgG_{1, κ} -Cy-Chrome and IgG_{1, κ} -APC served as isotype-specific controls. The expression of surface 'trafficking' markers by T cells was analysed using anti-human CD62L-FITC, anti-human CD49d-Cy-Chrome, anti-human CCR3-FITC and anti-human CCR5-FITC. IgG_{1, κ} -FITC, IgG_{1, κ} -Cy-Chrome, IgG_{2a, κ} -FITC and IgG_{2a, κ} -Cy-Chrome served as isotype-specific controls. Anti-human IL-4-Phycoerythrin (PE) and anti-human IFN- γ -APC were used to analyse intracellular cytokines with IgG_{1, κ} -PE and IgG_{1, κ} -APC as isotype-specific controls.

Cell surface marker analysis

On day 14, cultured cells were labelled with anti-CD4 and anti-CD8 antibodies or isotype-specific controls, washed and then labelled with anti-CD62L, anti-CD49d, anti-CCR3 and anti-CCR5 antibodies or isotype-specific controls. Percentages of CD4⁺ and CD8⁺ T cells expressing low levels of CD62L, high levels of CD49d, CCR3 or CCR5 were determined using a FACScaliber flow cytometer (BD Biosciences, San Jose, CA) and Cellquest software. The threshold for positive staining was taken as the point at which the test and isotype histograms crossed. Background values from the isotype control histograms were then subtracted. Absolute numbers of cell subsets in 14-day cultures were also calculated.

Intracellular cytokine staining

On day 14, cultured cells were stimulated (2.5×10^5 cells/well) in 96-well U bottom plates with immobilized anti-CD3 (OKT3; 10 μ g/ml) and rIL-2 (100 U/ml) in the presence of 10 μ g/ml Brefeldin A (Sigma) for 6 h at 37°C. Cells were surface stained as before, fixed in 4% paraformaldehyde/PBS and permeabilized using FACS permeabilizing solution (BD Biosciences). After washing, cells were double stained with anti-IL-4 and anti-IFN- γ antibodies or isotype-specific controls. Percentages of CD62L^{lo}, CD49d^{hi}, CCR3⁺ or CCR5⁺ T cells expressing IL-4 or IFN- γ were determined by flow cytometry. Absolute numbers of cell subsets in 14-day cultures were also calculated.

Statistics

Data were well approximated by a log-normal distribution and normalized via log-transformation prior to analysis. Statistical analysis was performed using Repeated Measures ANOVA with a Bonferroni adjustment for multiple comparisons. A two-sided *P*-value of 0.05 was considered statistically significant. Analysis was performed using InStat 2.0 software.

