Modulation of the Allergen-Specific T Cell Response

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SUMMARY

Allergen-specific T cells play a pivotal role in initiating and regulating the immune response to allergens. Atopic allergic individuals respond to allergen stimulation by predominant T cell secretion of IL-4 and IL-5 in contrast to non-atopic individuals where there is predominant IFN-γ secretion. In the treatment of allergic disease, allergen specific immunotherapy (SIT) is the only therapy that can modify the natural course of disease and potentially effect a cure. Conventional aeroallergen SIT involves the incremental administration of allergen extract to sensitised individuals to induce clinical tolerance. However efficacy of SIT varies with the allergen and there is a potential risk of serious side effects, so there is need to more fully understand the underlying mechanisms for clinically effective SIT. This will enable the design of more effective and safer allergen preparations and regimens, and thus use of SIT in a wider range of patients.

Studies have shown that effective SIT is accompanied by altered T cell responses, with decreased T cell proliferative responses to allergen and changes in cytokine production by circulating T cells. A shift from IL-4 and IL-5 to IFN-γ predominant is generally observed and increased IL-10 production has been reported. Since SIT is associated with the administration of higher concentrations of allergen than encountered naturally and antigen concentration can influence T cell priming, allergen concentration may be an important factor involved in altering T cell responses during SIT. This project firstly investigated the effects of different allergen concentrations on $CD4^+$ and $CD8^+$ T cell responses *in vitro*. As HDM is the major aeroallergen causing perennial rhinitis and asthma, it was chosen as the model allergen. Peripheral blood mononuclear cells

(PBMC) from house dust mite (HDM)-allergic individuals were cultured for 14 days in the presence of 1, 10 and 100 μg/ml HDM extract. Stimulation with high allergen concentrations in comparison to low was found to promote the expansion of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells, enhance apoptosis of IL-4-producing $CD4^+$ T cells and induce T cell anergy. In some high concentration cultures increased proportions of IL-10 positive $CD4^+$ T cell were also observed. These results indicate that administration of high allergen concentrations during SIT can promote allergen-specific Th1-type responses and possibly induce T regulatory cell function and point towards improved efficacy with the use of hypoallergenic preparations such as peptides or mutant allergens. These preparations can be administered safely at higher doses than natural extracts with considerably reduced risk of side effects.

As increased IFN-γ^+ cells have been identified in peripheral tissues following SIT, $CD4^+$ and $CD8^+$ T cell expression of adhesion molecules (CD62L and CD49d) and chemokine receptors (CCR3 and CCR5) was correlated with intracellular cytokine production. In 14-day cultures stimulated with high allergen concentration in comparison to low, increased numbers of IFN-γ positive $CD4^+$ and $CD8^+$ T cells expressing a peripheral tissue trafficking phenotype ie. $CD62L^-$, $CD49d^{hi}$ or $CCR5^+$ were detected. $CCR3^+$ T cell numbers were very low and did not differ between cultures. Decreased proportions of "peripheral tissue trafficking" $CD4^+$ T cells expressing IL-4 were also detected in high allergen concentration cultures. These results give further grounds for the importance of achieving higher allergen doses during SIT to increase IFN-γ production by allergen-specific $CD4^+$ and $CD8^+$ T cells expressing "peripheral tissue trafficking" surface markers. Increased IFN-γ production at sites of

allergen encounter could then down-regulate the clinical manifestations of allergic disease by reducing Th2-mediated inflammation.

In addition to examining the effects of high allergen concentration on T cell responses *in vitro*, changes in the allergen-specific T cell response during allergen immunotherapy were also investigated. A clinical trial of conventional HDM SIT was carried out at the Asthma and Allergy Clinic, Alfred Hospital, Melbourne, Australia. This clinical study provided an opportunity to investigate changes in T cell cytokine production and expression of "peripheral tissue trafficking" surface markers during *in vivo* treatment with high concentrations of allergen. Before and at 3- and 9-months of SIT, PBMC were cultured for 14-days with HDM extract at 25 μg/ml. Analysis of cytokine production by allergen-stimulated T cells at 9-months of SIT in comparison to pre-SIT, revealed increased proportions of IL-10 positive $CD4^+$ T cells, and decreased proportions of IL-4 positive $CD4^+$ and $CD8^+$ T cells. Increased proportions of "peripheral tissue trafficking" $CD4^+$ T cells expressing IL-10 were also detected at 9 months of SIT compared with baseline. IL-10 staining co-localised with $CD4^+CD25^+$ T cells consistent with a regulatory T cell phenotype. These results indicate that HDM-specific immunotherapy results in the induction of a population of $CD4^+$IL-10^+ T cells that express a peripheral tissue trafficking phenotype. As IL-10 can abate allergen-specific T cell responses and subsequent activation of effector cells, allergen preparations designed to enhance allergen-specific regulatory T cell IL-10 responses will lead to improved efficacy for immunotherapy.

In conclusion, the results presented in this thesis provide further insight into the underlying mechanisms for clinically successful SIT and will aid in the rational design of improved T cell targeted strategies for immunotherapy. Allergen derivatives with retained T cell reactivity but abrogated IgE binding that can be used safely at higher doses should increase the efficacy of SIT. Such derivatives include peptides containing dominant T cell epitopes of allergens and recombinant mutant allergen molecules. In addition, the functional T cell assays designed in this project could be used in the laboratory for monitoring clinical efficacy of SIT. These assays are currently not available.

DECLARATION

The work embodied in this thesis was conducted in the Department of Pathology and Immunology, Monash University, during 2000 to 2003. It contains no material which has been accepted for the award of any other degree or diploma in any other university or institution. To the best of my knowledge this thesis contains no material previously published or written by another person, except where specific reference has been made in the text.

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PUBLICATIONS

Results reported in this thesis have been published, submitted for publication and presented at scientific meetings as follows:

REFEREED JOURNAL ARTICLES

1. **Gardner, L. M.**, O'Hehir, R. E., Rolland, J. M. (2004) High dose allergen stimulation of T cells from house dust mite-allergic subjects induces expansion of IFN-γ^+ T cells, apoptosis of $CD4^+IL\text{-}4^+$ T cells and T cell anergy. Int. Arch. Allergy Immunol. **133**, 1-13.
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ABSTRACTS AND ORAL PRESENTATIONS

1. **Gardner, L. M.**, Thien, F. C., Douglass, J. A., Rolland, J. M., O'Hehir, R. E. "House dust mite immunotherapy increases IL-10$^+$ T cells with a peripheral trafficking phenotype". Alfred Science Symposium, Melbourne, Australia, 2003.
2. **Gardner, L. M.**, O'Hehir, R. E., Rolland, J. M. "Single cell analysis of high dose allergen-induced changes in human T cell cytokine and adhesion molecule expression: an *in vitro* model of allergen-specific immunotherapy". Allergy, Asthma and Hygiene Symposium, Keystone, Colorado, USA, 2003.
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6. **Gardner, L. M.**, O'Hehir, R. E., Rolland, J. M. "Increased IFN-γ production by allergen-specific T cells at higher allergen doses is associated with enhanced cell division". Australasian Society for Immunology, 31st Annual Meeting, Canberra, Australia, 2001.

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8. **Gardner, L. M.**, Burton, M.D., O'Hehir, R. E., Rolland, J. M. "Strategies for down-regulating the Th2-type cytokine profile of human allergen-specific T cells and promoting IFN-γ production". Australasian Society for Immunology, 30th Annual Meeting, Sydney, Australia, 2000.
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ABBREVIATIONS

AICD	activation-induced cell death
APC	antigen presenting cell
APL	altered peptide ligand
CCR	chemokine receptor
CD	cluster of differentiation molecules
CDR	complementary determining region
CFSE	carboxyfluorescein diacetate succinamidyl ester
CIIV	MHC class II vesicle
CLIP	class II-associated invariant chain peptide
CMV	cytomegalovirus
cpm	counts per minute
CTLA-4	cytotoxic T lymphocyte-associated molecule-4
°C	degrees celsius
DC	dendritic cell
DMSO	dimethylsulphoxide
DNA	deoxyribose nucleic acid
DTT	dithiothreitol
EAST	enzyme-allergosorbent test
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
EPR	early phase response
ER	endoplasmic reticulum

FasL	Fas-ligand
FADD	Fas-associated death domain
FBS	foetal bovine serum
FITC	fluorescein isothiocyanate
g	unit of gravity
GE	glove extract
GM-CSF	granulocyte macrophage colony stimulating factor
HCC	human CC chemokine
HDM	house dust mite
HEV	high endothelial venules
HLA	human leukocyte antigen
ICAM	intracellular adhesion molecule
ICOS	inducible T-cell costimulator
ICOSL	inducible T-cell costimulator ligand
IFN	interferon
Ig	immunoglobulin
Ii	invariant chain
IL-	interleukin
IP-10	inducible protein of 10 kDa
ISS	immunostimulatory sequences
I-TAC	interferon-induced T cell alpha chemoattractant
kDa	kilodalton
kg	kilogram
KLH	Keyhole Limpet Haemocyanin

L	litre
LFA	lymphocyte function-associated antigen
LPR	late phase response
LPS	lipopolysaccharide
M	molar
mAb	monoclonal antibody
MCP	macrophage chemotatic protein
MDC	monocyte-derived chemokine
MFI	mean fluorescence intensity
mg	milligram
MHC	major histocompatibility complex
Mig	monokine induced by IFN-γ
MIIC	MHC class II loading compartment
MIP-	macrophage inflammatory protein
ml	millilitre
mM	millimolar
MPL	monophosphoryl lipid A
µg	microgram
µl	microlitre
NFAT	nuclear factor of activated T cells
ng	nanogram
NK	natural killer
ODN	oligodeoxynucleotides
OVA	ovalbumin

PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PE	phycoerythrin
PI	propidium iodide
PIT	peptide immunotherapy
PHA	phytohaemagglutinin
PLA_2	phospholipase A_2
PMA	phorbol 12-myristate 13-acetate
QOL	quality of life
RANTES	regulated on activation normal T cell expressed and secreted
mRNA	messenger ribonucleic acid
SDF	stromal-derived factor
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SI	stimulation index
SIT	specific immunotherapy
SLC	secondary lymphoid-tissue chemokine
STAT	signal transducer and activator of transcription
TAP	transporter associated with antigen processing
TARC	thymus and activation-regulated chemokine
Tc	T cytotoxic
TCC	T cell clone

TCL	T cell line
TCR	T cell receptor
TECK	thymus-expressed chemokine
TEMED	N,N,N',N'-tetramethylethylene-diamine
TGF	transforming growth factor
^{3}H-thymidine	tritiated thymidine
Th	T helper
TLR	toll-like receptors
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
Tr	T regulatory
Tris	tris(hydroxymethyl)aminomethane
VCAM	vascular cell adhesion molecule
VLA	very late activation antigen
VLP	virus-like particle

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

1.1.1 Allergic Inflammation

With greater than 40% of the population being atopic in Australia and many other western countries, allergic diseases are a major health concern in our community (Burney *et al.*, 1997). IgE-mediated reactions occur upon exposure to innocuous antigens called allergens and result in the clinical manifestations of allergy including rhinitis, urticaria and asthma. Most allergens are common environmental proteins derived from house dust mites (HDM), grass and tree pollens, domestic pets and various moulds. Individuals with IgE to one or more of these common allergens are classified as atopic.

For an allergic reaction to occur, an atopic individual must firstly be sensitised to an allergen. Sensitisation predominantly occurs upon primary exposure to an allergen usually at mucosal surfaces. Allergen is taken up and processed by professional antigen-presenting cells (APC), typically dendritic cells (DC), at these sites (Figure 1.1). DC then migrate to the local lymph node where allergen is presented in the context of major histocompatibility (MHC) class II molecules to naïve T helper (Th) cells. Th cells play a pivotal role in the development of allergic disease because upon allergen presentation and recognition they respond with either a predominantly type 1 or type 2 polarised response. In atopic individuals Th cells differentiate to produce IL-4, IL-5 and IL-13 in

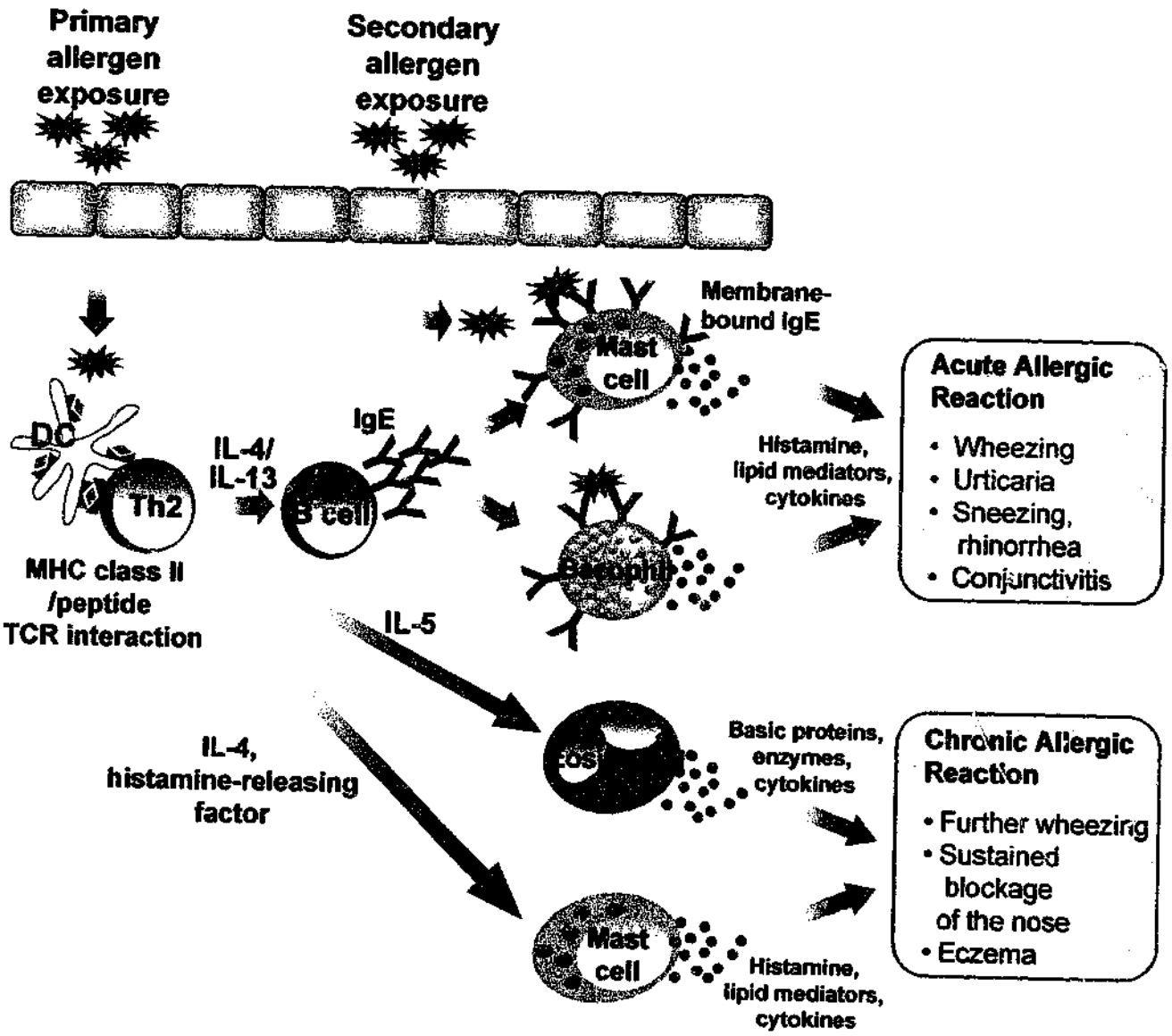


Figure 1.1 The allergen-specific Th cell plays a pivotal role in determining the clinical phenotype resulting from allergen encounter.

In allergic individuals, the allergen stimulated Th cell exhibits a Th2-polarised cytokine response with IL-4 driving IgE class switching by allergen-stimulated B cells and IL-5 promoting eosinophil migration and activation. Cross-linking of mast cell and basophil bound IgE by allergen on subsequent exposure triggers activation of these cells. The inflammatory mediators released from activated mast cells, basophils and eosinophils elicit the symptoms of acute and chronic allergic reactions.

a type 2 polarised response (O'Hehir *et al.*, 1993; Li *et al.*, 1996). Production of these cytokines leads to a series of events resulting in B cell production of allergen-specific IgE and inflammatory cell activation. The allergen-specific IgE produced can bind to high affinity surface IgE receptors (FcεRI) on mast cells and basophils (Ishizaka and Ishizaka, 1984). In non-atopic individuals Th cells produce predominantly IFN-γ, in a type 1 polarised response, resulting in B cell production of non-pathogenic allergen-specific IgG (Kemeny *et al.*, 1989).

B cell production of allergen-specific immunoglobulin requires allergen recognition, internalisation and processing. B cells present allergen as peptides via MHC class II molecules to Th cells. This cognate interaction in combination with the binding of T cell expressed CD40L to CD40 on B cells results in B cell activation. Under the control of Th cell cytokines, activated B cells then mature into antibody-secreting plasma cells. In the presence of IL-4 and IL-13 B cells undergo class-switching to produce IgE (O'Hehir *et al.*, 1989; Wills-Karp *et al.*, 1998).

When a sensitised individual re-encounters allergen acute allergic symptoms may become evident rapidly, often within 10 minutes. This early phase response (EPR) peaks at 30 minutes and resolves in 1-3 hours. The response is due to the release of preformed granule-associated mediators including histamine, tryptase, and newly generated mediators including cysteinyl leukotrienes and prostaglandins as well as chemotactic factors from mast cells triggered by allergen crosslinking IgE bound to high affinity FcεRI. Histamine is a fast-acting mediator that causes vasodilation, increased vasopermeability, contraction of bronchial smooth muscle and increased mucus

production. Although histamine effects are short lived, due to rapid metabolisation, allergic symptoms persist due to the effects of the newly generated mediators including cysteinyl leukotrienes and prostaglandins. The effects of leukotriene LTD_4 are similar to those of histamine, but enhanced. LTD_4 is also involved in eosinophil chemoattraction. PGD_2 is a stimulatory prostaglandin and plays a major role as a bronchoconstrictor. Chemoattractant factors including IL-5, IL-8 and TNF-α are also released and result in the infiltration of basophils, eosinophils and neutrophils. Clinical symptoms of the EPR include rhinitis, bronchoconstriction, dyspnoea, wheezing, coughing and skin itching.

Approximately 50% of adults and 70% of children who demonstrate an EPR to allergen will progress to the late phase response (LPR) (Holgate *et al.*, 2001). The LPR results from infiltration of inflammatory cells, importantly eosinophils and $CD4^+$ T cells, and the subsequent release of mediators and cytokines from these cells (Renz, 1995; Kay, 2001). This leads to further recruitment and activation of inflammatory cells at the site of the LPR (Figure 1.1). During this phase through the release of eosinophil major basic protein, cationic protein and eosinophil-derived neurotoxin, eosinophils are the principal cell mediating tissue damage. Eosinophils also produce a wide range of cytokines including IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-11, IL-12, TGF-β, TNF-α, GM-CSF, MIP-1α and RANTES which contribute to the ongoing allergic response (Hamid and Minshall, 2000). In addition, the release of pro-inflammatory cytokines including IL-4, IL-5, IL-6, IL-8, IL-13, GM-CSF and TNF-α from sensitised mast cells and IL-4 and IL-13 from infiltrating basophils are also important in the continuation of the LPR (Bingham and Austen, 2000; Hamid *et al.*, 2003). Chronic clinical manifestations of the LPR include asthma, eczema and nasal obstruction. Peak

symptoms are observed 6-12 hours after allergen exposure with resolution by 24 hours. Repeated allergen exposure results in chronic allergic inflammation (Galli and Lantz, 1998).

1.1.2 House Dust Mite Allergy

The HDM is the main source of aeroallergens. Within Australian and European communities prevalence of sensitisation to HDM allergens is in the order of 20% (Peat *et al.*, 1993; Burney *et al.*, 1997; Svanes *et al.*, 1999; Arlian and Platts-Mills, 2001). As well as HDM allergens being a major cause of perennial rhinitis and atopic dermatitis, research has shown that HDM exposure is a major risk factor for asthma (Sporik *et al.*, 1990; Sporik *et al.*, 1992; Peat *et al.*, 1993; Peat *et al.*, 1996; Miraglia Del Giudice *et al.*, 2002). A dose response relationship between HDM levels and increased risk of asthma has been demonstrated (Peat *et al.*, 1996).

World-wide there are 13 species of house dust mites, however the three most common mite species found in household dust are *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae* and *Euroglyphus maynei* (Arlian and Platts-Mills, 2001). These three species of mite are part of the *Pyroglyphidae* family. Over the past 20 years many mite allergens have been identified, cloned and sequenced. In 1980 Der p 1, a major allergen of *Dermatophagoides pteronyssinus*, was the first mite allergen to be characterised (Chapman and Platts-Mills, 1980). Group 1 mite allergens have high IgE-binding activities with binding frequencies of 80-100% of HDM-allergic patients. In total 19 allergenic proteins of HDM have now been identified (Thomas *et al.*, 2002). The biochemical and IgE-binding characteristics of these mite allergens are given in

Table 1.1. Interestingly many of these allergens function as enzymes and the contribution of these enzymes to the overall allergenicity of HDM is discussed in more detail later in this chapter.

1.1.3 Non-Specific Treatments for Allergy

Pharmacotherapy is the mainstay treatment for allergy. Therapeutic drugs have been designed to target inflammatory mediators released during an allergic reaction. There is a wide range of drugs including anti-histamines and decongestants which relieve sneezing, congestion, conjunctival irritation and tiredness associated with rhinitis. Corticosteroids are used extensively to reduce inflammation in chronic allergic disease. In asthma the use of bronchodilators such as β-agonists is an essential component of drug therapy. Although these therapies are effective at providing immediate symptom relief, they cannot effect a cure and frequently have associated side effects, which can include drowsiness and headache.

1.1.4 Specific Treatments for Allergy

Specific treatments are designed to target the allergen-specific response, which causes clinical manifestations in affected individuals. Allergen avoidance is a highly desirable treatment that is feasible for allergens such as certain foods or latex. However ubiquitous allergens such as HDM and pollens cannot be avoided and therefore the practicality of this treatment is limited.

Allergen-specific immunotherapy (SIT) is an attractive alternative involving the incremental administration of allergen extract to induce clinical tolerance (Bousquet *et*

Table 1.1 House dust mite allergens.

Group	Biochemical function	MW cDNA[1] (SDS-PAGE)	Species[2]	IgE binding[3]
1	Cysteine protease	25,000	Dp, Df, Dm, Ds, Em	80-100
2	Unknown	14,000	Dp, Df, Ds, Em, Ld, Tp, Gd, As	80-100
3	Trypsin	25000 (30,000)	Dp, Df, Ds, Em	16-100
4	a-Amylase	57,000	Dp, Em	40-46
5	Unknown	15,000	Dp, Bt, Ld	50-70
6	Chymotrypsin	25,000	Dp, Df	40
7	Unknown	25,000 (31,000, 29,000, 26,000)	Dp, Df, Lt	50
8	Glutathione-S-transferase	26,000	Dp	40
9	Collagenolytic serine protease	no cDNA, (30,000)	Dp	90
10	Tropomyosin	37,000	Dp, Df	50-95
11	Paramyosin	96,000 (92,000, 98,000)	Df, Bt	80
12	Unknown	14,000	Bt	50
13	Fatty acid-binding protein	15,000	Bt, Ld, As	10-23
14	Vitellogenin/apolipophorin-like	177,000 (variable)	Dp, Df, Em	90
15	98,000 Chitinase	62,500 (98,000, 105,000)	Df	70
16	Gelsolin	55	Df	35
17	Ca-binding EF protein	30	Df	35
18	Chitinase	60,000	Df	60
19	Anit-microbial peptide	7,000	Bt	10

[1] MW calculated from cDNA (SDS-PAGE of natural allergen, if different);
[2] Allergen described for the species designated by initials *Dermatophagoides pteronyssinus, Dermatophagoides farinae, Euroglyphus maynei, Dermatophagoides siboney, Dermatophagoides microceaus, Lepidoglyphus destructor, Blomia tropicalis, Tyrophagus putrescentiae, Glycophagus domesticus, Ascaris siro*;
[3] Binding frequency (% mite-allergic patients). (Thomas *et al.*, 2002)

al., 1998b). Although efficacy of SIT is high for venom-induced allergy, success rates for the more common aeroallergens such as HDM are lower. Moreover this treatment has associated side effects ranging from mild skin reactions at the site of injection to a risk of anaphylaxis which can be life threatening (Bousquet *et al.*, 1998b). Refinement of SIT to improve efficacy and safety requires an understanding of the fundamental mechanisms underlying regulation of the immune response to allergens. This literature review discusses the T cell response to allergen and examines the potential roles of antigen concentration, antigen form, antigen-presenting cell type and regulatory cells in modulating pathogenic immune responses to allergens. The use of different allergen preparations and adjuvants for more effective SIT are also considered.

1.2 T CELL RESPONSE TO ALLERGENS

1.2.1 T Cell Subsets

The majority of peripheral T cells can be divided into two subsets on the basis of surface expression of CD4 and CD8 molecules. $CD4^+$ T cells (Th cells) recognise antigen in the context of MHC class II molecules and secrete cytokines that primarily act on other leukocytes. MHC class II molecules contain extracellular antigenic-peptides and activate the immune system to destroy pathogens such as bacteria. $CD8^+$ T cells are T cytotoxic (Tc) cells that recognise antigen in the context of MHC class I molecules and kill antigen-bearing cells. MHC class I molecules contain mainly endogenous antigenic-peptides and thus this is an effective mechanism for killing cells infected with virus or intracellular pathogens.

$CD4^+$ and $CD8^+$ T cells are further subdivided on the basis of cytokine production.

Within the $CD4^+$ T cell compartment, Th1-type cells produce IFN-γ, IL-2 and TNF-β promoting cell-mediated immunity, whereas Th2-type cells produce IL-4, IL-5, IL-6, IL-9 and IL-13 promoting strong antibody responses and favouring eosinophil differentiation and activation (Romagnani, 2000). Th0-type cells produce cytokines of both patterns and mediate moderate effects of both Th1- and Th2-type cells. More recently two other $CD4^+$ T cell subsets with regulatory activity have been identified and include Th3 cells producing large amounts of TGF-β and Tr1 cells producing large amounts of IL-10 (Weiner, 2001; Levings *et al.*, 2002). In addition to subsets of $CD4^+$ T cells, it is now recognised that $CD8^+$ T cells can be divided into Tc1 cells producing IFN-γ but no IL-4, Tc2 cells producing IL-4 but no IFN-γ and Tc0 cells producing both IFN-γ and IL-4 (Mosmann *et al.*, 1997; Vukmanovic-Stejic *et al.*, 2000).

Of the different T cell populations, Th2-type cells are the most important subset involved in allergic inflammation. Upon allergen stimulation, the production of type 2 cytokines from $CD4^+$ T cells results in allergen-specific IgE production by B cells, mast cell growth and differentiation, and eosinophil development, survival, migration and activation (Figure 1.1). Therefore Th2-type cells are involved in initiation and maintenance of allergy. For these reasons, extensive research has been performed on the allergen-specific Th2-type cell and it is a major target for the design and refinement of allergen-specific therapeutics.

1.2.2 T Cell Receptor Recognition of MHC and Antigen

T cells express specific T cell receptors (TCR) that recognise antigen in the form of a peptide bound to a MHC molecule. Th cells generally recognise extracellular antigens

in the context of MHC class II molecules. MHC class II molecules are expressed predominantly on APC including DC, macrophages and B cells. Two polypeptide chains, α and β, non-covalently complex to form a MHC class II molecule. These molecules are synthesised and assembled, and become associated with an invariant chain (Ii) within the endoplasmic reticulum (ER) of a cell (Figure 1.2). The invariant chain inhibits peptide binding in the ER and is involved in transportation of the MHC class II molecules via the secretory pathway to endosomal compartments containing extracellular antigen-derived peptides (Sant and Miller, 1994). Within these compartments the invariant chain is cleaved by proteases. This results in the release of the MHC class II molecule bound to a short fragment of the invariant chain called CLIP (class II-associated invariant chain peptide). CLIP then dissociates allowing for peptide binding to the MHC class II molecule. MHC class II-peptide complexes are subsequently transported to the cell surface for antigen-presentation to $CD4^+$ T cells. Here the complexes are inserted into the plasma membrane, with the outer domains of the two chains forming the peptide-binding groove. The ends of this groove are open allowing for the binding of peptides of variable length (13-17 amino acid residues) (Rudensky *et al.*, 1991). Residues in the antigenic-peptide are anchored into pockets in the peptide-binding groove. These anchor residues form motifs common to most peptides bound to a specific MHC molecule. The range of peptides that can bind MHC class II molecules is dependent on the location, spacing, size and specificity of the pockets where anchor residues bind (Rammensee, 1995).

MHC class I molecules are expressed by most cell types in the body. These molecules consist of a large α chain inserted into the plasma membrane and a non-covalently

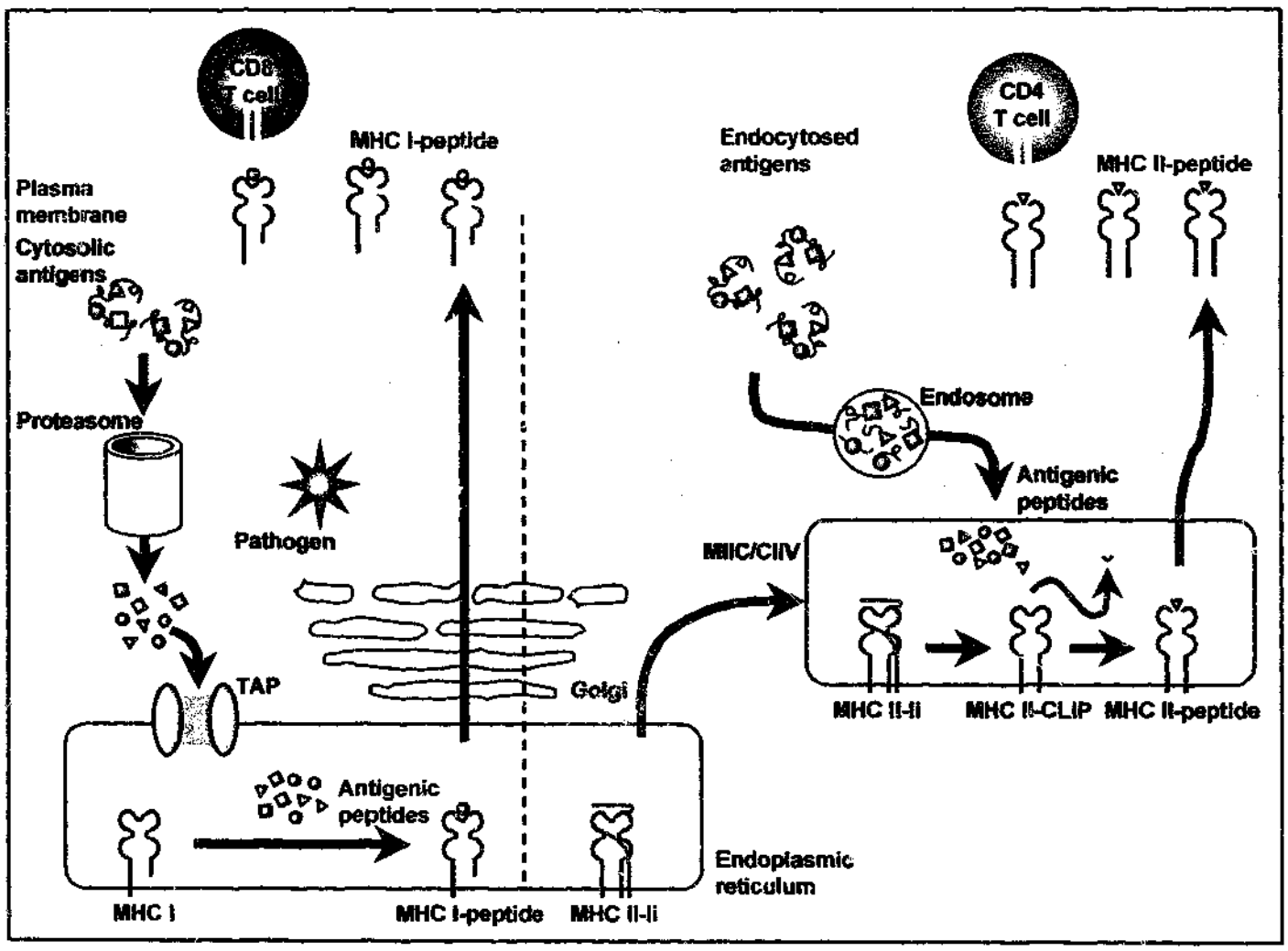


Figure 1.2 Different antigen-processing pathways for the MHC class I and class II molecules.
MHC class I molecules present peptides that are largely derived from endogenously synthesised proteins. These proteins are degraded into peptides by the proteasome. The peptides are then transported through the transporters of antigen-processing (TAP) molecules into the endoplasmic reticulum where they are loaded on MHC class I molecules. MHC class I-peptide complexes are then transported to the cell surface. In contrast, MHC class II molecules present proteins that enter the cell through the endocytic route. During assembly of the MHC class II molecules, they are prevented from binding to endogenous antigens in the endoplasmic reticulum by association with the invariant chain (Ii). Invariant chain-MHC class II complexes (MHC-Ii) move through the Golgi to the MIIC/CIIV compartment where the invariant chain is degraded to CLIP. CLIP is then removed from the CLIP-MHC class II complexes (MHC-CLIP) and exchanged for antigenic peptide. MHC class II-peptide complexes are then transported to the cell surface. (MIIC, MHC class II loading compartment; CIIV, MHC class II vesicles). This figure was adopted from Heath and Carbone, 2001.

bound smaller chain called β2-microglobulin. The outer two α chain domains form the peptide-binding groove of the MHC class I molecule. The peptides that bind MHC class I molecules are usually 8-10 amino acids in length. Synthesis and assembly of MHC class I molecules also occurs in the ER (Figure 1.2). In contrast to MHC class II molecules, class I molecules do not associate with an invariant chain. Instead endogenous peptides are loaded onto MHC class I molecules in the ER (York and Rock, 1996). Peptides move into the ER via transporters of antigen-processing (TAP) after endogenous antigen degradation by the proteasome (Shepherd *et al* , 1993; York and Rock, 1996). After peptide loading MHC class I-peptide complexes are transported to the plasma membrane for antigen-presentation to $CD8^+$ T cells.

TCR expressed by $CD4^+$ and $CD8^+$ T cells have defined specificities for MHC-peptide complexes. Most TCR consist of two polypeptide chains, α and β, linked by a disulphide bond. Approximately 5% of circulating T cells express TCR heterodimers consisting of γ and δ polypeptide chains. It appears that γδ T cells have an important immune function against bacterial pathogens at mucosal surfaces and in the skin (Aljurf *et al.*, 2002). As such αβ T cells, and not γδ T cells, are the focus of this research. Within the TCR there are three hypervariable peptide loops called complementarity determining regions (CDRs) that have been shown by mutational studies to be involved in ligand recognition (Nalefski *et al.*, 1992; White *et al.*, 1993; Wedderburn *et al.*, 1995). For most interactions studied, CDR1 and CDR2 interact with MHC α helices whereas CDR3, demonstrating a high level of diversity, interacts with the antigenic-peptide. Ligation of TCR with specific MHC-peptide complexes in addition to costimulatory molecule interaction results in T cell activation and proliferation.

1.2.3 T Cell Activation and Division

Naïve T cells are activated in draining lymph nodes through the recognition of antigen presented by DC that have migrated to the lymph node after antigen encounter in the periphery (Lambrecht *et al.*, 2000). Naïve T cell activation requires two signals with the first being sustained TCR interactions with specific MHC-peptide complexes and the second signal being provided by the ligation of costimulatory molecules expressed by T cells and APC. The CD28/B7 interaction is the best characterised costimulatory signal. B7 molecules including B7.1 (CD80) and B7.2 (CD86) are expressed by APC and are upregulated on DC during maturation (Turley *et al.*, 2000). T cells express CD28 and interaction with B7 molecules on DC in combination with TCR-MHC-peptide interactions results in the initiation of a cascade of intracellular signalling events leading to the activation of genes required for T cell proliferation and activation (Mueller *et al.*, 1989; Linsley and Ledbetter, 1993).

T cell division occurs rapidly after DC presentation of antigen. In a murine model used to study *in vivo* T cell division, antigen-specific T cells divided twice within two days after injecting antigen-loaded DC into the trachea of recipient mice (Lambrecht *et al.*, 2000). Four days after injection divided T cells had acquired an effector phenotype and thus had migrated from the lymph node to the periphery. Most dividing T cells acquire the ability to produce cytokines (Gett and Hodgkin, 1998), however in some circumstances cytokine production can occur independently of cell division (Auphan-Anezin *et al.*, 2003).

T cell division is driven by the cytokine IL-2, which is produced by activated T cells. IL-2 production by T cells requires the ligation of CD28 by B7. Thus in the absence of

CD28/B7 interactions, the ligation of MHC-peptide by TCR leads to T cell unresponsiveness which is also termed anergy (Schwartz, 2003). Further regulation of T cell division is provided by the interaction between T cell expressed CTLA-4 and B7 on APC. Upon T cell activation the expression of CTLA-4 is upregulated. CTLA-4 can bind B7 with stronger avidity than CD28 and delivers negative signals to activated T cells resulting in the down-regulation of T cell responses (Krummel and Allison, 1995; Boulougouris *et al.*, 1998). Through the regulation of T cell division, adaptive immune responses to antigens can be initiated, sustained and ceased. In this project allergen-driven T cell division is examined *in vitro* in order to determine the kinetics of proliferation and cytokine production of allergen-specific $CD4^+$ and $CD8^+$ T cells.

1.2.4 T Cell Trafficking

1.2.4.1 Introduction

T cells must traffic throughout the body in order to meet their specific antigen, become activated and exert their effector function. Naïve T cells home preferentially to lymphoid tissues, whereas activated effector T cells migrate to peripheral sites of inflammation (Figure 1.3). The migration of T cells from blood vessels into tissues involves interactions between T cell expressed adhesion molecules and ligands on endothelial cells (Westermann *et al.*, 2001). Selectins and integrins are adhesion molecules involved in T cell trafficking. Selectins mediate initial tethering of T cells to endothelial cells of blood vessels. Engagement of integrins to their ligands results in rolling and final arrest of T cells to vascular walls. Subsequent T cell migration into tissues is mediated by chemokine gradients. Chemokines are secreted polypeptides that bind to specific cell surface receptors. Adhesion molecule and chemokine receptor

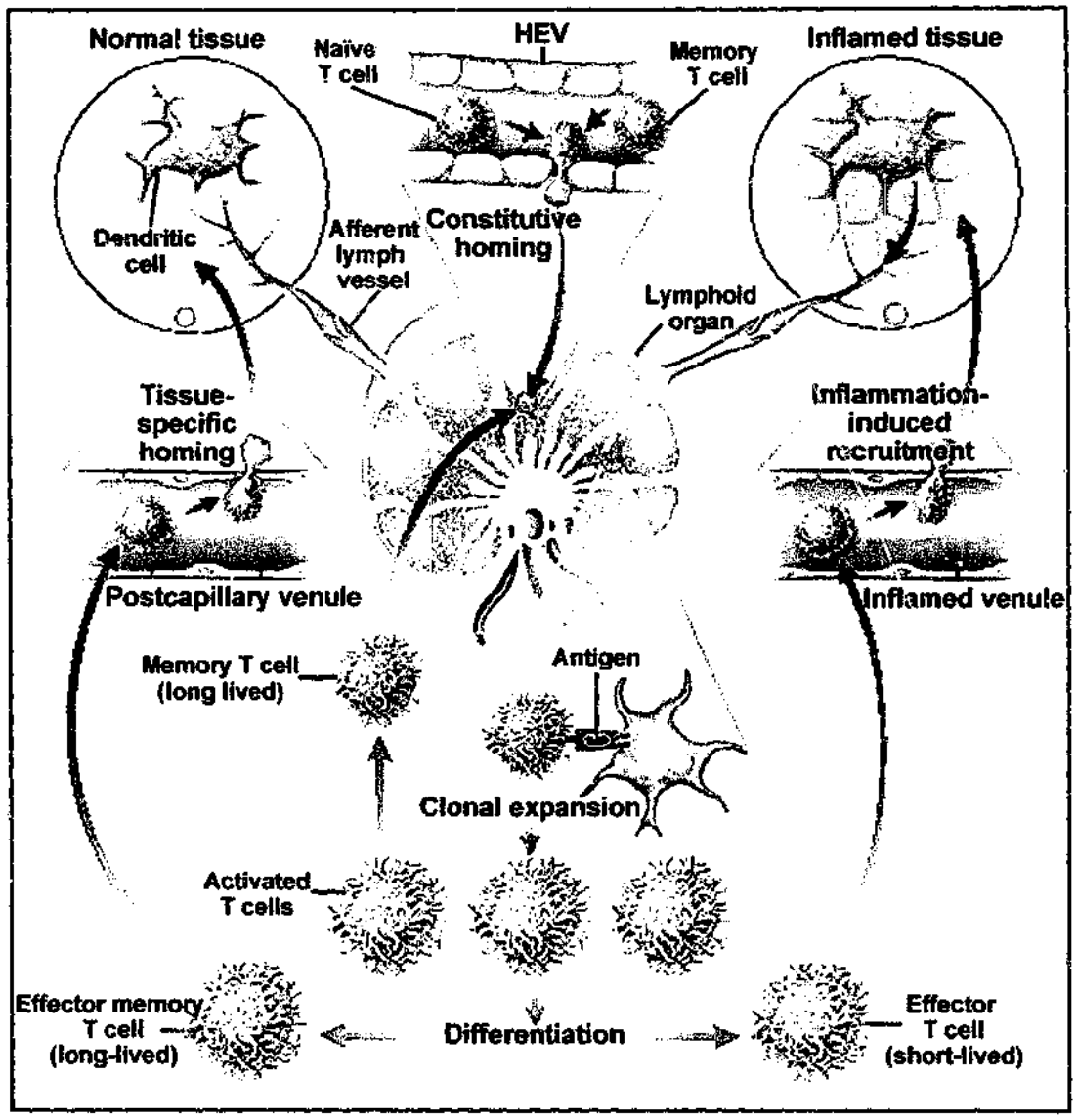


Figure 1.3 Migration of naïve and effector T cells throughout the body.
Naïve T cells home from the blood to lymph nodes and other secondary lymphoid tissues. Homing to lymph nodes occurs in high endothelial venules (HEV). Dendritic cells in the tissues collect antigenic material. In inflamed tissues the DC are mobilised to carry antigen to the lymph nodes where they stimulate antigen-specific T cells. Activated T cells proliferate and differentiate into effector T cells which express receptors that enable them to migrate to sites of inflammation. Although most effector T cells are short lived, a few antigen experienced cells survive for a long time and are divided into two groups: the effector memory T cells that migrate to peripheral tissues and the central memory T cells that migrate preferentially to lymphoid organs. (von Andrian and Mackay, 2000)

expression by T cells can be upregulated or lost depending on the maturity and activation state of the cell thereby influencing T cell trafficking to either lymphoid organs or sites of inflammation (von Andrian and Mackay, 2000).

1.2.4.2 Adhesion molecules

As mentioned earlier, naïve T cells encounter allergen presented by DC in the lymph nodes draining the site of allergic inflammation. T cells gain access to the lymph node through the binding of L-selectin (CD62L) to sulphated carbohydrates on CD34, a vascular addressin, expressed by high endothelial venules. Once T cells have entered the lymph node and are activated by antigen, L-selectin is shed from the cell surface and expression of integrins including the very late activation antigen-4 (VLA-4; CD49d) and lymphocyte function-associated antigen-1 (LFA-1) is upregulated (Chao *et al.*, 1997; Hamann *et al.*, 2000). VLA-4 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 (Figure 1.4). This change in adhesion molecule expression results in increased recruitment to peripheral tissues such as the lung (Hamann *et al.*, 2000). T cell expression of CD62L and CD49d following allergen treatment *in vitro* will be examined in this thesis to determine how allergen stimulation can alter adhesion molecule expression and thus affect the ability of T cells to traffic to sites of allergic inflammation.