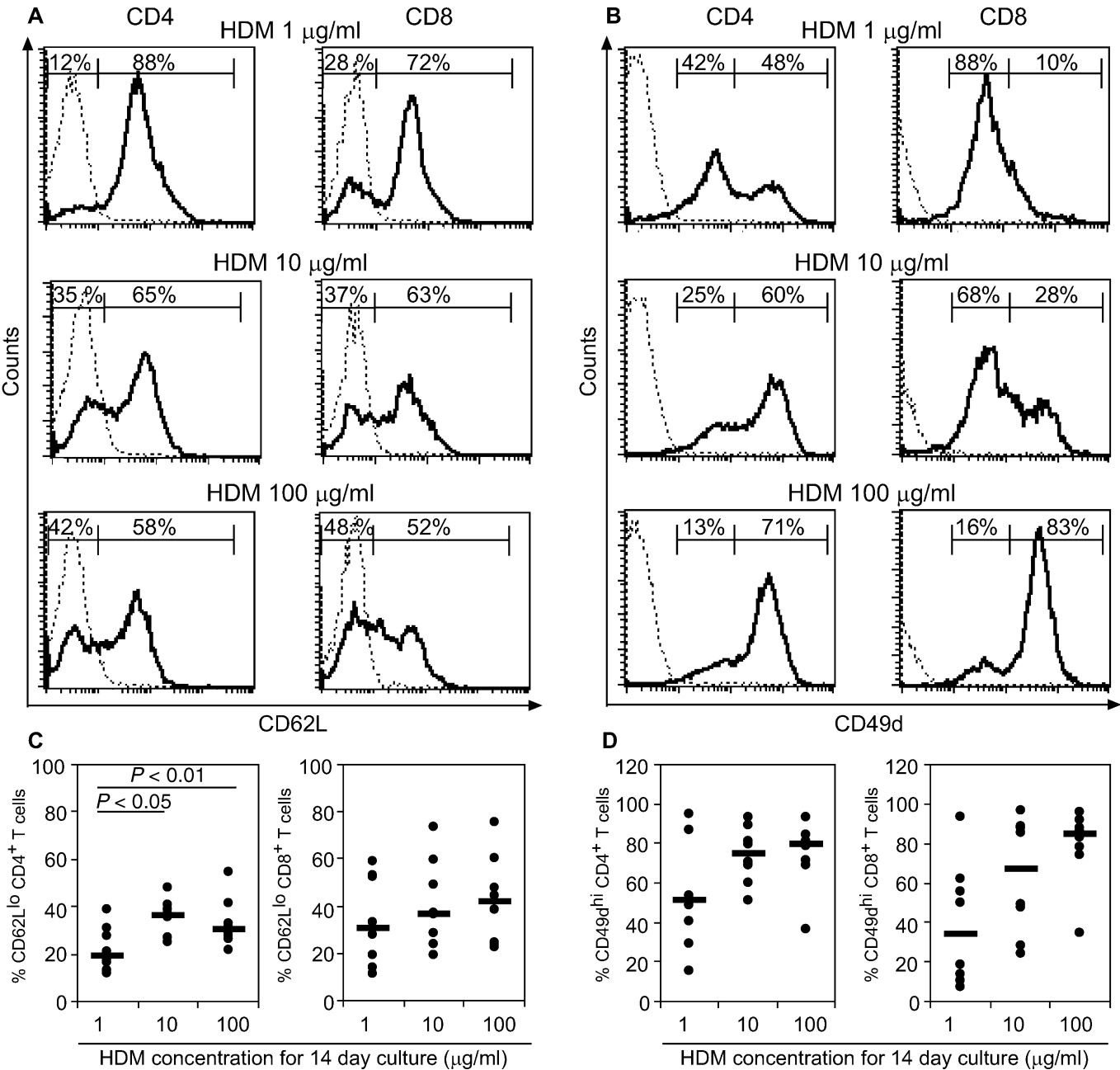


Figure 1. The effect of allergen concentration on CD4⁺ and CD8⁺ T cell expression of CD62L and CD49d. PBMC from eight HDM-allergic donors cultured with HDM extract (1, 10 and 100 $\mu\text{g/ml}$) for 14 days were harvested and stained with either anti-CD4 or anti-CD8 antibodies and then anti-CD62L or anti-CD49d antibodies (solid lines) or isotype controls (dotted lines) and analysed by flow cytometry. Representative profiles of CD62L (A) and CD49d (B) expression by CD4⁺ and CD8⁺ T cells from HDM-stimulated cultures of one HDM-allergic donor are shown. (C) The proportions of CD4⁺ T cells that expressed low levels of CD62L were greater in the 10 $\mu\text{g/ml}$ ($P < 0.05$) and 100 $\mu\text{g/ml}$ ($P < 0.01$) HDM extract-stimulated cultures in comparison to 1 $\mu\text{g/ml}$. There were greater proportions of CD8⁺ T cells that expressed low levels of CD62L in the 100 $\mu\text{g/ml}$ HDM-extract stimulated cultures in comparison to the 1 and 10 $\mu\text{g/ml}$ HDM-extract stimulated cultures ($P < 0.05$). (D) Greater proportions of CD8⁺ T cells expressing high levels of CD49d were observed in the 100 $\mu\text{g/ml}$ HDM extract-stimulated cultures in comparison with 1 $\mu\text{g/ml}$ ($P < 0.01$) and 10 $\mu\text{g/ml}$ ($P < 0.05$). In the graphs in (C) and (D) each symbol represents one HDM-allergic donor and the bars represent the median values.

Results

T cell expression of CD62L and CD49d

Pilot experiments revealed that approximately 10% of PBMC T cells expressed low levels of CD62L and 20% expressed high levels of CD49d (data not shown). To determine dose-dependent effects of repeated stimulation with allergen on expression of these markers, HDM-allergic donor PBMC were cultured for 14 days with different HDM extract concentrations. Representative profiles of CD62L and CD49d expression for one HDM-allergic donor are shown in Fig. 1A, B. Analysis of eight donors revealed greater proportions of CD62L^{lo} CD4⁺ ($P < 0.01$) and CD8⁺ ($P < 0.05$) T cells in the 100 μ g/ml HDM extract-stimulated cultures in comparison with 1 μ g/ml (Fig. 1C). However CD4⁺ CD62L^{lo} and CD8⁺ CD62L^{lo} T cell numbers did not differ significantly between cultures (Table 1). There were no significant differences in the proportions and numbers of CD4⁺ T cells expressing high levels of CD49d between cultures stimulated with different allergen concentrations. In contrast, greater proportions and numbers of CD8⁺ T cells expressing high levels of CD49d in cultures stimulated with 100 μ g/ml HDM extract in comparison with 1 μ g/ml (% $P < 0.01$; number $P < 0.05$) and 10 μ g/ml ($P < 0.05$) were detected.

T cell expression of CCR3 and CCR5

Expression of CCR3 and CCR5 by CD4⁺ and CD8⁺ T cells in 14-day cultures stimulated with either 1, 10 or 100 μ g/ml HDM extract was also examined to determine dose-dependent changes in chemokine receptor expression by T cells. Pilot experiments revealed that approximately 1% of PBMC T cells were CCR3⁺ and 10% were CCR5⁺ (data not shown). Representative profiles of CCR3 and CCR5 expression by CD4⁺ and CD8⁺ T cells in allergen-stimulated cultures of PBMC from one HDM-allergic donor are shown in Fig. 2A, B. Analysis of CCR3 revealed very low expression by a minor subset of T cells from most donors. No significant differences in the proportions or numbers of CD4⁺ and CD8⁺ T cells expressing CCR3 or CCR5 between cultures stimulated with different

concentrations of HDM extract were observed (Fig. 2C, D; Table 1).