1.2.4.3 Chemokine receptors

In humans, 40 chemokines and 18 chemokine receptors have been identified (von Andrian and Mackay, 2000; Zlotnik and Yoshie, 2000). Chemokines are divided into four subfamilies including CC chemokines that have the first two cysteines adjacent,

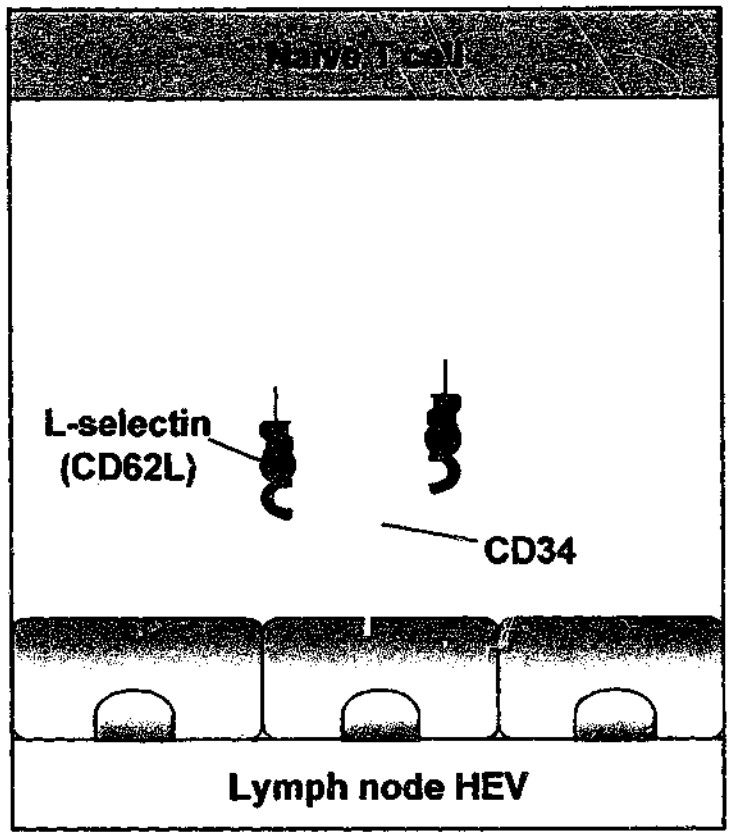


Figure 1.4 Effector T cells change their surface expression of adhesion molecules in order to home to peripheral sites of inflammation.
Naïve T cells home to lymph nodes through L-selectin (CD62L) binding to sulphated carbohydrates displayed by CD34 (upper panel). Upon antigen encounter T cells differentiate into effector cells, lose expression of CD62L and leave the lymph node. Effector T cells express VLA-4 (CD49d) and LFA-1 which bind to VCAM-1 and ICAM-1 respectively on peripheral vascular endothelium at sites of inflammation (lower panel). This figure was adapted from Janeway and Travers, 1997.

CXC chemokines that have the first two cysteines separated by one amino acid and XC and CX_3C chemokines (von Andrian and Mackay, 2000). The receptors for these families include the CCRs, the CXCRs and for the other two families each group has only one receptor (CX_3CR1 and XCR1).

T cells express different patterns of chemokine receptors depending on their differentiation and activation state thereby allowing for selective homing to different locations within the periphery (Table 1.2). For example CCR3 expression has been demonstrated on a subset of Th2-type cells (Sallusto *et al.*, 1997; Bonecchi *et al.*, 1998; Sallusto *et al.*, 1998a; Annunziato *et al.*, 1999) and expression of CCR5 largely on Th1-type cells (Odum *et al.*, 1999; Yamamoto *et al.*, 2000), although some studies have reported expression also by Th2-type cells (Sallusto *et al.*, 1998a; Nanki and Lipsky, 2000). In this way, T cells with different effector functions home to different sites of inflammation (O'Garra *et al.*, 1998; Sallusto *et al.*, 1998b), thereby altering the final outcome of the immune response.

With respect to human allergic inflammation, the involvement of a number of chemokines and chemokine receptors has been demonstrated. In the bronchoalveolar lavage fluid from atopic asthmatics, increased eotaxin, RANTES and MIP-1α production has been observed after allergen challenge (Cruikshank *et al.*, 1995; Holgate *et al.*, 1997; Lilly *et al.*, 2001). As RANTES and MIP-1α are the ligands for the receptor CCR5, and CCR5 is expressed by a population of resident T cells in the asthmatic lung, CCR5 may play a role in T cell recruitment to the lung (Campbell *et al.*, 2001). Expression of the chemokine receptor CCR3 and its ligand eotaxin are also

Table 1.2 Role of chemokine receptors and their ligands in T cell migration.

Biologic activity	Chemokine receptor[1]	Predominant chemokine agonists[2]
Migration of naïve T cells to lymph nodes and Peyer's patches	CCR7	SLC, MIP-3β
Migration of naïve T cells within lymphoid tissues	CXCR4	SDF-1α
Migration of central memory T cells to lymphoid tisses	CCR7	SLC, MIP-3β
Migration of memory T cells to the skin	CCR4	TARC, MDC-1
Migration of memory T cells to the gut	CCR9	TECK
Migration of memory cells to sites of inflammation	CCR2	MCP-1, -3, -4
	CCR5	RANTES, MIP-1α, -1β
Migration of effector T cells (Th1-type)	CCR2	MCP-1, -3, -4
	CCR5	RANTES, MIP-1α, -1β
	CXCR3	IP-10, Mig, I-TAC
Migration of effector T cells (Th2-type)	CCR3	Eotaxin-1, -2, -3; RANTES; MCP-2, -3, -4; HCC-2
	CCR4	TARC, MDC-1
	CCR8	I-309
	CXCR4	SDF-1α

[1]CCR denotes receptor for CC chemokine, CXCR receptor for CXC chemokine.

[2]Abbreviations for chemokines listed in the above table:
SLC: Secondary lymphoid-tissue chemokine
MIP: Macrophage inflammatory protein
SDF: Stroma-derived factor
TARC: Thymus and activation-regulated chemokine
MDC: Macrophage-derived chemokine
TECK: Thymus-expressed chemokine
MCP: Macrophage chemotatic protein
RANTES: Regulated on activation normal T cell expressed and secreted
IP-10: Inducible protein of 10 Kd
Mig: Monokine induced by interferon-γ
I-TAC Interferon-inducible T cell alpha chemoattractant
HCC: Human CC chemokine

This table was adapted from von Andrian and Mackay, 2000.

elevated in bronchial biopsies in atopic asthmatics in comparison to non-atopic controls (Ying *et al.*, 1997; Ying *et al.*, 1999). Ying and colleagues (1997) demonstrated that although the majority of CCR3 mRNA co-localised with eosinophils in the bronchial mucosa, a small population of $CD3^+$ T cells was positive for CCR3 mRNA. In a mouse model of allergic airway disease, polarised Th1 or Th2 cells were transferred into naïve mice (Lloyd *et al.*, 2000). These polarised Th2 cells expressed CCR3. After aeroallergen challenge, the murine lung tissue was examined for CCR3 expression by immunohistochemical staining. Increased expression of CCR3 in the lung was observed in recipients of polarised Th2 cells in comparison to those receiving Th1 cells. CCR3 expression co-localised with Th2 cells. The blocking of CCR3 resulted in reduced Th2 cell recruitment to the lung.

More recently, the role of CCR4 in T cell migration in allergy was investigated. For example, Panina-Bordignon and colleagues (2091) have shown increased numbers of T cells expressing the CCR4 protein after allergen challenge in bronchial biopsies from atopic asthmatics. A subset of these $CCR4^+$ T cells was positive for CCR8. In the same study increased production of the ligands for CCR4, i.e. TARC and MDC, by airway epithelial cells was identified providing further evidence for the involvement of CCR4 in the recruitment of T cells to the lung in allergic inflammation. These studies indicate that many chemokines and chemokine receptors are required for T cell migration to sites of allergic inflammation and could be potential targets for therapeutic intervention. T cell chemokine receptor expression following *in vitro* allergen challenge is examined in this thesis. The cytokine phenotype of T cells expressing chemokine receptors will be analysed to determine if different allergen stimulation protocols can result in differential chemokine receptor expression by allergen-specific Th1- and Th2-type cells.

1.2.5 T Cell Death

Within the body, apoptosis maintains peripheral T cell homeostasis and tolerance by counteracting the immense changes in T cell number and type during an immune response to antigen (Lenardo *et al.*, 1999). Two mechanisms for T cell death include antigen-induced (active) apoptosis and growth factor deprivation leading to passive apoptosis (Figure 1.5). The classic features of apoptosis include chromatin condensation, DNA fragmentation, cell shrinkage, plasma membrane blebbing and exposure of phosphatidylserine on the outer leaflet of the plasma membrane to facilitate phagocytosis (Janssen *et al.*, 2000b; Kagan *et al.*, 2003).

Activation-induced cell death (AICD) regulates antigen-specific T cell proliferation caused by continuous or repeated antigen stimulation. It is mediated by the binding of death factors including Fas ligand (FasL) and tumor necrosis factor (TNF) to their respective receptors, Fas and TNF receptor (TNFR). Expression of Fas and TNFR by T cells is induced by TCR-MHC-peptide interactions and thus TCR ligation is required indirectly for active apoptosis (Lenardo *et al.*, 1999). Ligation of Fas on a T cell by FasL results in the trimerisation of Fas on the surface of the cell. This brings together 'death domains' in the cytoplasmic tails of Fas that can then interact with an adapter protein called Fas-associated death domain (FADD). FADD then binds to a second 'death domain' in the apoptosis-initiating protease caspase-8. This activates caspase-8, which cleaves caspase-3 leading to a protease cascade (Muzio *et al.*, 1997). At the end of the caspase pathway, a caspase-activated DNase enters the nucleus of the cell and cleaves DNA resulting in apoptosis of the cell.

Passive apoptosis is mainly involved in the elimination of excess antigen-specific T

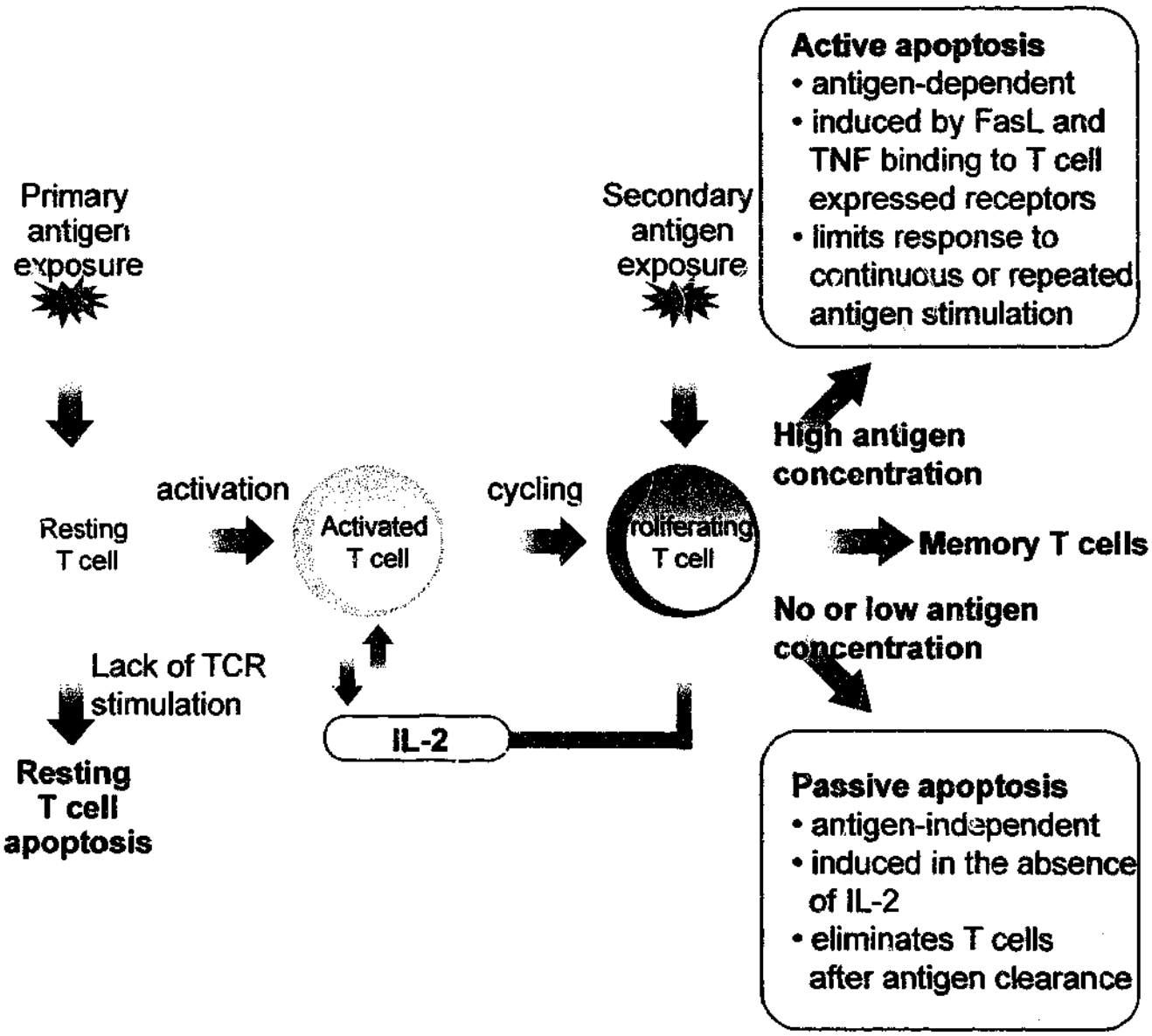


Figure 1.5 Pathways of active and passive T cell apoptosis.
In the presence of antigen resting T cells are activated, and begin to proliferate and produce IL-2. T cells cycling between activation and proliferation become susceptible to apoptosis and whether cell death ensues depends on the environmental conditions. Active apoptosis occurs if strong secondary TCR engagement is encountered. It can be induced by the interaction of FasL and/or TNF with surface receptors Fas and TNFR on T cells. Expression of Fas and TNFR is upregulated on T cells upon TCR signalling. Passive apoptosis occurs after cessation of antigen and IL-2 stimulation. A small number of cells escape both death pathways and these are believed to become the memory T cell population. This figure was adapted from Lenardo *et al.*, 1999.

cells after antigen clearance at the end of an immune response. In contrast to active apoptosis, this mechanism is antigen-independent and results from a lack of IL-2 required for T cell growth. The cellular processes for passive apoptosis are not fully defined, however it is believed to be caused by direct cytoplasmic activation of caspases, possibly due to mitochondrial damage (Lenardo *et al.*, 1999). There is no involvement of death factors or their receptors.

In this project apoptosis of allergen-specific T cells is analysed under different *in vitro* allergen stimulation protocols. This will be performed to determine whether pathogenic allergen-specific Th2-type cell responses can be down-regulated by the preferential induction of Th2-type cell apoptosis.

1.3 FACTORS ALTERING $CD4^+$ T CELL DIFFERENTIATION

$CD4^+$ T cell differentiation is a complex event and is dependent on many factors including the cytokine milieu, antigen concentration and form, APC-type, costimulation and regulatory cell function. The influence these factors have on Th cell differentiation and how they could be exploited to switch off allergen-specific Th2-type cell responses are discussed below.

1.3.1 Cytokine Milieu

Differentiation of naïve $CD4^+$ T cells to either Th1- or Th2-type effector cells is dependent in part on the cytokines present in the milieu surrounding the developing T cell. The priming of human $CD4^+$ T cells with antigen in the presence of IL-4 leads to the development of Th2-type cells (Maggi *et al.*, 1992; Palmer and van Seventer, 1997)

whereas IL-12 promotes Th1-type differentiation (Heufler *et al.*, 1996; Palmer and van Seventer, 1997). IFN-γ has been shown to enhance Th1-type differentiation by promoting increased IL-12 production by APC (Ma *et al.*, 1996) and inducing the up-regulation of the high affinity IL-12 receptor on responding T cells (Gollob *et al.*, 1997). Interestingly a recent report has also shown that IFN-γ can directly repress IL-4 expression in the human system, thereby inhibiting IL-4-induced Th2-type differentiation as previously shown in murine studies (Elser *et al.*, 2002). In contrast, IL-4 can decrease Th1-type differentiation by impairing T cell responsiveness to IL-12 through the down-regulation of the high affinity IL-12 receptor (Gollob *et al.*, 1997). Thus particular cytokine environments can either enhance or inhibit Th1- or Th2-type differentiation.

Other cytokines in the milieu can have inhibitory effects on the differentiation of both Th1- and Th2-type cells. For example, the cytokine IL-10 has an important regulatory role in human T cell differentiation and activation. IL-10 has been shown to reduce antigen-induced proliferation of human Th1 and Th2 clones (Del Prete *et al.*, 1993. In addition, production of IFN-γ by Th1 clones and of IL-4 and IL-5 by Th2 clones was also inhibited by IL-10 (Del Prete *et al.*, 1993. In the murine system IL-10 can inhibit IL-12 production by DC (Koch *et al.*, 1996). Hence IL-10 can alter the cytokine milieu by inhibiting the production of cytokines, which are important for naïve T cell differentiation, by effector T cells and APC.

1.3.2 Antigen Concentration

The concentration of antigen administered is well known to influence the degree and

type of immune response. Several studies have shown that in the case of soluble antigens, Th2-polarised responses are generated by priming with low concentrations of antigen but Th1-polarised responses result by priming higher antigen concentrations (Constant and Bottomly, 1997). However, few studies have examined the effect of antigen concentration on Th cell cytokine responses to allergens. In an *in vitro* study, Secrist and colleagues (1995) cultured freshly isolated atopic donor $CD4^+$ T cells with different concentrations of rye grass pollen (0.01-10 μg/ml) or HDM (0.02-20 μg/ml), and observed a dramatic decrease in IL-4 production at higher allergen concentrations. Similarly in an *in vivo* model, when mice were sensitised with 10 μg/ml or 1000 μg/ml ovalbumin (OVA), IL-4 and IL-5, but not IFN-γ, were detected in the bronchoalveolar lavage fluid of low concentration immunised mice, whereas IFN-γ was detected in the fluid of high concentration immunised mice (Sakai *et al.*, 1999).

Carballido and colleagues (1997) generated T cell clones (TCC) specific for phospholipase A_2 (PLA_2), the major allergen of bee venom. Analysis of cytokine production following stimulation of the TCC with different concentrations of anti-CD3, led to the conclusion that IFN-γ production by Th cells requires a higher threshold of stimulation than IL-4 production. With increased antigen concentration, an increased number of MHC-peptide complexes would be expected at the APC surface leading to multivalent cross-linking and increased TCR aggregation. From a study using a TCRαβ transgenic mouse model specific for a hemoglobin peptide, Grakoui and colleagues (1999) concluded that Th1 polarisation of both committed and naive T cells at high antigen concentrations was a result of activation of a critical number of cells to generate sufficient IFN-γ in the culture.

The differentiation of Th cells into Th1- and Th2-type can therefore be modulated by antigen concentration. Administration of high doses of allergen in SIT could cause a net shift in the allergen-specific Th cell cytokine production by a combination of the preferential expansion of IFN-γ^+ T cells, T cell anergy and deletion of $CD4^+IL\text{-}4^+$ T cells. As such a major focus of this thesis is to examine the cytokine profile of allergen-specific $CD4^+$ T cells stimulated *in vitro* with different concentrations of allergen. Changes in cytokine production will be examined at the single cell level using flow cytometry. In addition, T cell division and apoptosis will be investigated as potential factors involved in altering T cell cytokine production at different concentrations of allergen. If high allergen dose treatment can down-regulate allergen-specific Th2-type cell responses, the use of non-IgE binding but T cell reactive allergen preparations such as peptides and modified allergens will enable higher dose administration and thus more effective and safer allergen-SIT than currently possible with unfractionated extracts.

1.3.3 Antigen Form

The form of an antigen itself can also affect Th cell differentiation. In addition, by modifying antigen structure, the function of the molecule may be altered with consequent change in the immune response. Interestingly a large number of clinically relevant allergens are biologically active enzymes with the best analysed example being Der p 1. Der p 1 is a cysteine protease that is involved in mite digestion. It is suggested that this enzyme function plays a role in mucosal epithelium penetration by disrupting tight junctions (Wan *et al.*, 1999), and it can promote Th2-type responses by its effects on immune cells. Enzymatically active Der p 1 reduces DC IL-12 production by cleaving CD40 (Ghaemmaghami *et al.*, 2002) and stimulates mast cell and basophil IL-

4 production (Machado *et al.*, 1996) providing the cytokine milieu for Th2 differentiation. It also cleaves the α subunit of the IL-2 receptor (IL-2R, CD25) causing a dramatic reduction in anti-CD3-induced proliferation and IFN-γ secretion (Schulz *et al.*, 1998a) but increased IL-4 production (Ghaemmaghami *et al.*, 2001) by peripheral blood T cells. The enhanced IL-4 production promotes B cell IgE production (Ghaemmaghami and Shakib, 2002). Active Der p 1 also cleaves the low affinity IgE Fc receptor (CD23) from B cells further enhancing IgE production (Hewitt *et al.*, 1995; Schulz *et al.*, 1995). A similar promotion of Th2 responses by enzymically active Der p 1 was seen in murine studies with increased specific IgE (Gough *et al.*, 1999) and decreased IFN-γ production (Comoy *et al.*, 1998). Interestingly the bee venom allergen, PLA_2, also exhibits enzyme activity and has been shown to stimulate mast cell IL-4 production (Dudler *et al.*, 1995a; Machado *et al.*, 1996).

The structural components of allergens can also influence initiation and exacerbation of allergic immune responses. For example, removal of carbohydrates from the major Japanese cedar pollen allergen, Cry j 1, by periodate oxidation, resulted in reduced proliferation and production of IL-4 and IL-5 by Cry j 1-stimulated PBMC, T cell lines (TCL) and TCC (Okano *et al.*, 2001). Specific IgE reactivity against the carbohydrate moiety has been shown for several glycosylated allergens (Batanero *et al.*, 1996; Su *et al.*, 1996) and glycosylation-dependent T cell recognition of allergens is reported (Dudler *et al.*, 1995b; Corinti *et al.*, 1997; Okano *et al.*, 2001). In fact one study suggests that $CD8^+$ T cells may recognise a glycosylated antigen from *Parietaria judaica* pollen in an MHC-independent manner (Corinti *et al.*, 1997). However for other allergens, e.g. PLA_2 (Okano *et al.*, 1999) and Lol p 11 (van Ree *et al.*, 2000),

sugar residues show little relevance to IgE binding. Thus it appears that the contribution of the carbohydrate moiety to allergenicity depends on the allergen studied, presumably due to the composition and structure of the sugar residues (van Ree *et al.*, 2000).

Importantly, allergen form can influence the mode of antigen uptake by APC and in this way may determine the APC type. As discussed above, many allergens are glycoproteins and thus uptake via mannose receptors may occur. Mannose receptor mediated uptake of antigens by DC can result in a 100-fold increase in efficiency of antigen presentation to T cells compared with macropinocytosis (engulfment of large volumes of fluid and solutes) (Engering *et al.*, 1997). Increased antigen presentation might well alter subsequent Th cell cytokine responses. Studies have shown that Der p 1 and yeast *Malassezia furfur* allergens can be taken up by APC via the mannose receptor (Buentke *et al.*, 2000; Deslee Gt *et al.*, 2002), while other allergens may be taken up by macropinocytosis (soluble allergens) or phagocytosis (particulate allergens) (Sung *et al.*, 1999; Noirey *et al.*, 2000). Whether the T cell response to allergen differs depending on mode of uptake has not been assessed. With recent insight into the pivotal role of DC activation status and cytokine profile on Th cell differentiation, these issues should be resolved for refinement of more effective allergen preparations for SIT.

IgE-facilitated allergen uptake by DC via the low affinity IgE receptor is another mechanism by which allergen form could influence Th cell response. Preincubation of Der p 2 with HDM allergic donor serum has been shown to result in proliferation of Der p 2-specific TCC at a 1000-fold lower concentration than Der p 2 without serum using EBV-transformed B cells as APC (van der Heijden *et al.*, 1993). This process was

dependent on Der p 2 specific IgE in the donor serum. Following SIT for birch pollen allergy, serum IgG was shown to inhibit IgE-mediated allergen presentation resulting in decreased proliferation and IL-4, IL-5, IL-10, and IFN-γ production by allergen-specific T cells (van Neerven *et al.*, 1999).

Similarly, the structural integrity of an allergen can affect Th cell cytokine responses. When the tertiary structure of PLA_2 was disrupted by reduction and alkylation, there was preferential stimulation of IFN-γ production in 12-day PBMC cultures by this unrefolded form compared with IL-4 for the refolded form (Akdis *et al.*, 1998a). It was found that PLA_2 in its native form was processed and presented by monocytes and B cells whereas the denatured form of PLA_2 was processed and presented only by monocytes. There was pronounced IL-12 production by monocytes as APC but undetectable levels for B cells, thus accounting for the observed differences in Th cell cytokine responses to the two PLA_2 forms.

In addition to being able to divert Th cytokine profiles towards Th1, modified forms of allergens offer the potential for safer SIT if this alteration removes IgE reactivity. An alternative strategy is to use short peptides based on immunodominant T cell epitopes. Both approaches have been tested and are under current evaluation in model systems or clinical trials as will be discussed later.

1.3.4 Antigen-Presenting Cell Type

The APC can play a pivotal role in determining the T cell response to an antigen. Within the respiratory epithelium there is a contiguous network of DC which functions

as the 'first line of defence' in surveillance for inhaled antigens (Holt *et al.*, 1990). In the periphery, DC are generally in an immature state, specialised for antigen uptake. Interestingly, studies have shown that the state of maturity and location of the DC that presents antigen can influence Th cell differentiation. Stumbles *et al.* showed that when functionally immature rat respiratory tract DC were pulsed with OVA and adoptively transferred to naïve rats, splenic Th cell IL-4 production in response to subsequent OVA challenge was promoted, whereas DC matured by exposure to GM-CSF as APC induced increased T cell production of IFN-γ and undetectable IL-4 (Stumbles *et al.*, 1998). These cytokine differences were associated with altered production of IgG subclasses, with higher levels of IgG_1 OVA-specific antibodies in the case of immature DC and production of OVA-specific antibodies of all IgG subclasses with mature DC. In agreement with these results, Constant and colleagues showed strong IL-5 responses and little IFN-γ production in antigen-stimulated lung tissue cell cultures from mice immunised intranasally, whereas subcutaneous antigen delivery promoted preferential IFN-γ production (Constant *et al.*, 2002). Resident lung APC mediating T cell responses exhibited an immature DC surface marker phenotype. Codelivery of lipopolysaccharide with intranasal antigen resulted in a downregulation of IL-5 and IL-13 production with a slight increase in IFN-γ production resulting in a switch in net cytokine balance from Th2- to Th0-type. There was also an up-regulation of IL-12 mRNA. The results from both these studies indicate that under steady-state conditions in the respiratory tract the default pathway is Th2 biased and additional signals from the microenvironment are required by the DC to induce Th1 responses.

Several subsets of DC have been described and may differentially regulate Th cell

differentiation. Rissoan suggested that myeloid-derived DC, designated DC1, induce Th1 cell differentiation through the production of high levels of IL-12 whereas lymphoid DC, designated DC2, produce low levels of IL-12 and induce Th2 cell differentiation (Rissoan *et al.*, 1999). Human myeloid DC can be further subdivided based on CD1a expression. $CD1a^+$ monocyte-derived DC induced Th1 differentiation whereas $CD1a^-$ DC promoted Th2 differentiation (Chang *et al.*, 2000). The $CD1a^-$ DC lacked IL-12 production even after treatment with the normally Th1-provoking agents anti-CD40, lipopolysaccharide and IFN-γ.

Although DC are the most efficient APC for naïve T cells, B cells and monocytes may present allergen to T cells *in vivo*. Antigen conformation and concentration can determine which APC will take up an antigen, process and present it to T cells. As discussed previously, B cells and monocytes present different conformations of PLA_2 and consequently induce different Th cell cytokine profiles (Akdis *et al.*, 1998a). At low antigen concentration, antigen-specific B cells via their surface immunoglobulin antigen receptors are more efficient at antigen uptake than DC or monocytes by phagocytosis or endocytosis (Malynn *et al.*, 1985; Secrist *et al.*, 1995). Thus B cells may play a role in allergen uptake *in vivo* upon natural exposure. Human and murine studies have shown that B cells as APC preferentially induce Th2 cell differentiation (DeKruyff *et al.*, 1992; Secrist *et al.*, 1995; Macaulay *et al.*, 1997; Akdis *et al.*, 1998a). In comparison, antigen presentation by monocytes has been shown to induce Th1 cytokine production preferentially (Secrist *et al.*, 1995; Macaulay *et al.*, 1997; Akdis *et al.*, 1998a).

The mechanisms responsible for B cells and monocytes driving different Th cell differentiation are not fully understood but are likely to involve differential expression of cytokines and costimulatory molecules by the APC upon interaction with the T cell. Akdis and colleagues (1998a) showed that monocyte induced-Th1 differentiation correlated with high levels of IL-12 production whereas B cell induced-Th2 differentiation correlated with the inability of B cells to produce IL-12. Furthermore Skok and colleagues reported that OVA TCR transgenic T cells in spleen cell preparations showed increased numbers of IL-4 producing cells and decreased IFN-γ positive cells when stimulated by preactivated rather than resting B cells (Skok *et al.*, 1999). This response was blocked by addition of anti-IL-6 and anti-IL-10 monoclonal antibodies (mAb). Interestingly, the ability of the B cells to induce Th2 cell differentiation through the production of IL-6 and IL-10 was dependent on contact with IL-12 producing DC, suggesting a feedback loop. As discussed in the next section the expression of CD86 by an APC plays an important role in driving development of Th2 responses. B cells cultured with anti-CD86 mAb are unable to stimulate IL-4 production by antigen-specific $CD4^+$ T cells (Macaulay *et al.*, 1997). In addition, B cell induced-IL-4 production by $CD4^+$ T cells is dependent on interactions between CD40 on B cells and CD40L on T cells (Macaulay *et al.*, 1997; Skok *et al.*, 1999). These studies indicate that although different types of APC induce differential cytokine production from Th cells, there is a level of regulation of APC function that can determine the final outcome of Th cell differentiation.

1.3.5 Costimulation

In addition to specific engagement of MHC-peptide complexes with TCR, interactions

between costimulatory molecules on APC and Th cells are essential for Th cell activation. Of interest for control of allergic diseases are reports that show that the engagement of different costimulatory molecules during T cell activation can influence Th cell differentiation.

The importance of B7/CD28 interactions has been studied in a number of murine models of airway hyperresponsiveness. Allergen-sensitised mice treated with CTLA-4Ig, a fusion protein that binds to CD28 and blocks the binding of CD80 and CD86, have reduced levels of airway hyperresponsiveness, decreased eosinophil recruitment, reduction in Th2 cytokine production (IL-4, IL-5 and IL-13) and reduced levels of allergen-specific IgE (Krinzman *et al.*, 1996; Keane-Myers *et al.*, 1997; Tsuyuki *et al.*, 1997; Kasai *et al.*, 1998; Burr *et al.*, 2001). Similar results have been obtained in CD28 knockout mice (Mark *et al.*, 1998). Tsuyuki and colleagues found that blocking CD86 and not CD80 using mAbs gave comparable results to those seen using CTLA-4Ig (Tsuyuki *et al.*, 1997). These results suggested that CD86/CD28 interactions were essential for the development of Th2 responses. Interestingly Harris and colleagues showed that blockage of CD80 by Y100F-Ig, a CTLA4-Ig mutant that does not bind CD86, caused a reduction in eosinophil recruitment to the lungs but did not affect blood eosinophilia or the levels of Th2-dependent antibody (Harris *et al.*, 1997). In a more recent study by this group, Y100F-Ig was found to diminish Th cell IL-4 and IL-5 production after intranasal challenge with antigen but not their ability to migrate and accumulate in the lung (Harris *et al.*, 2001). Hence these T cells could traffic to the lungs upon challenge but once there they could not produce Th2 cytokines to induce eosinophil recruitment and antibody production.

The role of CD80 and CD86 costimulation in human allergic disease has also been studied. Reductions in allergen-specific proliferation and IL-5 and IL-13 production by PBMC from atopic individuals cultured with HDM extract in the presence of CTLA-4Ig and anti-CD86 mAb, but not anti-CD80 mAb, have been observed (Larche *et al.*, 1998; Van Neerven *et al.*, 1998; Jaffar *et al.*, 1999). Allergen-induced proliferation of bronchoalveolar lavage T cells was also inhibited by CD86 mAb and not CD80 mAb (Larche *et al.*, 1998), but secretion of IL-5 and IL-13 by allergen-stimulated asthmatic bronchial tissue was inhibited by both CD80 and CD86 mAbs (Jaffar *et al.*, 1999). Hence it seems that both CD80 and CD86 costimulation may be important in the development of the allergen-specific Th2-type response in humans although CD86 is the principal costimulatory molecule in PBMC cultures.

Another member of the CD28 family that can also influence Th cell differentiation is ICOS (inducible T-cell costimulator). ICOS is expressed by T cells rapidly after TCR stimulation and interacts with ICOS ligand (ICOSL) on resting B cells, monocytes and some dendritic cells (Sharpe and Freeman, 2002). Interestingly, ICOS is up-regulated on both Th1 and Th2 cells during the initial phase of differentiation but is then down-regulated on Th1 cells while remaining high on Th2 cells (Sharpe and Freeman, 2002). In murine studies of allergic airway disease, ICOS/ICOSL interactions play an important role in the regulation of effector Th2 cell responses (Gonzalo *et al.*, 2001; Tesciuba *et al.*, 2001). Treatment of mice with ICOS-Ig, to block ICOS costimulation, during sensitisation and challenge resulted in reduced airway inflammation with decreased numbers of eosinophils and lymphocytes in the lung tissue (Tesciuba *et al.*, 2001). In this model ICOS blockade did not prevent Th2 cell development, but greatly reduced effector Th2 cell cytokine production. Thus as ICOS costimulation is involved

in sustaining Th2 responses during allergic responses, blockade of ICOS may be a useful therapeutic for allergic disease in humans.

Th cell differentiation may be affected by other costimulatory molecule interactions. Two recent reports have demonstrated that LFA-1/ICAM-1 and LFA-1/ICAM-2 interactions inhibit IL-4 and IL-5 production by naïve $CD4^+$ T cells (Salomon and Bluestone, 1998; Luksch *et al.*, 1999). Blocking either LFA-1/ICAM-1 or LFA-1/ICAM-2 with mAbs resulted in a 15- to 40-fold increase in Th2 cytokine production and blocking both ICAM-1 and ICAM-2 resulted in a 100- to 1000-fold increase (Salomon and Bluestone, 1998). Interactions between OX40L (a member of the tumor necrosis factor superfamily) on APC and OX40 on T cells also differentially affects IL-4 and IFN-γ production (Flynn *et al.*, 1998; Ohshima *et al.*, 1998). Stimulation of naïve $CD4^+$ T cells with anti-OX40 mAb induced preferential IL-4 production and inhibited IL-12-induced IFN-γ production from $CD4^+$ T cells. The above studies suggest that costimulatory molecule interaction with CD28 or OX40 on an allergen-stimulated Th cell promotes Th2 differentiation and suppresses Th1 differentiation, whereas interaction with LFA-1 supports Th1 responses and suppresses Th2 responses. However, recent reports demonstrate that these relationships may be tempered by antigen concentration effects. Rogers and colleagues reported that the relationship between LFA-1 engagement and Th1 cell differentiation only occurred over a certain peptide concentration range and concluded that ligation of cell surface accessory receptors enables low concentrations of antigen to promote responses normally induced only by higher concentrations (Rogers and Croft, 2000). Th cell differentiation is thus controlled by both the level of expression of multiple accessory molecule pairs and the

number and affinity of peptide/MHC complexes. Thus in the design of new treatment strategies for allergic disease, costimulatory molecules should be seen as important regulatory switches that underlie the effects of antigen concentration and form on modulation of Th cell response.

1.3.6 Regulatory Cells

The concept of a regulatory T cell subset controlling normal and adverse immune responses has moved from controversy to dogma over the last two decades. Several regulatory cell populations are now described. In the field of allergy research, Rocklin *et al.* were the first to describe the generation of an antigen-specific "suppressor cell" during desensitisation with ragweed pollen (Rocklin *et al.*, 1980). This was followed by other reports implicating regulatory cells in the down-regulation of the immune response to allergens during SIT (Rivlin *et al.*, 1981; Hsieh *et al.*, 1987; Tilmant *et al.*, 1989). The first clear reports of a functional regulatory subset came from animal models of allergy in which $CD8^+$ T cells were shown to act as suppressor cells by inhibiting allergen-specific IgE production (McMenamin and Holt, 1993; Renz *et al.*, 1994; Holmes *et al.*, 1997). Suppressor function was mediated by IFN-γ production. Evidence for a role of suppressor $CD8^+$ T cells in mediating clinical tolerance to allergens remains controversial (Larche, 2000).

Another population of regulatory cells that has been described in mouse and human studies is the $CD4^+CD25^+$ T cell subset (Sakaguchi *et al.*, 1995; Taams *et al.*, 2001). These cells are functionally anergic as they do not proliferate or produce IL-2 upon TCR ligation, and they can suppress the proliferation of $CD4^+$ and $CD8^+$ T cells. The

mechanism of suppression *in vitro* is considered to be cell-contact dependent, however *in vivo*, production of IL-4, IL-10 and TGF-β may play a role (McHugh and Shevach, 2002). $CD4^+CD25^-$ T cells have been shown to play a pivotal role in the prevention of organ-specific autoimmunity and allograft rejection. Bellinghausen and colleagues recently reported on the existence and function of regulatory $CD4^+CD25^+$ T cells in the allergen-specific setting (Bellinghausen *et al.*, 2003). $CD4^+CD25^+$ T cells from allergic and non-allergic donors proliferated poorly and inhibited the proliferation of $CD4^+CD25^-$ T cells in the presence of allergen-pulsed DC. These $CD4^+CD25^+$ T cells also produced significantly less IL-4, IL-5, IL-10 and IFN-γ than $CD4^+CD25^-$ T cells and could inhibit the production of IL-4 and IFN-γ but not IL-10 by $CD4^+CD25^-$ T cells. The mechanism for suppression was investigated however the blocking of IL-10, TGF-β or CTLA-4 in these cultures did not reverse the inhibition of $CD4^+CD25^-$ T cell proliferation or cytokine production. Only high concentrations of IL-2 could reverse the inhibitory effects. These results indicate that allergic individuals possess $CD4^+CD25^+$ regulatory T cells that can inhibit allergen-specific T cell responses and further research is required to determine how to activate these cells *in vivo* to down-regulate pathogenic T cell responses.

Of interest in the control of allergic disease is the $CD4^+IL\text{-}10^+$ regulatory T cell subset. This phenotype is that of the Tr1 cells reported to prevent disease in a murine model of colitis (Groux *et al.*, 1997). Blocking IL-10 in these mice completely abrogated the protection from colitis (Groux, 2001). IL-10 production from Tr1 cells also reduced Th2 cell proliferation and cytokine secretion in a murine model of immediate-hypersensitivity (Cottrez *et al.*, 2000). This study suggests that Tr1 cells may play a

fundamental role in maintaining the balance of the immune system to prevent allergic disorders. Interestingly, IL-10 is produced by $CD4^+CD25^+$ T cells within a week of commencing bee venom immunotherapy and causes specific anergy in peripheral Th cells (Akdis *et al.*, 1998b; Akdis and Blaser, 1999). More recently the induction of IL-10-producing $CD4^+CD25^+$ T cells was also reported following grass pollen immunotherapy (Francis *et al.*, 2003). However whether this population of regulatory T cells expressing $CD25^+$ represent the same cell population identified by Bellinghausen and colleagues is not clear (Bellinghausen *et al.*, 2003). Hence full characterisation of this IL-10-producing population induced by SIT is required, as preferential induction of this subset would down-regulate both Th1- and Th2-mediated pathology at sites of allergen encounter. In this project IL-10 production by allergen-specific T cells is analysed *in vitro* following stimulation with different concentrations of allergen. Moreover $CD4^+$ T cell IL-10 production following HDM immunotherapy is examined.

Thus several factors relating to allergen concentration and form and mode of delivery during SIT may influence the T cell response to allergen (summarised in part in Figure 1.6). It is these factors that should be exploited when designing improved allergy vaccines that can switch off the allergen-specific Th2-type response.

1.4 ALLERGEN-SPECIFIC IMMUNOTHERAPY

1.4.1 Modulation of the T Cell Response by SIT

Introduced in 1911 by Noon and Cantab, allergen-specific immunotherapy is currently used to induce clinical tolerance to allergen in selected individuals (Noon and Cantab, 1911). This treatment has proved successful in reducing symptoms of allergy however

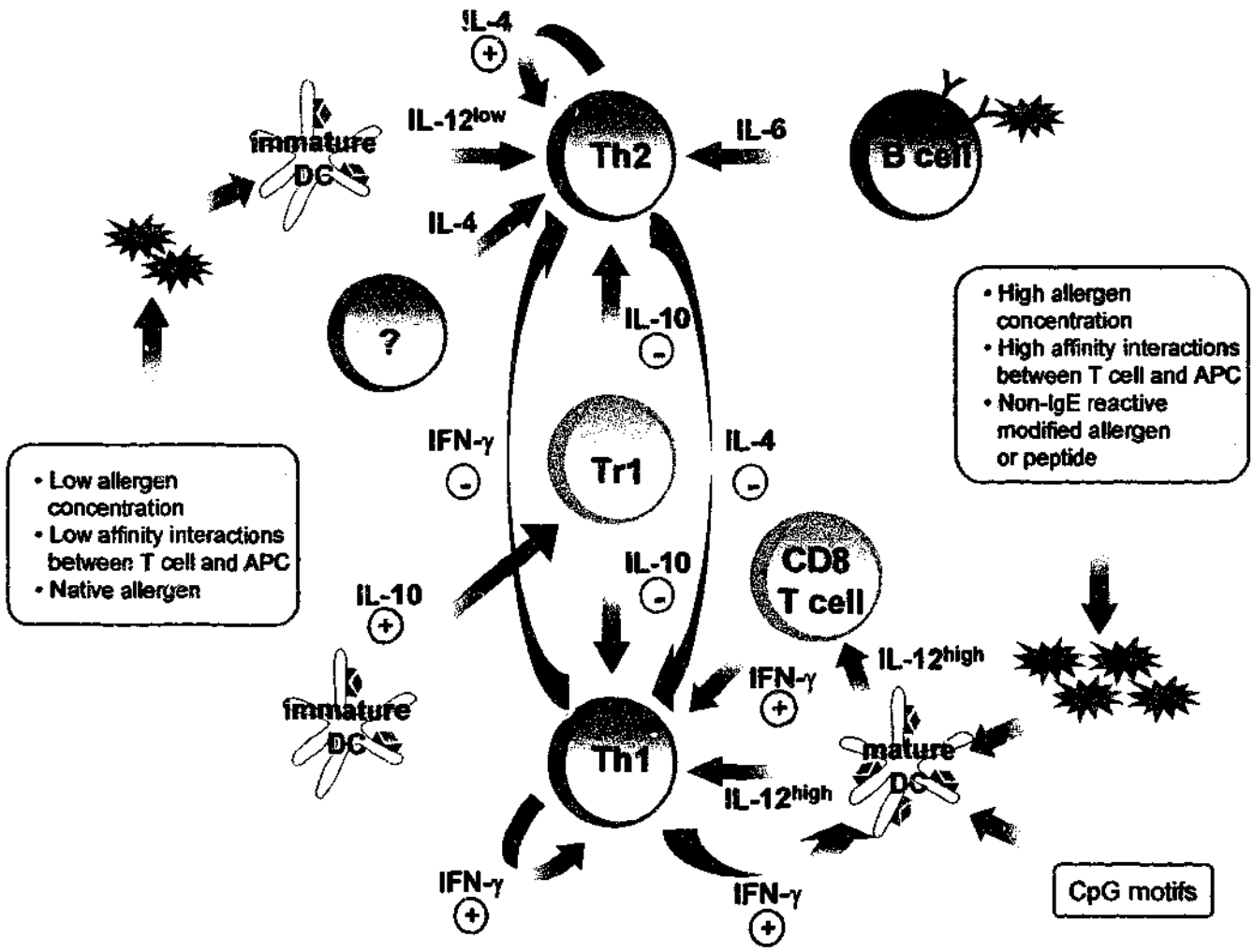


Figure 1.6 Factors that influence the T helper cell cytokine response to allergen.
The factors that can affect cytokine production by Th cells in response to allergen include those related to allergen concentration and affinity of interaction with specific receptors as well as the nature of the allergen. The cytokine milieu of a maturing T cells is also an important influence on T cell differentiation. IL-4 produced by an unknown cellular source can drive Th2-type responses. Expansion of an IL-10-producing regulatory T cell subset would down-regulate both Th2- and Th1-type inflammatory responses. Incorporation of these principles into the refinement of allergen specific immunotherapy will improve efficacy and safety of this treatment.

efficacy varies with the allergen and the risk of side effects is significant (Bousquet *et al.*, 1998b). Conventional SIT involves the administration of incremental doses of native-allergen extract until a maintenance dose it reached. This dose is continued for a period of 1-3 years until the individual becomes clinically tolerant to subsequent natural allergen exposure. Through the understanding of the underlying immune mechanisms involved in successful SIT, refinement of this treatment could lead to increased efficacy and safety. Therefore with the recognition of the important role of T cells in controlling allergic immune responses, T cell changes triggered in individuals receiving SIT has been under intensive investigation in the past ten years.