Increased numbers of IFN- γ -producing CD4⁺ CD62L^{lo} and CD4⁺ CD49d^{hi} T cells at high allergen concentration

To determine if repeated stimulation with different allergen concentrations could lead to alterations in the cytokine phenotype of peripheral tissue trafficking T cells, T cells expressing low levels of CD62L or high levels of CD49d in HDM-stimulated cultures were analysed for intracellular cytokines at day 14. Representative profiles of IL-4 vs IFN- γ for CD4⁺ and CD8⁺ T cells expressing either low levels of CD62L or high levels of CD49d in HDM-stimulated cultures for one HDM-allergic donor are shown in Fig. 3A, B. Analysis of 10 donors revealed greater proportions and numbers of CD4⁺ CD62L^{lo} T cells expressing IFN- γ in cultures stimulated with 100 μ g/ml HDM extract in comparison with 1 ($P < 0.01$; Fig. 3C, Table 2). In contrast, lower proportions of CD4⁺ CD62L^{lo} T cells expressing IL-4 were observed in cultures stimulated with 100 μ g/ml HDM extract in comparison with 1 μ g/ml ($P < 0.01$; Fig. 3C). No changes in CD4⁺ CD62L^{lo} IL-4⁺ T cell numbers were observed between cultures (Table 2). Although the proportions of IFN- γ or IL-4 positive CD8⁺ CD62L^{lo} T cells did not differ between cultures stimulated with different allergen concentrations (Fig. 3D), there were significantly greater numbers of CD8⁺ CD62L^{lo} IFN- γ ⁺ T cells in cultures stimulated with 100 μ g/ml HDM extract in comparison with 1 μ g/ml ($P < 0.05$), with no change in CD8⁺ CD62L^{lo} IL-4⁺ T cell number (Table 2).

Significantly greater proportions and numbers of CD4⁺ CD49d^{hi} T cells expressing IFN- γ were observed in cultures stimulated with 100 μ g/ml HDM extract in comparison with 1 μ g/ml ($P < 0.01$; Fig. 3E, Table 2). In contrast, significantly lower proportions of CD4⁺ CD49d^{hi} T cells expressing IL-4 were observed in cultures stimulated with 100 μ g/ml HDM extract in comparison with 1 μ g/ml ($P < 0.05$), although the CD4⁺ CD49d^{hi} IL-4⁺ T cell numbers did not differ between cultures (Table 2). While proportions of CD8⁺ CD49d^{hi} T cells expressing IFN- γ or IL-4 were unaffected by culture with different allergen concentrations (Fig. 3F), there were

Table 1. Median number and ranges in parentheses of T cells expressing low levels of CD62L, high levels of CD49d, CCR3 or CCR5 in HDM-allergic donor PBMC cultures ($n = 8$) stimulated for 14 days with 1, 10 or 100 μ g/ml HDM extract

HDM extract (μ g/ml)	CD4 ⁺ T cells ($\times 10^{-5}$)			CD8 ⁺ T cells ($\times 10^{-5}$)		
	1	10	100	1	10	100
CD62L ^{lo}	18.4 (6.7–47.2)	60.3 (16.5–83.9)	50.1 (7.0–142.4)	4.2 (0.8–39.6)	9.5 (2.7–59.0)	7.7 (1.7–76.6)
CD49d ^{hi}	60.3 (8.7–151.8)	111.3 (39.8–171.1)	103.5 (19.4–213.6)	3.6 (0.7–62.5)	14.6 (2.8–95.8)	26.7 (3.9–88.5)*, **
CCR3 ⁺	3.3 (0.1–28.2)	4.5 (0.1–39.4)	2.6 (0.1–37.7)	0.1 (0.1–3.7)	0.3 (0.1–1.2)	0.8 (0.1–5.2)
CCR5 ⁺	25 (0.1–40.6)	16.1 (0.1–85.0)	6.9 (0.1–81.3)	3.3 (0.2–32.2)	9.6 (2.2–49.9)	14.4 (1.2–82.1)

* $P < 0.05$ between 1 and 100 μ g/ml HDM extract; ** $P < 0.05$ between 10 and 100 μ g/ml HDM extract.