Irrespective of different immunotherapy preparations and regimens and the use of different experimental methods, SIT has been shown to consistently alter T cell cytokine production (Table 1.3). Analysis of Th2-type cytokine production from cultured cells obtained from peripheral blood of patients undergoing SIT, has demonstrated decreased production of IL-4 and where studied IL-5 (see Table 1.3). However in two *in vitro* studies analysing cytokine changes in grass pollen immunotherapy, Th2-type cytokine production remained unchanged (Wachholz *et al.*, 2002; Francis *et al.*, 2003). Analysis of Th1-type cytokines has revealed that IFN-γ production tends to be more variable with post-SIT production being increased, decreased or unchanged (see Table 1.3). Despite this inconsistency, successful SIT generally correlates with decreased IL-4:IFN-γ ratios with respect to cytokine production by circulating allergen-specific T cells (Secrist *et al.*, 1993; Jutel *et al.*, 1995a; McHugh *et al.*, 1995; Akoum *et al.*, 1996; Bellinghausen *et al.*, 1997; Ebner *et al.*, 1997; Kammerer *et al.*, 1997; Giannarini and Maggi, 1998; Oda *et al.*, 1998; van

Table 1.3 Summary of changes in allergen-specific T cell cytokine production and proliferation following allergen-specific immunotherapy.

Allergen	SIT regimen	Analysed cells	Cytokine Assay	Proliferation	IL-4	IFN-γ	IL-5	IL-2	IL-10	IL-12	IL-13	TGF-β	TNF-α	Author
Bee venom	Rush	TCL	ELISA	D	D	I	D	...	...	...	...	...	...	Jutel *et al*., 1995a
Bee venom	Rush	PBMC	ELISA	D	D	I	...	...	...	...	...	...	...	McHugh *et al*., 1995
Bee venom	Conventional	PBMC	ELISA	D	D	I (2-6 mths) D (>6 mths)	...	...	...	...	...	...	...	McHugh *et al*., 1995
Bee venom	Rush	Enriched T cells	ISH	D	D	I	...	...	...	...	...	...	...	Akoum *et al*., 1996
Bee venom	Rush	PBMC	ELISA	D	D	D	D	D	...	...	D	...	...	Akdis *et al*., 1996b
Bee venom	Conventional	Enriched CD4 + TCL	ELISA	Initial I then D	D (NS)	I	...	...	...	...	...	...	...	Kammerer *et al*., 1997
Bee venom	Rush	PBMC	ELISA	D	D	D	D	...	I	...	D	...	...	Akdis *et al*., 1998
Wasp/bee venom	Rush	PBMC	ELISA	D	D	I	...	...	I	...	...	...	...	Bellinghausen *et al*., 1997
Grass	Conventional	PBMC	...	D	...	...	...	...	...	...	...	...	...	Baskar *et al*., 1997
Grass	Conventional	PBMC/TCC	ELISA	D	D	NC	...	...	...	...	...	...	...	Ebner *et al*., 1997
Grass	Conventional	PBMC	ELISA	D	D	D (NS)	...	...	...	...	...	...	...	Giannarini and Maggi, 1998
Grass	Conventional	TCL	ELISA	I (4 mths)	D (1 mth) I (>4 mths)	I	D (2 wk) I (4 mths)	...	D (1 mth) I (>4 mths)	...	...	...	...	Moverare *et al*., 2000
Grass	Conventional	PBMC	ELISA	NC	...	NC	...	...	...	...	...	...	...	Wachholz *et al*., 2002
Grass	Conventional	TCL	ELISA	D	...	NP	NP	...	...	...	...	...	...	Eusebius *et al*., 2002
Grass	Conventional	PBMC	ELISA	NC	NC	NC	NC	...	I	...	NC	U	...	Francis *et al*., 2003
HDM/Grass	Conventional	TCL	ELISA	D	D	NC	...	NC	...	...	...	...	...	Secrist *et al*., 1993
HDM/Grass	Conventional	TCL	ELISA	...	D	NP	...	...	...	...	...	...	...	Benjaponpitak *et al*., 1999
HDM	Rush	PBMC	ICS	D	...	I	...	...	...	...	...	...	...	Lack *et al*., 1997
HDM	Conventional	PBMC	RT-PCR	...	D	D	...	...	...	...	...	...	...	O'Brien *et al*., 1997
HDM	Semi-rush	PBMC	ELISA	...	D	NC	D	D	...	NC	...	...	...	van Bever *et al*., 1998
HDM	Rush	PBMC/TCL/TCC	ELISA	D (3 mths) I (18 mths)	D	I	D	...	...	...	...	...	...	Oda *et al*., 1998
HDM	Conventional	PBMC	ELISA	...	...	D	D	...	...	...	...	...	D	Tanaka *et al*., 1998
HDM	Conventional	PBMC	ICS	...	D	I (3 mths) NC (12 mths)	...	...	...	...	...	...	...	Majori *et al*., 2000
HDM	Semi-rush	PBMC	ELISA	D	...	D	D	...	I	...	D	I	...	Jutel *et al*., 2003
Cat	Conventional	PBMC	ELISA	...	...	NC	D	...	...	NC	...	...	...	Meissner *et al*., 1999
Wasp venom	Conventional or rush	Skin biopsy (allergen-challenged)	ISH	...	D	NC	...	...	I	NC	NC	NC	...	Nasser *et al*., 2001
Grass	Conventional	Skin biopsy (allergen-challenged)	ISH	...	NC	I	NC	I	...	...	...	...	...	Varney *et al*., 1993
Grass	Conventional	Nasal biopsy (allergen-challenged)	ISH	...	NC	I	NC	NC	...	...	...	...	...	Durham *et al*., 1996
Grass	Conventional	Nasal biopsy (pollen season)	ISH	...	...	I	NC	...	...	...	...	...	...	Wachholz *et al*., 2002

NC, no change; D, decrease; I, increase; NS, not significant; U, undetectable; ICS, intracellular cytokine staining; ISH, In situ hybridisation; ..., not tested; NP, experiments were performed but the results were not published.

Bever *et al.*, 1998; Benjaponpitak *et al.*, 1999; Majori *et al.*, 2000; Eusebius *et al.*, 2002). Furthermore, this shift in cytokine production has been identified in peripheral tissues with increased IFN-γ $mRNA^+$ cell numbers being observed in nasal and skin biopsies following conventional grass pollen SIT (Varney *et al.*, 1993; Durham *et al.*, 1996; Wachholz *et al.*, 2002). Thus generally during successful SIT there is a shift from dominant Th2-type cytokine production to dominant Th1-type cytokine production by circulating and peripheral tissue T cells. 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 (Rolland and O'Hehir, 1998).

1.4.1.1 Deletion and anergy

As high-dose antigen treatment can induce clonal deletion via apoptosis (Vandenbark *et al.*, 2000), it is possible that high allergen concentrations administered during SIT could lead to the deletion of allergen-specific T cells. In agreement with this concept, a recent report demonstrated that IL-4-producing T cells from SIT-treated atopic patients cultured with specific allergen *ex vivo* undergo increased apoptosis in comparison to IFN-γ-producing T cells (Guerra *et al.*, 2001). This resulted in an overall shift from IL-4 predominant to IFN-γ predominant cytokine production by allergen-specific T cells.

Decreased allergen-specific T cell proliferative responses following immunotherapy together with reduced cloning efficiency (Ebner *et al.*, 1997) and altered cytokine production support a role for anergy induction in modulation of allergen-specific T cell response during SIT. Anergy, defined by diminution of antigen-specific proliferative response with reversal by IL-2 (Schwartz, 2003), has been induced in allergen-specific

cloned T cells by high dose peptide treatment (O'Hehir *et al.*, 1991). Administration of bee venom immunotherapy *in vivo* resulted in decreased allergen-specific T cell proliferation that was reversed by the addition of IL-2 *in vitro* (Muller *et al.*, 1998). The exact mechanism for the induction of anergy was unclear but increased production of the immunoregulatory cytokine IL-10 is one possibility (Groux *et al.*, 1996). Ligation of the IL-10R by IL-10 inhibits tyrosine phosphorylation of CD28, which is the first step of the CD28 signalling pathway (Akdis and Blaser, 2001). Thus costimulation through CD28 is inhibited resulting in induction of anergy. Interestingly, IL-10 positive T cells in blood are increased after venom, grass and HDM SIT (Bellinghausen *et al.*, 1997; Akdis *et al.*, 1998b; Francis *et al.*, 2003; Jutel *et al.*, 2003). In addition, increased numbers of IL-10 positive cells have been demonstrated in skin tissue at sites of allergen challenge following wasp venom SIT (Nasser *et al.*, 2001). The role of IL-10 in anergy induction post-SIT was demonstrated when the addition of anti-IL-10 neutralising antibody to allergen-stimulated cultures post-SIT resulted in restoration of allergen-specific proliferation and cytokine production to pre-SIT levels (Bellinghausen *et al.*, 1997; Akdis *et al.*, 1998b; Jutel *et al.*, 2003). As mentioned earlier, recent research has provided evidence that these IL-10-producing cells generated during clinically successful SIT represent a regulatory $CD4^+CD25^+$ T cell subset (Francis *et al.*, 2003; Jutel *et al.*, 2003). By targeting the generation of these IL-10-producing T cells during SIT, through the modification of allergen preparations, the efficiency of allergen tolerance induction may be enhanced.

1.4.1.2 Immune deviation

During SIT the shift from Th2-type cytokine production to Th1-type predominant could also be the result of allergen administration itself inducing immune deviation of T cell

cytokine production from IL-4 and IL-5 to IFN-γ. SIT is associated with the administration of considerably higher doses of allergen than are encountered naturally (Tovey *et al.*, 1981; Bousquet *et al.*, 1998a). As mentioned earlier in this chapter, high antigen concentration can increase IFN-γ production and decrease IL-4 production by T cells. Thus high dose allergen administration may be promoting IFN-γ production by allergen-specific T cells. In turn this production of IFN-γ will alter the cytokine milieu so that newly differentiating allergen-specific T cells will expand to also become IFN-γ-producers.

It is possible that immune deviation, anergy and deletion are all involved in the induction of clinical tolerance in allergic individuals during SIT. Each of these mechanisms could contribute to changes in allergen-specific T cell cytokine production, with decreased IL-4:IFN-γ ratios. The reduction in allergen-specific T cell proliferative responses could be due to anergy, deletion or both. Through the activation of regulatory T cells that produce IL-10, proliferation and cytokine production could be further reduced. Thus it is these mechanisms that require full investigation in order to devise new strategies for improved SIT.

1.4.2 New Approaches to SIT

1.4.2.1 Peptide immunotherapy

As clinically effective SIT is strongly associated with altered T cell responses to allergen, preparations that specifically target the allergen-specific T cell are currently being identified and evaluated. T cell epitope mapping of allergens is crucial for the design of these preparations. Although algorithms have been devised to predict T cell

epitopes in a molecular sequence, these are not reliable and for complete analysis of the T cell repertoire of the allergic population, functional T cell assays are required. For this, atopic donor PBMC or allergen-specific short-term TCL and/or TCC are cultured with overlapping synthetic peptides, usually 15 to 20 amino acids in length, spanning the entire sequence of the allergen molecule. Peptides that induce T cell proliferation contain T cell epitopes. T cell reactive sites of many allergens have now been mapped, e.g. PLA_2 from bee venom (Carballido *et al.*, 1993), Der p 1 and Der p 2 from HDM (O'Hehir *et al.*, 1993; Higgins *et al.*, 1994; O'Brien *et al.*, 1994; O'Brien *et al.*, 1995; Hales and Thomas, 1997), Fel d 1 from the domestic cat (Counsell *et al.*, 1996), Ole e 1 from Olive pollen (Cardaba *et al.*, 1998), Cry j 1 and Cry j 2 from Japanese cedar pollen (Hashiguchi *et al.*, 1996; Sone *et al.*, 1998), Hev b 3 and Hev b 5 from latex (Bohle *et al.*, 2000; de Silva *et al.*, 2000) and Lol p 1 and Lol p 5 from rye grass pollen (Blaher *et al.*, 1996; Burton *et al.*, 2002). These studies reveal numerous T cell reactive sites scattered throughout the allergen molecules, but with evidence for sites of dominance. Immunodominant regions of allergens can be used to design T cell targeted strategies for improved SIT. Suitable allergen preparations may comprise either peptides containing the T cell epitope or modified allergen molecules in which IgE reactivity is ablated but T cell epitopes are retained.

Peptides are an attractive alternative to the current whole extracts used in SIT due to their ease of standardisation as well as potential for increased efficacy and safety. Minimal core Th cell determinants are usually eight or nine residues in size, but flanking residues may play a role in stabilising the MHC molecule-peptide-T cell receptor interaction. Thus the size consistent with that found for naturally processed peptides for MHC class II presentation, i.e. 13-17 amino acids, would seem suitable for

selectively targeting allergen-specific T cells. In addition, peptide preparations will increase the safety of SIT because small peptides cannot cross-link surface bound IgE on mast cells and basophils. By reducing IgE-mediated side effects, peptide preparations could potentially be given at higher concentrations, thus repolarising T cell responses more effectively and in turn increasing the efficacy of this treatment.

An important consideration when selecting peptides for immunotherapy, is whether the peptide can be presented by different MHC class II molecules and therefore be suitable for treating an "outbred" human population. The identification of HLA "supertypes" comprising HLA groups that share largely overlapping peptide binding repertoires suggests that there are peptide sequences within antigens that can bind a range of MHC II molecules (Sette and Sidney, 1998; Southwood *et al.*, 1998). Evidence supporting this concept for allergen peptides was presented by Texier and colleagues who identified a region in PLA_2 which bound multiple MHC class II alleles (Texier *et al.*, 2000). This site contained immunodominant T cell epitopes (Carballido *et al.*, 1993). In addition, a promiscuous T cell epitope, that is presented to specific T cells by various MHC class II alleles, has been identified in the birch pollen major allergen Bet v 1 (Friedl-Hajek *et al.*, 1999). Allergen-specific Th cells generally appear to have diverse MHC class II binding restrictions (Haselden *et al.*, 2000). By designing allergen preparations based on promiscuous T cell epitopes, peptide immunotherapy can be developed for allergy. Peptides may well offer a gain in efficacy of SIT since they can load directly onto MHC class II molecules and hence be presented at higher frequency than peptides processed from the whole molecule by the APC (Santambrogio *et al.*, 1999).

In vitro and murine studies to evaluate peptide immunotherapy (PIT)

The potential efficacy of peptides in modulating allergen-specific T cell responses has been demonstrated in *in vitro* human studies and murine models of allergy. O'Hehir and colleagues first observed the tolerising effect of allergen-derived peptide treatment using a Der p 1 TCC (O'Hehir *et al.*, 1991). Pre-treatment with a supraoptimal concentration of the dominant T cell epitope Der p 1(89-117) rendered the TCC unresponsive to restimulation with immunogenic concentrations of peptide. Interestingly, the anergic TCC could produce IFN-γ on restimulation, however IL-4 production was abolished. Providing a molecular basis for such high dose peptide induced T cell anergy, Faith and co-workers demonstrated defective TCR transmembrane signalling due to an abrogation in the activity of $p56^{lck}$ and ZAP-70 tyrosine kinases in a similarly anergised PLA_2-specific $CD4^+$ TCC (Faith *et al.*, 1997).

Efficacy of PIT has been demonstrated in several murine models of allergy. In a model of HDM allergy, inhalation of a dominant T cell epitope Der p 1 peptide inhibited specific-T cell responses to the whole protein in both naïve and sensitised mice (Hoyne *et al.*, 1993). Further analysis of this tolerance induction revealed an overall downregulation of IL-4 and IFN-γ by Th cells rather than a shift from Th2 to Th1 cytokine production (Hoyne *et al.*, 1997). In this model intramolecular epitope suppression, in which T cell responses to all epitopes on an antigen are inhibited by a single peptide, was observed. Intramolecular epitope suppression has also been shown using immunodominant peptides of Fel d 1, Cry j 2 and PLA_2 (Briner *et al.*, 1993; Hirahara *et al.*, 1998; Astori *et al.*, 2000). Interestingly, Hoyne and colleagues demonstrated that unresponsive T cells are not rendered anergic or deleted because the

suppression could be "uncoupled" by subsequent immunisation with a minor epitope in adjuvant (Hoyne *et al.*, 1997). The authors suggested that a regulatory $CD4^+$ T cell population mediated local suppression of naïve cells through the recognition of linked epitopes on the same APC; there was evidence that suppression occurred via cell-to-cell contact rather than by the production of cytokines. The identity of the cell surface molecules mediating this interaction is not clear, but the Notch signalling pathway has been proposed. Notch functions as a receptor for two different families of ligands, Delta and Serrate, and these interactions play an important role in regulating cell growth and differentiation in general (Artavanis-Tsakonas *et al.*, 1999). Hoyne and colleagues observed a marked increase in the numbers of Delta-1 expressing cells after tolerance induction by delivery of Der p 1 peptide via the respiratory mucosa (Hoyne *et al.*, 1999), and antigen presentation by APC over-expressing Serrate-1 induced naïve $CD4^+$ T cells to become regulatory cells (Hoyne *et al.*, 2000). Thus peptide immunotherapy may modulate allergen-specific Th cell responses by directly inducing Th cell anergy at the level of the APC or by inducing the action of $CD4^+$ T regulatory cells.

Clinical trials of peptide immunotherapy

Clinical trials of allergen-specific PIT have been performed for some allergens. In early studies on cat allergen immunotherapy, two 27-mer peptides containing the dominant T cell epitopes of the principal allergen of cat dander, Fel d 1, were synthesised and incorporated into a vaccine (ALLERVAX® CAT). Patients receiving subcutaneous injections of high concentrations of peptide (750 μg) showed clinical improvement and decreased symptoms (Norman *et al.*, 1996; Marcotte *et al.*, 1998; Pene *et al.*, 1998). There was reduced allergen-stimulated Th cell IL-4 production, but no change in IFN-γ

production and no evidence for allergen-specific T cell anergy after treatment (Marcotte *et al.*, 1998; Pene *et al.*, 1998). Although these large peptides induced basophil histamine release in less than 1% of cat allergic subjects, adverse reactions to vaccine treatment were seen in some patients (Norman *et al.*, 1996). In a clinical trial by Simons and colleagues, these same Fel d 1 peptides were found to be ineffective at reducing allergic symptoms and no change in the production of IL-4, IFN-γ and IL-10 by PBMC was observed (Simons *et al.*, 1996). However a lower dose of peptides (250 μg) was administered in this study. More recently, intradermal injection of smaller Fel d 1 peptides of 16-17 amino acids in length has been shown to inhibit early IgE-mediated response to allergen challenge (Haselden *et al.*, 1999). However 9 out of 40 subjects experienced late asthmatic reactions mediated by T cells after peptide injection. Continuing on from this study, a placebo-controlled, double-blind trial of a mixture of 12 short overlapping peptides of Fel d 1 was performed (Oldfield *et al.*, 2002). By gradually increasing the dose of peptide administered, starting at 0.1 μg/ml, late phase skin reactions to whole cat dander were inhibited without late asthmatic reactions. Patients receiving peptide had significantly reduced allergen-induced PBMC proliferation and production of IL-4, IL-13 and IFN-γ, while IL-10 production was increased. Thus the use of peptide SIT for cat allergy is promising.

Clinically effective PIT without serious side effects has also been reported for bee venom allergy by Muller and colleagues (1998). The PIT preparation consisted of three immunodominant peptides of PLA_2: PLA_2(45-62), PLA_2(82-92) and PLA_2(113-124). Peptides were administered subcutaneously in an equimolar mixture to five patients starting at 0.1 μg. Successive doses of 1, 3, 6, 12, 25 and 50 μg were given weekly until

a maintenance dose of 100 μg was reached and continued for another two weeks. Post-PIT all patients tolerated subcutaneous provocation with purified PLA_2 without systemic side effects. Clinically successful immunotherapy correlated with the induction of specific anergy of peripheral T cells to PLA_2 and peptides and decreased IL-4, IL-5 and IFN-γ production. In two patients mild systemic allergic symptoms were observed after bee sting challenge. For both these patients, decreases in specific proliferation and cytokine production were either less pronounced or insignificant. These results indicate that the peptides of PLA_2 used may not be immunodominant for these two individuals. The final dose of peptides injected into these five patients corresponded to a 40-times higher amount of PLA_2 than given for conventional bee venom SIT. This demonstrates that given in peptide form, higher concentrations of allergen can be tolerated by patients with potentially increased efficacy of SIT.

Altered peptide ligands for immunotherapy

An alternative strategy based on dominant T cell epitope peptides is the use of altered peptide ligands (APL) for SIT preparations. The alteration is usually substitution of a single amino acid at a putative T cell contact residue. This substitution results in the formation of peptides that are T cell antagonists or partial agonists. Antagonistic peptides are unable to elicit T cell signalling and thus inhibit T cell activation whereas partial agonists elicit some but not all the signals required for T cell activation (Boutin *et al.*, 1997; De Palma *et al.*, 2000).

Evidence for the downregulation of allergen-specific T cell responses by APL comes from *in vitro* studies. Single amino acid substituted peptides based on T cell epitopes of

Cry j 1, Der p 1 and Par j 1 have been found to be antagonistic to specific TCL and TCC (Ikagawa *et al.*, 1996; Fasler *et al.*, 1998; De Palma *et al.*, 1999). These APL inhibited T cell proliferative responses to the native peptide. Furthermore the peptide analogue of the Par j 1 epitope was able to inhibit proliferation of TCL to the whole Par j 1 molecule providing evidence for linked suppression (De Palma *et al.*, 1999). In addition to inhibiting T cell responses, co-cultures of Der p 1-specific TCC and B cells with the antagonistic Der p 1 epitope peptide resulted in an inability of the T cells to provide help for B cell IgE production (Fasler *et al.*, 1998). This was attributed to the APL inhibiting the induction of CD40L expression on Th cells, which is required for Th cell/B cell interactions.

Partial agonist peptides also modulate allergen-specific T cell responses. Increased IFN-γ production with no alteration in T cell proliferation and IL-4 production was demonstrated for APL based on epitopes of Cry j 1, Der p 2 and Der f 1 (Ikagawa *et al.*, 1996; Matsuoka *et al.*, 1996; Tsitoura *et al.*, 1996). Interestingly, enhanced T cell production of IFN-γ induced by a Der f 1 APL, was the result of increased APC production of IL-12 (Matsuoka *et al.*, 1996). This lead the authors to suggest that upon T cell/APC interactions, mediated through the TCR, APL can directly transmit signals to affect the APC response. A partial agonist based on a PLA_2 epitope reduced proliferation of a specific TCC and decreased IL-4 production without affecting IFN-γ production (Faith *et al.*, 1999). In the absence of APC, this PLA_2 APL also induced T cell anergy and reduced IL-4, IL-5 and IL-13 production. Anergised T cells displayed altered TCR signalling due to the lack of tyrosine phosphorylation of ZAP-70. In another study, a Der p 2 epitope APL induced unresponsiveness in a specific TCC at

lower concentrations than the native peptide (Verhoef and Lamb, 2000). During tolerance induction, this APL was 22% more potent in inducing IL-10 production than the native peptide. These results indicate that partial agonist peptides can shift the cytokine profile of allergen-specific T cells from Th2- to Th1-predominant and under the correct conditions induce anergy.

Although *in vitro* studies investigating immune modulation by APL are promising, these antagonistic or partial agonist peptides have not been clinically tested for allergen immunotherapy in humans to date. However a study by Janssen and colleagues investigated the effect of APL in a murine model of allergic asthma (Janssen *et al.*, 2000a). In this model, BALB/c mice were sensitised and challenged with OVA to induce airway hyperresponsiveness, eosinophilia and OVA-specific IgE production. Cells isolated from the lymph nodes draining the lungs produced IL-4 and IL-5 *in vitro*. Previously defined Th2- and Th1-skewing partial agonist peptides of OVA were then used for immunotherapy by subcutaneous injection into sensitised mice. Administration with either the native peptide or the Th2-skewing APL resulted in increased eosinophilia upon OVA challenge. In contrast administration with the Th1-skewing APL resulted in decreased eosinophilia and reduced IL-4 and IL-5 production by cells isolated from the lymph nodes draining the lungs upon OVA challenge. The results from this study suggest that the use of Th1-skewing APL instead of native peptide may improve the efficacy of PIT.

1.4.2.2 Modified allergen preparations

An alternative strategy to the use of peptides for T cell targeted SIT is to use modified allergens. These have the advantage of containing the multiple T cell epitopes which

have been identified throughout allergen molecules and they could also be designed to target particular APC by their chemical nature. To overcome the risk of IgE-mediated side effects with current whole allergen preparations, several different approaches have been explored to render allergens hypoallergenic but still effective for SIT.

Chemically modified allergens

An early strategy for the generation of effective but hypoallergenic SIT preparations was to treat the allergen with chemicals such as glutaraldehyde or formaldehyde. Allergens modified in this way were termed 'allergoids'. In 1970 Marsh and colleagues developed a rye grass pollen group one allergoid by formaldehyde treatment (Marsh *et al.*, 1970), and in 1981 they produced ragweed pollen allergoids by formaldehyde and glutaraldehyde treatment with reduced IgE binding and histamine release from leukocytes (Marsh *et al.*, 1981). Clinical trials of allergoids of ragweed pollen, mixed grass pollen, Parietaria pollen and dust mite allergens demonstrated that the preparations could be well tolerated and were effective at reducing allergic symptoms (Metzger *et al.*, 1981; Norman *et al.*, 1982; Meriney *et al.*, 1986; Bousquet *et al.*, 1988; Tari *et al.*, 1997; Passalacqua *et al.*, 1998; Ariano *et al.*, 1999; Negro *et al.*, 1999). Although increased production of IgG_4 blocking antibody, decreased IgE production and reduced histamine release were demonstrated, changes in the T cell response following clinically effective SIT were not analysed in these studies (Tari *et al.*, 1997; Ariano *et al.*, 1999; Negro *et al.*, 1999). Modulation of T cell responses by allergoids has however been investigated *in vitro* (Dormann *et al.*, 1998; Kahlert *et al.*, 2000). In these studies allergoids induced weaker proliferative responses and reduced production of IL-5 and IFN-γ by specific TCL and TCC in comparison to native allergen. These results suggest

that allergoids can down-regulate production of both Th2 and Th1 cytokines. Kahlert and colleagues demonstrated that allergoids also affect immune responses at the level of the APC (Kahlert *et al.*, 2000). Recombinant Phl p 5b allergoids were selectively presented by DC and monocytes but not by B cells. It was suggested that B cell presentation was inhibited as a consequence of destruction of IgE binding epitopes on the allergoid.

Recently, T cell reactivity was addressed for the first time during allergoid SIT for birch pollen allergy (Klimek *et al.*, 1999). Although treatment was clinically effective, allergen-induced T cell proliferation and production of IL-4, IL-5, IFN-γ and IL-10 remained unaffected during SIT. Nasal secretions showed decreased levels of IL-5 and increased levels of IFN-γ following SIT. Although this suggests a shift from Th2- to Th1-type cytokine production, the cellular source of the cytokines recovered in the nasal secretions was not identified. Thus more studies are required to assess the ability of allergoid preparations to down-regulate pathogenic allergen-specific T cell responses.

Allergen isoforms

For some allergens, natural forms with reduced IgE reactivity may exist as isoforms. These usually have only minor sequence differences and would therefore be expected to exhibit similar T cell reactivity to IgE reactive forms. Studying nine isoforms of the major birch tree pollen allergen Bet v 1, Ferreira and colleagues identified three isoforms which had low or no IgE-binding activity (Bet v 1d, g and l) (Ferreira *et al.*, 1996). Of these isoforms two, Bet v 1d and Bet v 1l, were tested for T cell reactivity and found to induce similar responses in comparison to naturally purified Bet v 1. In

addition, the d and l isoforms showed no or very weak urticarial skin reactions in allergic subjects upon skin prick testing. This research indicates the potential for the use of hypoallergenic isoforms in immunotherapy, however the extent to which isoforms with reduced IgE binding exist for other allergens is not clear.

Mutant allergens

An alternative approach is to use recombinant technology to produce allergen variants with reduced IgE reactivity and retained T cell responses. Site-directed mutagenesis provides a controlled system for disrupting residues important for protein tertiary structure or IgE recognition. For allergen molecules which contain intramolecular disulphide bonds, mutation of cysteine residues is a logical strategy for disruption of conformation and thus IgE binding. Cysteine variants prepared for HDM allergens Der p 2, Der f 2 and Lep d 2, cow dander allergen Bos d 2 and Par j 1 showed reduced or abolished IgE binding, retained T cell reactivity and, where tested, reduced skin prick test response (Olsson *et al.*, 1998; Smith *et al.*, 1998; Kauppinen *et al.*, 1999; Korematsu *et al.*, 2000; Bonura *et al.*, 2001). Targeting other critical residues for IgE binding by site-directed mutagenesis, hypoallergenic mutants have been prepared for Bet v 1, Lol p 5 and Ara h 1, 2, and 3 (Ferreira *et al.*, 1998; Singh *et al.*, 1999; Rabjohn *et al.*, 2002; Swoboda *et al.*, 2002). For Phl p 5b, deletion mutants have been produced (Schramm *et al.*, 1999). By making deletions within putative IgE binding regions but outside T cell epitopes, variants were produced with reduced IgE binding, decreased histamine releasing capacity and conserved T cell reactivity. Another recombinant strategy involves the production of hypoallergenic recombinant allergen oligomers (Vrtala *et al.*, 1999) or fragments (Zeiler *et al.*, 1997; Vrtala *et al.*, 2000). Overlapping fragments, encoding approximately half of the allergen molecule, produced from Bet v

1 and Bos d 2, have shown reduced IgE binding capacity most likely due to the loss of their native folding within the entire allergen molecule (Zeiler *et al.*, 1997; Vrtala *et al.*, 2000). In addition, the Bet v 1 fragments retained T cell reactivity in comparison to recombinant wildtype Bet v 1. Interestingly these fragments induced differential cytokine production, with the C-terminal fragment inducing IFN-γ and the N-terminal fragment inducing IL-4, IL-5 and IL-13 (Vrtala *et al.*, 2000).

Evaluation of recombinant hypoallergenic preparations as potential immunomodulators in mouse models is limited. Yasue and colleagues showed that administration of a hypoallergenic Der f 2 mutant could suppress immediate allergic reactions in a mouse model of allergic bronchial asthma more effectively than the wild type recombinant Der f 2 (Yasue *et al.*, 1998). Although preliminary results are encouraging, further studies are required to validate the use of hypoallergenic variants as SIT preparations.

1.4.2.3 DNA vaccines

As for the general field of vaccine technology, DNA vaccines have attracted attention for allergen immunotherapy. Of interest was the finding that bacterial DNA elicits strong Th1 cell responses in mice due to the presence of immunostimulatory sequences (ISS) containing CpG motifs (Krieg, 2002). As a result bacterial DNA has been assessed as an immunoregulator and an adjuvant for the modulation of allergen-specific Th2-type responses. Allergen-encoding DNA vaccines have also been tested for efficacy (Spiegelberg and Raz, 2002).

Bacterial DNA as an immunoregulator and adjuvant

Administration of bacterial oligodeoxynucleotides containing CpG motifs (CpG-ODN) before allergen sensitisation in mice has been shown to induce predominant allergen-specific Th1 cell responses for the allergens Bet v 1, Cry j 1, Cry j 2, Aed a 2, Amb a 1 and OVA (Broide and Raz, 1999; Jahn-Schmid *et al.*, 1999; Kohama *et al.*, 1999; Sur *et al.*, 1999; Tighe *et al.*, 2000; Peng *et al.*, 2001). Increased levels of serum specific-IgG_{2a} and increased production of IFN-γ by *in vitro* allergen-stimulated splenocytes confirmed induction of Th1 responses in these models. Suppression of IgE production (Kohama *et al.*, 1999; Sur *et al.*, 1999; Tighe *et al.*, 2000) and decreased production of Th2 cytokines were also observed (Broide and Raz, 1999; Kohama *et al.*, 1999; Peng *et al.*, 2001). Reduced eosinophilia and airway hyperresponsiveness has been reported in sensitised mice given CpG-ODN before allergen challenge (Broide *et al.*, 1998; Broide and Raz, 1999; Sur *et al.*, 1999; Broide *et al.*, 2001). Using cytokine neutralisation, Broide and colleagues demonstrated that CpG-ODN-mediated reduction in eosinophilia in a murine model of asthma was partially dependent on IL-12, IFN-α/β and IFN-γ (Broide *et al.*, 1998). As NK cells have previously been demonstrated to produce high levels of IFN-γ in the presence of CpG-ODN (Chace *et al.*, 1997), Broide and colleagues investigated the role of NK cells in reducing eosinophilia (Broide *et al.*, 2001). NK cell depletion did not affect the reduction in eosinophilia induced by CpG-ODN treatment. However the ability of CpG-ODN to affect other cells within the immune system may account for these results.

CpG-ODN stimulation of B cells results in the up-regulation of surface markers including MHC class II, CD40 and CD16/23 and downregulation of CD23 (Fcε

receptor) (Martin-Orozco *et al.*, 1999). On macrophages, CpG-ODN induce up-regulation of MHC class I, CD40, CD80 and ICAM-1 (Martin-Orozco *et al.*, 1999). Interestingly CpG-ODN-induced IL-12 production from macrophages is responsible for enhanced NK cell production of IFN-γ (Chace *et al.*, 1997). More investigation is required to fully understand the Th1 promoting activity of CpG-ODN.

A limited number of studies have investigated the ability of CpG-ODN to modulate human Th2 allergen-specific immune responses. Bohle and colleagues stimulated atopic and non-atopic PBMC with CpG-ODN *in vitro* and observed increased production of IFN-γ for both groups (Bohle *et al.*, 1999). The IFN-γ production was attributed to NK cells and inhibition experiments indicated that, as in the mouse, this was IL-12 dependent. Further analysis demonstrated that CpG-ODN-treated monocyte-derived DC produce IL-12 and IL-18 (Pichyangkul *et al.*, 2001). Importantly, Marshall and colleagues demonstrated that CpG-ODN can alter established allergen-specific Th responses *in vitro* (Marshall *et al.*, 2001). Allergic donor PBMC were cultured for six days in the presence of Amb a 1 and then cultured with either Amb a 1 alone or Amb a 1 linked to a CpG-ODN. Restimulation with Amb a 1 resulted in a predominant Th2 cytokine profile whereas restimulation with the CpG-ODN linked Amb a 1 resulted in predominant Th1 cytokine profile. Increased IFN-γ production induced by the CpG-ODN in this study was again shown to be IL-12 dependent.

As yet the anti-allergy effects of CpG-ODN in humans has not been assessed. However care must be taken when utilising bacterial DNA as it can cause pathological inflammatory responses and toxic shock due to the over-production of TNF-α by

activated APC (Lipford *et al.*, 1997). Thus further research is required to assess the safety and the full capacity of CpG-ODN to down-regulate established human allergen-specific Th2-type cell responses.

DNA vaccines encoding allergen genes

The direct administration of DNA encoding allergen proteins is also a focus of current research. In 1996 Raz and colleagues demonstrated that injection into mice of naked plasmid DNA encoding beta-galactosidase elicited predominant T cell IFN-γ production in comparison to injection of the beta-galactosidase protein which elicited predominant T cell IL-4 and IL-5 production (Raz *et al.*, 1996). The induction of allergen-specific Th1 responses in mice and rats by administration of plasmid DNA encoding the allergen of interest has now been demonstrated for Der p 5, Der f 11, Hev b 5, Bet v 1a, Cry j 1, bovine beta-lactoglobulin and peptides containing T cell epitopes of Der p 1 and PLA_2 (Hsu *et al.*, 1996; Slater *et al.*, 1998; Hartl *et al.*, 1999; Li *et al.*, 1999; Toda *et al.*, 2000; Adel-Patient *et al.*, 2001; Jilek *et al.*, 2001; Kwon *et al.*, 2001; Draghi *et al.*, 2002; Peng *et al.*, 2002). In comparison to the native protein, injection of DNA encoding the allergen leads to reduced levels of allergen-specific IgE (Hsu *et al.*, 1996; Hartl *et al.*, 1999; Li *et al.*, 1999; Toda *et al.*, 2000; Adel-Patient *et al.*, 2001; Jilek *et al.*, 2001; Kwon *et al.*, 2001; Peng *et al.*, 2002). DNA vaccines may also modulate ongoing allergic responses. In mice sensitised to bee venom allergens, therapeutic treatment with DNA plasmids containing PLA_2 T cell epitope peptides, resulted in a decreased incidence of anaphylaxis which was associated with increased IFN-γ and IL-10 production and decreased IL-4 production (Jilek *et al.*, 2001). Furthermore, treatment of Der f 11 sensitised mice with a Der f 11 encoding DNA plasmid resulted in reduced

levels of allergen-specific IgE and increased levels of allergen-specific IgG_{2a} (Peng *et al.*, 2002). These studies indicate that DNA vaccines can down-regulate the Th2 biased immune response to allergens. However initial trials for the treatment of malaria in humans using DNA vaccines produced very low antigen-specific immune responses in comparison to the strong responses seen in murine models. Thus the use of DNA vaccines for human disease management requires optimisation.

More recently a number of new strategies have been employed to increase the effectiveness of DNA vaccines. Toda and colleagues designed two DNA vaccines that encode a T cell epitope of Cry j 1 and an invariant chain for the delivery of the epitope peptide to the MHC class II loading pathway (Toda *et al.*, 2002). Pre-treatment of mice with these vaccines inhibited Cry j 2-specific IgE responses and promoted Cry j 2-specific IgG_{2a} responses in Cry j 2 sensitised mice. In addition, splenocytes from vaccinated mice produced large amounts of IFN-γ when stimulated with Cry j 2 *in vitro*. Roy and colleagues investigated a new delivery system in which plasmid DNA containing the Ara h 2 gene was complexed to chitosan, a natural biocompatible polysaccharide, to form nanoparticles that can be administered via the oral route (Roy *et al.*, 1999). This vaccine reduced the incidence of anaphylaxis in a murine model of peanut allergen-induced hypersensitivity. Injection of the nanoparticles also resulted in increased allergen-specific IgG_{2a} and secretory IgA and decreased IgE. This new strategy offers the use of a non-viral vector for immunotherapy preparations.

1.4.2.4 Adjuvants

In parallel with refinement of allergen preparations for SIT are studies on adjuvants or

carriers to promote efficacy of SIT. Adjuvants play an important role in targeting the site for immunomodulation and in determining the type of immune response elicited. While alum has been the mainstay adjuvant for allergen vaccines for many years due to its safety and depot effects, it may not be the best adjuvant for down-regulating Th2-mediated disease. Alum is in fact used in many mouse models to drive Th2 responses. Consideration of other adjuvants for promoting Th1-biased responses at mucosal sites is warranted. Improved clinical efficacy and shorter treatment regimens are additional potential outcomes.

Bacterial components

A number of studies have investigated the influence of conjugating allergen to bacterial components on humoral and cellular immune responses. Jahn-Schmid and colleagues tested the efficiency of crystalline bacterial surface layers to drive Th1 cytokine production by human allergen-specific T cells (Jahn-Schmid *et al.*, 1997). TCL from birch pollen allergic donors generated in the presence of surface layers conjugated to recombinant Bet v 1 had higher IFN-γ:IL-4 ratios than TCL generated against the unconjugated recombinant Bet v 1. The Th1-polarised phenotype was associated with increased PBMC IL-12 production induced by conjugate stimulation. As the allergen-surface layer conjugates are 1-5 μm in size the authors proposed that the potent Th1 shifting ability is due to preferential macrophage antigen uptake and subsequent IL-12 production. Using another strategy, the dominant T cell epitope Der p 1 (111-139) was inserted within a permissive loop of the bacterial superoxide dismutase and expressed in *Mycobacterium vaccae* (Hetzel *et al.*, 1998). The immunisation of mice with the recombinant bacteria led to the development of a Der p 1 (111-139)-specific Th1

response. Allergen conjugated to the mucosa-binding β subunit of cholera toxin administered via the oral route was found by Rask and colleagues to suppress allergen-specific IgE production in sensitised mice (Rask *et al.*, 2000). Another potential bacterial component adjuvant, is immunostimulatory monophosphoryl lipid A (MPL) isolated from *Salmonella minnesota* R595. Administration of antigen and MPL leads to systemic immunity characteristic of a Th1 response (Baldridge *et al.*, 2000; Wheeler *et al.*, 2001). Injection of allergen with MPL to allergen-sensitised rats prevented secondary IgE responses (Wheeler *et al.*, 2001). In humans a new allergy vaccine comprising a tyrosine-adsorbed glutaraldehyde-modified grass pollen extract with MPL adjuvant was recently trialled in a controlled clinical study (Drachenberg *et al.*, 2001). After only four preseasonal subcutaneous injections this well tolerated vaccine was efficacious, in terms of reduced symptoms and skin prick test scores. More research is needed to analyse changes in T cell reactivity during SIT using MPL as an adjuvant. Nevertheless many bacterial components can drive allergen-specific Th1 responses and suppress ongoing allergen-specific IgE responses and thus their use as adjuvants in SIT is promising.

Virus-like particles

Viral components have been trialled as immunomodulators. In particular, virus-like particles (VLP) offer promise for allergen immunotherapy. VLP containing the p1 protein of the yeast retrotransposon Ty have been used to present allergen, as peptide, to the immune system and elicit cell mediated responses. VLP containing 300 copies of a fusion protein comprising a relevant dominant T cell epitope peptide linked to the p1 protein have been administered to Der p 1 and Asp f 2 sensitised mice (Hirschberg *et*

al., 1999; Svirshchevskaya *et al.*, 2002). Reduced allergen-specific splenocyte IL-5 production was observed for Der p 1 sensitised mice injected intraperitoneally with Der p 1 (111-139)-VLP in comparison to native Der p 1 (Hirschberg *et al.*, 1999). Der p 1 (111-139)-VLP treated mice displayed decreased T cell proliferation to both Der p 1 (111-139) and Der p 1, leading the authors to suggest that VLP peptide administration renders Th2 cells anergic and provides evidence for linked suppression (Hirschberg *et al.*, 1999). T cell anergy was seen in mice injected subcutaneously with Asp f 2 peptide-VLP (Svirshchevskaya *et al.*, 2002). Thus through the delivery of high dose peptide, VLP could be used to "turn off" the adverse T cell response to allergen.

Recombinant cytokines

Recombinant cytokines are another possibility for SIT adjuvants that can direct the allergen-specific immune response away from Th2 predominant. Immunisation of BALB/c mice with recombinant IL-12-OVA fusion protein induced anti-OVA IgG_{2a} antibody and increased levels of OVA-specific IFN-γ production from lymph node cells *in vitro* (Kim *et al.*, 1997). Immunisation of mice with a recombinant IL-18-OVA fusion protein induced increased IFN-γ production by OVA-stimulated splenocytes (Maecker *et al.*, 2001a). Both fusion proteins reversed established predominant Th2 OVA responses in mice (Kim *et al.*, 1997; Maecker *et al.*, 2001a). In addition, Kumar and colleagues showed that mice receiving plasmid DNA encoding IL-12 and IFN-γ intramuscularly in combination with subcutaneous allergen injection, produced increased allergen-specific IgG_{2a} antibody and decreased total serum IgE compared with control mice (Kumar *et al.*, 2001). Allergen-stimulated splenocytes from co-immunised mice produced high levels of IFN-γ and IL-2 and low levels of IL-4 indicative of a Th1

response. These mice had reduced methacholine-induced airway hyperresponsiveness and abolished lung inflammation in comparison to mice immunised with only allergen. Thus using Th1-promoting cytokines either as recombinant proteins or encoded in DNA plasmids in conjunction with allergen administration is an interesting alternative adjuvant strategy for SIT.