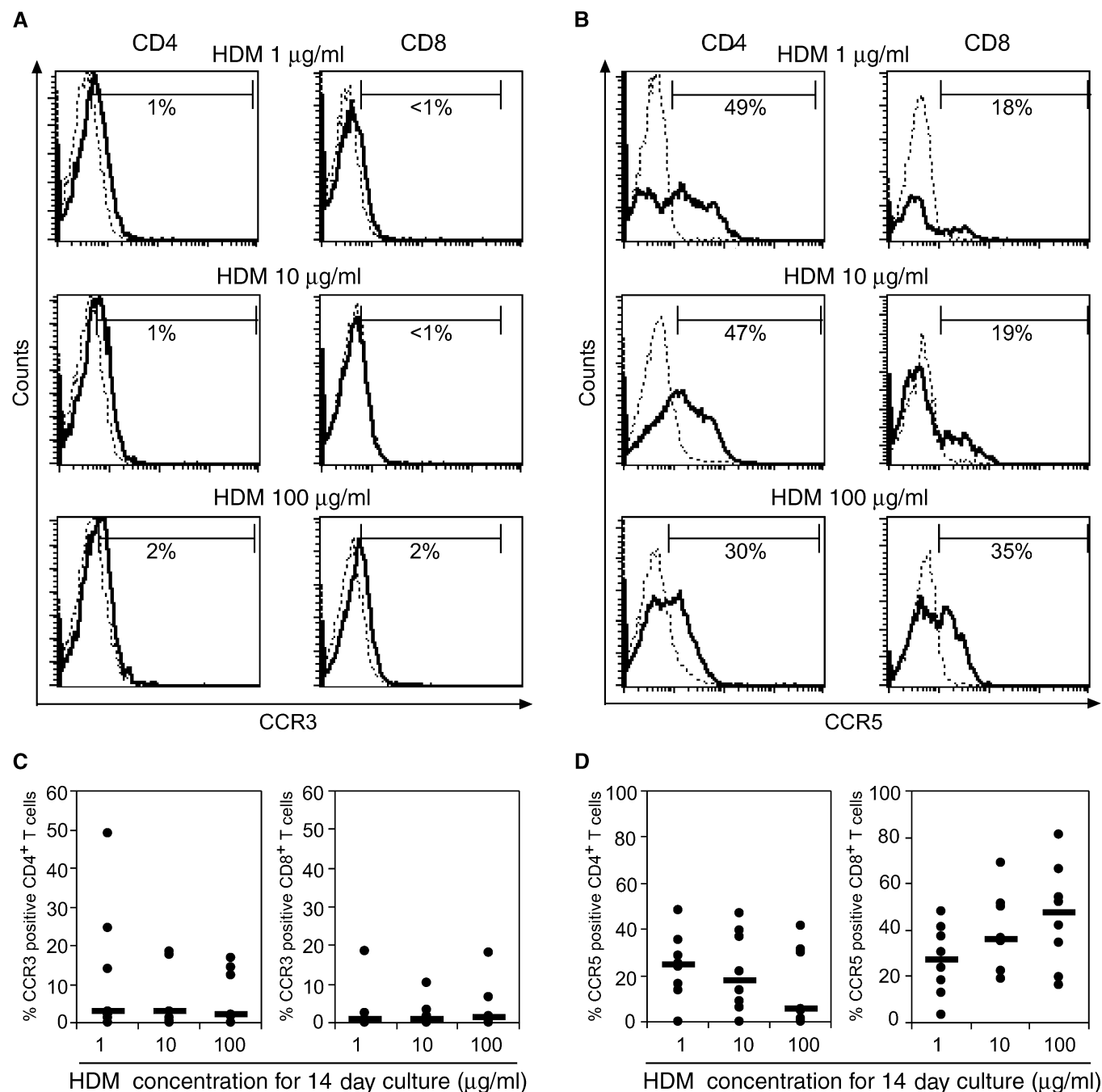
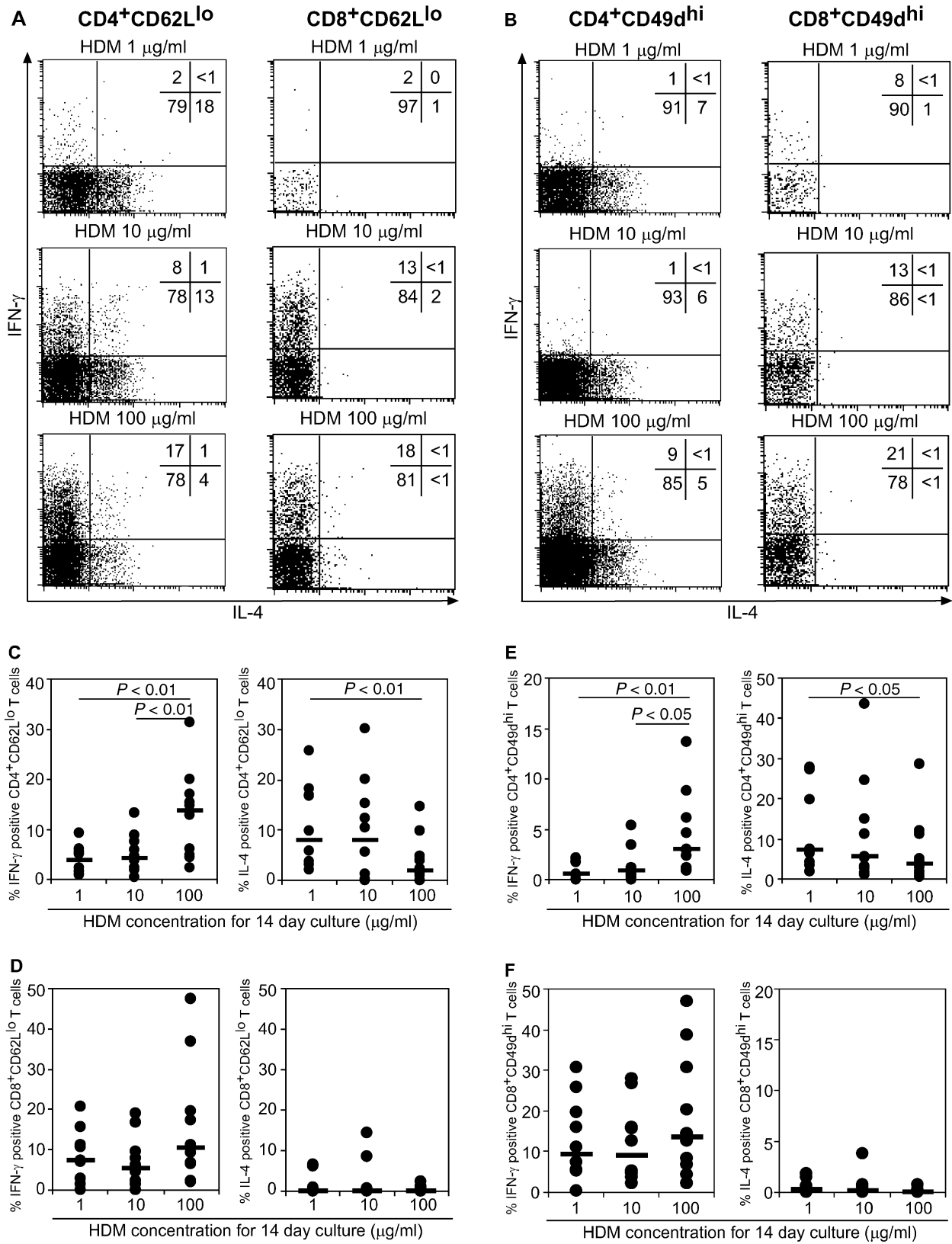