Liposomes

Liposomes are another type of adjuvant/carrier that could possibly be used for allergen delivery in SIT. Liposomes are non-toxic, biodegradable lipid vesicles that can incorporate allergen. In 1991, Arora and colleagues demonstrated that liposomes can be used as a non-immunogenic vehicle for antigen presentation *in vivo*. Furthermore, administration of liposome-entrapped allergen into allergen-sensitised mice can reduce specific IgE production (Arora and Gangal, 1992). In 1998, Sehra and colleagues demonstrated that intraperitoneal immunisation of naïve mice with liposome-entrapped *Artemisia scoparia* pollen induced increased serum levels of IFN-γ and IgG_{2a} compared with mice immunised with free allergen (Sehra *et al.*, 1998a). Upon intravenous allergen challenge, liposome-immunised mice had reduced histamine release and were protected from fatal systemic reactions. Dominant Th1 responses characterised by increased IFN-γ and decreased IL-4 production have also been shown in mice immunised with allergen-entrapped liposomes (Sehra *et al.*, 1998b; Nigam *et al.*, 2002). Macrophages were required for processing of the liposome-entrapped allergen (Sehra *et al.*, 1998b). Presumably, IL-12 production from macrophages diverts the response to Th1. By inhibiting IgE binding to allergen, the lipid coating of the liposomes would prevent the Th2-promoting uptake of allergen by B cells and IgE-bearing DC. In

humans, liposome-allergen delivery has been trialled in a one year double-blind, placebo controlled study for HDM allergy (Basomba *et al.*, 2002). Encouraging results with reduced symptoms and a high level of safety were achieved however T cell responses were not analysed. Thus the use of liposomes as adjuvants for SIT is promising. As liposomes protect the allergen from IgE binding upon administration, the utilisation of this adjuvant could potentially increase the safety of allergen immunotherapy.

1.5 SUMMARY AND AIMS

T cells orchestrate the immune response to allergens. In allergic individuals, allergen-stimulated T cells produce IL-4, IL-5 and IL-13, driving allergen-specific IgE production by B cells and maturation and activation of mast cells, basophils and eosinophils. Clinical manifestations of allergy include rhinitis, urticaria and asthma. With approximately 40% of the population in Australia being atopic and hence at risk of developing allergy, allergic disease is a major health concern in our community. Conventional SIT is the only treatment for allergic diseases that potentially modifies the natural course of allergy. However the efficacy of SIT varies with the allergen and there is a potential risk of harmful side effects. Elucidation of the underlying immune basis for specific immunomodulation of the pathogenic Th2-biased response to allergens points to new strategies for improved efficacy and safety of allergen immunotherapy.

A fundamental knowledge of factors influencing the type and magnitude of the immune response to an allergen provides a rational basis for refinement of the form and mode of delivery of allergen in SIT. Factors that can alter allergen-specific $CD4^+$ T cell responses include antigen concentration, antigen form, APC-type, costimulatory molecule interactions and regulatory cell function. It is important to understand how these factors modulate $CD4^+$ T cell activation, division, differentiation and apoptosis in order to devise strategies to down-regulate allergen-induced Th2 responses.

A variety of different allergen preparations are currently being explored for more selective targeting of the allergen-specific $CD4^+$ T cell. These include peptides, hypoallergenic allergen forms and DNA vaccines. In addition the ability of different

allergen preparations and adjuvants in targeting particular APC at different tissue sites in order to modulate responses at sites of allergen encounter are being examined. Knowledge gained from this research would permit development of more practical delivery of SIT via oral, sublingual or intranasal routes.

During clinically successful SIT, T cell responses are modified, usually with deviation from IL-4 and IL-5 cytokine production to predominant IFN-γ production and increased IFN-γ^+ cell numbers at sites of allergen challenge. As conventional SIT is associated with the administration of higher allergen concentrations than are encountered naturally, allergen concentration may be playing an important role in inducing altered T cell responses during SIT. The objective of this project was to design an *in vitro* culture system in which HDM-allergic donor PBMC were stimulated with different allergen concentrations, and to examine T cell cytokine (IL-4, IFN-γ and IL-10) production at the single cell level using intracellular cytokine staining. This culture system was used to determine the effects of different concentrations of allergen on 1) allergen-specific T cell production of IL-4, IFN-γ and IL-10, proliferation and apoptosis; 2) the expression of adhesion molecules (CD62L and CD49d) and chemokine receptors (CCR3 and CCR5) by allergen-specific T cells and to correlate these parameters with cytokine production. The induction of a regulatory T cell population was examined by analysis of $CD4^+IL\text{-}10^+$ T cells. As an *in vivo* comparison of the results obtained from the *in vitro* culture system, T cell changes during HDM-specific immunotherapy were also analysed.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Tissue Culture Reagents

DMSO	Sigma Chemical Company, USA
Ficoll-Paque (research grade)	Pharmacia, Sweden
Foetal Bovine Serum (FBS)	Gibco BRL, USA
Freeze dried house dust mite	Allergy Therapeutics Limited, England
Human AB^+ serum	Sigma Chemical Company, USA
Latex glove extract (GE)	Laboratory stocks
Keyhole Limpet Haemocyanin (KLH)	Sigma Chemical Company, USA
Mouse anti-human CD3 (OKT3)	Laboratory stocks
Penicillin, Streptomycin and L-glutamine	Gibco BRL Life Technologies, USA
Phytohaemagglutinin (PHA)	Wellcome Diagnostics, England
Recombinant human IL-2 (rIL-2)	Cetus Corporation, USA
Recombinant human IL-10 (rIL-10)	PharMingen, USA
RPMI 1640	Gibco BRL Life Technologies, USA
Sodium heparin (preservative free)	David Bull Laboratories, Australia

2.1.2 Flow Cytometric Reagents

Annexin-V-FITC	PharMingen, USA
Annexin-V-PE	PharMingen, USA

Brefeldin A	Sigma Chemical Company, USA
5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE)	Molecular Probes, Oregon
Mouse anti-human CCR5-FITC	PharMingen, USA
Mouse anti-human CD25-FITC	PharMingen, USA
Mouse anti-human CD3-FITC	PharMingen, USA
Mouse anti-human CD4-Allophycocyanin	PharMingen, USA
Mouse anti-human CD4-CyChrome	PharMingen, USA
Mouse anti-human CD4-FITC	PharMingen, USA
Mouse anti-human CD4-PE	PharMingen, USA
Mouse anti-human CD49d-CyChrome	PharMingen, USA
Mouse anti-human CD56-PE	PharMingen, USA
Mouse anti-human CD62L-FITC	PharMingen, USA
Mouse anti-human CD8-Allophycocyanin	PharMingen, USA
Mouse anti-human CD8-CyChrome	PharMingen, USA
Mouse anti-human CD8-FITC	PharMingen, USA
Mouse anti-human CD8β-FITC	PharMingen, USA
Mouse anti-human αβ TCR-PE	PharMingen, USA
Mouse anti-human γδ TCR-Allophycocyanin	PharMingen, USA
Mouse anti-human IFN-γ-Allophycocyanin	PharMingen, USA
Mouse anti-human IL-4-PE	PharMingen, USA
Mouse IgG_1-Allophycocyanin isotype control	PharMingen, USA
Mouse IgG_1-Allophycocyanin isotype control (intracellular)	PharMingen, USA
Mouse IgG_1-CyChrome isotype control	PharMingen, USA

Mouse IgG_1-FITC isotype control	PharMingen, USA
Mouse IgG_{2a}-FITC isotype control	PharMingen, USA
Mouse IgG_1-PE isotype control	PharMingen, USA
Mouse IgG_1-PE isotype control (intracellular)	PharMingen, USA
Permeabilising solution (10x)	Becton Dickinson, USA
Propidium iodide	Sigma Chemical Company, USA
Rabbit anti-human active caspase-3-FITC	PharMingen, USA
Rat anti-human CCR3-FITC	R&D Systems, USA
Rat anti-human IL-10-PE	PharMingen, USA
Rat IgG_1-PE isotype control (intracellular)	PharMingen, USA
Rat IgG_{2a}-FITC isotype control	R&D Systems, USA

2.1.3 General Reagents

Counting Bags	Wallac, UK
Printed glass fibre mats	Wallac, UK

2.1.4 General Chemicals

Acrylamide/BIS (29:1), premixed powder, electrophoresis grade	BIO-RAD Laboratories, USA
Ammonium persulphate	BIO-RAD Laboratories, USA
Aqueous counting scintillant (ASCII)	Ajax Chemicals, Australia
Bromophenol blue	BDH Laboratory Supplies, UK
Calcium chloride ($CaCl_2$)	Merck, Australia
Dithiothreitol (DTT)	Sigma Chemical Company, USA
Glycine	Ajax Chemicals, Australia

Glycerol	Merck, Australia
HEPES	Sigma Chemical Company, USA
Hydrogen chloride (HCl)	Ajax Chemicals, Australia
Paraformaldehyde	Sigma Chemical Company, USA
Propidium iodide	Sigma Chemical Company. USA
Sodium azide (NaN_3)	BDH Laboratory Supplies, UK
Sodium chloride (NaCl)	Ajax Chemicals, Australia
Sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$)	Sigma Chemical Company, USA
Sodium dodecyl sulphate (SDS)	Bio-Rad Laboratories, USA
Sodium hydroxide (NaOH)	Merck, Australia
Sodium phosphate (Na_2HPO_4)	Sigma Chemical Company, USA
N,N,N',N'-Tetramethylethylene-diamine (TEMED)	Bio-Rad Laboratories, USA
^{3}H-thymidine	Amersham Biosciences, UK
Tris (hydroxymethyl) aminomethane (Tris)	Bio-Rad Laboratories, USA
Trypan blue solution (0.4 %)	Sigma Chemical Company, USA

2.1.5 Buffers and Solutions

All buffers and solutions were prepared in Milli Q (Millipore, USA) H_2O unless otherwise stated.

Ammonium persulphate (5% w/v)

A 5% ammonium persulphate solution was prepared by adding 100 mg ammonium persulphate to 2 ml H_2O. This solution was stored at 4°C for up to 3-4 days.

Annexin-V binding buffer

A 10x stock solution of Annexin-V binding buffer was prepared by dissolving 4.77 g HEPES, 16.4 g NaCl and 740 µg $CaCl_2$ in 150 ml H_2O. The pH was adjusted to 7.4 and the volume made up to 200 ml with H_2O. This solution was stored at 4°C and diluted ten-fold with H_2O prior to use.

Brefeldin A

A 1 mg/ml solution of Brefeldin A was prepared by dissolving 5 mg Brefeldin A in 5 ml sterile DMSO. This solution was stored at –20°C in 100 µl aliquots.

CFSE

A 1 mM solution of CFSE was prepared by dissolving 25 mg CFSE in 44.9 ml of sterile DMSO. This solution was stored at either –70°C in 500 µl aliquots or –20°C in 40 µl aliquots.

FBS/15 % DMSO

50 ml sterile DMSO was added to 450 ml heat-inactivated FBS and stored at –20°C in 10 ml aliquots.

4% Paraformaldehyde, pH 7.5

4 g of paraformaldehyde was added to 90 ml of PBS and the solution was heated in a water bath to 65°C to dissolve the paraformaldehyde. Once dissolved the pH was adjusted to 7.5 with NaOH and the volume made up to 100 ml. This solution was stored at 4°C.

Permeabilising solution (1x)

1 ml permeabilising solution (10x) was diluted in 9 ml of H_2O just prior to use.

Phosphate buffered saline (PBS), pH 7.2

A 10x stock solution was prepared by dissolving 85 g NaCl, 3.9 g $NaH_2PO_4.2H_2O$ and 10.7 g Na_2HPO_4 in 1 L H_2O. This was stored at room temperature and diluted ten-fold prior to use.

PHA (400 µg/ml)

2 mg lyophilised PHA was reconstituted with 5 ml H_2O under sterile conditions to give a final concentration of 400 µg/ml. This was then aliquoted into 50 µl lots and stored at -20°C.

Propidium iodide (PI) (50 µg/ml)

A 10x stock solution was prepared by dissolving 5 mg propidium iodide in 10 ml H_2O and stored at 4°C. Prior to use this solution was diluted ten-fold (50 µg/ml) with H_2O and 10 µl was added to 100 µl cell suspension 5 minutes before flow cytometry.

RPMI 1640/10 U/ml sodium heparin/ L-glutamine, penicillin and streptomycin (Heparin medium)

5000 units of sterile preservative free sodium heparin and 2 mmol/L L-glutamine with 100 IU/ml penicillin/streptomycin was added to 495 ml of RPMI 1640 medium and stored at 4°C protected from light.

RPMI 1640/5% human AB^+ serum/ L-glutamine, penicillin and streptomycin (Complete medium)

To 470 ml of RPMI 1640 medium 25 ml of heat inactivated human AB^+ serum and 2 mmol/L L-glutamine with 100 IU/ml penicillin/streptomycin were added and the medium stored at 4°C protected from light.

RPMI 1640/L-glutamine, penicillin and streptomycin (Wash medium)

To 495 ml of RPMI 1640 medium 2 mmol/L L-glutamine with 100 IU/ml penicillin/streptomycin were added and the medium stored at 4°C protected from light.

Separating gel buffer

30.3 g Tris was added to 150 ml H_20 and the pH was adjusted to 8.8 with HCL. The volume was then made up to 250 ml with H_20 and the buffer was stored at 4°C.

SDS (10% w/v)

A 10% (w/v) SDS solution was prepared by the addition of 10 g SDS to 100 ml H_20.

SDS running buffer (10x stock solution)

A 10x stock solution was prepared by dissolving 30.3 g Tris, 144 g glycine and 10 g SDS in 1 L H_2O. This solution was stored at room temperature and diluted ten-fold prior to use.

SDS sample buffer (reducing)

SDS sample buffer was prepared by the addition of 2.5 ml "Stacking Gel Buffer", 4 ml 10% (w/v) SDS, 78 mg DTT for reducing conditions, 2 ml glycerol, bromophenol blue and H_2O to give a total volume of 10 ml. The buffer was stored at room temperature.

1 M Sodium azide

6.5 g of NaN_3 was carefully added to 100 ml of H_2O in a fume hood and the resulting solution stored at room temperature.

Stacking gel buffer, pH 6.8

This buffer was prepared by the addition of 11.4 g Tris to 150 ml H_2O. Following adjustment of the pH to 6.8 with HCl the volume was made up to 250 ml with H_2O and stored at 4°C.

^{3}H-thymidine

100 µl 1 mCi/ml ^{3}H-thymidine stock solution was added to 900 ml sterile PBS and stored at 4°C. 10 µl of this solution was aliquoted to each well (final concentration of 1 µCi/well).

Wash buffer (1% FBS/0.02% NaN_3/PBS)

5 ml of FBS and 1.54 ml of 1 M NaN_3 solution was added to 443.46 ml of PBS and the solution stored at 4°C.

2.2 STUDY POPULATION

Peripheral blood was taken from HDM allergic donors recruited from the Alfred Hospital Allergy Clinic, Melbourne, Australia and the Department of Pathology and Immunology, Monash University, 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 > 3 mm) and/or EAST (Kallestad Allercoat Sanofi-

Pasteur Diagnostics, score > 2). Non-atopic controls were also used in this study and were selected on the basis of negative skin prick tests to a number of common environmental allergens including HDM.

2.3 HDM EXTRACT PREPARATION

An aqueous extract of HDM, *Dermatophagoides pteronyssinus*, was prepared by reconstituting 100 mg freeze-dried HDM, kindly provided by Allergy Therapeutics Limited (England), in 100 ml PBS. For use in tissue culture, the 1 mg/ml HDM extract was sterilised by passage through a 0.2 µm syringe filter. The extract was stored at -20°C in 1 ml aliquots.

2.4 ANALYSIS OF HDM EXTRACT BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

2.4.1 Preparation of 14% SDS-Polyacrylamide Mini Gels

A 14% resolving gel solution was prepared by mixing 7 ml 50% Acrylamide/BIS, 9 ml separating gel buffer, 250 µl 10% w/v SDS, 7.7 ml H_2O, 625 µl 10 % (w/v) ammonium persulphate solution and 6.25 µl TEMED. The gel solution was immediately poured into 4 gel cassettes (Invitrogen, USA) up to approximately 2 cm from the top of the cassette and H_2O was overlaid onto the gel solution to exclude atmospheric oxygen which interferes with polymerisation. The gels polymerised over a time period of approximately 1 hour. The water at the top of the gels was then poured off and replaced with a 4% stacking gel. The stacking gel consisted of 1 ml 50% Acrylamide/BIS, 4.2 ml stacking gel buffer, 6.3 ml H_2O, 5 µl TEMED and 1 ml 10 % (w/v) ammonium persulphate solution and was used for 4 mini gels. A plastic template was then inserted

into the stacking gel to create wells in the gel for sample loading. The gels were allowed to stand for 1 hour to polymerise.

2.4.2 SDS-PAGE

Gels were loaded on a XCELL II Mini Cell (Novex, USA) electrophoresis unit and SDS running buffer was added. HDM extract samples were diluted in H_20 to achieve the required protein concentration, reducing SDS-sample buffer was added and the samples were boiled for 5 minutes. Samples (10 µl) were run alongside Benchmark™ pre-stained molecular weight markers (1.25 µl). The gel was run at a constant voltage of 125 volts for approximately 1½ hours or until the dye front reached the bottom of the gel. After electrophoresis, the gel was silver stained using the SilverXpress™ Silver Staining Kit (Invitrogen, USA) according to the manufacturer's instructions.

2.5 CELL CULTURE

2.5.1 Mononuclear Cell Separation from Peripheral Blood

Peripheral blood (30-100 ml) was obtained from subjects by venipuncture in 50 ml syringes coated with sodium heparin to prevent clotting. Blood was firstly diluted 1:1 in warm (37°C) RPMI/heparin medium. The diluted blood (25 ml) was gently layered on to 15 ml of Ficoll-Paque in 50 ml polypropylene tubes (Greiner, Germany). With the brake off, samples were then centrifuged at 652 g for 25 minutes at room temperature. Using sterile disposable plastic pipettes, PBMC layers were taken off the Ficoll and resuspended in 20 ml RPMI/heparin medium in fresh 50 ml tubes. Cells were pelleted by centrifugation at 452 g for 15 minutes. Cells were resuspended in 50 ml of RPMI/heparin medium, and centrifuged at 329 g for 10 minutes. Separated PBMC were resuspended in complete medium, and using a haemocytometer the viable cell number

was calculated by trypan blue exclusion. HDM extract-stimulated cultures were established from the PBMC and the remaining PBMC were frozen and stored in liquid N_2 for use as APC or in other cultures.

2.5.2 Freezing of PBMC or T Cell Cultures

To store PBMC and T cell cultures for later use, cells were pelleted by centrifugation at 329 g for 10 minutes, resuspended with complete medium and diluted 1:1 with ice cold FBS/15% DMSO to give a final concentration of 0.5-1 x 10^7 cells/ml. The cell suspension was divided into polypropylene cryovials (Greiner, Germany; 1 ml/vial). Vials were placed into a freezing container, "Mr Frosty" (Nalgene, USA), that was transferred to a -80°C freezer for a minimum of 4 hours. Vials were then placed in liquid N_2 for long term storage. When cells from the liquid N_2 were required, they were defrosted at 37°C in a water bath, resuspended in 10 ml cold (4°C) RPMI wash medium and centrifuged at 329 g for 10 minutes to remove DMSO. Cells were then resuspended in complete medium ready for use in assays.

2.5.3 PBMC Proliferation Assays

PBMC (1 x 10^5/well), either freshly isolated or from stocks stored in liquid nitrogen, were incubated in 96-well round-bottom tissue culture plates (Linbro, ICN Biomedicals, USA) in triplicate in the presence of complete medium and HDM extract at various concentrations, medium alone as the negative control or PHA (2 µg/ml) as the positive control for 7 days at 37°C in 5% CO_2 in a humidified incubator. Cultures were pulsed with 1 µCi ^{3}H-thymidine (Amersham Biosciences, UK) for the last 16 hours of incubation and then harvested onto printed glass fibre filters (Wallac, UK) using a 96-well automatic cell harvester (Skatron, UK). Proliferation as correlated with ^{3}H-

thymidine incorporation was measured by liquid scintillation spectroscopy using a Wallac 1205 β-counter and results expressed as Δ cpm (the mean cpm of the triplicates with antigen minus the mean cpm of the triplicates without antigen) or as a stimulation index (SI; the mean cpm of the triplicates with antigen divided by the mean cpm of the triplicates without antigen). Proliferative responses were considered positive if the SI was ≥2.5.

2.5.4 Generation of 14-day HDM Extract-Stimulated Cultures

PBMC (2.5 x 10^6/well), either freshly isolated or from stocks stored in liquid nitrogen, were cultured in 24-well tissue culture plates (Costar, USA) in complete medium with HDM extract at either 1, 10, 25 or 100 μg/ml in a final volume of 2 ml/well for 7 days at 37°C in 5% CO_2 in a humidified incubator. A 'no allergen' control culture 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 x 10^6 cells/ml) were restimulated with HDM extract at 1, 10, 25 or 100 μg/ml in the presence of washed irradiated (3000 rads) autologous PBMC (from liquid nitrogen stocks; 1 x 10^6/ml) as a source of APC in fresh 24-well plates. On day 8 of culture rIL-2 (25 U/ml) was added, and on day 11 of culture 1 ml of culture medium was removed and replaced with fresh medium and rIL-2 (25 U/ml). On day 14, cultured cells were used in T cell assays for the detection of proliferation and cytokine production (as described below) or frozen for future use.

2.5.5 Oligoclonal T Cell Proliferation Assays

T cells (5 x10^4/well) from 14-day HDM extract-stimulated cultures were incubated in 96-well round-bottom plates in triplicate with equal numbers of washed irradiated (3000 rads) autologous PBMC as APC in the presence of HDM extract at various

concentrations 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 negative controls. After 48 hours culture at 37°C in 5% CO_2 in a humidified incubator, cells were pulsed with 1 µCi ^{3}H-thymidine and harvested 16 hours later onto printed glass fibre filters using a 96-well automatic cell harvester. Proliferation as correlated with ^{3}H-thymidine incorporation was measured by liquid scintillation spectroscopy.

2.5.6 Allergen Mitogenicity Assays

Mitogenicity of the HDM extract was assessed by culture of HDM extract with oligoclonal latex-reactive T cells and APC. Fourteen-day GE-stimulated T cells (5 x 10^4/well), generously donated by Neeru Eusebius, were incubated in 96-well round-bottom plates in triplicate with equal numbers of washed irradiated (3000 rads) autologous PBMC and HDM extract at various concentrations 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 negative controls. T cells cultured in the presence of IL-2 (100 U/ml) were included as a positive control. Cultures were incubated for 48 hours, pulsed for another 16 hours with ^{3}H-thymidine (1 µCi/well) and harvested onto printed glass fibre filters with a 96-well automatic cell harvester. ^{3}H-thymidine incorporation was measured by liquid scintillation spectroscopy.

2.5.7 Allergen Toxicity Assays

Toxicity of the HDM extract was excluded by culture of HDM extract with oligoclonal latex-reactive T cells and IL-2. Fourteen-day GE-stimulated T cells (5 x 10^4/well), generously donated by Neeru Eusebius, were incubated in 96-well round-bottom plates in triplicate with HDM extract at various concentrations in the presence of rIL-2 (100

U/ml) in a final volume of 200 µl/well. No APC were included in assay cultures. Cultures of T cells in the absence of antigen were included as a negative control and cultures of T cells in the presence of IL-2 (100 U/ml) were included as a positive control. Cultures were incubated for 48 hours, pulsed for another 16 hours with ^{3}H-thymidine (1 µCi/well) and harvested onto printed glass fibre filters with a 96-well automatic cell harvester. ^{3}H-thymidine incorporation was measured by liquid scintillation spectroscopy.

2.5.8 Assessing HDM Extract for Non-Specific Induction of Cytokine Production

To test for non-specific induction of cytokine production by the HDM extract, 14-day GE-stimulated T cells (7.5 x 10^5/well) were incubated in 48-well culture plates 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 5% CO_2. Cultures of T cells and APC with GE (40 µg/ml), IL-2 (100 U/ml), HDM extract (100 µg/ml), or without antigen were included as controls. After culture cells were analysed for intracellular IL-4 and IFN-γ (Section 2.6.3.1).

2.5.9 Assessing the Enzyme Activity of HDM Extract

Fourteen-day GE-stimulated T cells (1 x 10^6 cells/well) were cultured with complete medium in the presence of IL-2 (100 U/ml) in a 48-well tissue culture plate for 24 hours at 37°C in 5% CO_2 in a total volume of 750 µl. After 24 hours, HDM extract at 1, 10 and 100 µg/ml and KLH at 1, 10 and 100 µg/ml were added in a volume of 250 µl to different wells of the IL-2-stimulated T cells. A negative control containing no antigen was also used. The T cells were cultured for another 24 hours at 37°C in 5% CO_2,

harvested for surface staining with anti-CD4-Allophycocyanin and anti-CD25-FITC and analysis by flow cytometry (Section 2.6.1).

2.5.10 Endotoxin Detection in HDM Extract

Endotoxin levels in HDM extracts were determined using the QCL-1000™ endotoxin detection kit (BioWhittaker, Norway) according to the manufacturer's instructions.

2.6 FLOW CYTOMETRY

All fluorochrome-labelled monoclonal antibodies used in surface staining and intracellular cytokine staining (Sections 2.6.1-2.6.3) were titrated prior to use to determine the optimal antibody dilution for detection by flow cytometry.

2.6.1 Cell Surface Staining

Surface marker staining of PBMC and T cells from *in vitro* cultures was performed in round-bottom 96-well plates (Costar, USA). Before staining, cells (0.5×10^6/well) were washed once in wash buffer and pelleted by centrifugation (329 g, 5 minutes, 4°C). Cells were then incubated with appropriate fluorochrome-labelled monoclonal antibodies or relevant isotype controls (10 µl/well) for 15 minutes in the dark. After washing in wash buffer and pelleting by centrifugation (329 g, 5 minutes), cells were resuspended in wash buffer and transferred into tubes for flow cytometry. The percentage of stained cells was determined from 30,000 events using a Becton Dickinson FACScalibur flow cytometer and Cellquest software.

2.6.2 Cell Surface Staining for Adhesion Molecules and Chemokine Receptors

Surface marker staining of 14-day HDM extract-stimulated cell cultures was performed

in round-bottom 96-well plates (Costar, USA). Before staining, cells (0.5 x 10^6/well) were washed once in wash buffer and pelleted by centrifugation (329 g, 5 minutes, 4°C). Cells were then incubated with either anti-CD4-Allophycocyanin or anti-CD4-Cy-Chrome and anti-CD8-FITC or anti-CD8-CyChrome and isotype-specific controls ($IgG_{1,\kappa}$-FITC, $IgG_{1,\kappa}$-Cy-Chrome and $IgG_{1,\kappa}$-Allophycocyanin) at 10 µl/well for 15 minutes in the dark. After washing in wash buffer and pelleting by centrifugation (329 g, 5 minutes), cells were then labelled for 15 minutes with anti-CD62L-FITC, anti-CD49d-CyChrome, anti-CCR3-FITC and anti-CCR5-FITC (10 µl/well). Mouse $IgG_{1,\kappa}$-FITC, mouse $IgG_{1,\kappa}$-CyChrome, rat $IgG_{2a,\kappa}$-FITC and mouse $IgG_{2a,\kappa}$-FITC served as isotype-specific controls. After the incubation, the cells were washed again, resuspended in 200 µl of wash buffer and transferred into tubes for flow cytometry. The percentages of $CD4^+$ and $CD8^+$ T cells expressing $CD62L^-$, $CD49d^{hi}$, $CCR3^+$ or $CCR5^+$ was determined from 30,000 events by flow cytometer and Cellquest software. The threshold for positive staining of adhesion molecules and chemokine receptors 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.

2.6.3 Intracellular Cytokine Staining

2.6.3.1 Intracellular IL-4 and IFN-γ

Fourteen-day HDM extract-stimulated cultures were first stimulated with immobilised anti-CD3 mAb and rIL-2 before staining for intracellular IL-4 and IFN-γ. For this, anti-CD3 mAb (OKT-3, 10 µg/ml in sterile PBS) was firstly coated (30 µl/well) on to wells of a 96-well round-bottom tissue culture plate for 1 hour at 37°C and unbound antibody was then removed by washing with PBS. Cultured cells (2.5 x 10^5 cells/well) were

added to these wells and incubated in the presence of rIL-2 (100 U/ml) and 10 µg/ml Brefeldin A in a final volume of 200 µl/well for 6 hours at 37°C in 5% CO_2. A stimulation period of 6 hours was found to be optimal for the detection of IL-4 and IFN-γ by $CD4^+$ T cells (see Section 3.3.4.1). After incubation, cells were pelleted by centrifugation at 329 g for 5 minutes, resuspended in wash buffer and transferred to a fresh 96-well round-bottom plate (0.5 x 10^6/well) for staining. The following steps for intracellular cytokine detection were performed at room temperature and cells were incubated in the dark. Cells were surface stained by incubation with anti-CD4-CyChrome and anti-CD8-FITC or appropriately-labelled isotype controls (10 µl/well) for 15 minutes. In experiments where the expression of surface "trafficking" markers by cytokine (IL-4 or IFN-γ) positive T cells was analysed, the cells were labelled at this point with anti-CD62L, anti-CD49d, anti-CCR3 and anti-CCR5 antibodies or the appropriately labelled isotype controls as described in Section 2.6.2. Cells were then washed by addition of 180 µl of wash buffer, and pelleted by centrifugation at 329 g for 5 minutes. Cells were then fixed for 20 minutes in 50 µl 4% paraformaldehyde, washed by addition of 150 µl of wash buffer, centrifuged at 329 g for 5 minutes, and permeabilised for 20 minutes by addition of 50 µl of 1x permeabilising solution. After washing as for the fixation step, cells were double labelled with anti-IL-4-PE (31.25 ng/well) and anti-IFN-γ-Allophycocyanin (62.5 ng/well) or appropriately labelled isotype controls in a 10 µl total volume for 35 minutes at room temperature. Cells were washed as for surface staining, resuspended in 150 µl wash buffer and transferred to tubes for analysis. Percentages of cytokine-positive $CD4^+$ and $CD8^+$ T cells were determined by flow cytometry and Cellquest software.

2.6.3.2 Intracellular IL-10

Intracellular IL-10 was detected in T cells from 14-day HDM extract-stimulated cultures. For this, the wells in a 96-well round-bottom tissue culture plate were coated with anti-CD3 mAb (10 μg/ml) as for the detection of IL-4 and IFN-γ. Cultured cells (2.5×10^5 cells/well) were then added to the 96-well round-bottom plate and in the presence of rIL-2 (100 U/ml) were incubated for 20 hours at 37°C. Brefeldin A (10 μg/ml) was added to the cells for the last 4 hours of stimulation. A stimulation period of 20 hours was found to be optimal for the detection of IL-10 by $CD4^+$ T cells (see Section 3.3.4.2). After incubation, cells were pelleted by centrifugation at 329 g for 5 minutes, resuspended in wash buffer and transferred to a fresh 96-well round-bottom plate (0.5×10^6/well) for staining. Cells were then surface stained for CD4 and CD8. In experiments where the expression of surface "trafficking" markers by cytokine (IL-10) positive T cells was analysed, the cells were labelled at this point with anti-CD62L, anti-CD49d, anti-CCR3 and anti-CCR5 antibodies or the appropriately labelled isotype controls as described in Section 2.6.2. Following surface marker staining, the cells were fixed and permeabilised as for IL-4 and IFN-γ detection (Section 2.6.3.1). After washing by addition of 150 μl of wash buffer and pelleting by centrifugation at 329 g for 5 minutes, cells were stained with anti-IL-10-PE (62.5 ng/well) or the relevant isotype control (10 μl/well) for 35 minutes at room temperature. Cells were washed again by addition of 180 μl of wash buffer, then pelleted at 329 g for 5 minutes, resuspended in 150 μl wash buffer and transferred to tubes for analysis. Percentages of IL-10-positive $CD4^+$ and $CD8^+$ T cells were determined by flow cytometry and Cellquest software.

2.6.4 Analysis of T Cell Division

2.6.4.1 CFSE labelling

PBMC or cultured cells were labelled with 1 μM CFSE to analyse cell division. For this, cells (1 x 10^7 cells/ml) were washed twice in sterile PBS and centrifuged at 329 g for 10 minutes. After the supernatant was removed, freshly prepared CFSE (1 μM; in sterile PBS) was added to cell samples. Immediately after CFSE was added to a cell pellet, the sample was vortexed gently. Cells were then incubated with CFSE for 10 minutes at 37°C. During the incubation samples were vortexed gently, twice. To cease staining, cells were washed by the addition of a 10x volume of complete medium and centrifugation at 329 g for 10 minutes. Cells were washed a further two times in complete medium to ensure there was minimal residual CFSE.

2.6.4.2 Analysis of T cell division in HDM extract-stimulated cultures

After 7 days of PBMC culture with HDM extract (1, 10 and 100 μg/ml) cells were labelled with CFSE (Section 2.6.4.1) to analyse cell division. 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. Optimisation experiments revealed that this positive control was required because defined peaks of CFSE intensity were not detected for T cells stimulated with HDM extract (See Section 3.3.4.3). Hence the positive control cultures were run in parallel to enable the setting of division interval positions for allergen-stimulated $CD4^+$ T cells according to CFSE fluorescence intensity peaks for positive control culture $CD4^+$ T cells. After culture cells were harvested, surface stained with either anti-CD4-CyChrome or anti-CD8-CyChrome or the appropriately labelled isotype controls (see Section 2.6.1) and analysed by flow

cytometry. In other experiments cytokine production by dividing T cells was analysed by intracellular cytokine detection as detailed in Section 2.6.3.

2.6.5 Analysis of Apoptosis

2.6.5.1 Annexin-V staining

Apoptotic PBMC or cultured cells were analysed by Annexin-V and propidium iodide staining and flow cytometry. The method for this procedure had previously been determined in the laboratory (Lina Papalia, personal communication). Cells were placed in tubes (0.5 x 10^6 cells/tube) for staining, washed in 500 µl wash buffer and pelleted by centrifugation at 329 g for 5 minutes. Cells were then incubated with anti-CD4-Allophycocyanin (10 µl/tube) for 15 minutes in the dark at room temperature. A control sample was stained with a relevant isotype control. Cells were then washed by the addition of 500 µl wash buffer and centrifuged at 329 g for 5 minutes. Pelleted cells were resuspended at 1 x 10^6 cells/ml in Annexin-V binding buffer and 100 µl of this cell suspension was transferred to a fresh tube and 5 µl of Annexin-V-FITC or Annexin-V-PE was added. Cells were incubated for 15 minutes in the dark at room temperature and propidium iodide (concentration in 10 µl/tube of 50 µg/ml) was added to the stained cells 5 minutes before cytometric analysis. The percentage of Annexin-V^+PI^-, early apoptotic, and Annexin-V^+PI^+, late apoptotic, $CD4^+$ T cells was then determined from 30000 events by flow cytometry and Cellquest software.

2.6.5.2 Intracellular active caspase-3 staining

Intracellular anti-active caspase-3 staining was used to determine the cytokine profile of apoptotic T cells in 14-day HDM extract-stimulated cultures and thus was performed in parallel with intracellular IL-4 and IFN-γ staining. The method used for intracellular

cytokine detection was found to be satisfactory for the detection of active caspase-3 (Section 3.3.4.4). Cultured cells were harvested and stimulated in a 96-well round-bottom plate (2.5 x 10^5 cells/well) with immobilised anti-CD3 (10 µg/ml) and rIL-2 (100 U/ml) for 6 hours at 37°C in the presence of 10 µg/ml Brefeldin A. After stimulation, cells were washed and transferred to a fresh 96-well round-bottom plate (0.5 x 10^6 cells/well) and surface labelled with anti-CD4 (10 µl/well) for 15 minutes at room temperature in the dark. Cells were then fixed and permeabilised as for intracellular cytokine detection. Cells were then triple stained with anti-active caspase-3-FITC (7.5 µl/well), anti-IL-4-PE (31.25 ng/well) and anti-IFN-γ-Allophycocyanin (62.5 ng/well) in a total volume of 10 µl per well for 35 minutes at room temperature in the dark. The anti-active caspase-3 antibody binds the 17 kDa active form of caspase-3 and not the proenzyme caspase-3. The percentages of active caspase-3 positive $CD4^+IL\text{-}4^+$ and $CD4^+IFN\text{-}\gamma^+$ T cells were determined by flow cytometry and Cellquest software.

2.7 STATISTICAL ANALYSIS

Data that were found to be well approximated by a log normal distribution were normalised via log-transformation prior to analysis. Statistical analysis was performed using Repeated Measures ANOVA with a Bonferroni adjustment for multiple comparisons. In some analyses group comparisons were made using the non-parametric Wilcoxon Signed Rank test. Statistical significance between groups was analysed using a Mann Whitney test and correlations were assessed using the Spearman Rank test. A two-sided p-value of 0.05 was considered statistically significant. InStat 2.0 software was used for statistical analyses.

CHAPTER 3

CHARACTERISATION OF HOUSE DUST MITE EXTRACT AND OPTIMISATION OF T CELL ASSAYS

3.1 INTRODUCTION

This study was designed to investigate the effect of allergen concentration on T cell responses *in vitro*. In this chapter the preparation and characterisation of the allergen extract to be used for the stimulation of T cells *in vitro* is presented. HDM extract was chosen as a model allergen. The optimisation of assays required for the analysis of T cell responses is also described in this chapter. In previous studies, T cell cytokine responses to different allergen concentrations were analysed by measuring cytokine levels in supernatants by ELISA (Secrist *et al.*, 1995; Carballido *et al.*, 1997). Using this method the net outcome of cytokine production is determined. In the current study, T cell responses were analysed at the single cell level to clearly dissect mechanisms by which allergen concentration alters the T cell response. Cytokine production was assessed by intracellular cytokine staining and flow cytometry. By CFSE cell labelling and subsequent analysis by flow cytometry, T cell division was also examined at the single cell level. The analysis of apoptosis was performed using two flow cytometric techniques. The first involved labelling phosphatidyl serine exposed on the surface of cells undergoing apoptosis with Annexin-V. The second method involved labelling intracellular active caspase-3 in apoptotic cells and was coupled with intracellular cytokine staining to determine the cytokine phenotype of T cells undergoing programmed cell death.

3.2 EXPERIMENTAL PROCEDURES

For the optimisation of T cell assays, a panel of 14 donors was chosen based on the selection criteria described in Section 2.2. The clinical characteristics of these donors are presented in Table 3.1. Of the panel, 13 were HDM allergic, one of whom had received HDM-immunotherapy in the past 5 years, and the other donor was non-atopic. Refer to Chapter 2 for the methods used in this chapter.

Table 3.1 Characterisitics of donors used in this chapter.

Patient	Sex	Age	Rhinitis	Asthma	HDM skin test reactivity (wheal mm)	HDM EAST score (AEU/ml)	Received HDM SIT in the past 5 years
A1	F	42	Y	Y	11	5 (61.9)	N
A2	F	55	Y	Y	21	...	N
A3	F	22	Y	Y	16.5	...	N
A4	M	23	N	Y	15 x 8	...	N
A5	M	52	Y	Y	16 x 10	...	N
A6	F	22	N	Y	7	...	N
A7	F	40	Y	Y	13	...	N
A8	F	22	N	Y	10	3 (8.0)	N
A9	F	26	Y	N	8	...	N
A12	F	25	Y	Y	10	2 (2.5)	N
A19	M	31	...	...	8	...	N
A31	F	27	Y	Y	15	...	N
D1	F	31	N	Y	12 x 10	...	Y
N1	M	25	N	N	0	0	N

A, atopic; D, desensitised; N, non-atopic; F, female; M, male; ..., not available; Y, yes; N, no

3.3 RESULTS

3.3.1 Analysis of the HDM Extract Used in T Cell Cultures

The HDM extract, which was to be used in T cell cultures was prepared as described in Section 2.3 of this thesis. The proteins in this extract were resolved by 14% SDS-PAGE and visualised by silver staining (Figure 3.1). The HDM extract consists of many protein bands revealing its heterogeneous nature. The large intensely stained band at approximately 25 kDa is presumably Der p 1, the major allergen. Affinity purified Der p 1 has been shown in our laboratory to run at the same position as this large band (data not shown) and the relative molecular mass of this band is consistent with the previously determined molecular mass of Der p 1 (Thomas *et al.*, 2002). These results indicate that the HDM extract used as a model allergen in this research had a similar protein profile to that of previously described HDM extracts (O'Hehir *et al.*, 1987; Thomas *et al.*, 2002).

3.3.2 Assessing the Suitability of the HDM Extract for Use in T Cell Cultures

In this section the mitogenicity, toxicity, enzyme activity and endotoxin content of the HDM extract were determined to ensure that the extract was suitable for allergen-specific stimulation of T cells *in vitro*.

3.3.2.1 Assessing mitogenicity and toxicity of HDM extract

The HDM extract was tested for non-specific T cell activation in a mitogenicity assay. Latex-specific T cells were cultured in the presence of APC and HDM extract at 1, 10 or 100 μg/ml (Figure 3.2a). The HDM extract did not induce proliferation of latex-specific T cells (SI<2.5 at all HDM concentrations) indicating that it was not mitogenic. To ensure that HDM was not toxic to T cells, latex-specific T cells were cultured with

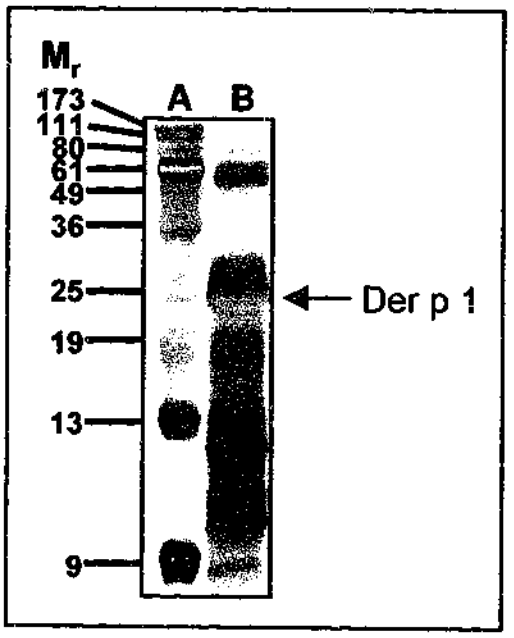


Figure 3.1 SDS-PAGE analysis of HDM extract.
The HDM extract was resolved under reducing conditions by 14% SDS-PAGE and proteins were visualised by silver staining. To lane A 1.25 µl Benchmark™ pre-stained molecular weight markers were added. Along side this in lane B 10 µl HDM extract was added. The HDM extract had previously been diluted in reducing SDS-sample buffer and boiled for 5 minutes. The gel was run at a constant voltage of 125 volts for approximately 1½ hours. After electrophoresis, the gel was silver stained using the SilverXpress™ silver staining kit. The approximate position of Der p 1 is indicated.

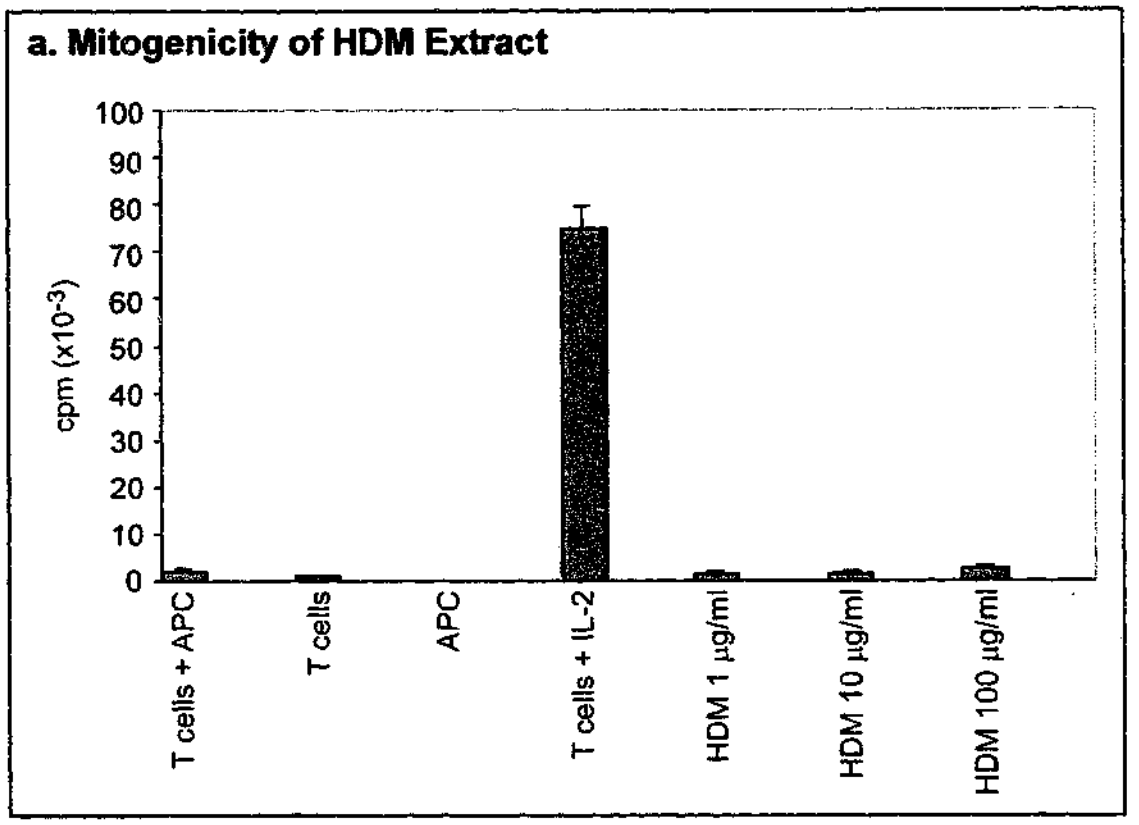


Figure 3.2 Analysis of mitogenicity and toxicity of HDM extract.
(a) Mitogenicity of the HDM extract was assessed by culture of oligoclonal latex-reactive T cells in the presence of APC and HDM extract. Latex-specific T cells were incubated in 96-well round-bottom plates in triplicate with equal numbers of washed irradiated autologous PBMC and HDM extract at 1, 10 and 100 µg/ml. Cultures were incubated at 37°C, 5% CO_2 for 48 hours, and pulsed for another 16 hours with ^{3}H-thymidine. Cells were harvested and ^{3}H-thymidine incorporation was measured by liquid scintillation spectroscopy and expressed as cpm. Mean values for triplicate cultures plus standard deviation are shown.
(b) Toxicity of the HDM extract was assessed by culture of oligoclonal latex-reactive T cells in the presence of IL-2 and HDM extract. Latex-specific T cells were incubated in 96-well round-bottom plates in triplicate in the presence of no antigen or 1, 10 or 100 µg/ml HDM extract with IL-2 (100 U/ml). Cultures were incubated at 37°C in 5% CO_2 for 48 hours, and pulsed for another 16 hours with ^{3}H-thymidine. Cells were harvested and ^{3}H-thymidine incorporation was measured by liquid scintillation spectroscopy and expressed as cpm. Mean values for triplicate cultures plus standard deviation are shown.