Figure 2. The effect of allergen concentration on CD4⁺ and CD8⁺ T cell expression of CCR3 and CCR5. PBMC from eight HDM-allergic donors cultured with HDM extract (1, 10 and 100 µg/ml) for 14 days were harvested and stained with either anti-CD4 or anti-CD8 antibodies and then anti-CCR3 or anti-CCR5 antibodies (solid lines) or isotype controls (dotted lines) and analysed by flow cytometry. Representative profiles of CCR3 (A) and CCR5 (B) expression by CD4⁺ and CD8⁺ T cells from HDM-stimulated cultures of one HDM-allergic donor are shown. (C) The proportions of CD4⁺ and CD8⁺ T cells that were CCR3⁺ did not differ between cultures stimulated with different concentrations of allergen. (D) There was a trend for decreased proportions of CD4⁺ T cells expressing CCR5 ($P = 0.09$) and increased proportions of CD8⁺ T cells expressing CCR5 ($P = 0.1$) in the 100 µg/ml HDM extract-stimulated cultures in comparison with 1 µg/ml however statistical significance was not reached. In the graphs in (C) and (D) each symbol represents one HDM-allergic donor and the bars represent the median values.



significantly greater numbers of CD8⁺ CD49d^{hi} IFN- γ ⁺ T cells in cultures stimulated with 100 μ g/ml HDM extract in comparison with 1 μ g/ml ($P < 0.01$), with no change in CD8⁺ CD49d^{hi} IL-4⁺ T cell number (Table 2).

Increased numbers of IFN- γ -producing CD4⁺ CCR5⁺ T cells at high allergen concentration

The effect of repeated stimulation with different allergen concentrations on the cytokine profiles of T cells expressing CCR3 and CCR5 chemokine receptors was analysed on 14-day HDM-stimulated cultures. After the 6-h anti-CD3 stimulation required for the detection of intracellular cytokines, CCR3 expression was down-regulated such that CCR3 staining intensity was equal to or less than the isotype control (data not shown). Thus the cytokine phenotype of CD4⁺ CCR3⁺ and CD8⁺ CCR3⁺ T cells was not determined. CCR5 expression was not affected in this way; representative intracellular IL-4 and IFN- γ profiles for CCR5⁺ T cells of one HDM-allergic donor shown in Fig. 4A. Although only a subset of CD4⁺ T cells expressed low levels of CCR5, significantly greater proportions ($P < 0.001$; Fig. 4B) and numbers ($P < 0.05$) of CD4⁺ CCR5⁺ T cells expressed IFN- γ in cultures stimulated with 100 μ g/ml HDM extract in comparison with 1 μ g/ml (Table 2). In contrast,

significantly lower proportions ($P < 0.01$) and numbers ($P < 0.05$) of CD4⁺ CCR5⁺ T cells expressing IL-4 were observed in cultures stimulated with 100 μ g/ml HDM extract in comparison with 1 μ g/ml. Although proportions of IFN- γ or IL-4 positive CD8⁺ CCR5⁺ T cells were unaffected by culture with different allergen concentrations (Fig. 4C), there were significantly greater numbers of CD8⁺ CCR5⁺ IFN- γ ⁺ T cells in cultures stimulated with 100 μ g/ml HDM extract in comparison with 1 μ g/ml ($P < 0.05$; Table 2). No changes in CD8⁺ CCR5⁺ IL-4⁺ T cell numbers were observed between cultures.