IL-2 in the presence of HDM extract at 1, 10 or 100 μg/ml (Figure 3.2b). The HDM extract did not cause a reduction in IL-2-induced proliferation of the latex-specific T cells indicating that it was not toxic.

3.3.2.2 Assessing enzyme activity of the HDM extract

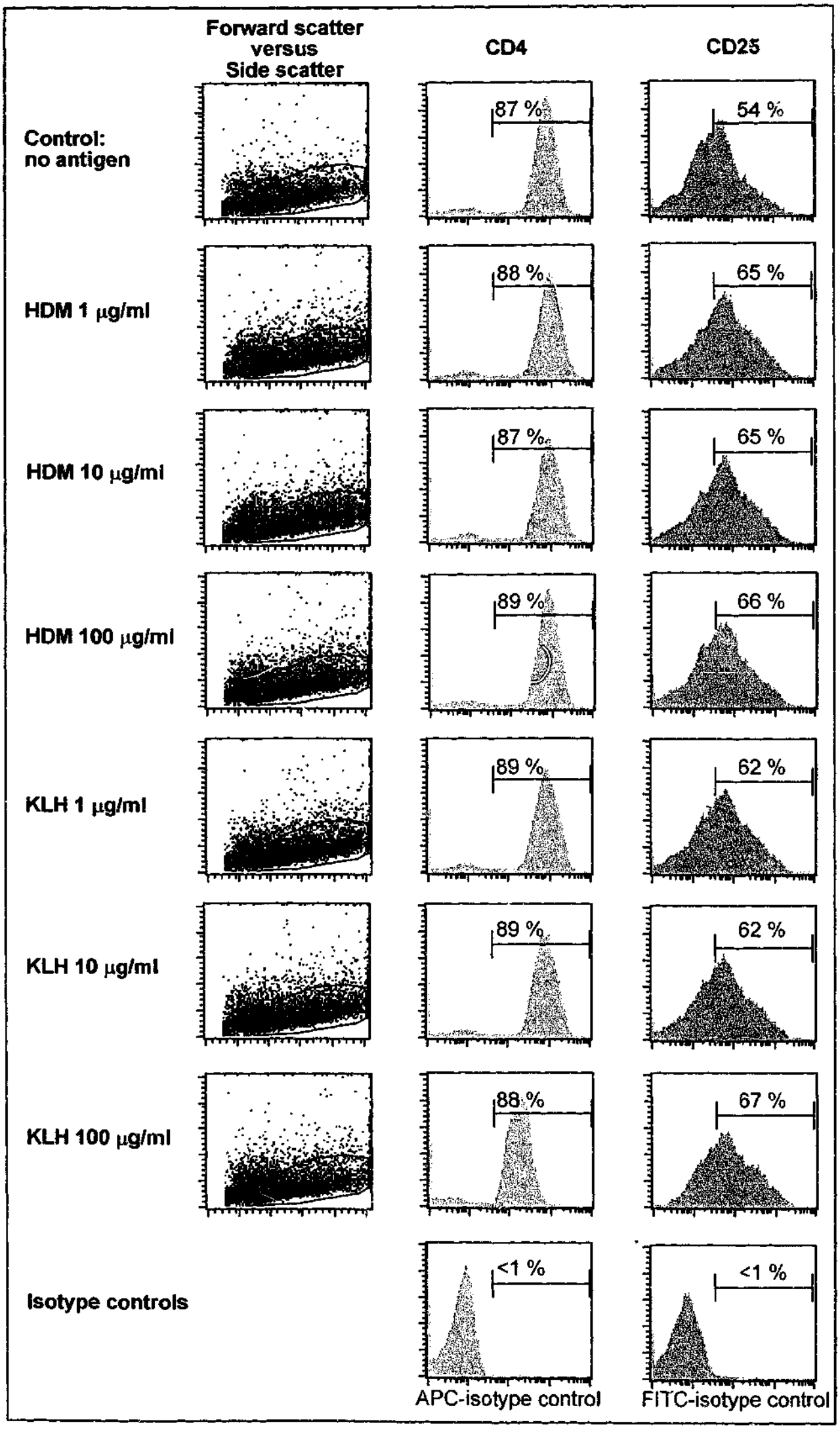
As mentioned above HDM extract consists of many proteins, including the protease Der p 1. Der p 1 can cleave a number of surface proteins, such as CD25, expressed by human leukocytes (Schulz *et al.*, 1998a; Ghaemmaghami *et al.*, 2002). As the cleavage of CD25 from Th cells has been shown to alter Th cell cytokine production in response to HDM (Ghaemmaghami *et al.*, 2001), an experiment was devised to detect CD25 cleavage from $CD4^+$ T cells cultured with the HDM extract used in this project. Latex-specific T cells were cultured in the presence of IL-2 to up-regulate CD25 expression. HDM extract, at 1, 10 or 100 μg/ml, was then added to the IL-2-stimulated T cells and 24 hours later CD25 expression by $CD4^+$ T cells was analysed by flow cytometry. In Figure 3.3 it can be seen that CD25 expression by $CD4^+$ T cells in the HDM extract was not reduced compared with levels of expression seen for the no antigen control or the non-enzyme protein, KLH, control. Thus with respect to CD25 cleavage the HDM extract was not enzymically active.

3.3.2.3 Assessing the levels of endotoxin in HDM extract

Endotoxin levels in HDM extract were undetectable (<3 U/ml) as determined by the BioWhittaker QCL-1000™ endotoxin detection kit.

Figure 3.3 Assessing the enzyme activity of HDM extract.

Latex-specific T cells were cultured with complete medium in the presence of IL-2 (100 U/ml) in a 48-well tissue culture plate for 24 hours at 37°C in 5% CO_2. HDM extract at 1, 10 and 100 μg/ml and, as a control, KLH at 1, 10 and 100 μg/ml were then added to the IL-2-stimulated T cells. A negative control with no antigen added was also used. Following another 24-hour culture, T cells were harvested, washed and surface stained with anti-CD4-APC and anti-CD25-FITC antibodies or the appropriately labelled isotype controls. Labelled cells were then analysed by flow cytometry. CD25 expression by $CD4^+$ T cells was analysed by gating on the lymphocyte population as determined by forward scatter versus side scatter and the $CD4^+$ cells within this gate. Markers indicating positive staining are set according to minimal (<1%) isotype control staining. Percentages of positive cells are indicated and were determined from the acquisition of 30000 events by the flow cytometer.


Forward scatter versus Side scatter
CD4
CD25
Control: no antigen
87 %
54 %
HDM 1 µg/ml
88 %
65 %
HDM 10 µg/ml
87 %
65 %
HDM 100 µg/ml
89 %
66 %
KLH 1 µg/ml
89 %
62 %
KLH 10 µg/ml
89 %
62 %
KLH 100 µg/ml
88 %
67 %
Isotype controls
<1 %
<1 %
APC-isotype control
FITC-isotype control

3.3.3 Phenotypic Analysis of Leukocytes after 14-Day Culture with HDM Extract

After determining that the HDM extract was suitable for *in vitro* T cell cultures, pilot experiments were performed to analyse the cell types present in 14-day HDM-extract stimulated PBMC cultures. As HDM extract is an exogenous antigen and is presented predominantly via MHC class II molecules to $CD4^+$ T cells, the proportions of $CD4^-$ T cells were analysed over the two-week culture period. After culture of HDM allergic donor (A9) PBMC for 7 days of stimulation with HDM extract, the proportion of $CD4^+$ T cells was 56% (Figure 3.4a). After 14 days of stimulation with HDM extract the $CD4^+$ T cell population had expanded as the proportion of $CD4^+$ T cells had increased to 86% (Figure 3.4b). Similar results were observed when this experiment was repeated on PBMC from another HDM-allergic donor (A6).

In addition to $CD4^-$ T cells, other types of leukocytes including $CD8^+$ T cells, B cells and monocytes were analysed by flow cytometry in the 14-day HDM extract-stimulated cultures (Figure 3.5a). The proportions of B cells (3%) and monocytes (<1%) were found to be low and as expected the proportion of $CD4^+$ T cells was high (85%). Interestingly 12% of cells in the culture at day 14 stained positive for CD8. The antibody used to stain these $CD8^+$ cells was an anti-CD8α antibody, which can label a number of different cell types including $CD8^+$ T cells, NK and NKT cells. Hence in a follow-up experiment $CD8\alpha^+$ cells were labelled with a panel of other cell surface markers to accurately determine their phenotype. $CD4^-$ cells were also analysed in this experiment as human monocytes express CD4 at low levels. Flow cytometric analysis revealed that virtually all the $CD4^+$ and $CD8\alpha^+$ cells were $CD3^+$ $\alpha\beta TRC^+$ $\gamma\delta TCR^-$ T cells (Figure 3.6). The $CD4^+$ T cells were $CD8\beta^-$ whereas the $CD8\alpha^+$ T cells were $CD8\beta^+$. In another experiment the proportion of NK cells in the 14-day HDM extract-

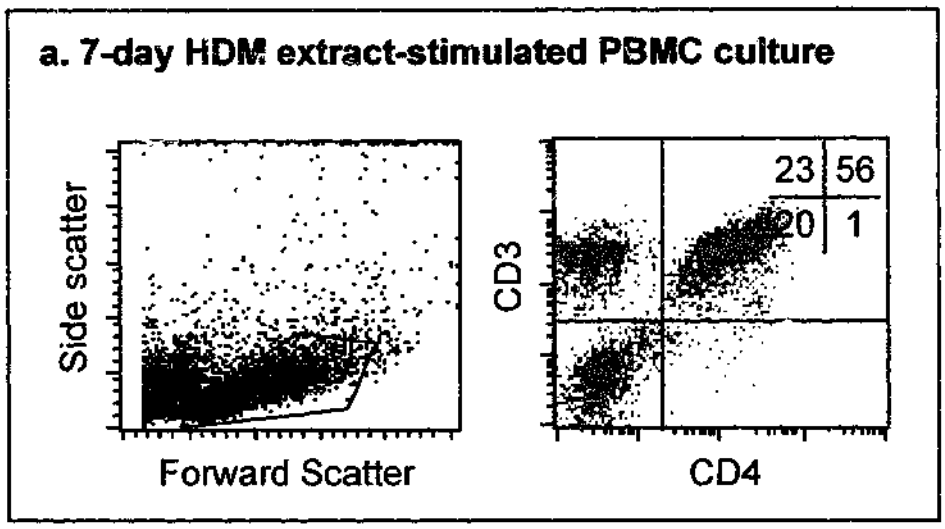
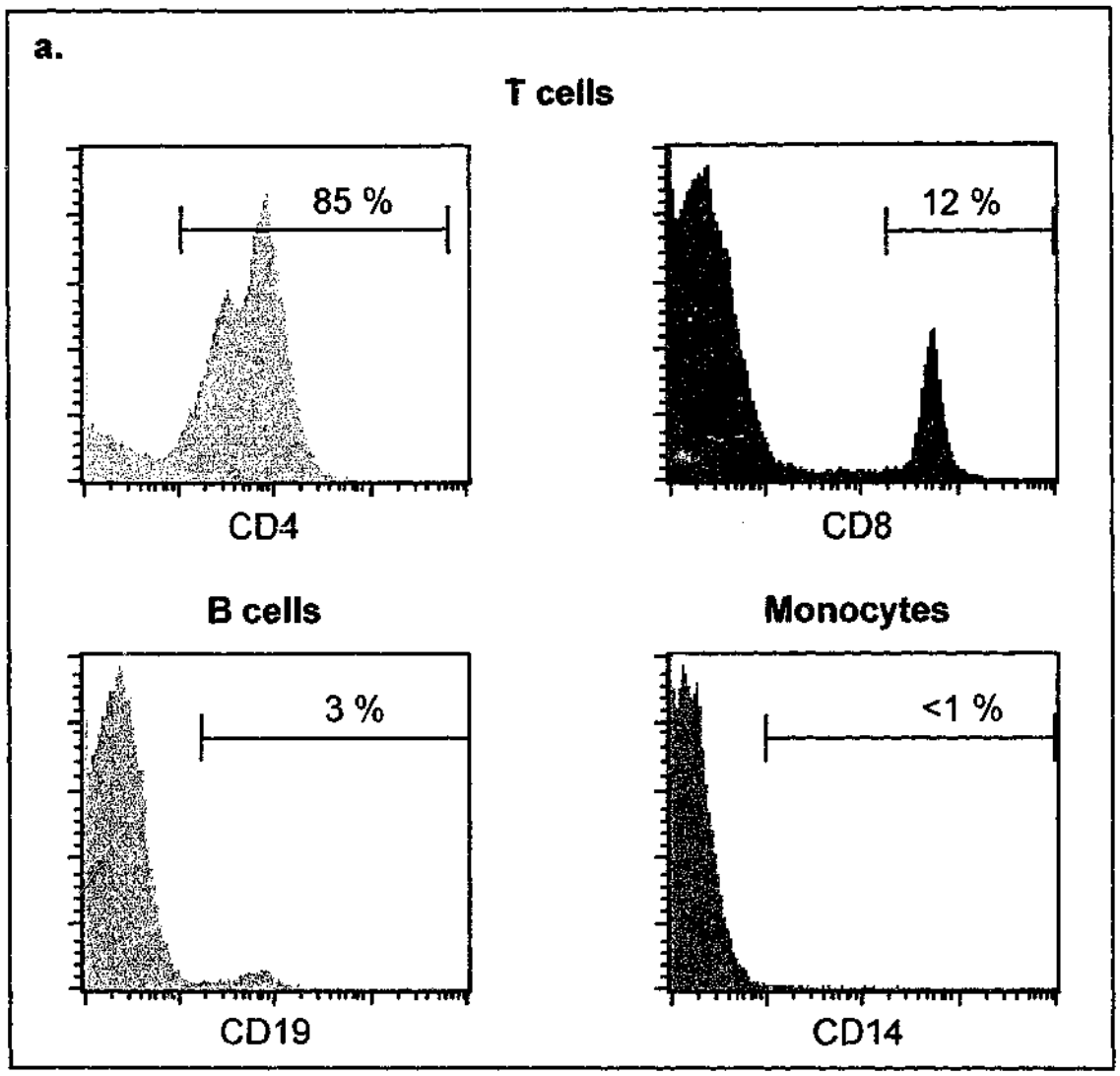


Figure 3.4 $CD4^+$ T cell expansion in 14-day HDM extract-stimulated cultures.
HDM-allergic donor PBMC (A9) were cultured in the presence of 25 µg/ml HDM extract at 37°C in 5% CO_2 for 7 days. At day 7, the cells were restimulated with 25 µg/ml HDM extract and irradiated PBMC as APC and cultured for another 7 days. At day 7 **(a)** and day 14 **(b)** cultured cells were stained with anti-CD4 and anti-CD3 antibodies and analysed by flow cytometry. The CD3 versus CD4 dot plots shown are gated on the lymphocyte population as determined by forward scatter versus side scatter. Quadrant markers were set according to minimal (<1%) isotype control staining (not shown). Quadrant percentages are indicated and were determined from the acquisition of 30000 events by the flow cytometer. This experiment was repeated on PBMC from another HDM-allergic donor (A6) achieving similar results.



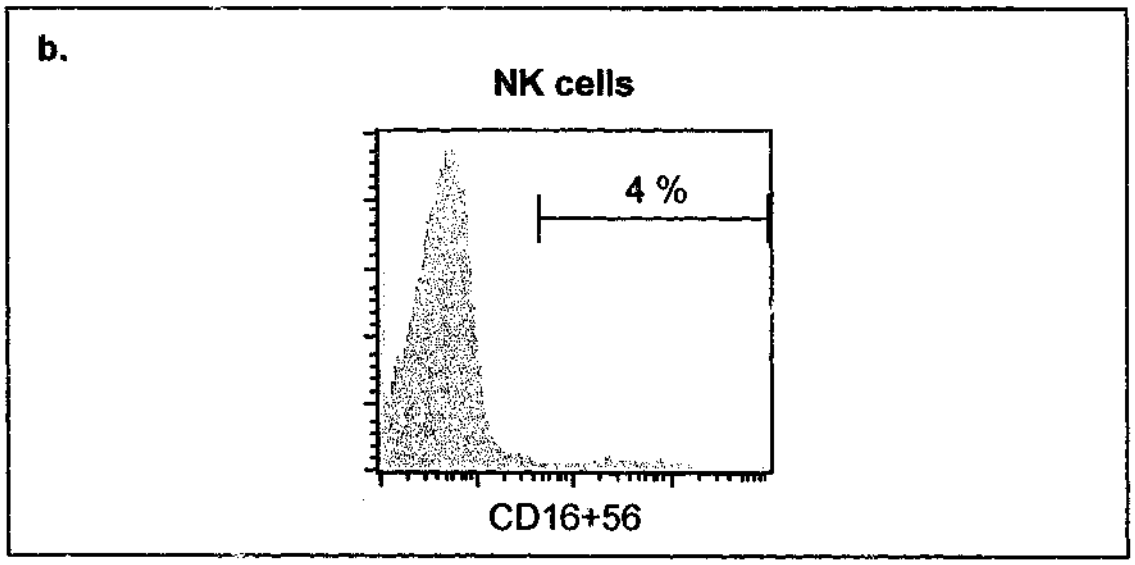


Figure 3.5 Phenotypic analysis of leukocytes present in 14-day HDM extract-stimulated cultures.

HDM-allergic donor PBMC (A3) were cultured in the presence of 25 µg/ml HDM extract at 37°C in 5% CO_2 for 7 days. At day 7, the cells were restimulated with 25 µg/ml HDM extract and irradiated PBMC as APC and cultured for another 7 days. **(a)** At day 14, cultured cells were stained with anti-CD4, anti-CD8 (T cell markers), anti-CD19 (B cell marker) and anti-CD14 (monocyte marker) antibodies and analysed by flow cytometry. **(b)** In a separate experiment 14-day HDM extract-stimulated cells generated from PBMC from donor A9 were stained with CD16+56 (NK cell markers) antibodies. The histograms shown are gated on the lymphocyte population as determined by forward scatter versus side scatter. Markers indicating positive staining are set according to minimal (<1%) isotype control staining (not shown). Percentages of positive cells are indicated and were determined from the acquisition of 30000 events by the flow cytometer.

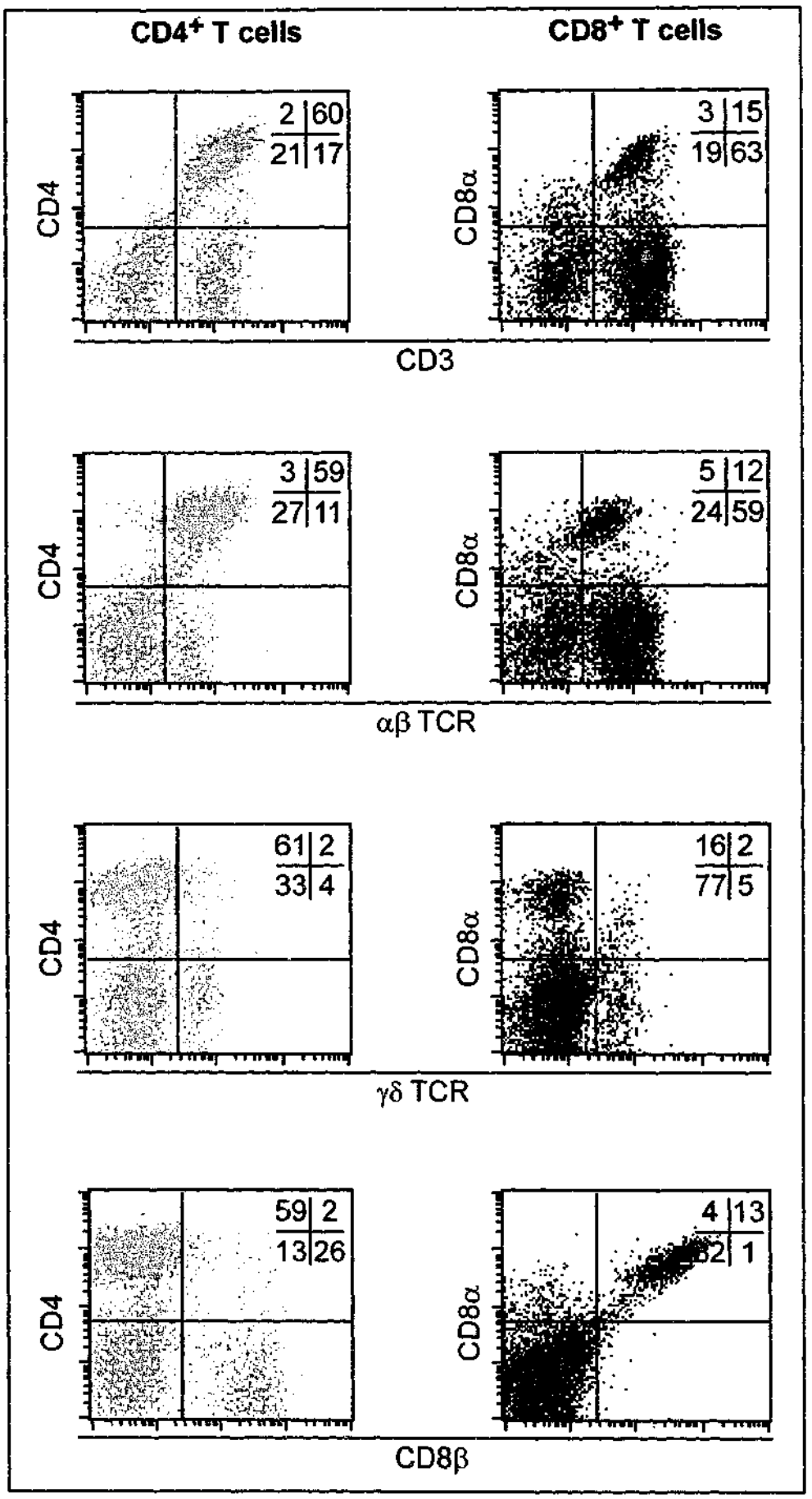


Figure 3.6 Phenotypic analysis of $CD4^+$ and $CD8^+$ cells present in 14-day HDM extract-stimulated cultures.

HDM-allergic donor PBMC (A7) were cultured in the presence of 25 µg/ml HDM extract at 37°C in 5% CO_2 for 7 days. At day 7, the cells were restimulated with 25 µg/ml HDM extract and irradiated PBMC as APC and cultured for another 7 days. At day 14 cells were stained with anti-CD4, anti-CD8α, anti-CD8β, anti-CD3, anti-αβTCR and anti-γδTCR and analysed by flow cytometry. The dot plots shown are gated on the lymphocyte population as determined by forward scatter versus side scatter. Quadrant markers indicating positive staining are set according to minimal (<1%) isotype control staining (not shown). Quadrant percentages are indicated and were determined from the acquisition of 30000 events by the flow cytometer.

stimulated culture was determined using the cell surface markers CD16 and CD56. The proportions of NK cells were found to be low (4%; Figure 3.5b).

3.3.4 Optimisation of T Cell Assays for Analysis of Cytokine Production, Cell Division and Apoptosis

3.3.4.1 Optimisation of detection of T cell intracellular IL-4 and IFN-γ by flow cytometry

In order to analyse T cell cytokine production at the single cell level in HDM extract-stimulated cultures, intracellular cytokine staining for IL-4 and IFN-γ was optimised. As discussed by Carter *et al.*, it can be very difficult to detect intracellular cytokines in T cells and a stimulation step using a polyclonal activator can be required to enhance cytokine levels (Carter and Swain, 1997). OKT3 $F(ab)_2$ stimulation of T cells pre-cultured with allergen has been shown to increase T cell cytokine production without altering the balance of IL-4 and IFN-γ production (Jutel *et al.*, 1995b). The addition of exogenous IL-2 to anti-CD3 mAb stimulated T cells also enhances cytokine production without skewing cytokine profiles (Demeure *et al.*, 1994). Therefore in this study, T cells were stimulated through the TCR with immobilised anti-CD3 mAb in the presence of IL-2 to detect allergen-specific T cell intracellular cytokines. The following section describes preliminary experiments performed to optimise the stimulation time and concentration of anti-CD3 mAb, and the type of costimulation required for intracellular cytokine detection.

Previous studies analysing intracellular IL-4 and IFN-γ for human T cells have reported that these cytokines can be detected after 4 to 10 hours of stimulation with polyclonal activators (Jung *et al.*, 1995; Prussin and Metcalfe, 1995). Therefore T cells from 14-

day HDM extract-stimulated cultures (from donors A6 and A5) were stimulated in the presence of immobilised anti-CD3 mAb (10 μg/ml) and IL-2 (100U/ml) for different lengths of time (2-10 hours) according to the protocol described in Section 2.6.3.1 (Figure 3.7). At the 6-hour time point the proportions of both IL-4 and IFN-γ positive $CD4^+$ T cells were maximal. Although the surface expression of CD3 by $CD4^+$ T cells was down-regulated from 76% at 2 hours of stimulation to 21% at 6 hours, the expression of CD4 remained unchanged. Thus 6 hours incubation allowed for the accurate gating of $CD4^-$ T cells for analysis of cytokine staining on this population of cells.

The strength of anti-CD3 stimulation required for optimal cytokine production was then determined by altering the concentration of anti-CD3 mAb coated on to the wells of the 96-well plate. In this experiment, HDM-allergic donor T cells (A12) were stimulated for 6 hours in the presence of anti-CD3 mAb at concentrations ranging from 0-15 μg/ml and IL-2 (100 U/ml) (Figure 3.8). The proportions of cytokine positive ($IL\text{-}4^+$ and $IFN\text{-}\gamma^+$) $CD4^+$ T cells were greater at concentrations between 7.5 and 15 μg/ml anti-CD3 mAb in comparison to concentrations less than 7.5 μg/ml. Only minimal variation in the proportions of $IFN\text{-}\gamma^+$ and $IL\text{-}4^+$ cells was observed for concentrations of anti-CD3 mAb between 7.5 and 15 μg/ml. CD4 expression also remained unchanged at this concentration range. CD3 expression was down-regulated from 99% in the absence of anti-CD3 mAb to 79% at 7.5 and 10 μg/ml anti-CD3 mAb and to 65% or lower at concentrations greater than 10 μg/ml. As cytokine production and CD4 expression were similar for anti-CD3 mAb concentrations between 7 and 15 μg/ml, an optimal concentration of 10 μg/ml was chosen.

Figure 3.7 Optimisation of anti-CD3 mAb stimulation time required for the detection of T cell intracellular IL-4 and IFN-γ.

HDM-allergic donor PBMC (A6) were cultured in the presence of 25 μg/ml HDM extract at 37°C in 5% CO_2 for 7 days. At day 7, the cells were restimulated with 25 μg/ml HDM extract and irradiated PBMC as APC and cultured for another 7 days. At day 14, cells were stimulated in the presence of plate bound anti-CD3 (10 μg/ml) and IL-2 (100 U/ml) at 37°C in 5% CO_2 for 2, 4, 6, 8 and 10 hours. Brefeldin A (10 μg/ml) was added with 2 hours stimulation remaining. Following labelling with anti-CD4, anti-CD3, anti-IL-4 and anti-IFN-γ antibodies or the appropriately labelled isotype controls, cells were analysed by flow cytometry. The CD4 histograms are gated on the lymphocyte population as determined by forward scatter versus side scatter. The CD3 histograms show $CD4^+$ T cell expression of CD3. Markers and percentages shown on these histograms indicate positive staining and were determined according to minimal (<1%) isotype control staining. The IL-4 versus IFN-γ dot plots and the isotype control dot plot are gated on the $CD4^+$ T cell population. Quadrant markers indicating positive staining are set according to minimal (<1%) isotype control staining. Quadrant percentages are indicated in the top right-hand corner of each plot and were determined from the acquisition of 30000 events by the flow cytometer. This experiment was repeated on PBMC from another HDM-allergic donor (A5) achieving similar results.

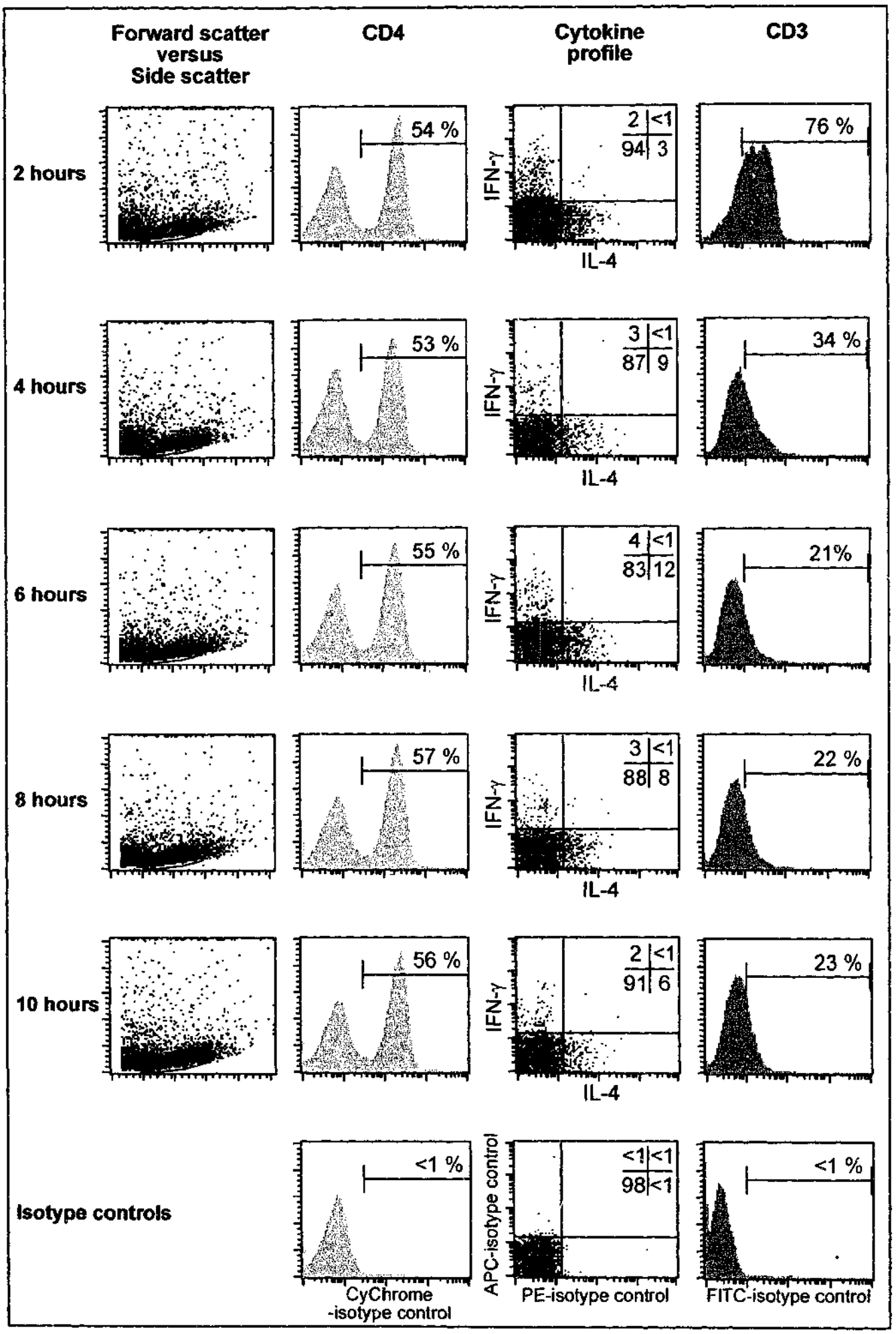
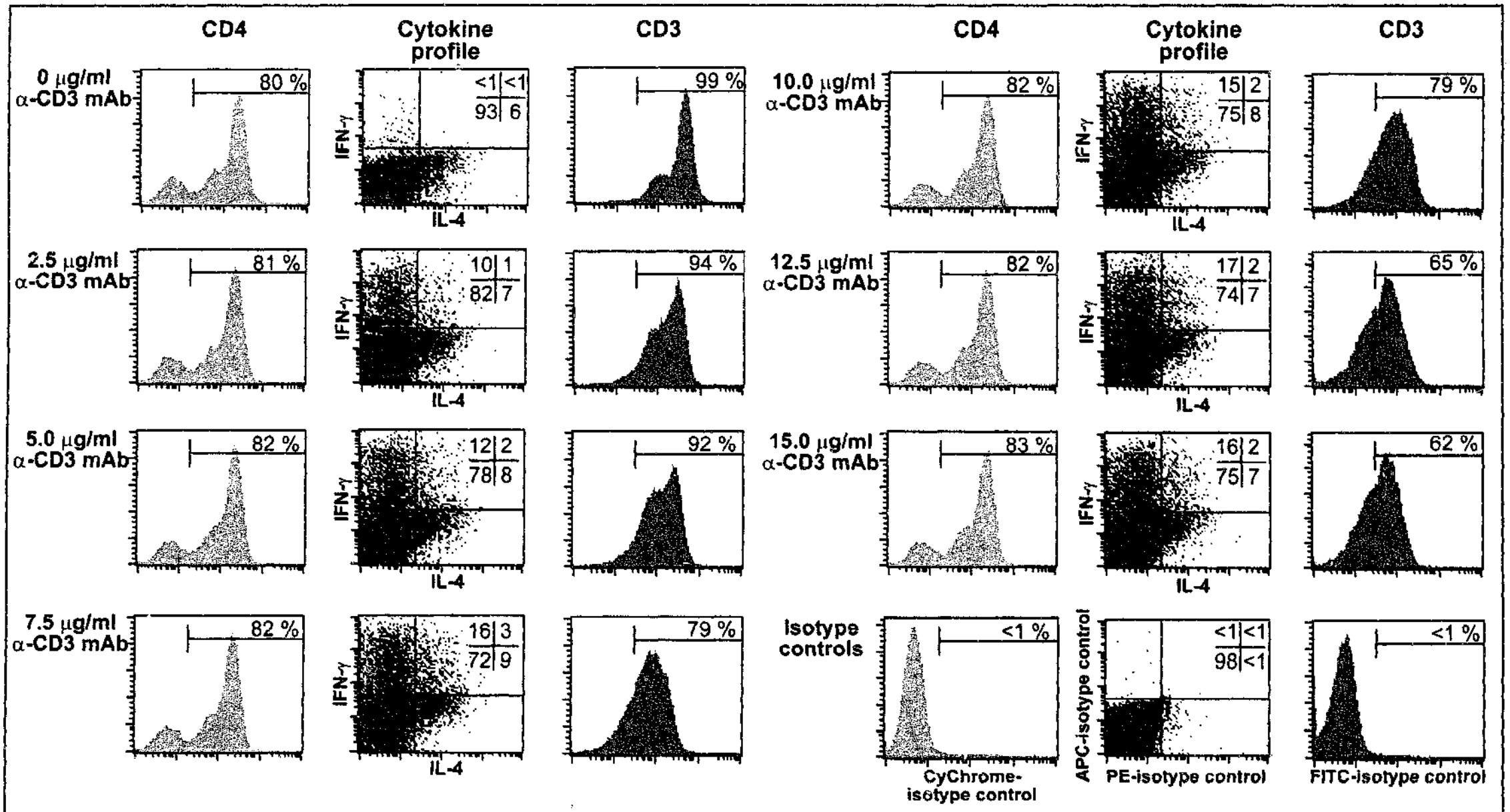

Forward scatter versus Side scatter
CD4
Cytokine profile
CD3
2 hours
54 %
2 <1
94 3
IFN-γ
IL-4
76 %
4 hours
53 %
3 <1
87 9
IFN-γ
IL-4
34 %
6 hours
55 %
4 <1
83 12
IFN-γ
IL-4
21%
8 hours
57 %
3 <1
88 8
IFN-γ
IL-4
22 %
10 hours
56 %
2 <1
91 6
IFN-γ
IL-4
23 %
Isotype controls
<1 %
CyChrome -isotype control
<1 <1
98 <1
APC-isotype control
PE-isotype control
<1 %
FITC-isotype control

Figure 3.8 Optimisation of the concentration of anti-CD3 mAb used to stimulate T cells for the detection of intracellular IL-4 and IFN-γ.

HDM-allergic donor PBMC (A12) were cultured in the presence of 25 μg/ml HDM extract at 37°C in 5% CO_2 for 7 days. At day 7, the cells were restimulated with 25 μg/ml HDM extract and irradiated PBMC as APC and cultured for another 7 days. At day 14, cells were stimulated with different concentrations of anti-CD3 mAb (0, 1, 2.5, 5, 7.5, 10, 12.5 and 15 μg/ml) and IL-2 (100 U/ml) in the presence of Brefeldin A (10 μg/ml) at 37°C in 5% CO_2 for 6 hours. Following labelling with anti-CD4, anti-CD3, anti-IL-4 and anti-IFN-γ antibodies or the appropriately labelled isotype controls, cells were analysed by flow cytometry. The CD4 histograms are gated on the lymphocyte population as determined by forward scatter versus side scatter. The CD3 histograms show $CD4^+$ T cell expression of CD3. Markers and percentages shown on these histograms indicate positive staining and were determined according to minimal (<1%) isotype control staining. The IL-4 versus IFN-γ dot plots and the isotype control dot plot are gated on the $CD4^+$ T cell population. Quadrant markers indicating positive staining are set according to minimal (<1%) isotype control staining. Quadrant percentages are indicated in the top right-hand corner of each plot and were determined from the acquisition of 30000 events by the flow cytometer.


CD4
Cytokine profile
CD3
0 µg/ml α-CD3 mAb
80 %
<1 | <1
93 | 6
99 %
10.0 µg/ml α-CD3 mAb
82 %
15 | 2
75 | 8
79 %
2.5 µg/ml α-CD3 mAb
81 %
10 | 1
82 | 7
94 %
12.5 µg/ml α-CD3 mAb
82 %
17 | 2
74 | 7
65 %
5.0 µg/ml α-CD3 mAb
82 %
12 | 2
78 | 8
92 %
15.0 µg/ml α-CD3 mAb
83 %
16 | 2
75 | 7
62 %
7.5 µg/ml α-CD3 mAb
82 %
16 | 3
72 | 9
79 %
Isotype controls
<1 %
<1 | <1
98 | <1
<1 %
IFN-γ
IL-4
APC-isotype control
CyChrome-isotype control
PE-isotype control
FITC-isotype control

To determine if the proportions of anti-CD3-induced IL-4$^+$ and IFN-γ$^+$ T cells could be increased further, T cells were stimulated in the presence of immobilised anti-CD3 mAb with different concentrations of soluble anti-CD28 mAb, as a costimulator, in comparison to IL-2 (100 U/ml) (Figure 3.9). Previous experiments in the laboratory had determined that stimulation of T cells with anti-CD3 mAb in the presence of IL-2 (100 U/ml) in comparison to no IL-2 resulted in enhanced cytokine detection (Leanne Gardner, B.Sc. Honours). Of the concentrations of anti-CD28 mAb tested, 5 μg/ml was the only concentration which resulted in proportions of IL-4 and IFN-γ positive cells greater than those detected for anti-CD3 stimulation without anti-CD28. As the total proportion of cytokine positive CD4$^+$ T cells was greater in the anti-CD3/IL-2 stimulation in comparison to the anti-CD3/anti-CD28 (5 μg/ml) stimulation, IL-2 (100 U/ml) was chosen instead of anti-CD28 mAb to enhance anti-CD3-induced cytokine production. Thus the optimal protocol for the detection of intracellular IL-4 and IFN-γ was determined to be: 6 hour stimulation of allergen-specific T cells with 10 μg/ml immobilised anti-CD3 mAb in the presence of IL-2 (100 U/ml) and Brefeldin A (10 μg/ml) at 37°C in 5% CO_2.

The binding specificity of the anti-IL-4 and anti-IFN-γ antibodies has been previously demonstrated in our laboratory. The anti-IL-4 antibody was pre-incubated with rhIL-4 and the anti-IFN-γ antibody was pre-incubated with rhIFN-γ. Blocking of these antibodies resulted in the inhibition of CD4$^+$ T cell IL-4 and IFN-γ staining, respectively (data not shown).

The optimised protocol for intracellular IL-4 and IFN-γ detection was then used to analyse cytokine production by CD4$^+$ T cells from 14-day HDM extract-stimulated

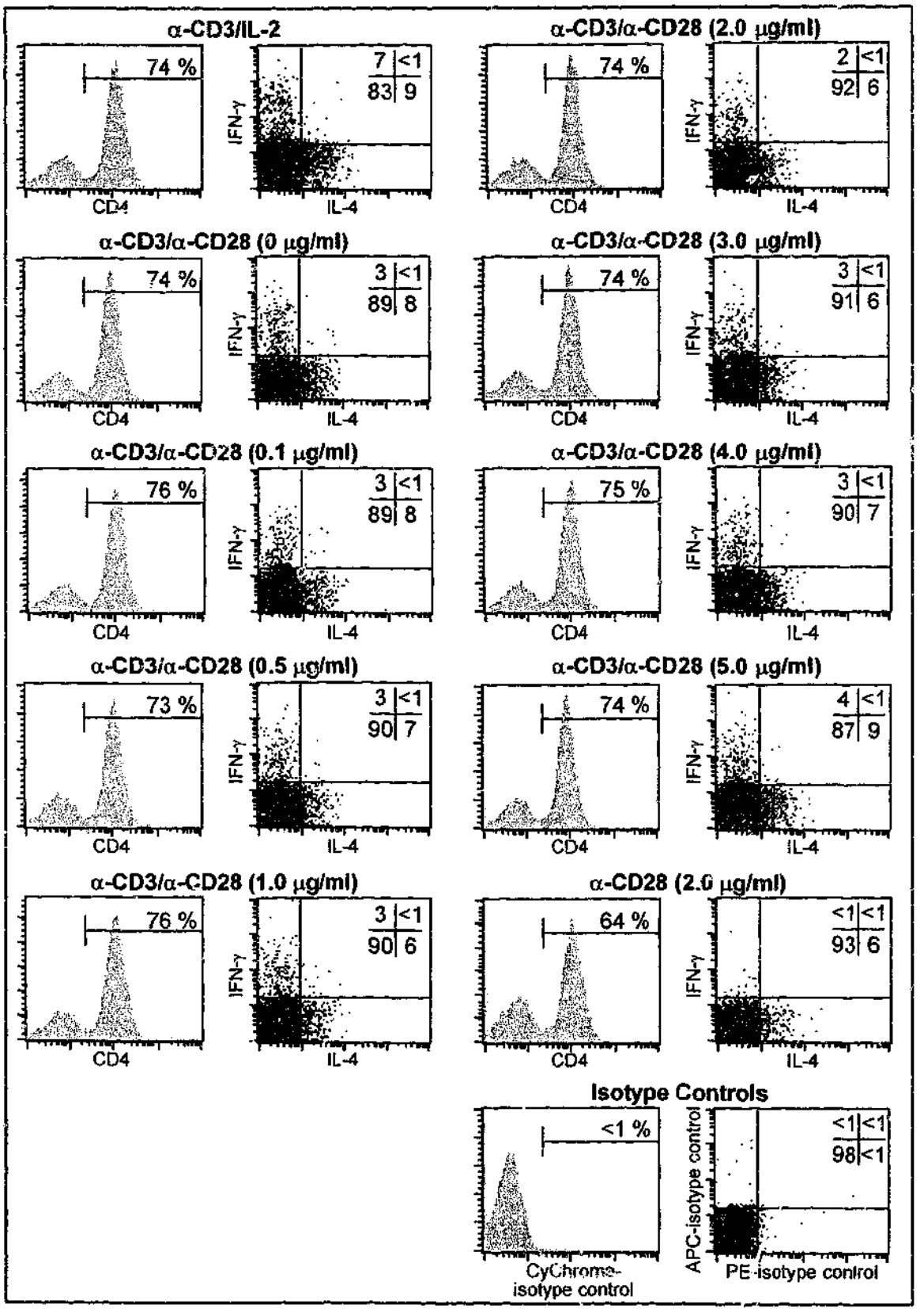


Figure 3.9 Optimisation of the type of costimulation used in combination with anti-CD3 mAb to stimulate T cells for the detection of intracellular IL-4 and IFN-γ.

HDM-allergic donor PBMC (A8) were cultured in the presence of 25 μg/ml HDM extract at 37°C in 5% CO_2 for 7 days. At day 7, the cells were restimulated with 25 μg/ml HDM extract and irradiated PBMC as APC and cultured for another 7 days. At day 14 cells were stimulated with anti-CD3 mAb (10 μg/ml) and either different concentrations of anti-CD28 mAb (0, 0.1, 1, 2.5 and 5 μg/ml) or IL-2 (100 U/ml) in the presence of Brefeldin A (10 μg/ml) at 37°C in 5% CO_2 for 6 hours. Following labelling with anti-CD4, anti-IL-4 and anti-IFN-γ antibodies or the appropriately labelled isotype controls, cells were analysed by flow cytometry. The CD4 histograms are gated on the lymphocyte population determined by forward scatter versus side scatter. The markers and percentages shown on these histograms indicate positive staining and were determined according to minimal (<1%) isotype control staining. The IL-4 versus IFN-γ dot plots and the isotype control dot plot are gated on the $CD4^+$ T cell population. Quadrant markers indicating positive staining are set according to minimal (<1%) isotype control staining. Quadrant percentages are indicated in the top right-hand corner of each plot and were determined from the acquisition of 30000 events by the flow cytometer.

cultures generated from PBMC from one HDM-allergic donor (A31) and one non-atopic donor (N1) (Figure 3.10). The cytokine profile of $CD4^+$ T cells from the allergic donor showed IL-4 predominance with 31% IL-4^+ and 6% IFN-γ^+. In contrast the cytokine profile of $CD4^+$ T cells from the non-atopic donor showed IFN-γ^+ predominance with 2% IL-4^+ and 33% IFN-γ^+. These cytokine profiles are consistent with previous reports detecting allergen-induced cytokine production from atopic and non-atopic donors using ELISA and limiting dilution (O'Hehir *et al.*, 1993; Li *et al.*, 1996).

As the aim of this research was to determine the effect of repeated allergen stimulation at different concentrations on T cell responses, IL-4 and IFN-γ production was compared at day 7 of culture after one stimulation and at day 14 of culture after a second stimulation (Figure 3.11). The proportions of IL-4 and IFN-γ positive $CD4^+$ T cells were greater at day 14 in comparison to day 7. These results indicate that repeated allergen stimulation results in an expansion of $CD4^+$ T cells and in increased proportions of cytokine-producing $CD4^+$ T cells.

While performing the intracellular cytokine optimisation experiments it was noticed that within the $CD4^+$ T cell population, a minor population expressed CD4 at a lower intensity (Figure 3.12a). In unstimulated control cultures the $CD4^{lo}$ population was more apparent and co-expressed IL-4 and Annexin-V. Further analysis indicated that before the 6-hour incubation only 4% of the total $CD4^+$ T cell population expressed $CD4^{lo}$ (Figure 3.12b). Following the 6-hour anti-CD3/IL-2 stimulation 18% of the $CD4^+$ T cells were $CD4^{lo}$ and without stimulation 25% were $CD4^{lo}$ (Figure 3.12b). As shown in Figure 3.12b, large proportions of these $CD4^{lo}$ cells before and after the 6-hour

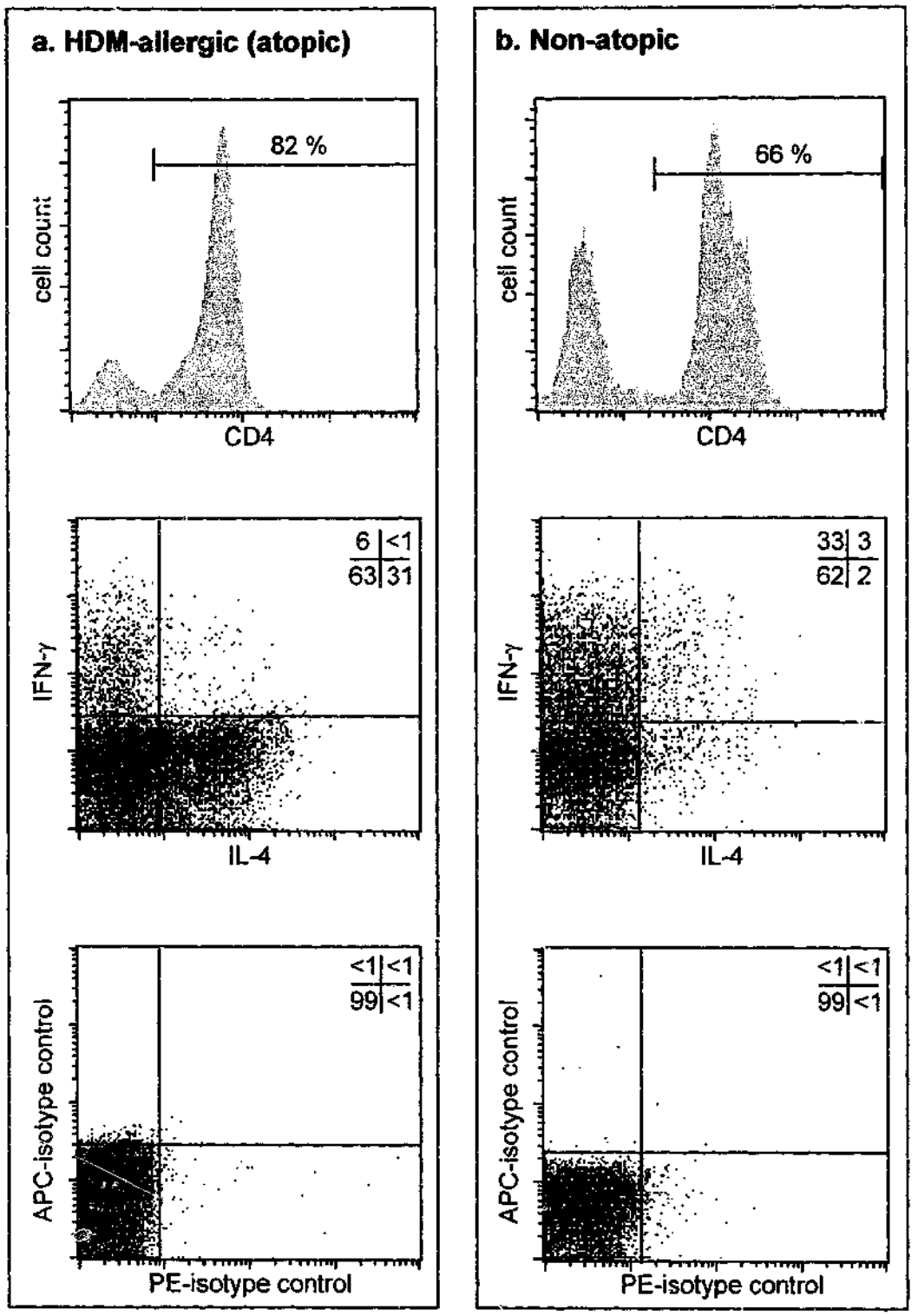


Figure 3.10 Assessing the IL-4 and IFN-γ profiles of $CD4^+$ T cells present in 14-day HDM extract-stimulated cultures generated from PBMC from an atopic donor and a non-atopic donor.

PBMC from a HDM-allergic donor (A31) and a non-atopic donor (N1) were cultured in the presence of 25 μg/ml HDM extract at 37°C in 5% CO_2 for 7 days. At day 7, the cells were restimulated with 25 μg/ml HDM extract and irradiated PBMC as APC and cultured for another 7 days. At day 14 cells were stimulated with anti-CD3/IL-2 in the presence of Brefeldin A (10 μg/ml) at 37°C in 5% CO_2 for 6 hours. Following labelling with anti-CD4, anti-IL-4 and anti-IFN-γ antibodies or the appropriately labelled isotype controls, cells were analysed by flow cytometry. The CD4 histograms are gated on the lymphocyte population as determined by forward scatter versus side scatter. The markers and percentages shown on these histograms indicate positive staining. The IL-4 versus IFN-γ dot plots and the corresponding isotype control dot plots are gated on the $CD4^+$ T cell population. Quadrant markers indicating positive staining are set according to minimal (<1%) isotype control staining. Quadrant percentages are indicated and were determined from the acquisition of 30000 events by the flow cytometer.

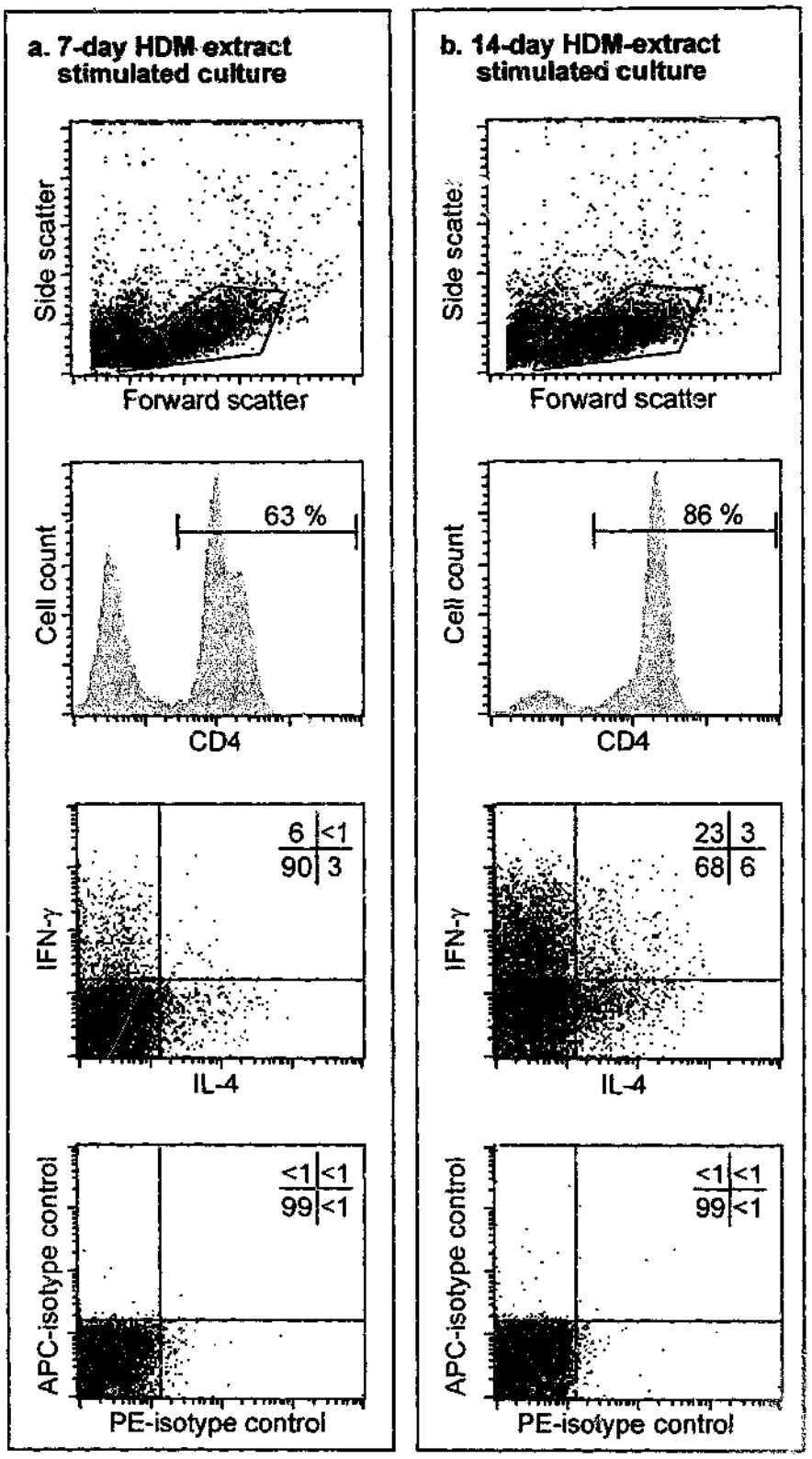


Figure 3.11 $CD4^+$ T cell intracellular IL-4 and IFN-γ staining at 7 and 14 days of culture with HDM extract.

HDM-allergic donor PBMC (A9) were cultured in the presence of 25 μg/ml HDM extract at 37°C in 5% CO_2 for 7 days. At day 7, the cells were restimulated with 25 μg/ml HDM extract and irradiated PBMC as APC and cultured for another 7 days. At day 7 **(a)** and day 14 **(b)** a portion of cultured cells were stimulated with anti-CD3/IL-2 in the presence of Brefeldin A (10 μg/ml) at 37°C in 5% CO_2 for 6 hours. Cells were then stained with anti-CD4, anti-IL-4 and anti-IFN-γ antibodies or the appropriately labelled isotype controls and analysed by flow cytometry. The CD4 profiles are gated on the lymphocyte population as determined by forward scatter versus side scatter. The markers and percentages shown on these histograms indicate positive staining. The IL-4 versus IFN-γ dot plots and the corresponding isotype control dot plots are gated on $CD4^+$ T cell population. Quadrant markers indicating positive staining are set according to minimal (<1%) isotype control staining. Quadrant percentages are indicated and were determined from the acquisition of 30000 events by the flow cytometer.

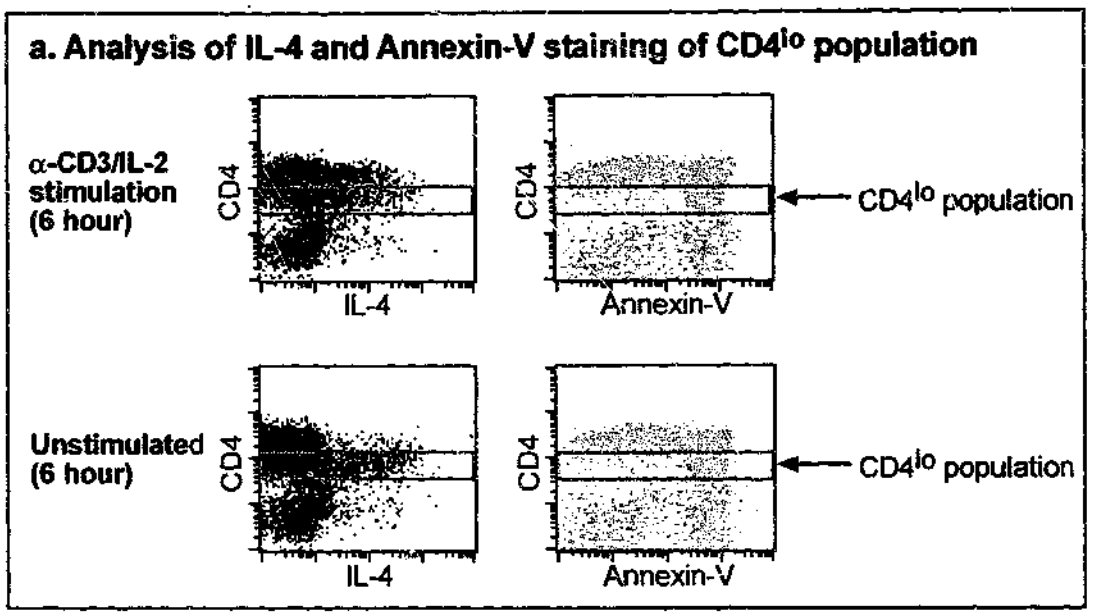


Figure 3.12 Analysis of $CD4^{lo}$ T cells after 6 hour incubation for cytokine detection.
HDM-allergic donor PBMC (A9) were cultured in the presence of 25 μg/ml HDM extract at 37°C in 5% CO_2 for 7 days. At day 7, the cells were restimulated with 25 μg/ml HDM extract and irradiated PBMC as APC and cultured for another 7 days.
(a) At day 14, cultured cells were incubated with or without anti-CD3/IL-2 in the presence of Brefeldin A (10 μg/ml) at 37°C in 5% CO_2 for 6 hours. A portion of cells was immediately stained with anti-CD4 antibodies and Annexin-V and analysed by flow cytometry. A second portion of cells was labelled with anti-CD4 antibodies and then stained for intracellular IL-4 and analysed by flow cytometry. Dot plots of CD4 versus IL-4 and CD4 versus Annexin-V for the 6-hour stimulation with anti-CD3/IL-2 and the 6-hour unstimulated control are shown with the $CD4^{lo}$ population highlighted in a red box. These profiles were generated by gating on the lymphocyte population as determined by forward scatter versus side scatter.
(b) At day 14, cultured cells were stained with anti-CD4 antibodies and Annexin-V either before the 6 hour incubation and after a 6-hour incubation either with or without anti-CD3/IL-2 in the presence of Brefeldin A (10 μg/ml) at 37°C in 5% CO_2. Cells were then analysed by flow cytometry. Histograms of Annexin-V staining gated on $CD4^{lo}$ and $CD4^{hi}$ cell populations are shown. The markers and percentages shown on these histograms indicate positive staining. This experiment was repeated on PBMC from a non-atopic donor (N1) achieving similar results.

incubation were double positive for Annexin-V. Some $CD4^{hi}$ cells also stained positive for Annexin-V. Similar results were seen when this experiment was repeated on a 14-day HDM extract-stimulated culture generated from non-atopic donor PBMC (N1). These results indicate that T cell apoptosis was induced in 14-day HDM extract-stimulated cultures during the incubation required for intracellular cytokine detection. As apoptosis was greater in the 6-hour incubation without anti-CD3/IL-2 stimulation in comparison to anti-CD3/IL-2 stimulation and was associated with IL-4 production, an unstimulated control could not be used. In addition, since it was not possible to clearly "gate" out the $CD4^{lo}$ cells during FACS analysis, it was decided to include the total $CD4^{+}$ population for cytokine detection in subsequent comparisons of different culture conditions.

3.3.4.2 Optimisation of detection of T cell intracellular IL-10 staining by flow cytometry

Previous reports have demonstrated that detection of intracellular IL-10 requires a longer period of T cell stimulation with polyclonal activators than required for the detection of IL-4 and IFN-γ (Akdis *et al.*, 1998b; Francis *et al.*, 2003). Hence T cells from 14-day HDM extract-stimulated cultures were stimulated in the presence of anti-CD3 mAb (10 μg/ml) for different lengths of time, ranging from 4 to 12 hours in the first experiment and 8 to 24 hours in the second experiment. In these experiments, stimulation of cultured cells with anti-CD3 mAb and either anti-CD28 mAb (1 μg/ml) or IL-2 (100 U/ml) were compared for optimal T cell IL-10 production. The method for intracellular IL-10 detection is described in Section 2.6.3.2 of this thesis.

In the first experiment the proportions of IL-10 positive $CD4^{+}$ T cells increased from less than 1% at 4 hours to 2% at 12 hours in the presence of either anti-CD3/IL-2 or

anti-CD3/anti-CD28 (Figure 3.13). The proportions of IL-10 positive $CD4^+$ T cells in the unstimulated control at any time point were less than 1%. In the second experiment $CD4^+$ T cell IL-10 production was analysed at longer stimulation times ranging from 8 to 24 hours (Figure 3.14). Proportions of IL-10 positive $CD4^+$ T cells increased in the presence of both anti-CD3/IL-2 and anti-CD3/anti-CD28 from 8 hours to 20 hours and then decreased at the 22 and 24 hour time points. At the optimal time point of 20 hours, stimulation with anti-CD3/IL-2 resulted in a higher proportion of IL-10 positive $CD4^+$ T cells (20%), in comparison to stimulation with anti-CD3/anti-CD28 (15%). Thus for optimal detection of IL-10, allergen-specific T cells were cultured for 20 hours at 37°C in 5% CO_2 with 10 μg/ml immobilised anti-CD3 mAb and IL-2 (100 U/ml) with Brefeldin A (10 μg/ml) being added for the last 4 hours of stimulation.

To ensure the binding specificity of the anti-IL-10 antibody, the anti-IL-10 antibody was pre-incubated with rhIL-10. As shown in Figure 3.15, blocking of the anti-IL-10 antibody resulted in the inhibition of $CD4^+$ T cell IL-10 staining.

3.3.4.3 Optimisation of CFSE cell labelling and analysis for the detection of T cell division

In this study T cell division was detected by labelling cell cultures with the fluorescent dye CFSE that is divided equally between daughter cells upon cell division (Lyons and Parish, 1994). This technique allows for the analysis of cell division at the single cell level by flow cytometry. The following section presents experiments performed to optimise the analysis of allergen-specific T cell division using this technique.

Firstly the concentration of CFSE required to satisfactorily label cells in order to detect division by flow cytometry was determined. Hence PBMC were labelled with different

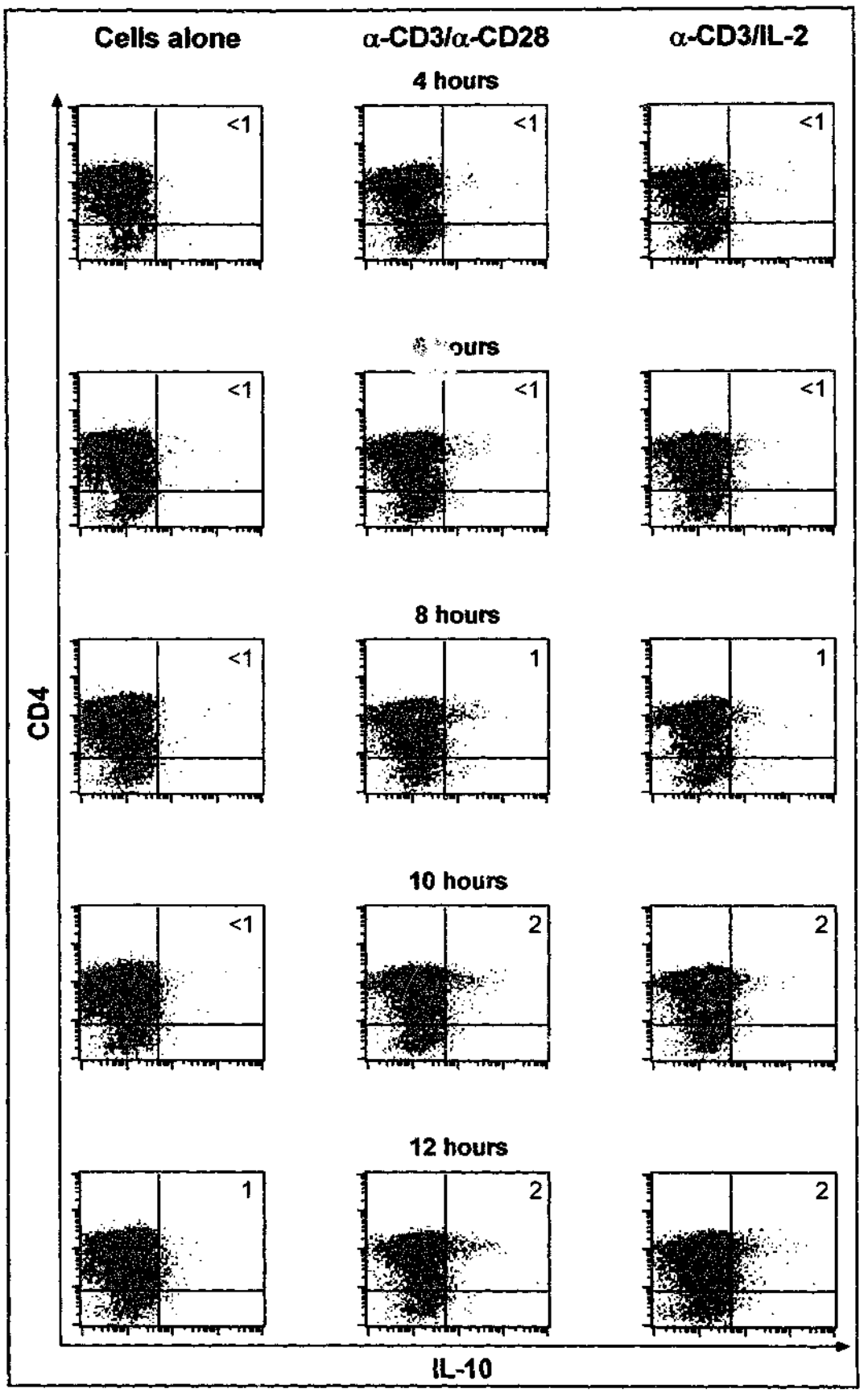
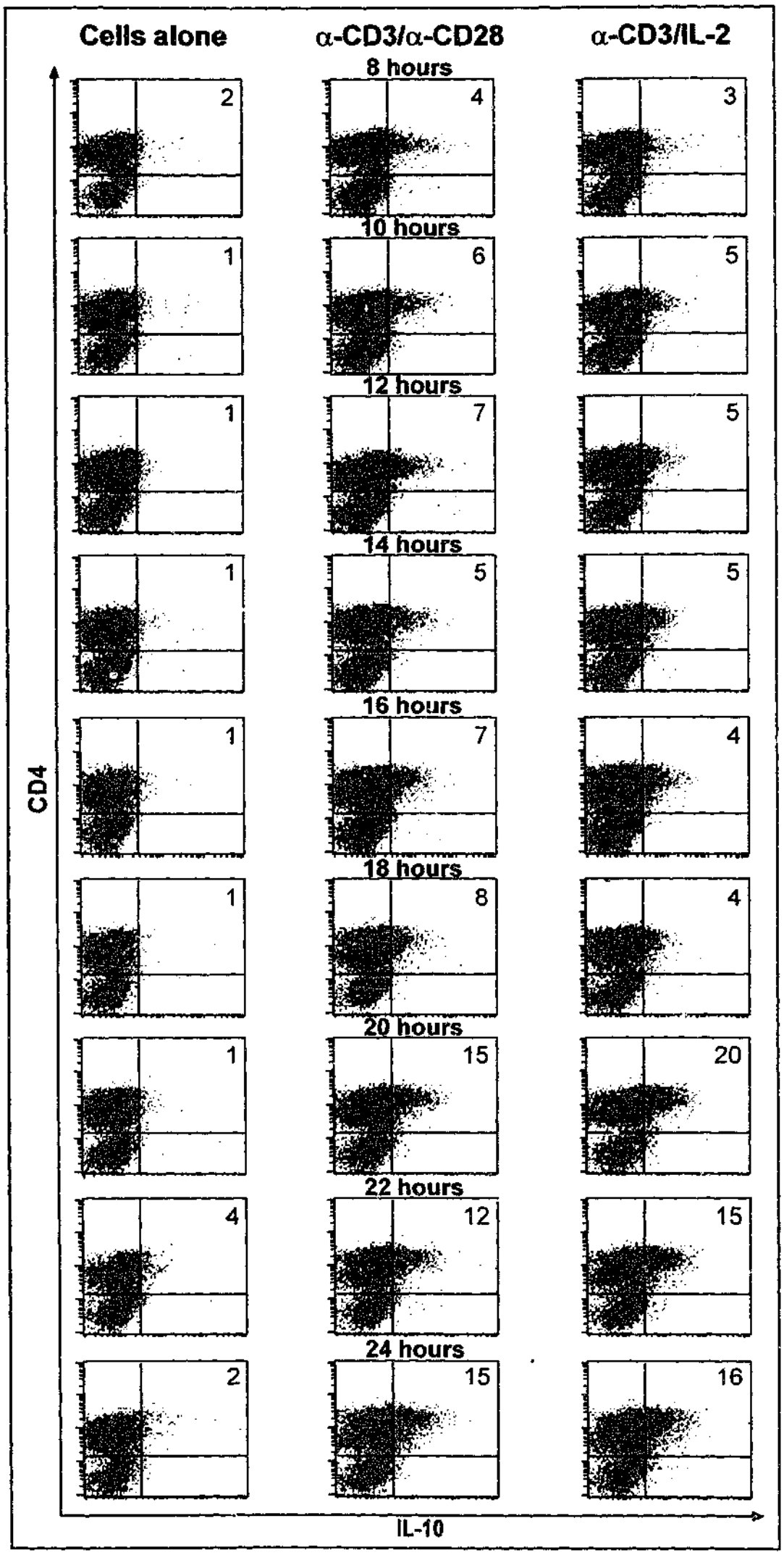


Figure 3.13 Optimisation of anti-CD3 stimulation time (4 to 12 hours) and the type of costimulation required for the detection of T cell intracellular IL-10.
HDM-allergic donor PBMC (A4) were cultured in the presence of 25 μg/ml HDM extract at 37°C in 5% CO_2 for 7 days. At day 7, the cells were restimulated with 25 μg/ml HDM extract and irradiated PBMC as APC and cultured for another 7 days. At day 14 cells were cultured in the presence of no stimulus or plate bound anti-CD3 (10 μg/ml) with either anti-CD28 (1 μg/ml) or IL-2 at 37°C in 5% CO_2 for 4, 6, 8, 10 and 12 hours. Brefeldin A (10 μg/ml) was added with 4 hours stimulation remaining. Following labelling with anti-CD4 and anti-IL-10 antibodies, cells were analysed by flow cytometry. The CD4 versus IL-10 dot plots are gated on the lymphocyte population as determined by forward scatter versus side scatter. Quadrant markers were set according to minimal (<1%) isotype control staining (not shown). The percentages of $IL\text{-}10^{+}CD4^{+}$ T cells are indicated in the top right-hand corner of each plot and were determined from the acquisition of 30000 events by the flow cytometer.

Figure 3.14 Optimisation of anti-CD3 stimulation time (8 to 24 hours) and the type of costimulation required for the detection of intracellular IL-10 from T cells.

HDM-allergic donor PBMC (A2) were cultured in the presence of 25 μg/ml HDM extract at 37°C in 5% CO_2 for 7 days. At day 7, the cells were restimulated with 25 μg/ml HDM extract and irradiated PBMC as APC and cultured for another 7 days. At day 14 cells were cultured alone or in the presence of plate bound anti-CD3 (10 μg/ml) with either anti-CD28 (1 μg/ml) or IL-2 (100 U/ml) at 37°C in 5% CO_2 for 8, 10, 12, 14, 16, 18, 20, 22 and 24 hours. Brefeldin A (10 μg/ml) was added with 4 hours stimulation remaining. Following labelling with anti-CD4 and anti-IL-10 antibodies, cells were analysed by flow cytometry. The CD4 versus IL-10 dot plots are gated on the lymphocyte population as determined by forward scatter versus side scatter. Quadrant markers were set according to minimal (<1%) isotype control staining (not shown). The percentages of $IL\text{-}10^{+}CD4^{+}$ T cells are indicated in the top right-hand corner of each plot and were determined from the acquisition of 30000 events by the flow cytometer. This experiment was repeated on PBMC from another HDM-allergic donor (A4) achieving similar results.


Cells alone
α-CD3/α-CD28
α-CD3/IL-2
8 hours
10 hours
12 hours
14 hours
16 hours
18 hours
20 hours
22 hours
24 hours
2
4
3
1
6
5
1
7
5
1
5
5
1
7
4
1
8
4
1
15
20
4
12
15
2
15
16
CD4
IL-10

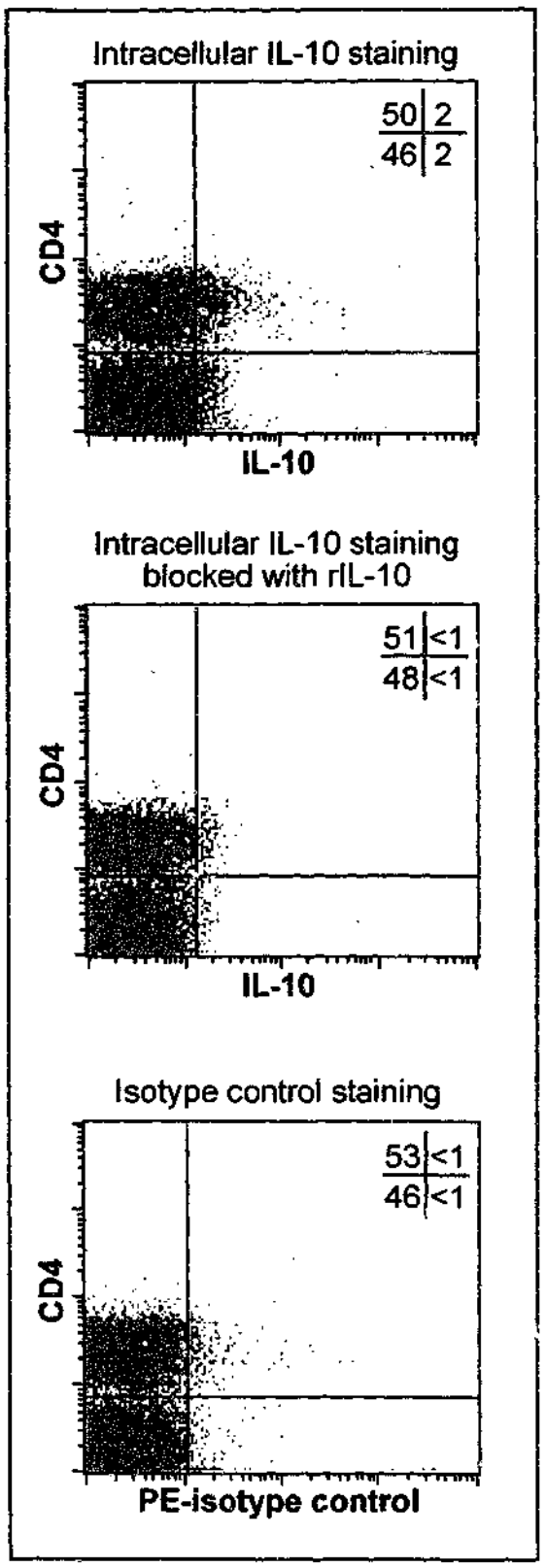


Figure 3.15 Specificity of intracellular IL-10 staining.
HDM-allergic donor PBMC (D1) were cultured in the presence of 25 μg/ml HDM extract at 37°C in 5% CO_2 for 7 days. At day 7, the cells were restimulated with 25 μg/ml HDM extract and irradiated PBMC as APC and cultured for another 7 days. At day 14, cells were stimulated in the presence of anti-CD3/IL-2 at 37°C in 5% CO_2 for 20 hours. Brefeldin A (10 μg/ml) was added with 4 hours stimulation remaining. Culture cells were labelled with anti-CD4 antibodies and then with either anti-IL-10 antibodies or anti-IL-10 antibodies that had been pre-incubated with rhIL-10 or the appropriately labelled isotype control. Cells were analysed by flow cytometry. The CD4 versus IL-10 dot plots and the CD4 versus isotype control dot plot are gated on the lymphocyte population as determined by forward scatter versus side scatter. Quadrant markers were set according to minimal (<1%) isotype control staining. Quadrant percentages are indicated in the top right-hand corner of each plot and were determined from the acquisition of 30000 events by the flow cytometer.

concentrations of CFSE ranging from 0 to 2.5 μM as described in Section 2.6.4.1. The CFSE-labelled PBMC were then cultured in the presence of unlabelled PBMC overnight. The cells did not receive any form of stimulation during this time as the fluorescence intensity of the CFSE-labelled PBMC was to be compared with the intensity of unlabelled cells. The following day, cells were labelled with anti-CD4 antibodies for the analysis of Th cell division.

For CFSE concentrations equal to or less than 0.1 μM the fluorescence intensity of the CFSE-labelled PBMC was not at high enough levels to analyse highly dividing daughter cells that would contain less CFSE and thus exhibit lower fluorescence intensities (Figure 3.16). At a concentration of 2.5 μM the fluorescence intensity of the CFSE-labelled cells was too high to detect the undivided cell population. The levels of intensity of the unlabelled PBMC in this sample were higher than the levels seen for the lower CFSE concentrations tested. This is most likely due to the presence of residual free CFSE, which stained the unlabelled PBMC. To ensure this did not occur in subsequent CFSE staining experiments, an additional washing step after CFSE cell labelling was added to the protocol. A concentration of 1 μM CFSE was chosen as the optimal staining concentration because the separation of fluorescence peaks for the CFSE-labelled cells and the unlabelled cells was maximal.

When analysing the CFSE profiles of HDM extract-stimulated $CD4^+$ T cells it was discovered that defined peaks marking cell division intervals were not observed (Figure 3.17). Thus a positive control for cell division was trialled to determine whether defined peaks in CFSE intensity could be detected. PBMC cultured for 7 days with HDM extract were labelled with CFSE and re-stimulated either in the presence of HDM

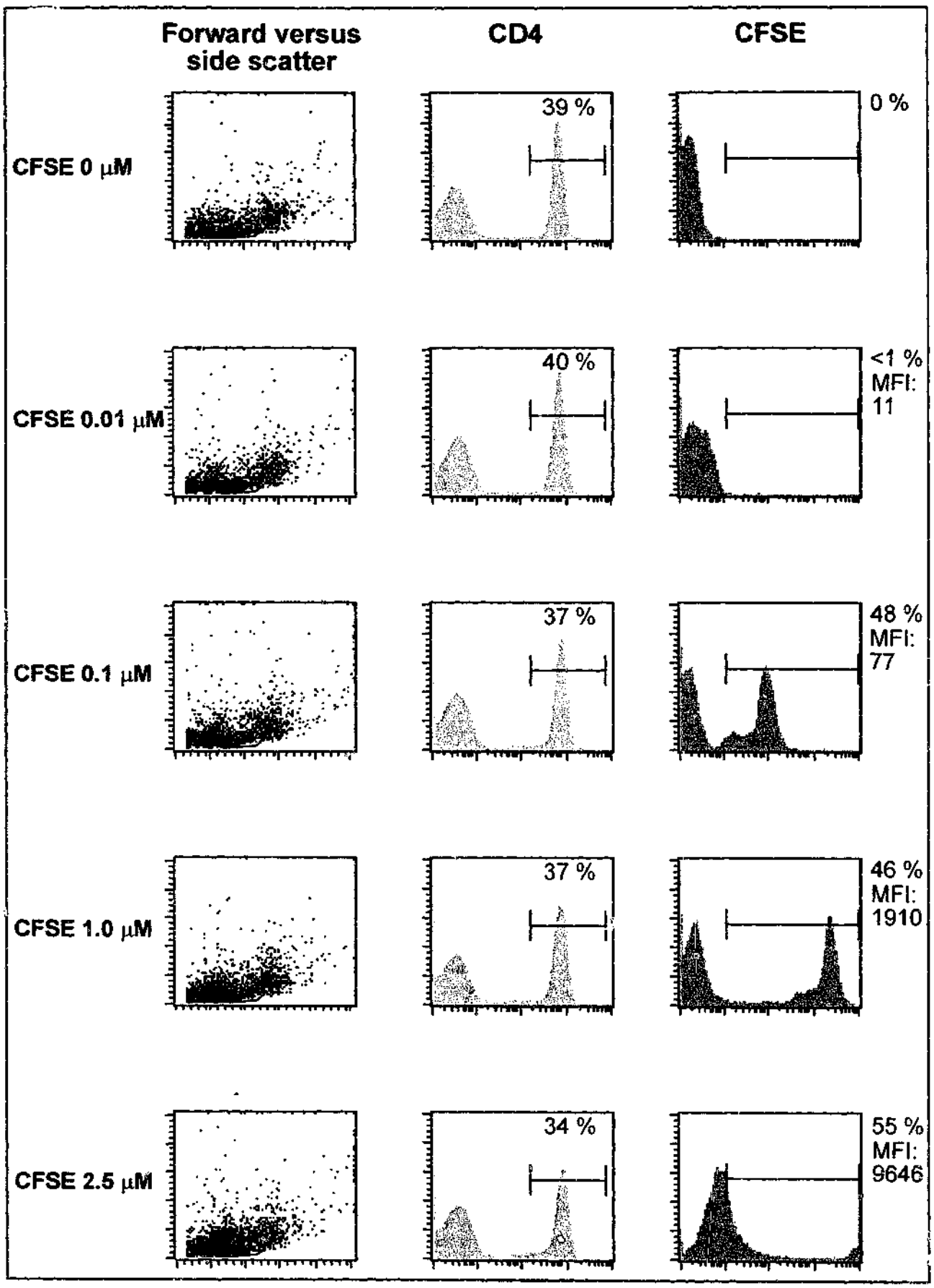


Figure 3.16 Optimisation of the concentration of CFSE required for the analysis of T cell division.

PBMC from donor A8 were labelled 0, 0.01, 0.1, 1 and 2.5 μM CFSE and cultured in the presence of unlabelled PBMC at 37°C in 5% CO_2 for 24 hours. Following culture, cells were labelled with anti-CD4 antibodies and analysed by flow cytometry. The CD4 histograms are gated on the lymphocyte population as determined by forward scatter versus side scatter. The CFSE histograms, gated on the $CD4^+$ T cell population, are also shown. Markers were set based on the intensity of the unstained cells. The percentages shown on these histograms indicate positive CFSE staining. The mean fluorescence intensities (MFI) for positively stained cells are also shown. Percentages and MFI were determined from the acquisition of 30000 events by the flow cytometer.

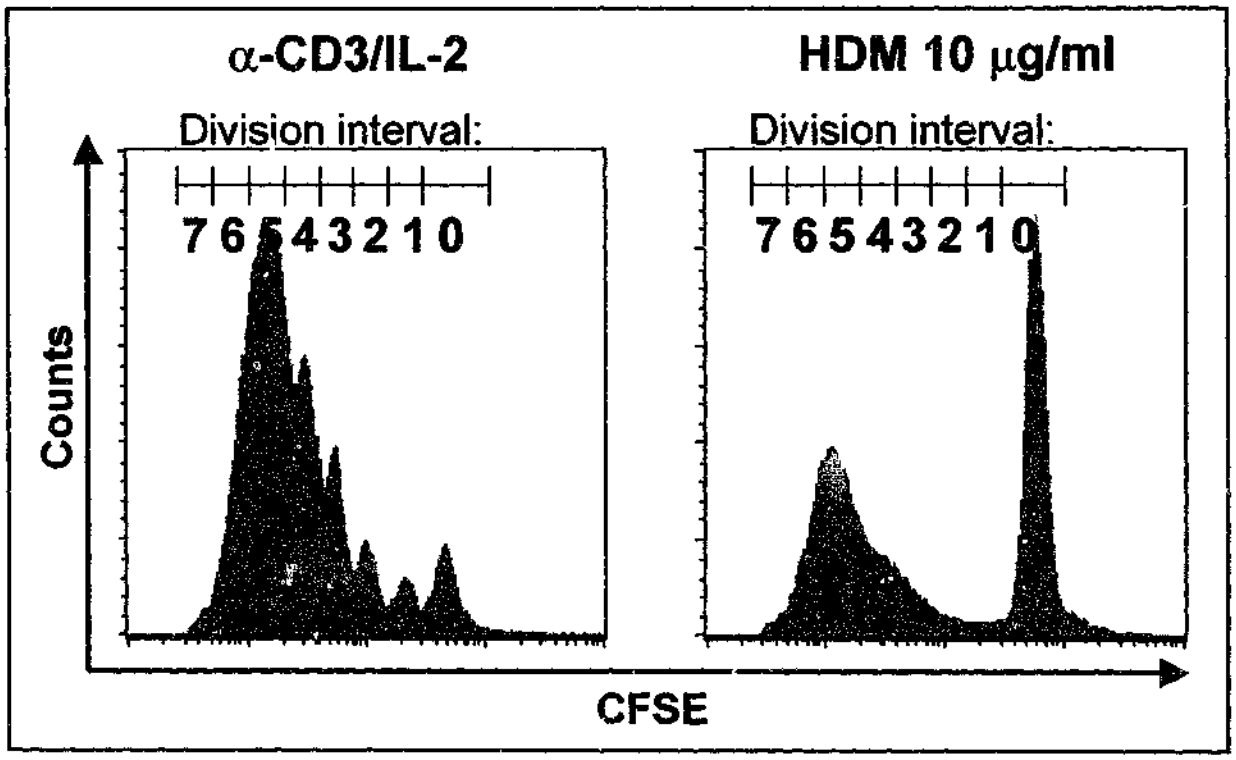


Figure 3.17 Determination of T cell division by analysis of CFSE staining.
HDM-allergic donor PBMC (A2) were cultured in the presence of 10 μg/ml HDM extract at 37°C in 5% CO_2 for 7 days. At day 7, cultured cells were labelled with 1 μM CFSE and then restimulated either in the presence of HDM extract and unlabelled APC, or anti-CD3/IL-2 as a positive control. After 5 days, cultured cells were harvested, washed, stained with anti-CD4 antibodies and analysed by flow cytometry. The histograms show CFSE profiles of $CD4^+$ T cells with division intervals marked. These division intervals were determined from the CFSE profile of $CD4^+$ T cells in the positive CD3/IL-2 control and copied on to the CFSE profile of $CD4^+$ T cells in the HDM extract-stimulated culture.

extract and APC, or anti-CD3/IL-2 as a positive control (see Section 2.6.4.2). In the positive control, the $CD4^+$ T cells have undergone ≥5 rounds of division represented by the defined peaks of CFSE fluorescence intensity (Figure 3.17). In subsequent analysis of cell division in allergen-stimulated T cell cultures, this positive control culture was run in parallel to enable the setting of division interval positions for allergen-stimulated $CD4^+$ T cells.