Discussion

In subjects with allergic rhinitis, increased CD4⁺ T cell numbers (22) with local T cell expression of IL-4 and IL-5 are a feature of nasal late-phase responses to allergens (23–26). During clinically successful SIT, IFN- γ ⁺ cell numbers increase at sites of allergen challenge (8–10). Since IFN- γ can inhibit IL-4-induced expression of the germline epsilon transcript in human B cells and consequently prevent IgE production (27), this mechanism could explain the observed reduction in IgE-mediated inflammatory cell activation and decreased local allergic inflammation following SIT (8). Here we demonstrate that culturing HDM-allergic donor PBMC for 14 days with high allergen concentration in comparison with low results in increased numbers of ‘peripheral tissue trafficking’ T cells expressing IFN- γ . Our results suggest that allergen concentration may be a key factor in promoting SIT-induced alterations in the cytokine phenotype of T cells trafficking to peripheral sites of allergen encounter.

In our study, altered cytokine production by ‘peripheral tissue trafficking’ T cells was consistently observed at an allergen concentration greater than 10 μ g/ml. Relating the allergen concentration in *in vitro* cultures to that delivered to T cells *in vivo* during SIT is difficult. However allergen doses administered for SIT (approximately 10 μ g/ml) would be substantially higher than the pg/l air concentrations estimated to be encountered naturally (16, 17). Hence, we focussed on analysing T cell responses upon stimulation with 1–100 μ g/ml HDM. Unfortunately, analysis of T cell responses at allergen concentrations lower than 1 μ g/ml could not be undertaken because T cell survival in these lower concentration cultures could not be maintained. Nevertheless, our results argue strongly for improved efficacy of SIT using high doses of allergen.

By examining the cytokine profiles of CD4⁺ and CD8⁺ T cells expressing low levels of CD62L or high levels of CD49d, we observed cytokine skewing to IFN- γ predominant at high allergen concentration. In agreement with our results, Pacheco and colleagues observed CD49d up-regulation on human T cells after allergen stimulation *in vitro* (28) and animal models have confirmed the

Figure 3. Cytokine profile of CD4⁺ and CD8⁺ T cells expressing either low levels of CD62L or high levels of CD49d in cultures stimulated with different allergen concentrations. PBMC from 10 HDM-allergic donors cultured with HDM extract (1, 10 and 100 μ g/ml) for 14 days were harvested and stimulated with anti-CD3/IL-2 in the presence of Brefeldin A for 6 h. Cells were analysed by flow cytometry following labelling with anti-CD4, anti-CD8, anti-CD62L, anti-CD49d, anti-IL-4 and anti-IFN- γ antibodies. Representative profiles of IL-4 and IFN- γ expression by CD4⁺ CD62L^{lo} and CD8⁺ CD62L^{lo} T cells (A) and CD4⁺ CD49d^{hi} and CD8⁺ CD49d^{hi} T cells (B) in HDM-stimulated cultures for one HDM-allergic donor are shown. (C) Proportions of CD4⁺ CD62L^{lo} T cells expressing IFN- γ were greater in cultures stimulated with 100 μ g/ml HDM extract in comparison with 1 and 10 μ g/ml ($P < 0.01$). Proportions of CD4⁺ CD62L^{lo} T cells expressing IL-4 were lower in the 100 μ g/ml HDM extract-stimulated cultures in comparison with 1 μ g/ml ($P < 0.01$). (D) Proportions of CD8⁺ CD62L^{lo} T cells that were expressing either IFN- γ or IL-4 did not differ between cultures stimulated with different concentrations of allergen. (E) Proportions of CD4⁺ CD49d^{hi} T cells expressing IFN- γ were greater in cultures stimulated with 100 μ g/ml HDM extract in comparison with 1 μ g/ml ($P < 0.01$) and 10 μ g/ml ($P < 0.05$). Proportions of CD4⁺ CD49d^{hi} T cells expressing IL-4 were lower in the 100 μ g/ml HDM extract-stimulated cultures in comparison with 1 μ g/ml ($P < 0.05$). (F) Proportions of CD8⁺ CD49d^{hi} T cells expressing either IFN- γ or IL-4 did not differ between cultures stimulated with different concentrations of allergen. In the graphs in (B), (C), (E) and (F) each symbol represents one HDM-allergic donor and the bars represent the median values.

Table 2. Median number and ranges in parentheses of cytokine positive T cells expressing low levels of CD62L, high levels of CD49d or CCR5 in 14-day HDM extract (1, 10 or 100 μ g/ml) stimulated cultures ($n = 10$) after 6 h incubation with anti-CD3 and IL-2 in the presence of Brefeldin A