3.3.4.4 Intracellular active caspase-3 analysis for the detection of T cell apoptosis

The initial strategy for the analysis of T cell apoptosis at the single cell level by flow cytometry was to use Annexin-V to label phosphatidyl serine exposed on the surface of cells undergoing apoptosis (Vermes *et al.*, 1995). This method had previously been optimised in our laboratory (Personal Communication, Lina Papalia). However as Annexin-V staining cannot be performed on cells that have been fixed and permeabilised, this method cannot be used to determine the cytokine phenotype of apoptotic T cells. Instead T cells can be labelled for an intracellular enzyme called caspase-3 that is active in cells undergoing apoptosis. Intracellular active caspase-3 labelling was tested using the same method for the detection of intracellular IL-4 and IFN-γ in allergen-stimulated T cells. Cells from a 14-day HDM extract-stimulated culture were incubated in the presence of anti-CD3/IL-2 and Brefeldin A for 6 hours and labelled for CD4 and intracellular IL-4, IFN-γ and active caspase-3 (see Section 2.6.5.2). By gating on $CD4^+IL\text{-}4^+$ T cells and $CD4^+IFN\text{-}\gamma^+$ T cells, active caspase-3 labelling of cytokine positive cells was analysed (Figure 3.18). In this particular experiment 41% of $CD4^+IL\text{-}4^+$ T cells and 5% $CD4^+IFN\text{-}\gamma^+$ T cells were caspase-3 positive. Thus the same protocol for intracellular cytokine staining was shown to be suitable for intracellular active caspase-3 staining.

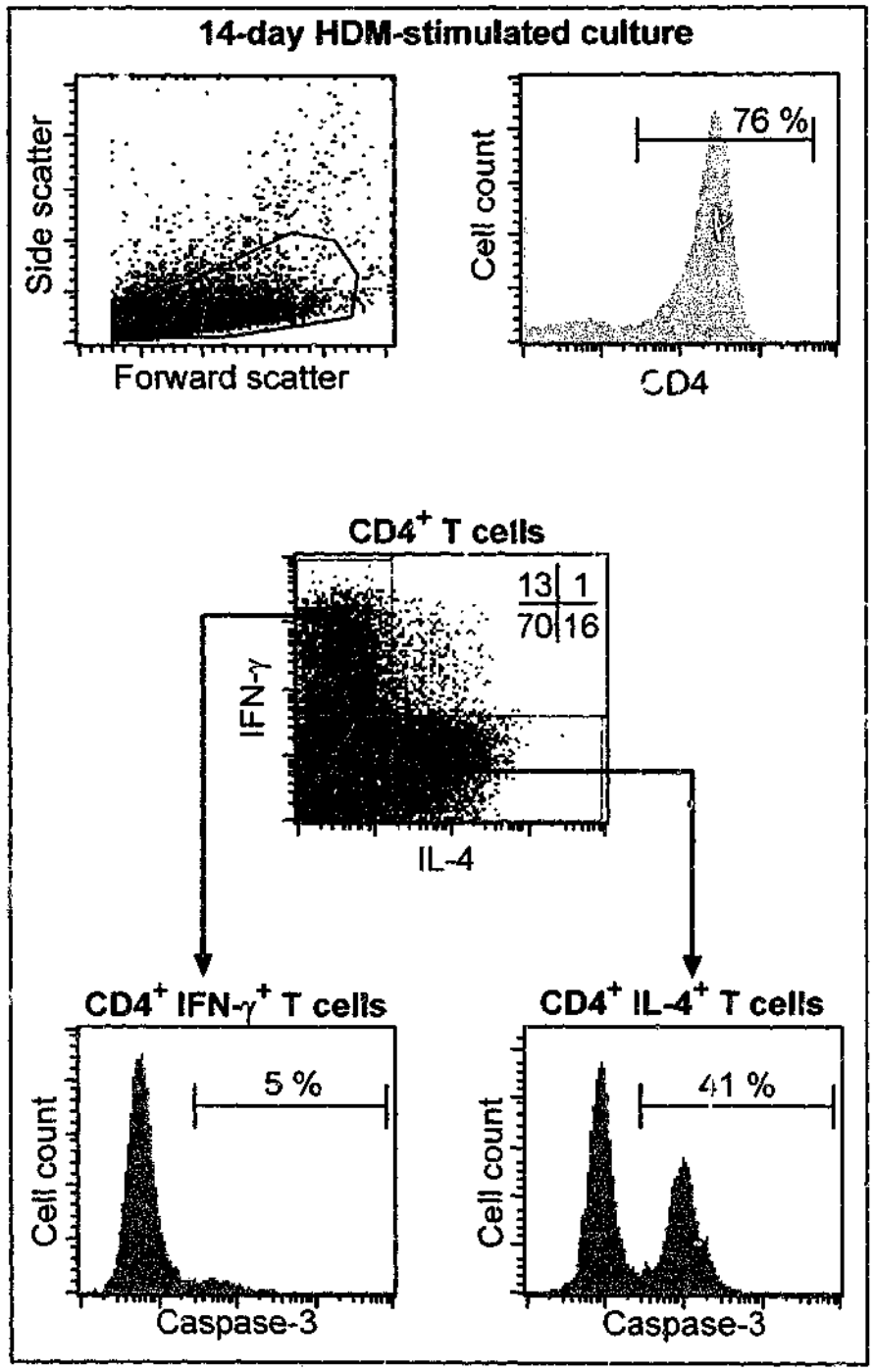


Figure 3.18 Active caspase-3 staining for the analysis of T cell apoptosis.
HDM-allergic donor PBMC (A1) were cultured in the presence of 25 μg/ml HDM extract at 37°C in 5% CO_2 for 7 days. At day 7, the cells were restimulated with 25 μg/ml HDM extract and irradiated PBMC as APC and cultured for another 7 days. At day 14, cells were stimulated with anti-CD3/IL-2 in the presence of Brefeldin A (10 μg/ml). Cells were then labelled with anti-CD4, anti-IL-4, anti-IFN-γ and anti-active caspase-3 antibodies. The CD4 histogram is gated on the lymphocyte population as determined by forward scatter versus side scatter. The IL-4 versus IFN-γ dot plot of $CD4^+$ T cells was used to analyse active caspase-3 staining of $CD4^+IL\text{-}4^+$ T cells and $CD4^+IFN\text{-}\gamma^+$ T cells. Positive active caspase-3 staining is shown by markers with the percentage of positive cells determined from the acquisition of 30000 events by the flow cytometer indicated above the marker.

3.4 DISCUSSION

This chapter presents the characterisation of the allergen extract for T cell stimulation and the optimisation of methods for examining allergen-simulated T cell responses. The preparation and analysis of the HDM extract for use in T cell cultures is first described. The aqueous extract consisted of a freeze-dried preparation of whole mites (*Dermatophagoides pteronyssinus*) reconstituted in PBS. Analysis by SDS-PAGE revealed that the extract contains many proteins, with a predominant component corresponding to Der p 1 (Thomas *et al.*, 2002). This preparation was not toxic or mitogenic to T cells *in vitro* and contained undetectable levels of endotoxin. As naturally purified Der p 1 from similar HDM extracts has been found to be enzymically active (Schulz *et al.*, 1998b), the enzyme activity of the HDM extract was assessed. Previously, a continuous rate assay using the fluorogenic peptide substrate N-tertbutoxy-carbonyl (Boc)-Gln-Ala-Arg-7-amino-4-methylcoumarin has been used to determine the enzyme activity of Der p 1 (Schulz *et al.*, 1998b). However, as the main concern in this project was the observation that Der p 1 cleavage of CD25 can result in altered T cell cytokine production (Ghaemmaghami *et al.*, 2001), flow cytometry was used to analyse CD25 expression on T cells cultured with the extract. The HDM extract did not induce a down-regulation in T cell expression of CD25, suggesting that the Der p 1 enzyme function would not confound the results of this study. These observations indicate that this HDM extract was suitable for stimulation of T cells *in vitro*.

As reported previously, *in vitro* stimulation of allergic donor PBMC with allergen results in the preferential proliferation of $CD4^+$ T cells (O'Hehir *et al.*, 1987); proportions of $CD4^+$ T cell in 14-day HDM extract-stimulated cultures were increased in comparison to 7-day cultures. This proliferation is consistent with the expansion of allergen-specific Th cells. Hence it seems reasonable to expect high proportions of

allergen-specific $CD4^+$ T cells following 14 days culture with HDM extract. However it is possible that some non-specific $CD4^+$ T cells may have survived the culturing period due to bystander activation. With further developments in MHC class II tetramer technology, the proportions of allergen-specific T cells after *in vitro* allergen stimulation could be determined.

The observation that a population of $CD8^+$ T cells was also present in 14-day HDM extract-stimulated cultures was unexpected. The recent report of human MHC class I-restricted T cell epitopes of the allergen Der p 1 identified using MHC class I tetramers (Seneviratne *et al.*, 2002), suggests that peptides may also be presented by MHC class I to $CD8^+$ T cells in the immune response to allergens. These observations lead to further investigation of $CD8^+$ T cell responses to HDM extract in this project (Chapter 4).

This chapter also details the optimisation of T cell assays used for the analysis of allergen-specific T cell cytokine production, proliferation and apoptosis *in vitro*. These assays examine T cell responses at the single cell level and were required for dissecting the mechanisms for HDM-induced changes in the allergen-specific T cell response. Protocols for the optimal detection of T cell intracellular cytokines including IL-4, IFN-γ and IL-10 were established. Using the optimised method for intracellular IL-4 and IFN-γ detection, the cytokine profiles of $CD4^+$ T cells in 14-day HDM extract-stimulated cultures generated with PBMC from an HDM-allergic donor and a non-atopic donor were assessed. In agreement with previous reports, the cytokine profile of allergic donor $CD4^+$ T cells was IL-4 predominant whereas the cytokine profile of non-atopic donor $CD4^+$ T cells was IFN-γ predominant (O'Hehir *et al.*, 1989; Byron *et al.*, 1994; Li *et al.*, 1996). Interestingly, analysis of intracellular cytokine production at a

single cell level showed that most of the individual cytokine-producing T cells in the cultures expressed either IL-4 or IFN-γ and very few cells expressed both cytokines. This observation is an example of the additional information that can be gained through the analysis of T cell cytokine synthesis at the single cell level rather than analysis of cytokines in culture supernatants by ELISA.

Analysis of T cell cytokine production in 14-day HDM extract-stimulated cultures by flow cytometry, revealed that approximately one third of the $CD4^+$ T cells in HDM extract-stimulated cultures stained positive for intracellular cytokines (IL-4 and IFN-γ). That a greater proportion were not cytokine positive could be due to the transient nature of T cell cytokine production (Richter *et al.*, 1999). T cells enter a refractory phase following activation and cytokine production (Pala *et al.*, 2000), and hence at a given point in time some cells may be producing cytokines whereas others may have entered a refractory phase. Additionally, heterogeneity in phase of the cell cycle of cultured T cells could also alter the proportions of cytokine-producing T cells detected. This heterogeneity exists because the time required for T cells to enter the first division following stimulation is variable (Gett and Hodgkin, 1998), and has been shown to be dependent on the levels of IL-2 receptor expression and the availability of T cell activating cytokines (Cantrell and Smith, 1984). Thus if a population of T cells is not synchronously in cell cycle, it is not likely that following 6 hours of activation with anti-CD3 and IL-2 all T cells would stain positive for cytokine production.

The detection of T cell intracellular cytokines in 14-day HDM extract-stimulated cultures required a 6-hour stimulation with anti-CD3/IL-2. In these experiments a 6-hour unstimulated control culture was run in parallel. However in this control culture a minor population of $CD4^+$ T cells was found to stain positive for IL-4. These T cells

down-regulated the expression of CD4 to become $CD4^{lo}$ and co-expressed Annexin-V indicating that they were undergoing apoptosis. Down-regulation of CD4 expression by human T cells during apoptosis has previously been demonstrated (Kabelitz *et al.*, 1996; Regamey *et al.*, 1999). Recently *in vitro* stimulation of PBMC from healthy controls with "apoptosis-inducing toxins" resulted in intracellular IL-4 expression by apoptotic cells (Stein *et al.*, 2000). Stein *et al.* suggested that apoptotic T cells produce IL-4 in an effort to inhibit apoptosis and support cell survival.

Analysis of cell division at the single cell level using CFSE cell labelling and flow cytometry was also described in this chapter. This assay was used for the analysis of allergen-induced T cell proliferation and in combination with intracellular cytokine staining, was useful for analysing cytokine synthesis by dividing T cells. The optimum concentration of CFSE used to label cells for cell division analysis by flow cytometry was determined. Interestingly, when HDM extract-stimulated T cells were labelled with CFSE and analysed for division 3 or 5 days after re-stimulation with HDM extract, clear peaks of CFSE fluorescence intensity marking cell division intervals were not seen, unlike cultures stimulated with anti-CD3/IL-2. Possibilities for this observation include uneven labelling of the T cells with CFSE due to inadequate mixing of the cell suspension or the incorporation of different quantities of CFSE into T cells due to differences in T cell size. Difficulties in labelling T cell lines due to the heterogeneity of cell size has been previously reported (Hasbold *et al.*, 1999). However in the present study, defined peaks of CFSE fluorescence intensity were observed for control cultures in which T cells were stimulated with anti-CD3/IL-2 indicating that cell size and inadequate mixing were not causing this problem. Another possible explanation is that of the heterogeneity of the T cell response to the HDM extract. Defined peaks of CFSE fluorescence intensity may not have formed for T cells in these cultures because the T

cells were responding to different antigens, at different doses and with different specificities within the extract resulting in uneven cell division rates. In contrast, T cells in the anti-CD3/IL-2 stimulated cultures were all activated with the same stimulus for the same length of time thus leading to even cell division rates and defined peaks of CFSE fluorescence intensity.

As an overall T cell cytokine response can be altered by the apoptosis of a particular T cell subset, techniques for the analysis of programmed T cell death in allergen-stimulated cultures by flow cytometry were assessed. Initially apoptosis was analysed by labelling phosphatidyl serine which is translocated from the inner to the outer leaflet of cells undergoing apoptotsis (Vermes *et al.*, 1995). However a drawback of this method is that the cells can not be treated with fixing agents and hence it can not be used for the detection of cytokines and apoptosis at the same time. Therefore another flow cytometric method for analysis of apoptosis was employed. This method involved labelling intracellular active caspase-3 in cells undergoing apoptosis. Caspase-3 is expressed as a proenzyme in the cytosol of a cell. When a cell receives a proapoptotic signal, a cascade of effector caspases is activated which leads to eventual cell death (Thornberry and Lazebnik, 1998). Apoptosis can be initiated either through the binding of death receptors resulting in the activation of caspase-8 or induced by cytotoxic agents resulting in the activation of caspase-9 (Thornberry and Lazebnik, 1998). Active caspase-8 and caspase-9 can cleave the caspase-3 proenzyme thereby releasing active caspase-3 (Li *et al.*, 1997; Muzio *et al.*, 1997), which in turn then cleaves several target proteins leading to apoptosis. As active caspase-3 is involved in both of these pathways, it can be used as an intracellular marker of apoptotic cells. In this project intracellular active caspase-3 staining was trialled using the same method for the detection of

intracellular IL-4 and IFN-γ. This protocol allowed for the determination of the cytokine phenotype (IL-4 and IFN-γ) of T cells undergoing apoptosis.

In this chapter a panel of *in vitro* T cell assays were established for the detection of T cell cytokine production, proliferation and apoptosis in HDM extract-stimulated PBMC cultures. These assays were used throughout this project to investigate the underlying mechanisms for SIT-induced changes in the allergen-specific T cell response.

CHAPTER 4

ANALYSIS OF T CELL RESPONSES TO DIFFERENT ALLERGEN CONCENTRATIONS[1]

4.1 INTRODUCTION

Allergen-specific immunotherapy involves the repeated administration of allergen extract and is a current treatment for the symptoms of allergy in severely affected individuals (Bousquet *et al.*, 1998b). 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 HDM allergy (Bousquet *et al.*, 1998b). 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-γ dominance (Jutel *et al.*, 1995a; McHugh *et al.*, 1995; Akdis *et al.*, 1996; Bellinghausen *et al.*, 1997; Ebner *et al.*, 1997; Kammerer *et al.*, 1997; O'Brien *et al.*, 1997; Eusebius *et al.*, 2002). 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 (Rolland and O'Hehir, 1998). Induction of $CD4^+$ T cell production of IL-10 has been shown during SIT and can result in decreased allergen-specific proliferation and cytokine production (Akdis *et al.*, 1998b; Francis *et al.*, 2003; Jutel *et al.*, 2003). Changes in $CD8^+$ T cell cytokine production during treatment are poorly studied.

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[1] This chapter is based on the publication: **Leanne M. Gardner**, Robyn E. O'Hehir, Jennifer M. Rolland (2004) High dose allergen stimulation of T cells from house dust mite-allergic subjects induces expansion of IFN-γ^+ T cells, apoptosis of $CD4^+IL\text{-}4^+$ T cells and T cell anergy. Int. Arch. Allergy Immunol. **133**, 1-13.

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-γ production at higher antigen doses (Constant *et al.*, 1995; Hosken *et al.*, 1995; Rogers *et al.*, 1998; Grakoui *et al.*, 1999; Rogers and Croft, 1999) but the effect of antigen concentration on IL-4 production seems to be more variable (Hosken *et al.*, 1995; Grakoui *et al.*, 1999; Rogers and Croft, 1999), presumably due to differences in duration of Th cell stimulation (Rogers and Croft, 1999). Cell division also plays a critical role in regulating cytokine production by Th cells (Gett and Hodgkin, 1998) and this may be influenced by antigen concentration (Ben-Sasson *et al.*, 2001).

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 (Secrist *et al.*, 1995; Carballido *et al.*, 1997). To more clearly dissect the mechanisms by which allergen concentration alters T cell functional phenotype, cytokine responses at a single cell level were analysed by flow cytometry. IL-4, IFN-γ and IL-10 production by allergen stimulated $CD4^+$ and $CD8^+$ T cells were examined using intracellular cytokine staining. 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.

4.2 EXPERIMENTAL PROCEDURES

In this study PBMC were separated from peripheral blood of HDM-allergic donors recruited according to the selection criteria detailed in Section 2.2. The clinical characteristics of these patients are shown in Table 4.1. PBMC were cultured for 14 days in the presence of 1, 10 or 100 μg/ml HDM extract. To determine dose dependent changes on T cell cytokine production, 14-day cultures were stimulated for 6 hours with anti-CD3/IL-2 for the detection of T cell intracellular IL-4 and IFN-γ. T cell production of IL-10 was also examined after a 20-hour stimulation with anti-CD3/IL-2. To further examine the effects of allergen concentration on T cell cytokine production, T cell division was analysed by CFSE cell labelling and flow cytometry. In addition, T cell apoptosis was analysed to determine whether programmed cell death of a particular population of T cells was contributing to overall T cell cytokine profiles observed at different allergen concentrations. For this, intracellular active caspase-3 staining was performed to detect apoptosis of cytokine-producing T cells. T cell proliferative responses to an immunogenic dose of HDM extract were also determined following the 14-day culture of PBMC with different concentrations of HDM extract. The methods for cell culture, flow cytometry, T cell proliferation assays, the analysis of cell division and apoptosis, and statistical analyses are described in Sections 2.5, 2.6 and 2.7 of this thesis.

Table 4.1 Characterisitics of donors used in this study.

Patient	Sex	Age	Rhinitis	Asthma	HDM skin test reactivity (wheal mm)	HDM EAST score (AEU/ml)
A1	F	42	Y	Y	11	5 (61.9)
A2	F	55	Y	Y	21	...
A3	F	22	Y	Y	16.5	...
A10	M	43	N	Y	14	...
A11	F	36	Y	Y	21 x 10	6
A12	F	25	Y	Y	10	2 (2.5)
A13	F	28	Y	Y	17	4 (18.2)
A14	M	32	N	Y	15	5 (51.8)
A15	M	40	N	Y	11	...
A16	F	28	Y	Y	11	...
A17	M	32	N	Y	21	...
A18	F	25	Y	Y	11	...
A19	M	31	...	...	8	...
A20	F	34	...	...	13 x 9	...
A21	F	25	N	Y	12	3
A22	F	36	N	Y	11	...
A23	M	25	N	N	8 x 12	2 (3.3)
A24	F	21	N	Y	12 x 8	...
A25	F	34	Y	Y	10	5 (64.6)
A26	F	30	Y	N	10	...
A27	F	67	Y	Y	15	3 (5.9)

A, atopic; F, female; M, male; ..., not available; Y, yes; N, no

4.3 RESULTS

4.3.1 High Allergen Concentration Increases the Proportion and Number of $CD4^+IFN\text{-}\gamma^+$ T Cells and $CD8^+IFN\text{-}\gamma^+$ T Cells, but Decreases the Proportion of $CD4^+IL\text{-}4^+$ 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 4.1a. Repeating this experiment on PBMC from another 9 donors revealed significantly higher $CD4^+IFN\text{-}\gamma^+$ 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 ($p<0.05$) (Figure 4.1b). In addition significantly lower $CD4^+IL\text{-}4^+$ 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. Very few $CD4^+IFN\text{-}\gamma^+IL\text{-}4^+$ T cells were observed. $CD8^+IFN\text{-}\gamma^+$ T cell proportions were significantly higher in cultures stimulated with 100 μg/ml HDM extract in comparison to 1 μg/ml ($p<0.01$; Figure 4.1c). 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, $CD4^+IFN\text{-}\gamma^+$ and $CD8^+IFN\text{-}\gamma^+$ T cells were significantly higher in cultures stimulated with 100 μg/ml HDM extract in comparison to 1 μg/ml ($p<0.05$; Figure 4.1b, c). $CD4^+IL\text{-}4^+$ and $CD8^+IL\text{-}4^+$ T cell numbers did not differ significantly between cultures. No dose-dependent changes were found for total $CD4^+$ and $CD8^+$ T cell numbers or cytokine negative $CD4^+$ T cell number (Figure 4.2a, b).

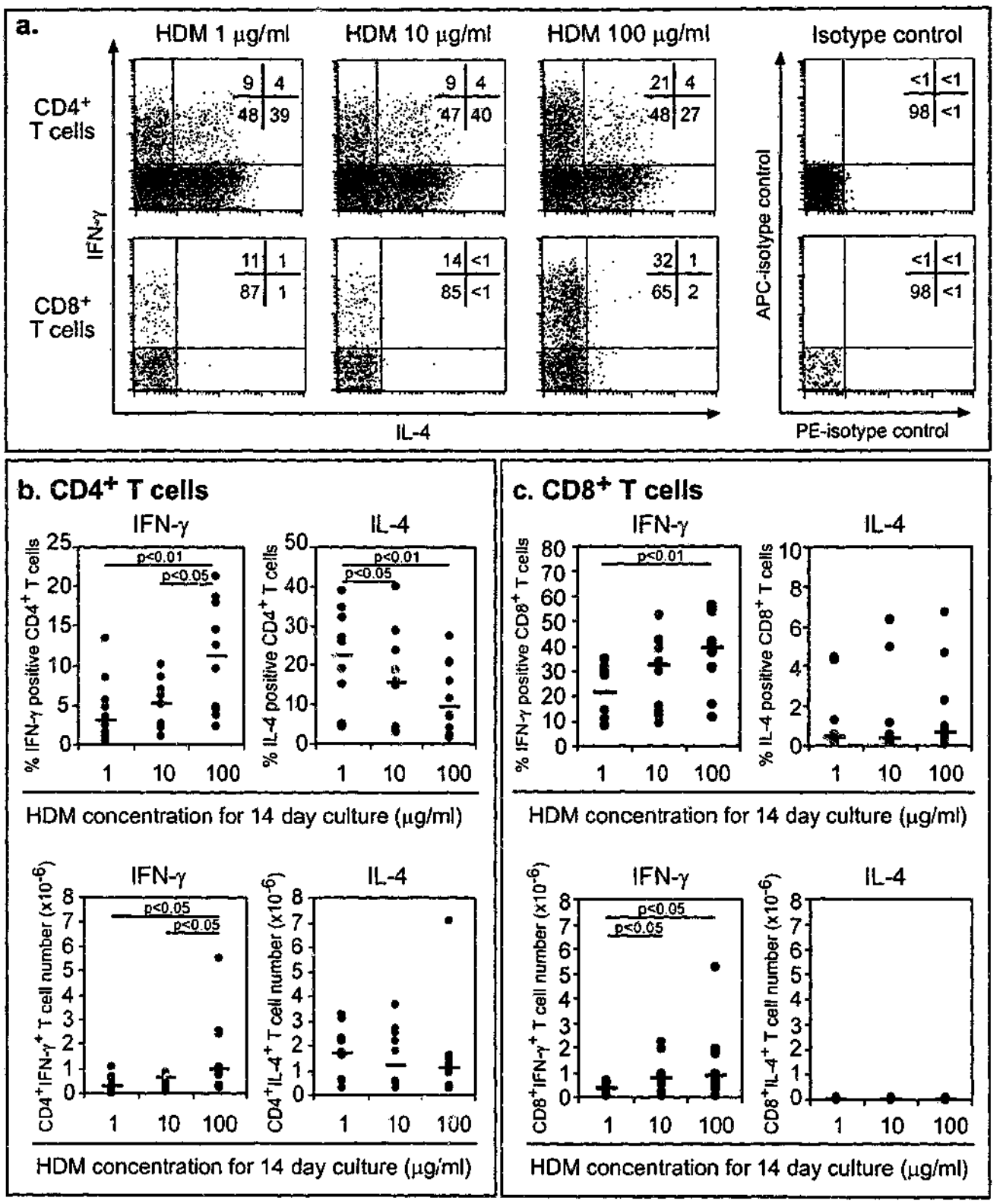
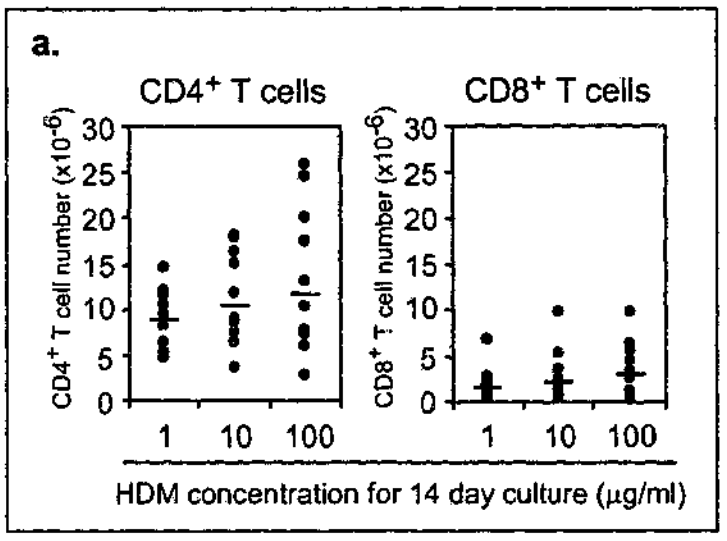


Figure 4.1 Effect of allergen concentration on $CD4^+$ and $CD8^+$ T cell cytokine profiles. PBMC from 10 HDM-allergic donors were cultured with HDM extract (1, 10 and 100 µg/ml) for 14 days, stimulated with anti-CD3/IL-2 in the presence of Brefeldin A for 6 hours and analysed by flow cytometry following labelling with anti-CD4, anti-CD8, anti-IL-4 and anti-IFN-γ antibodies. **(a)** $CD4^+$ and $CD8^+$ T cell cytokine profiles for one representative HDM-allergic donor. Quadrant markers indicating positive staining are set according to minimal (<1%) isotype control staining. Quadrant percentages are indicated in the top right-hand corner of each plot and were determined from the acquisition of 30000 events by the flow cytometer. **(b)** The proportion and numbers of cytokine positive $CD4^+$ T cells. The proportion and numbers of IFN-γ positive $CD4^+$ T cells were significantly greater at 100 µg/ml HDM extract in comparison to 1 µg/ml (% $p<0.01$; number $p<0.05$) and 10 µg/ml ($p<0.05$) HDM extract. The percentages of IL-4 positive $CD4^+$ 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)** The proportion and numbers of cytokine positive $CD8^+$ T cells. The proportions and numbers 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 (% $p<0.01$; number $p<0.05$). There were also increased numbers of IFN-γ positive $CD8^+$ T cells in cultures stimulated with 10 µg/ml HDM extract in comparison to 1 µg/ml ($p<0.05$). Each symbol in the graphs in represents one HDM-allergic donor and the bars represent the medians.



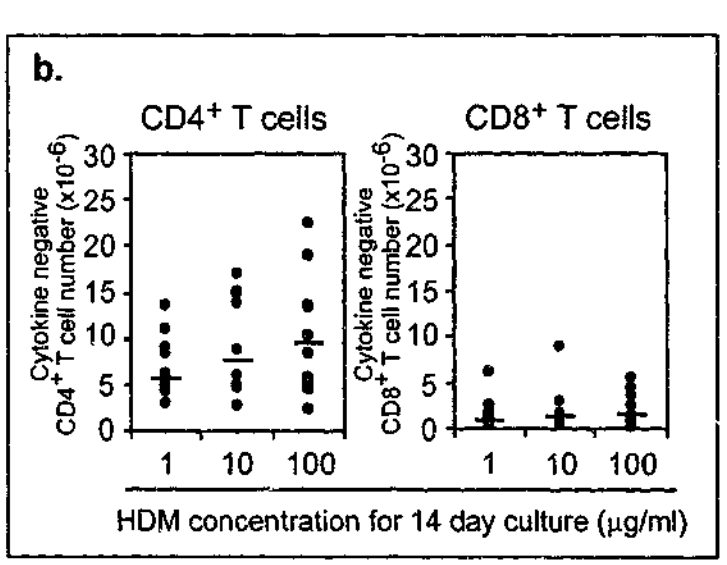


Figure 4.2 Effect of allergen concentration on $CD4^+$ and $CD8^+$ T cell numbers and cytokine negative $CD4^+$ and $CD8^+$ T cell numbers in 14-day HDM extract-stimulated cultures. PBMC from 10 HDM-allergic donors were cultured with HDM extract (1, 10 and 100 μg/ml) for 14 days, stimulated with anti-CD3/IL-2 in the presence of Brefeldin A for 6 hours and analysed by flow cytometry following labelling with anti-CD4, anti-CD8, anti-IL-4 and anti-IFN-γ antibodies. **(a)** Summary of the total numbers of $CD4^+$ and $CD8^+$ T cells in allergen-stimulated cultures at day 14. **(b)** Summary of the total numbers of cytokine negative (IL-4 and IFN-γ negative) $CD4^+$ and $CD8^+$ T cells in allergen-stimulated cultures at day 14.

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 μg/ml HDM extract for 4 days. Latex-specific T cells did not proliferate in the presence of 1, 10 or 100 μg/ml HDM extract, demonstrating that the extract did not have mitogenic properties (refer to Chapter 3, Figure 3.2). Furthermore, to exclude that the HDM extract could non-specifically activate T cells to produce cytokines, IL-4 and IFN-γ 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-γ by GE-stimulated $CD4^+$ T cells (Figure 4.3a, b). 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 μg/ml indicating that the HDM extract was not toxic (see Figure 3.2).

4.3.2 High Allergen Concentration Increases $CD4^+$ T cell Division and Alters the Relationship Between IFN-γ Production and Cell Division Number

The possible mechanisms involved in skewing cytokine production of $CD4^+$ T cells towards IFN-γ predominance after repeated stimulation with high HDM extract concentration over 14 days were next investigated. Cell division has been shown to play an important role in regulating cytokine production by murine T cells (Gett and Hodgkin, 1998). Hence T cell division was examined in 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, whether the relationship between cell division and cytokine production could be altered by different allergen concentrations was assessed. 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 (see Section 3.3.4.3). CFSE profiles of HDM extract-

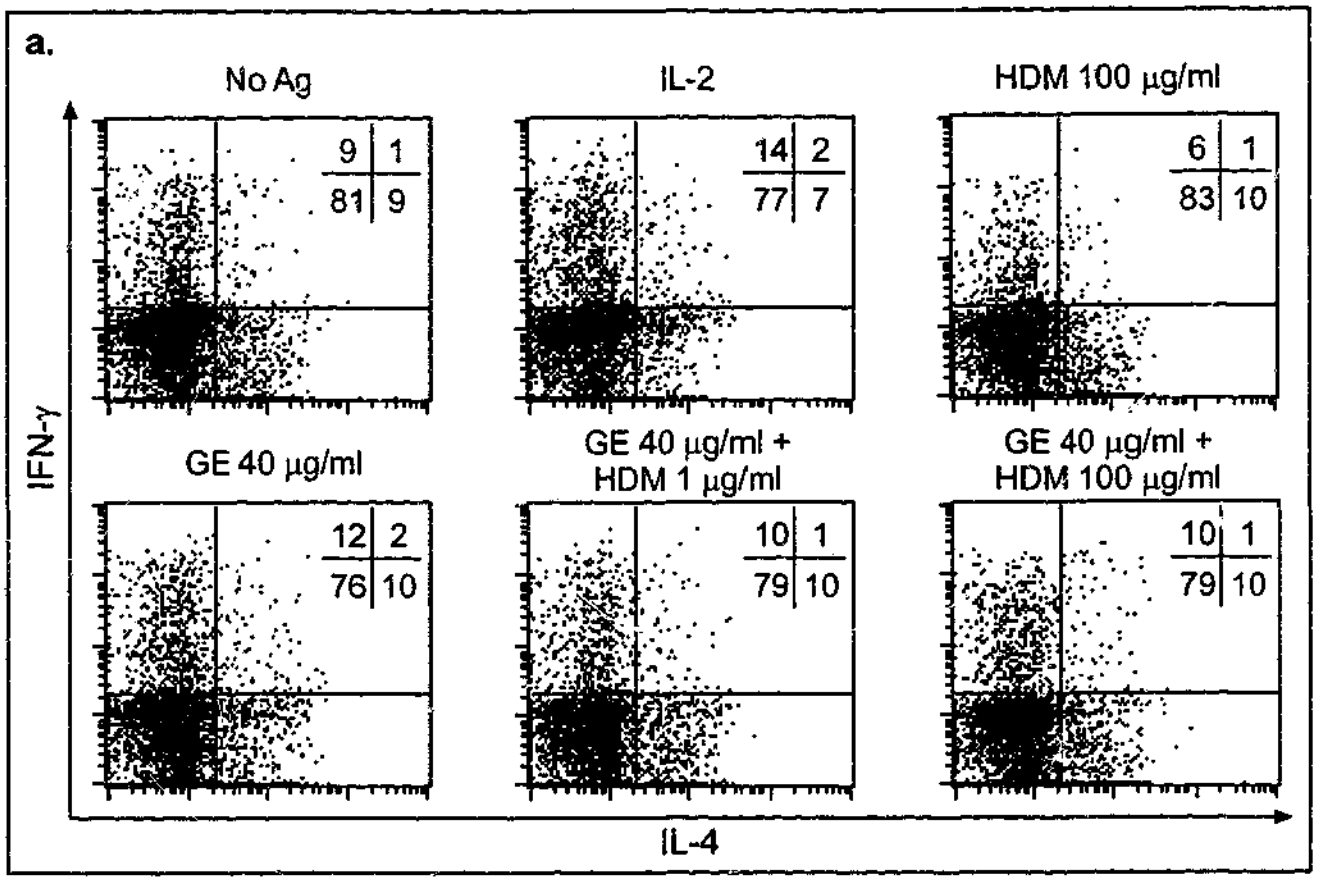


Figure 4.3 Assessing non-specific T cell cytokine production induced by HDM extract. Latex-specific T cells were cultured for two weeks in the presence of GE and irradiated PBMC as APC. Latex-specific T cells were then cultured with equal numbers of washed irradiated autologous PBMC in the presence of 40 µg/ml GE with either 0, 1 or 100 µg/ml HDM extract (final volume 1 ml/well) for 4 days at 37°C in a 5% CO_2. 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 4 days, cells were stimulated for 6 hours with anti-CD3/IL-2 and analysed by flow cytometry following staining with anti-CD4, anti-IL-4 and anti-IFN-γ antibodies. **(a)** IL-4 and IFN-γ dot plots of GE-specific $CD4^+$ T cells stimulated in the presence of no antigen, IL-2, GE, HDM extract or GE with HDM extract. Quadrant markers indicating positive staining are set according to minimal (<1%) isotype control staining. Quadrant percentages are indicated in the top right-hand corner of each plot and were determined from the acquisition of 30000 events by the flow cytometer. **(b)** Culture of GE-specific $CD4^+$ T cells with GE and HDM extract did not result in an alteration of IL-4 or IFN-γ production.

stimulated cultures at day 10 and 12 are shown for one HDM-allergic donor (Figure 4.4a). Repeating this experiment on another 5 donors revealed increased numbers of $CD4^+$ T cells undergoing division in higher HDM concentration cultures (Figure 4.4b; $p<0.05$ for division 6 in 100 μg/ml HDM extract-stimulated cultures in comparison to 1 μg/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 4.5a and a summary of results for 6 HDM allergic donors is shown in Figure 4.5b. Culturing PBMC with 100 μg/ml HDM extract altered the relationship between $CD4^+IFN\text{-}\gamma^+$ T cell proportion and cell division interval in comparison to that observed for lower allergen concentration cultures. $CD4^+IFN\text{-}\gamma^+$ T cell proportions in cultures stimulated with 100 μg/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-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 lower concentration cultures. In contrast HDM concentration did not alter the relationship between $CD4^+IL\text{-}4^+$ T cell proportion and cell division number. $CD4^+IL\text{-}4^+$ T cell proportions peaked at division intervals 4 or 5 for all concentrations tested.

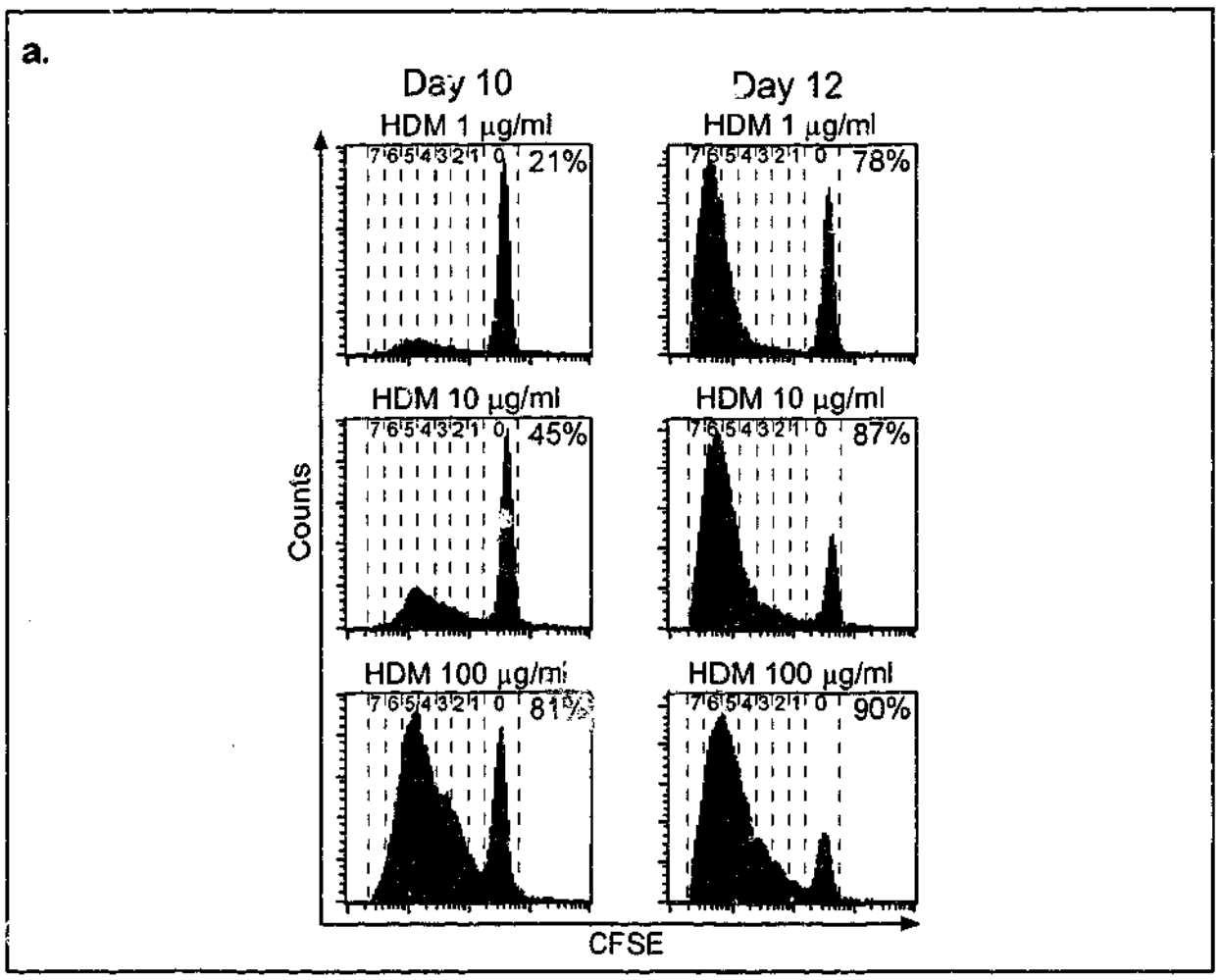


Figure 4.4 $CD4^+$ T cell division in allergen stimulated cultures.
Seven day HDM extract-stimulated (1, 10 and 100 µg/ml) cultures were labelled with CFSE and restimulated with HDM extract and APC. **(a)** Histograms of CFSE fluorescence gated on $CD4^+$ T cells at days 10 and 12 are shown for one donor with the percentage of cells that have undergone 1 or more divisions shown in the top right hand corner of each plot and the dotted lines representing division intervals. These intervals were determined from the anti-CD3 (10 µg/ml) and rIL-2 (100 U/ml) positive control. **(b)** Performing this experiment on six HDM-allergic donors revealed increased division of $CD4^+$ T cells in the higher HDM concentration cultures in comparison to the 1 µg/ml HDM extract-stimulated cultures at day 10 and 12. This increase reached statistical significance when the 100 µg/ml HDM extract-stimulated cultures were compared to the 1 µg/ml HDM extract-stimulated cultures at division 6 on day 12 (*: $p<0.05$). The mean number of $CD4^+$ T cells and standard error at each division interval for six HDM-allergic donors are shown.