HDM extract (μ g/ml)	CD4 ⁺ T cells ($\times 10^{-5}$)			CD8 ⁺ T cells ($\times 10^{-5}$)		
	1	10	100	1	10	100
CD62L ^{lo} IFN- γ ⁺	0.9 (0.2–4.1)	2.9 (0.3–4.6)	7.9** (1.2–17.8)	0.3 (0.1–2.4)	1.1 (0.1–2.6)	1.4* (0.1–11.2)
CD49L ^{hi} IFN- γ ⁺	0.4 (0.1–2.5)	1.2 (0.1–3.9)	3.0** (0.1–26.2)	0.8 (0.1–3.1)	1.7 (0.1–3.5)	1.9** (0.5–32.9)
CCR5 ⁺ IFN- γ ⁺	0.8 (0.1–3.9)	1.9 (0.4–5.6)	4.0* (0.5–12.7)	0.4 (0.1–7.2)	1.1 (0.1–9.8)	1.7* (0.1–18.1)
CD62L ^{lo} IL-4 ⁺	3.5 (0.4–9.8)	4.1 (0.1–10.9)	1.7 (0.1–8.3)	0.1 (0.1–0.3)	0.1 (0.1–1.2)	0.1 (0.1–0.5)
CD49d ^{hi} IL-4 ⁺	5.1 (1.9–25.5)	5.4 (1.8–50.1)	4.6 (0.3–55.1)	0.1 (0.1–0.1)	0.1 (0.1–3.5)	0.1 (0.1–0.1)
CCR5 ⁺ IL-4 ⁺	3.9 (1.0–15.8)	3.2 (0.7–11.4)	1.7* (0.2–14.3)	0.1 (0.1–0.2)	0.1 (0.1–0.8)	0.1 (0.1–0.9)

* $P < 0.05$ and ** $P < 0.01$ between 1 and 100 μ g/ml HDM extract.

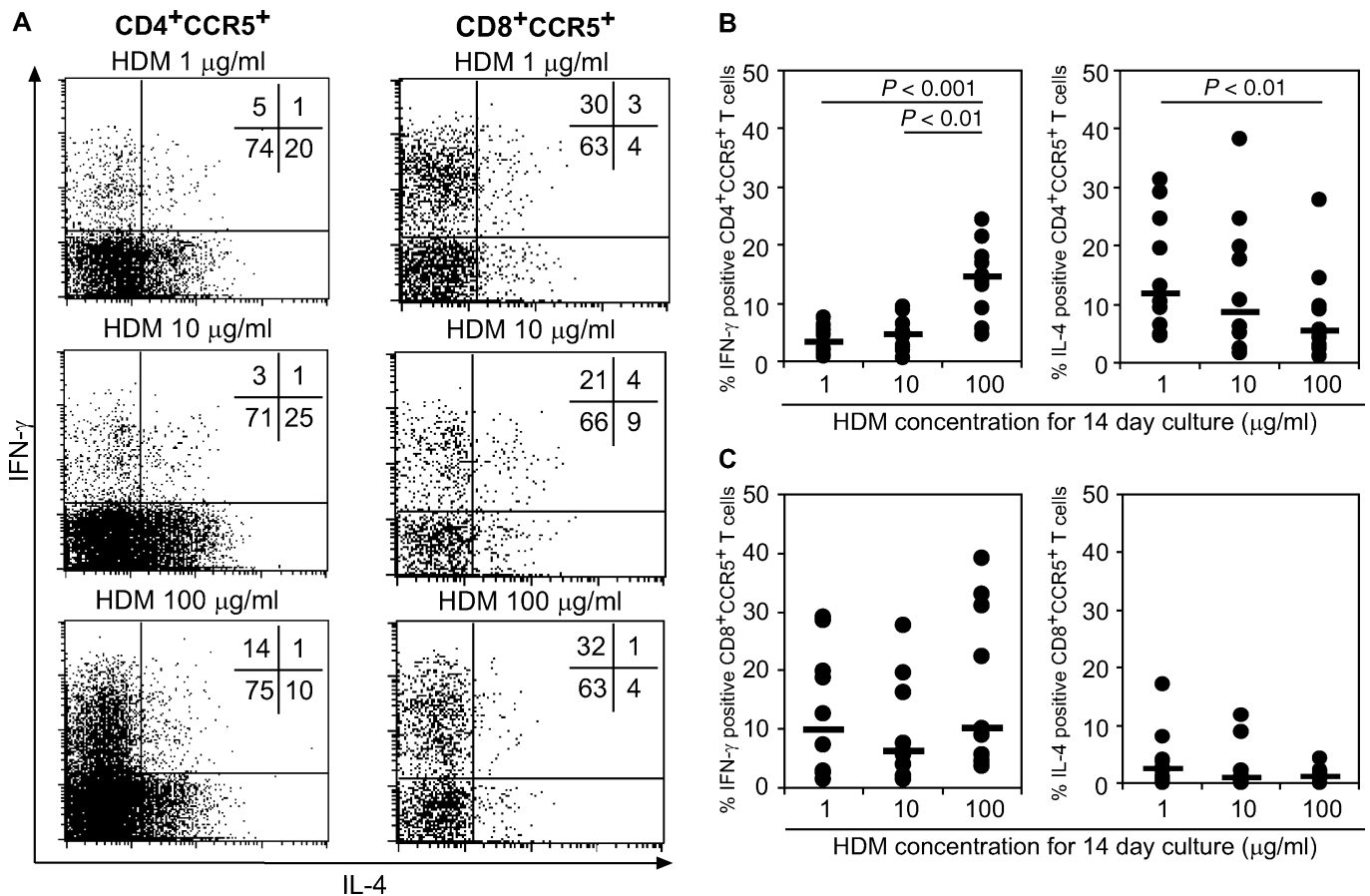


Figure 4. Cytokine profile of CD4⁺ CCR5⁺ and CD8⁺ CCR5⁺ T cells in cultures stimulated with different allergen concentrations. PBMC from 10 HDM-allergic donors cultured with HDM extract (1, 10 and 100 μ g/ml) for 14 days were harvested and stimulated with anti-CD3/IL-2 in the presence of Brefeldin A for 6 h. Cells were analysed by flow cytometry following labelling with anti-CD4, anti-CD8, anti-CCR5, anti-IL-4 and anti-IFN- γ antibodies. Representative profiles of IL-4 and IFN- γ expression by CD4⁺ CCR5⁺ and CD8⁺ CCR5⁺ T cells (A) in HDM-stimulated cultures for one HDM-allergic donor are shown. (B) Proportions of CD4⁺ CCR5⁺ T cells expressing IFN- γ were greater in cultures stimulated with 100 μ g/ml HDM extract in comparison with 1 μ g/ml ($P < 0.001$) and 10 μ g/ml ($P < 0.01$). Proportions of CD4⁺ CCR5⁺ T cells expressing IL-4 were lower in the 100 μ g/ml HDM extract-stimulated cultures in comparison with 1 μ g/ml ($P < 0.01$). (C) Proportions of CD8⁺ CCR5⁺ T cells expressing either IFN- γ or IL-4 did not differ between cultures stimulated with different concentrations of allergen. In the graphs in (B) and (C) each symbol represents one HDM-allergic donor and the bars represent the median values.

importance of CD49d in T cell migration into sites of peripheral allergen challenge by *in vivo* blocking experiments (29, 30). Hence high dose allergen administration

during SIT may induce a population of effector Th1-type cells in the circulation that have lost CD62L required for migration into lymphoid tissues but upregulated expres-

sion of CD49d for binding to VCAM-1 on endothelial cells thus enabling migration into peripheral tissues.

Expression of chemokine receptors by antigen-stimulated T cells and the production of chemokines at sites of inflammation mediate homing of activated T cells into peripheral tissues. Allergen-specific T cell expression of CCR3 was analysed in this study because production of its ligand, eotaxin, is reported to be increased after allergen challenge in bronchoalveolar lavage fluid of atopic asthmatics (21). As CCR3 expression is upregulated in the presence of IL-4 (31), we expected to observe increased T cell expression of CCR3 due to allergen-induced IL-4 production in HDM extract-stimulated cultures. However CCR3 expression by allergen-stimulated T cells was not altered and only very small proportions of T cells expressed this marker. Thus we believe that CCR3 plays only a limited role in determining the T cell contribution to the cytokine milieu at peripheral sites of allergen encounter. CCR5 was also examined in this study. In contrast to previous findings that CCR5 is expressed by only Th1 cells (15), but in agreement with Sallusto and colleagues (13), CCR5 expression was detected on IFN- γ ⁺ and IL-4⁺ T cells. As the chemokine ligands for CCR5, i.e. RANTES and MIP1- α , are also detected in the bronchoalveolar lavage fluid from atopic asthmatics after allergen challenge (32), CCR5⁺ T cells could potentially migrate via chemokine gradients into lung tissue in asthmatics. This conclusion is supported by the demonstration of CCR5 expression by a population of resident T cells in the asthmatic lung (33). In our study, at low allergen concentration increased numbers of CD4⁺ CCR5⁺ T cells expressed IL-4 whereas at high allergen concentration increased numbers of CD4⁺ CCR5⁺ T cells expressed IFN- γ . Hence after high dose allergen administration during SIT, CCR5 may be playing an important role in homing CD4⁺ IFN- γ ⁺ T cells to sites of allergic inflammation. Recent studies suggest other chemokine receptors may also be important for trafficking of Th cells. For example, increased

numbers of CCR4 mRNA⁺ cells were found in atopic asthmatic lungs following allergen challenge (34). Thus several chemokines and chemokine receptors are likely to be involved in T cell inflammation during an allergic response and additional studies are required to determine whether any could be potential targets for therapeutic intervention.

Der p 1-specific CD8⁺ T cells have recently been identified (35). We observed increased numbers of CD8⁺ IFN- γ ⁺ T cells with a 'peripheral tissue trafficking' phenotype at high HDM concentration. This was a surprising observation since in the few studies that have analysed changes in CD8⁺ T cell cytokine production during SIT, CD8⁺ T cell populations were minor and produced decreased or unchanged IFN- γ (36, 37). However we have previously shown that expansion of CD8⁺ IFN- γ ⁺ T cells in allergen-stimulated cultures does occur at sufficiently high doses (18). These results again highlight the advantage of using high dose allergen administration in SIT to achieve immune deviation in peripheral tissues.

In conclusion, this study provides evidence that allergen concentration can influence the production of IFN- γ and IL-4 by human T cells expressing adhesion molecules and chemokine receptors required for peripheral T cell trafficking. High allergen concentration promotes IFN- γ production by allergen-specific 'peripheral tissue trafficking' T cells. Thus the use of hypoallergenic allergen preparations in SIT will lead to more effective treatment by permitting high dose administration.

Acknowledgments

This work was funded by the Co-operative Research Centre for Asthma, Australia and Alfred Hospital Research Trusts. We thank Michael Bailey for his assistance with statistical analysis and Cathy Fraser and Lorraine Baxter for collection of patient clinical data and blood samples.

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