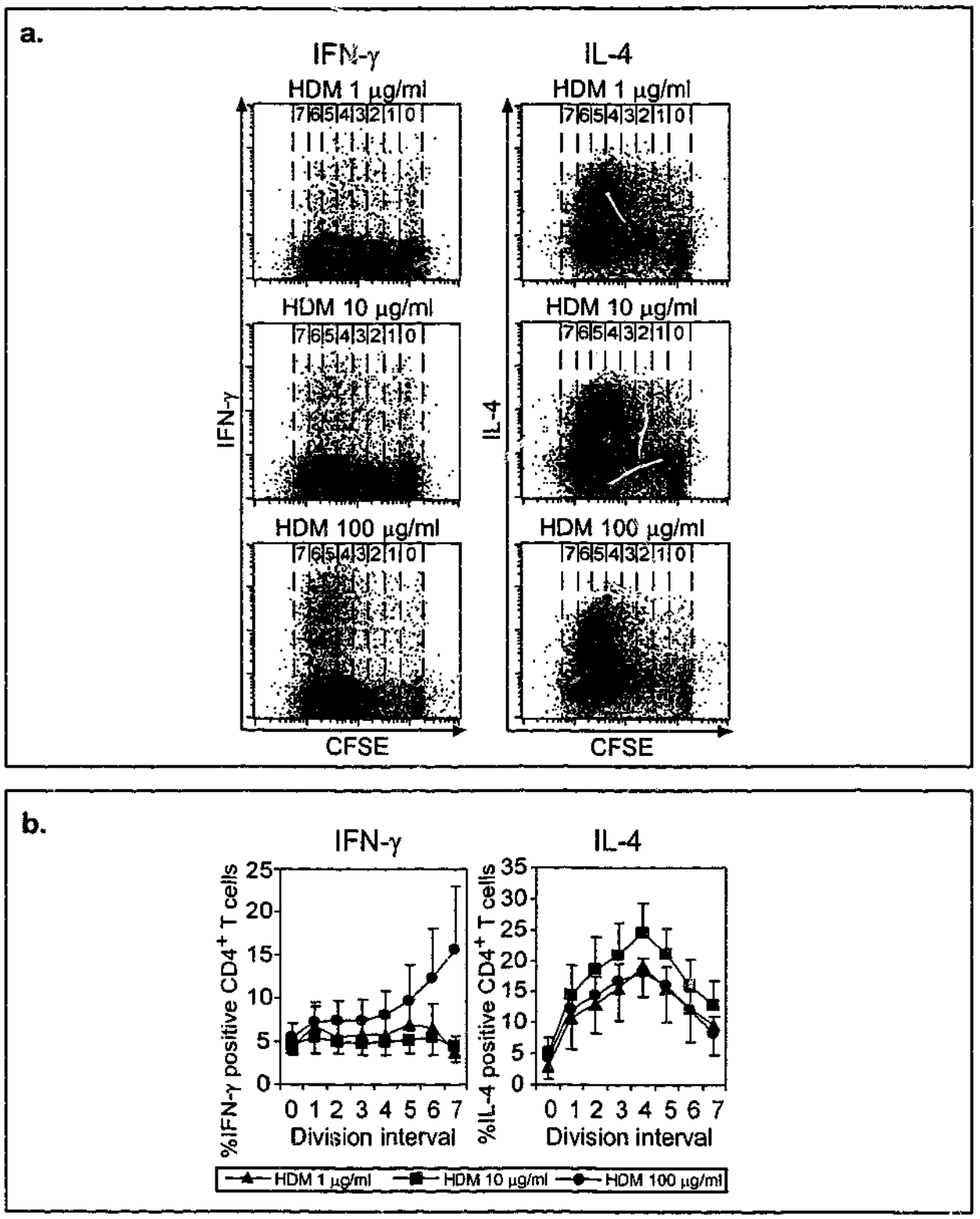


Figure 4.5 High allergen concentration alters the relationship between cell division and IFN-γ production but not IL-4 production.
Seven day HDM extract-stimulated (1, 10 and 100 µg/ml) cultures were labelled with CFSE and restimulated with HDM extract and APC. On day 12, CFSE labelled cultures were stimulated with anti-CD3/IL-2 in the presence of Brefeldin A for 6 hours, labelled with anti-CD4, anti-IL-4 and anti-IFN-γ antibodies and analysed by flow cytometry. **(a)** Representative profiles of CFSE fluorescence versus IFN-γ, and versus IL-4 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. **(b)** The mean percentages of $CD4^+IFN\text{-}\gamma^+$ T cells and $CD4^+IL\text{-}4^+$ T cells 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.

4.3.3 $CD8^+$ T Cells Divide and Produce IFN-γ in HDM-Stimulated Cultures

To determine whether the relationship between $CD8^+$ T cell division and IFN-γ 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 profiles of $CD8^+$ T cells in HDM-stimulated cultures on days 10 and 12 for one HDM-allergic donor are shown in Figure 4.6a. 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, and there was a trend towards increased $CD8^+$ T cell division in cultures stimulated with 10 and 100 μg/ml HDM extract (Figure 4.6b). As $CD8^+$ T cells produced minimal IL-4 at any HDM concentration, shown previously in Figure 4.1c, dividing cells were only analysed for IFN-γ production (Figure 4.6c, 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.

4.3.4 Increased Th2-type 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 (Figure 4.4b) without an overall increase in $CD4^+$ T cell number (Figure 4.2a), 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 4.7a demonstrate increased proportions of active caspase-3 positive $CD4^+IL\text{-}4^+$ cells but not $CD4^+IFN\text{-}\gamma^+$ T cells with increasing HDM extract concentration. There was a significant increase ($p<0.05$) in the proportion of

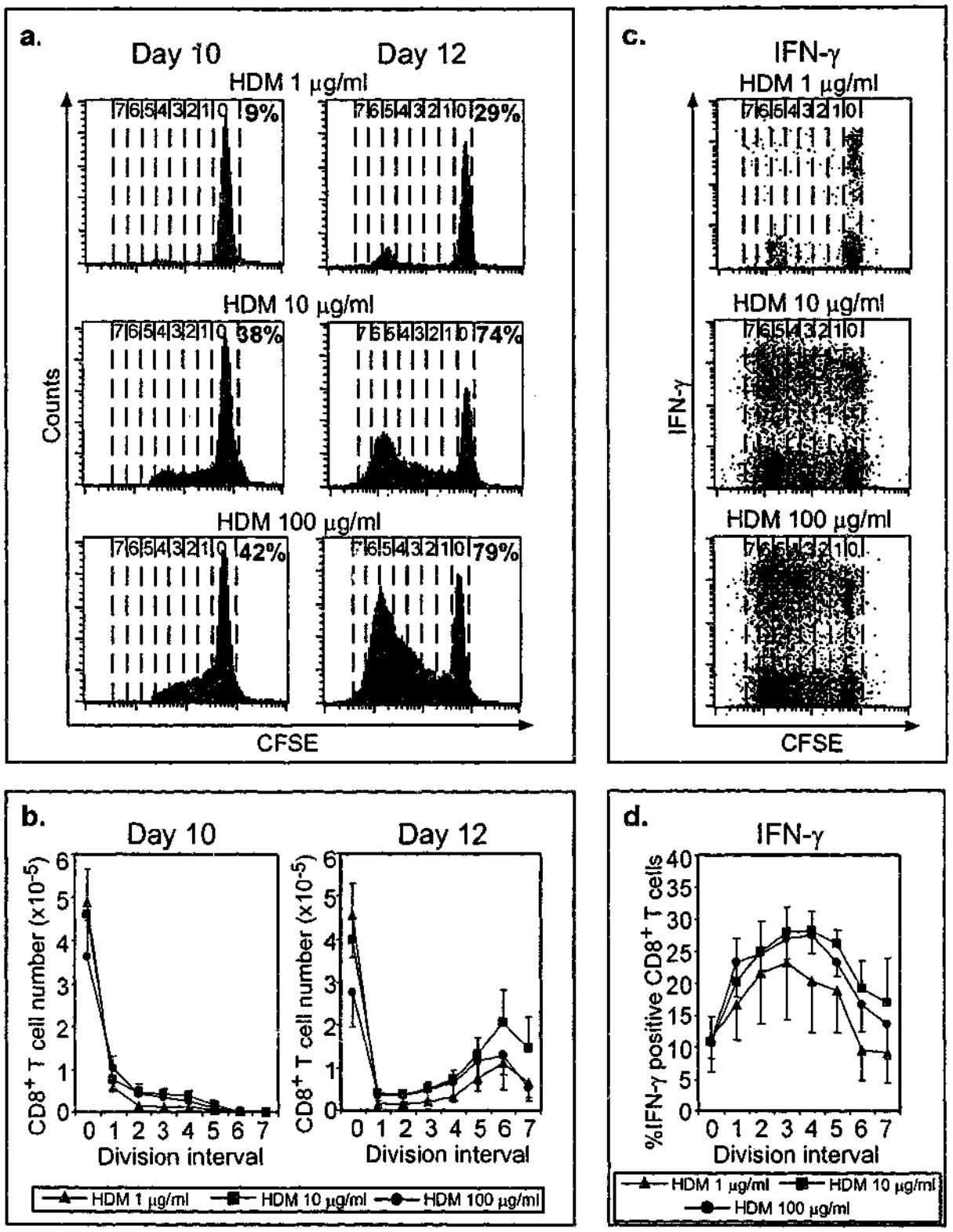


Figure 4.6 CD8$^+$ T cells divide and produce IFN-γ in HDM-stimulated cultures.
PBMC, cultured with HDM extract (1, 10 and 100 µg/ml) for 7 days, were harvested, labelled with CFSE and restimulated with HDM extract (1, 10 and 100 µg/ml) and APC. **(a)** Representative histograms of CFSE fluorescence gated on CD8$^+$ T cells from HDM-stimulated cultures on days 10 and 12 are shown with the percentage of cells that have undergone 1 or more divisions in the top right hand corner of each plot. 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. **(b)** 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 µg/ml HDM-stimulated cultures in comparison to 1 µg/ml at day 12. The mean number and standard error of CD8$^+$ T cells at each division interval are shown. **(c)** Representative profiles of CFSE fluorescence versus IFN-γ were generated by gating on CD8$^+$ T cells from cultured cells labelled with anti-CD8 and anti-IFN-γ antibodies at day 12 after stimulation with anti-CD3/IL-2 in the presence of Brefeldin A for 6 hours. 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. **(d)** The mean percentage and standard error of IFN-γ positive CD8$^+$ T cells at each division interval in HDM-stimulated cultures for the 4 donors are graphed.

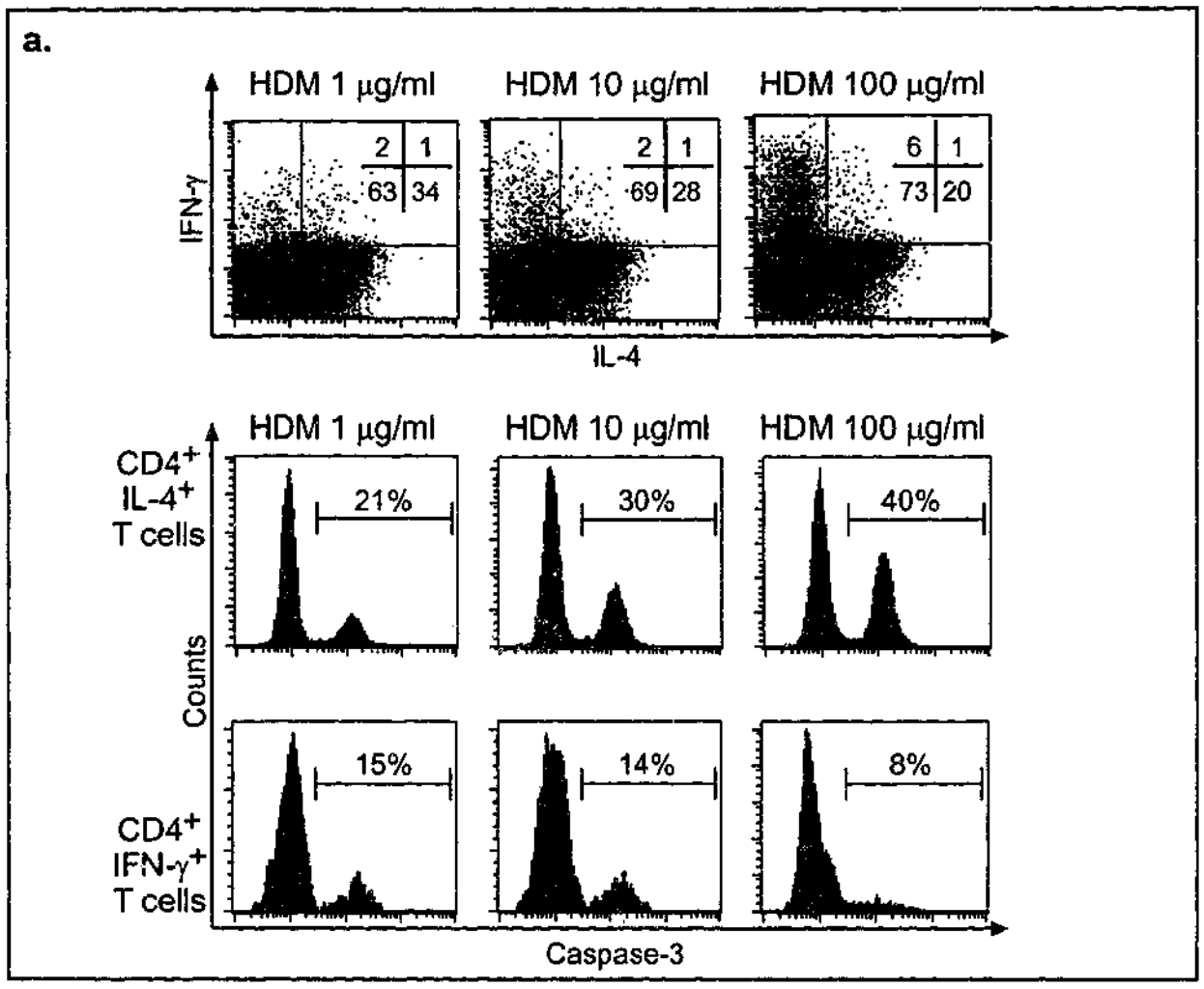


Figure 4.7 Effect of allergen concentration on apoptosis of cytokine-positive $CD4^+$ T cells. PBMC from 8 HDM-allergic donors were cultured with HDM extract (1, 10 and 100 µg/ml) for 14 days. Cultured cells were stimulated with anti-CD3/IL-2 in the presence of Brefeldin A for 6 hours and analysed by flow cytometry following labelling with anti-CD4, anti-active caspase-3, anti-IL-4 and anti-IFN-γ antibodies. **(a)** Cytokine profiles of $CD4^+$ T cells and histograms of active caspase-3 staining for $CD4^+IL\text{-}4^+$ and $CD4^+IFN\text{-}\gamma^+$ T cells for one representative HDM-allergic donor. Quadrant and marker percentages indicate positive staining determined from the acquisition of 30000 events by the flow cytometer. **(b)** Summary of the proportions of active caspase-3 positive $CD4^+IL\text{-}4^+$ T cells and $CD4^+IFN\text{-}\gamma^+$ T cells. The percentage of active caspase-3 positive $CD4^+IL\text{-}4^+$ T cells increased significantly at 100 µg/ml HDM extract in comparison to 1 µg/ml HDM extract ($p<0.05$). In the graphs each symbol represents one HDM-allergic donor and the bars represent the median values for the 8 donors.

active caspase-3 positive $CD4^+IL\text{-}4^+$ T cells in cultures stimulated at 100 μg/ml HDM extract in comparison to those stimulated at 1 μg/ml (Figure 4.7b). Allergen concentration did not alter proportions of active caspase-3 positive $CD4^+IFN\text{-}\gamma^+$ T cells which were low at all concentrations (Figure 4.7b).

Apoptosis of a subset of $CD8^+$ T cells was also considered. The results for one HDM allergic-donor are shown in Figure 4.8a. The percentage of active caspase-3 positive $CD8^+IFN\text{-}\gamma^+$ T cells was increased in cultures stimulated with HDM extract at 10 μg/ml ($p<0.05$) and 100 μg/ml (not significant) in comparison to 1 μg/ml (Figure 4.8b). Although the proportions of active caspase-3 positive $CD8^+IL\text{-}4^+$ T cells were unaffected by allergen concentration, approximately 80% of the $CD8^+IL\text{-}4^+$ T cells in each HDM extract-stimulated culture were undergoing apoptosis (Figure 4.8b).

4.3.5 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 (O'Hehir *et al.*, 1991), whether anergy could be induced by stimulation with high concentrations of HDM extract over the 14-day culture period was examined. After 14 days culture of 10 HDM-allergic donor PBMC with 1, 10 or 100 μg/ml HDM extract, cells were restimulated with an immunogenic concentration of HDM extract (10 μg/ml) or IL-2 (100 U/ml). The results for one HDM allergic-donor are shown in Figure 4.9a. Pre-treatment with 100 μg/ml HDM extract resulted in significantly lower proliferative response to 10 μg/ml HDM extract compared to pre-treatment with 1 μg/ml ($p<0.01$) or 10 μg/ml ($p<0.05$) HDM extract

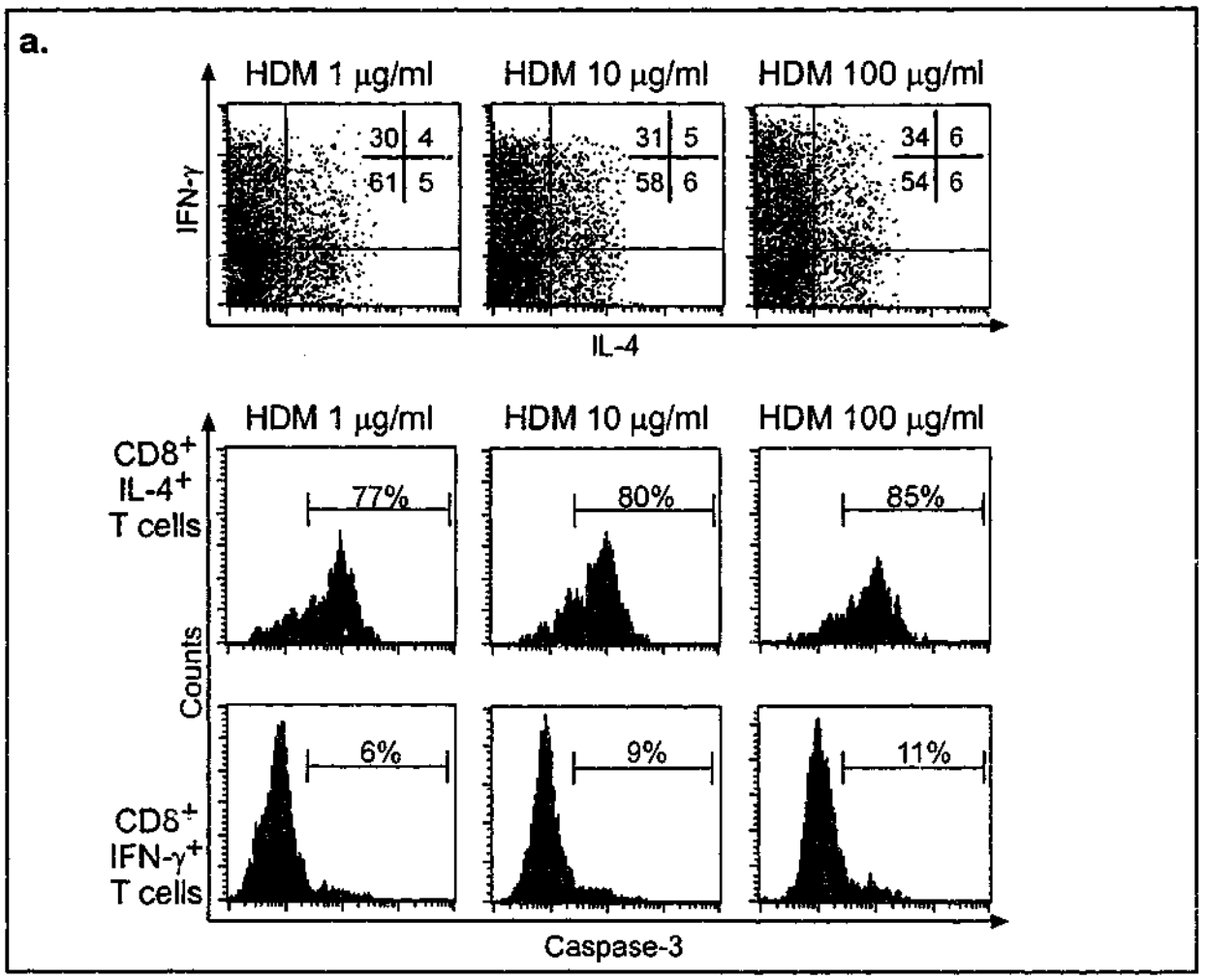


Figure 4.8 Effect of allergen concentration on apoptosis of cytokine-positive $CD8^+$ T cells. PBMC from 8 HDM-allergic donors were cultured with HDM extract (1, 10 and 100 µg/ml) for 14 days. Cultured cells were stimulated with anti-CD3/IL-2 in the presence of Brefeldin A for 6 hours and analysed by flow cytometry following labelling with anti-CD8, anti-active caspase-3, anti-IL-4 and anti-IFN-γ antibodies. **(a)** Cytokine profiles of $CD8^+$ T cells and histograms of active caspase-3 staining for $CD8^+IL\text{-}4^+$ and $CD8^+IFN\text{-}\gamma^+$ T cells for one representative HDM-allergic donor. Quadrant and marker percentages indicate positive staining determined from the acquisition of 30000 events by the flow cytometer. **(b)** Summary of the proportions of active caspase-3 positive $CD8^+IL\text{-}4^+$ T cells and $CD8^+IFN\text{-}\gamma^+$ T cells. There was a significant increase in the proportions of active caspase-3 positive $CD8^+IFN\text{-}\gamma^+$ T cells in cultures stimulated with 10 µg/ml HDM extract in comparison to 1 µg/ml. In the graphs each symbol represents one HDM-allergic donor and the bars represent the median values for the 8 donors.

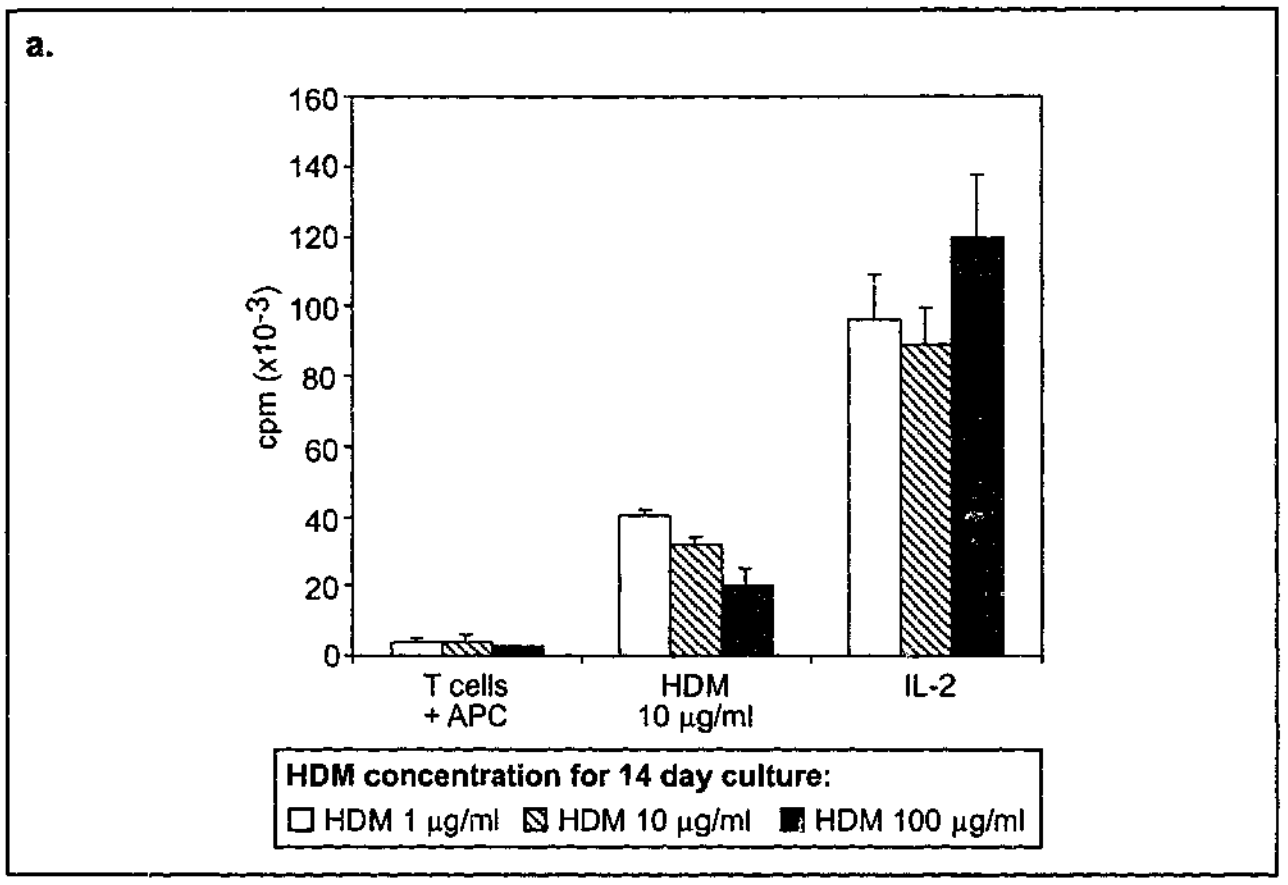


Figure 4.9 Decreased allergen-specific proliferation by cultures stimulated with high allergen concentration for 14 days.

PBMC from 10 HDM-allergic donors were cultured with 1, 10 and 100 µg/ml HDM extract for 14 days and restimulated in the presence of irradiated PBMC as APC and either 10 µg/ml HDM extract or IL-2 (100 U/ml) or no antigen. After 72 hours, proliferation as correlated with ^{3}H-thymidine incorporation (Δ cpm) was determined. **(a)** Proliferative responses (cpm) to HDM extract and IL-2 of 14-day PBMC cultures stimulated with different allergen concentrations generated from one HDM-allergic donor. **(b)** Summary of the proliferative responses of 14-day PBMC cultures stimulated with different allergen concentrations generated from 10 HDM-allergic donors. Cells pretreated with 100 µg/ml HDM extract had significantly decreased proliferation upon restimulation with an immunogenic concentration of allergen in comparison to cells pretreated with 1 µg/ml ($p<0.01$) and 10 µg/ml ($p<0.05$) HDM extract. Each symbol in the graphs represents one HDM-allergic donor and the bars represent the medians.

(Figure 4.9b). Cells pre-treated with 1, 10 and 100 μg/ml HDM extract showed similar proliferative responses to IL-2 (Figure 4.9b).

4.3.6 $CD4^+$ T Cell IL-10 Production is Unaffected by Allergen Concentration

Increased T cell IL-10 production has been shown to decrease or inhibit allergen-specific T cell proliferation during SIT (Akdis *et al.*, 1998b; Jutel *et al.*, 2003). Hence induction of increased IL-10 production by $CD4^+$ T cells at high allergen concentration was investigated. PBMC from 10 HDM-allergic donors were cultured for 14 days with different concentrations HDM extract and $CD4^+$ T cells were analysed for intracellular IL-10. Results for one HDM-allergic donor are shown in Figure 4.10a and a summary of data for the 10 HDM-allergic donors is shown in Figure 4.10b. There were no significant changes in the proportions and numbers of IL-10 positive $CD4^+$ T cells in cultures stimulated with different concentrations of HDM extract.

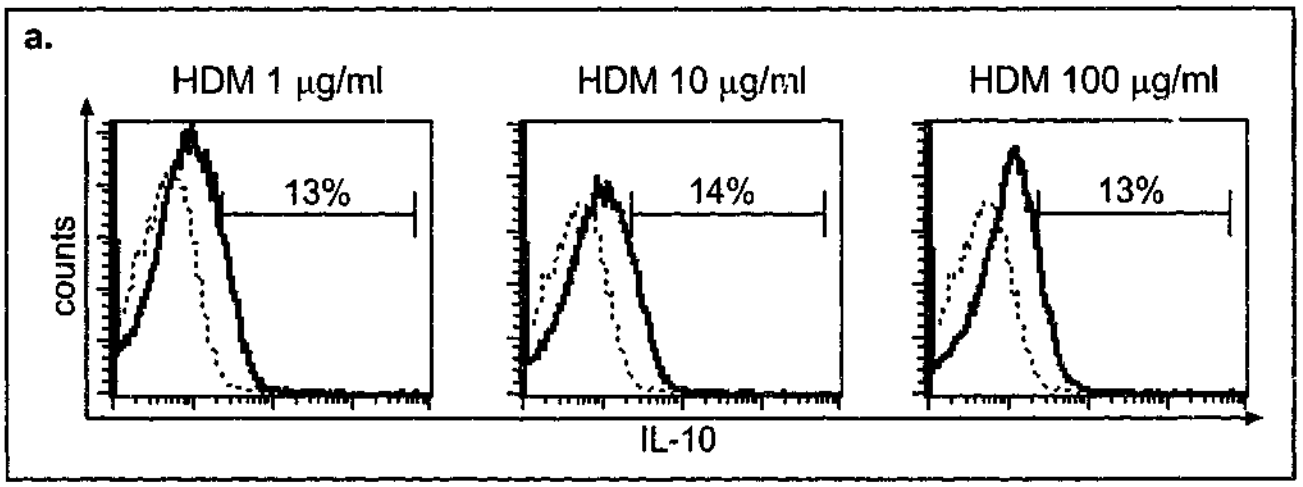


Figure 4.10 Effect of allergen concentration on IL-10 production by $CD4^+$ T cells.
PBMC from 10 HDM-allergic donors were cultured with HDM extract (1, 10 and 100 µg/ml) for 14 days, stimulated with anti-CD3/IL-2 for 20 hours with Brefeldin A added with 4 hours of stimulation remaining. Cells were analysed by flow cytometry following labelling with anti-CD4, and anti-IL-10 antibodies. **(a)** $CD4^+$ T cell histograms of IL-10 staining (solid line) for one representative HDM-allergic donor. Markers indicating positive staining are set according to minimal (<1%) isotype control staining (dotted line). Percentages indicated above the markers and were determined from the acquisition of 30000 events by the flow cytometer. **(b)** Summary of the proportion and numbers of IL-10 positive $CD4^+$ T cells for 10 HDM-allergic donors. Each symbol in the graphs in represents one HDM-allergic donor and the bars represent the medians.

4.4 DISCUSSION

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 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 considerably higher doses of allergen than are encountered naturally (Tovey *et al.*, 1981; Bousquet *et al.*, 1998a), the effect of allergen concentration on cytokine production, division and apoptosis of allergen-specific T cells *in vitro* was investigated. Using intracellular cytokine staining and flow cytometry T cell changes were analysed at the single cell level. Culturing cells in the presence of high allergen concentration in comparison to lower concentrations resulted in increased $CD4^+IFN\text{-}\gamma^+$ and $CD8^+IFN\text{-}\gamma^+$ T cell numbers with no difference in $CD4^+IL\text{-}10^+$, $CD4^+IL\text{-}4^+$ and $CD8^+IL\text{-}4^+$ T cell numbers, although $CD4^+IL\text{-}4^+$ T cell proportions were decreased. CFSE cell labelling demonstrated an association between increased IFN-γ 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-γ by allergen-specific T cells could lead to a reduction in allergen-induced pathology. In human B cells, IFN-γ inhibits IL-4-induced expression of the germline epsilon transcript and consequently prevents IgE production (Venkataraman *et al.*, 1999). Via this mechanism IFN-γ, 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-γ reversed bronchial hyperresponsiveness and bronchoalveolar lavage eosinophilia (Huang *et al.*, 2001). Increased IFN-γ positive T cell numbers at sites of allergen challenge have been demonstrated following clinically successful immunotherapy (Durham *et al.*, 1996). 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-γ production (Constant and Bottomly, 1997). 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 (Ben-Sasson *et al.*, 2001). In agreement, IFN-γ production by more highly dividing $CD4^+$ T cells at high allergen concentration than for lower allergen concentrations was observed. 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 lipopolysaccharide (LPS), peptidoglycan and other specific pathogen-associated microbial products, can bind Toll-like receptors (TLR) expressed by human monocytes, dendritic cells (DC), B cells and T cells

(Kadowaki *et al.*, 2001; Zarember and Godowski, 2002). Binding of these ligands to TLRs on APC results in the production of inflammatory cytokines such as IL-12, TNF-α and IL-6 (Kadowaki *et al.*, 2001; Zarember and Godowski, 2002), thus altering the cytokine milieu of antigen-stimulated T cells and influencing Th differentiation. Polyclonal activators may only be playing a minor role, if any, in the cell culture system used in the present study as LPS levels in the HDM extract were shown to be undetectable, and IL-4 and IFN-γ production by latex-specific T cells was not enhanced by culture with glove extract in combination with HDM extract. Furthermore, pilot experiments showed a similar skewing of T cell cytokine production at high concentrations using 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 (Rolland and O'Hehir, 1998). The induction of T cell anergy has been suggested as a possible mechanism. (Rolland and O'Hehir, 1998; Larche, 2001). Anergy, defined by diminution of antigen-specific proliferative response with reversal by IL-2 (Schwartz, 2003), has been induced in allergen-specific cloned T cells by high dose peptide treatment and can result in altered T cell cytokine production (O'Hehir *et al.*, 1991). In the present study, 14-day pre-treatment 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 (Groux *et al.*, 1996). IL-10 positive cells have been shown to be increased in skin at sites of allergen challenge and in the blood after venom and grass pollen SIT (Akdis *et al.*, 1998b; Nasser *et al.*, 2001; Francis *et al.*, 2003; Jutel *et al.*, 2003). However increased IL-10 production at high allergen concentration was not

observed suggesting that it may not be playing a role in anergy induction in the cultures in this study. The culture conditions may not have been ideal for enhanced T cell IL-10-production at high allergen concentration. T cell production of IL-10 has been reported to be influenced by APC type, costimulation and the cytokine milieu. For example repetitive stimulation of T cells with immature DC instead of mature DC favours IL-10 production (Jonuleit *et al.*, 2000). In addition interactions between effector T cells and professional APC through ICOS and ICOSL enhances IL-10 release whereas interactions with CD28 and B7 ligands attenuates IL-10 release (Witsch *et al.*, 2002). Moreover IL-10 production can be inhibited by Th1 (IFN-γ and IL-12) and Th2 (IL-4) cytokines (Barrat *et al.*, 2002). It is these factors that could be exploited to enhance the effectiveness of high dose allergen administration to induce IL-10 production by $CD4^+$ T cell *in vivo*.

As apoptosis can follow anergy induction after high dose antigen treatment (Vandenbark *et al.*, 2000), deletion of allergen-specific Th cells could also lead to in part the reduced proliferative responses seen in 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-γ cytokine production. In support of this proposed mechanism, increased apoptosis of IL-4-producing $CD4^+$ T cells at high allergen concentration with no change in the number of apoptotic IFN-γ-producing $CD4^+$ T cells was observed. The results of the present study differ from previous research which suggested that Th1 cells had increased susceptibility to apoptosis (Janssen *et al.*, 2000c). 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 (Janssen *et al.*, 2000c). 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 the culture system used in the present study could address this issue. Nevertheless, the results observed in the present study 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 (Guerra *et al.*, 2001). 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-γ 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 (Seneviratne *et al.*, 2002), 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 (Renz *et al.*, 1994). In addition IFN-γ produced by $CD8^+$ T cells transferred into sensitised rats inhibited allergen-induced eosinophilic inflammation (Suzuki *et al.*, 2002). Of note, IFN-γ production by $CD8^+$ T cells after fewer cell divisions in comparison to $CD4^+$ T cells was observed in the present study, suggesting that the $CD8^+$ T cell IFN-γ 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-γ production by $CD8^+$ T cells was shown to occur after TCR stimulation independently of cell division indicating that

there are different thresholds for IFN-γ production and cell division in $CD8^+$ T cells (Auphan-Anezin *et al.*, 2003). This could explain why a change in the relationship between $CD8^+$ T cell division and IFN-γ production at high allergen concentration as seen for the $CD4^+$ T cells was not observed in this study. Although $CD8^+$ T cell IFN-γ production has been shown in some studies to be decreased or unchanged in individuals receiving SIT (Majori *et al.*, 2000; O'Brien *et al.*, 2000), the results from this present study suggest that this may be antigen dose related. The induction of regulatory $CD8^+$ T cells producing IFN-γ 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-γ in addition to Th2 mediated inflammation (Levings *et al.*, 2002).

In conclusion this study indicates 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 (Muller *et al.*, 1998; Larche, 2001) or mutant allergens (Ferreira *et al.*, 1996; Smith and Chapman, 1996) should permit further refinement of efficacy and safety of this potentially curative treatment.

CHAPTER 5

T CELL EXPRESSION OF SURFACE MARKERS FOR PERIPHERAL TISSUE TRAFFICKING AT DIFFERENT ALLERGEN CONCENTRATIONS[1]

5.1 INTRODUCTION

During clinically successful SIT, T cell responses are modified, with deviation from IL-4, IL-5 cytokine production to IFN-γ predominant (McHugh *et al.*, 1995; Ebner *et al.*, 1997; Eusebius *et al.*, 2002) and increased IFN-γ^+ cell numbers at sites of allergen challenge (Varney *et al.*, 1993; Durham *et al.*, 1996; Wachholz *et al.*, 2002). Mechanisms for cytokine changes during SIT include immune deviation (McHugh *et al.*, 1995; Ebner *et al.*, 1997; Eusebius *et al.*, 2002), T cell anergy (McHugh *et al.*, 1995; Ebner *et al.*, 1997; Eusebius *et al.*, 2002), induction of T regulatory cells (Francis *et al.*, 2003; Jutel *et al.*, 2003), and enhanced allergen-induced apoptosis (Guerra *et al.*, 2001), however it is not clear whether SIT promotes trafficking of IFN-γ^+ T cells to peripheral tissues.

Trafficking of naïve and effector T cells from blood vessels into tissues is dependent on interactions between T cell expressed adhesion molecules and ligands on endothelial cells (Westermann *et al.*, 2001). Naïve T cells gain access to lymph nodes via the binding of L-selectin (CD62L) to sulphated carbohydrates on CD34, a vascular

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[1] This chapter is based on the publication: **Leanne M. Gardner**, Lisa Spyroglou, Robyn E. O'Hehir and Jennifer M. Rolland (2003) House dust mite-allergic donor peripheral blood T cells expressing markers for peripheral tissue trafficking comprise increased numbers of IFN-γ^+ cells after *in vitro* stimulation with high allergen concentration. Immunology and Cell Biology (submitted).

addressin, expressed by high endothelial venules. Upon entry to the lymph node and after antigen activation, CD62L is shed from T cells and expression of the integrins LFA-1 and VLA-4 (CD49d) is upregulated (Chao *et al.*, 1997; Hamann *et al.*, 2000). CD49d and LFA-1 bind VCAM-1 and ICAM-1, respectively, on activated peripheral vascular endothelium. This change in adhesion molecule expression accompanies changes in T cell trafficking properties, with reduced recirculation through lymphoid tissue and increased movement into peripheral tissues such as the lung (Hamann *et al.*, 2000). T cell migration into tissues occurs under the influence of chemokine gradients. Differential chemokine receptor expression by T cell subsets has been observed. Expression of CCR3, which binds eotaxin, MCP-3, 4 and RANTES, has been demonstrated on a subset of Th2-type cells (Sallusto *et al.*, 1997; Bonecchi *et al.*, 1998; Sallusto *et al.*, 1998a; Annunziato *et al.*, 1999) whereas expression of CCR5, which binds MIP-1α, MIP-1β and RANTES, is largely on Th1-type cells (Odum *et al.*, 1999; Yamamoto *et al.*, 2000), although some studies report expression by Th2-type cells (Sallusto *et al.*, 1998a; Nanki and Lipsky, 2000). Differences in chemokine receptor expression by Th1- and Th2-type cells has been suggested to result in differential recruitment to sites of inflammation (O'Garra *et al.*, 1998; Sallusto *et al.*, 1998b).

Aeroallergen SIT is associated with administration of considerably higher allergen concentrations than encountered naturally (Tovey *et al.*, 1981; Bousquet *et al.*, 1998a). As shown in Chapter 4, stimulation of allergic-donor PBMC with high allergen concentration in comparison to low resulted in preferential expansion of IFN-γ-producing cells. This observation is consistent with the well recognised influence of antigen concentration on Th cell differentiation (Constant *et al.*, 1995; Hosken *et al.*, 1995; Rogers *et al.*, 1998; Grakoui *et al.*, 1999; Rogers and Croft, 1999). The current study determined whether repeated stimulation of 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. T cell expression of the adhesion molecules CD62L and CD49d was analysed because allergen-activated T cells expressing $CD62L^-$ or $CD49d^{hi}$ would represent T cell populations that could potentially traffic to peripheral tissue and, CD49d has been reported to enable T cell movement into lung tissue after allergen challenge (Pacheco *et al.*, 1998). Expression of CCR3 and CCR5 was also examined because a number of ligands for these receptors including eotaxin, RANTES and MIP-1α, are increased in bronchoalveolar lavage fluid from atopic asthmatics after allergen challenge (Lilly *et al.*, 2001) and T cells expressing CCR3 or CCR5 have been identified in the lungs of allergic individuals (Ying *et al.*, 1997; Campbell *et al.*, 2001). Here the proportions and numbers of T cells co-expressing a "peripheral tissue trafficking" phenotype ($CD62L^-$, $CD49d^{hi}$, $CCR3^+$ or $CCR5^+$) and intracellular IL-4 or IFN-γ in 14-day PBMC cultures stimulated with different allergen concentrations were analysed. This was performed to determine if high allergen dose could induce increased numbers of "peripheral tissue trafficking" allergen-specific Th1-type cells that could potentially migrate to peripheral tissues and upon subsequent allergen encounter produce cytokines to down-regulate Th2-type responses in the tissues.

5.2 EXPERIMENTAL PROCEDURES

In this study PBMC were separated from peripheral blood of 10 HDM-allergic donors recruited according to the selection criteria detailed in Section 2.2. The clinical characteristics of these patients are shown in Table 5.1. PBMC were cultured for 14 days in the presence of 1, 10 or 100 μg/ml HDM extract. On day 14 of culture, T cell expression of CD62L, CD49d, CCR3 and CCR5 was analysed by flow cytometry. In addition, cultured cells were stimulated for 6 hours with anti-CD3/IL-2 in order to detect intracellular IL-4 and IFN-γ in T cells expressing peripheral tissue trafficking phenotypes ($CD62L^-$, $CD49d^{hi}$, $CCR3^+$ or $CCR5^+$). Details for cell culture methods, flow cytometric protocols and statistical analyses used in this study Sections 2.5, 2.6 and 2.7 of this thesis.

Table 5.1 Characterisitics of donors used in this study

Patient	Sex	Age	Rhinitis	Asthma	HDM skin test reactivity (wheal mm)	HDM EAST score (AEU/ml)
A2	F	55	Y	Y	21	...
A10	M	43	N	Y	14	...
A11	F	36	Y	Y	21 x 10	6
A12	F	25	Y	Y	10	2 (2.5)
A16	F	28	Y	Y	11	...
A17	M	32	N	Y	21	...
A18	F	25	Y	Y	11	...
A19	M	31	...	...	8	...
A28	F	29	Y	N	15	...
A29	M	54	Y	Y	11	4 (42)

A, atopic; F, female; M, male; ..., not available; Y, yes; N, no

5.3 RESULTS

5.3.1 T Cell Expression of CD62L and CD49d in PBMC Cultures Stimulated with Different Allergen Concentrations

To determine dose-dependent effects of repeated stimulation with allergen on expression of CD62L and CD49d, flow cytometry was performed on HDM-allergic donor PBMC after culture for 14 days with different concentrations of HDM extract. Representative profiles of CD62L and CD49d expression by $CD4^+$ and $CD8^+$ T cells in HDM extract-stimulated cultures of PBMC from one HDM-allergic donor are shown in Figure 5.1a, b and the results for 8 HDM-allergic donors are summarised in Figure 5.1c, d. Analysis of CD62L expression revealed greater proportions of CD62L negative $CD4^+$ T cells in the 10 μg/ml ($p<0.05$) and 100 μg/ml ($p<0.01$) HDM extract-stimulated cultures in comparison to 1 μg/ml (Figures 5.1c). There were greater proportions of CD62L negative $CD8^+$ T cells in the 100 μg/ml HDM extract-stimulated cultures in comparison to the 1 and 10 μg/ml HDM-extract stimulated cultures ($p<0.05$). However $CD4^+CD62L^-$ and $CD8^+CD62L^-$ T cell numbers did not differ significantly between cultures stimulated with different allergen concentrations (Table 5.2). There was a trend for increased proportions of $CD49d^{hi}$ $CD4^+$ T cells in the 10 μg/ml ($p=0.053$) and 100 μg/ml ($p=0.09$) HDM extract-stimulated cultures in comparison to 1 μg/ml however statistical significance was not reached (Figure 5.1d). There was no significant difference in $CD4^+CD49d^{hi}$ T cell numbers between cultures stimulated with different concentrations of allergen. There were greater proportions and numbers of $CD49d^{hi}$ $CD8^+$ T cells in cultures stimulated with 100 μg/ml HDM extract in comparison to 1 μg/ml (% $p<0.01$; number $p<0.05$) and 10 μg/ml (% and number $p<0.05$).

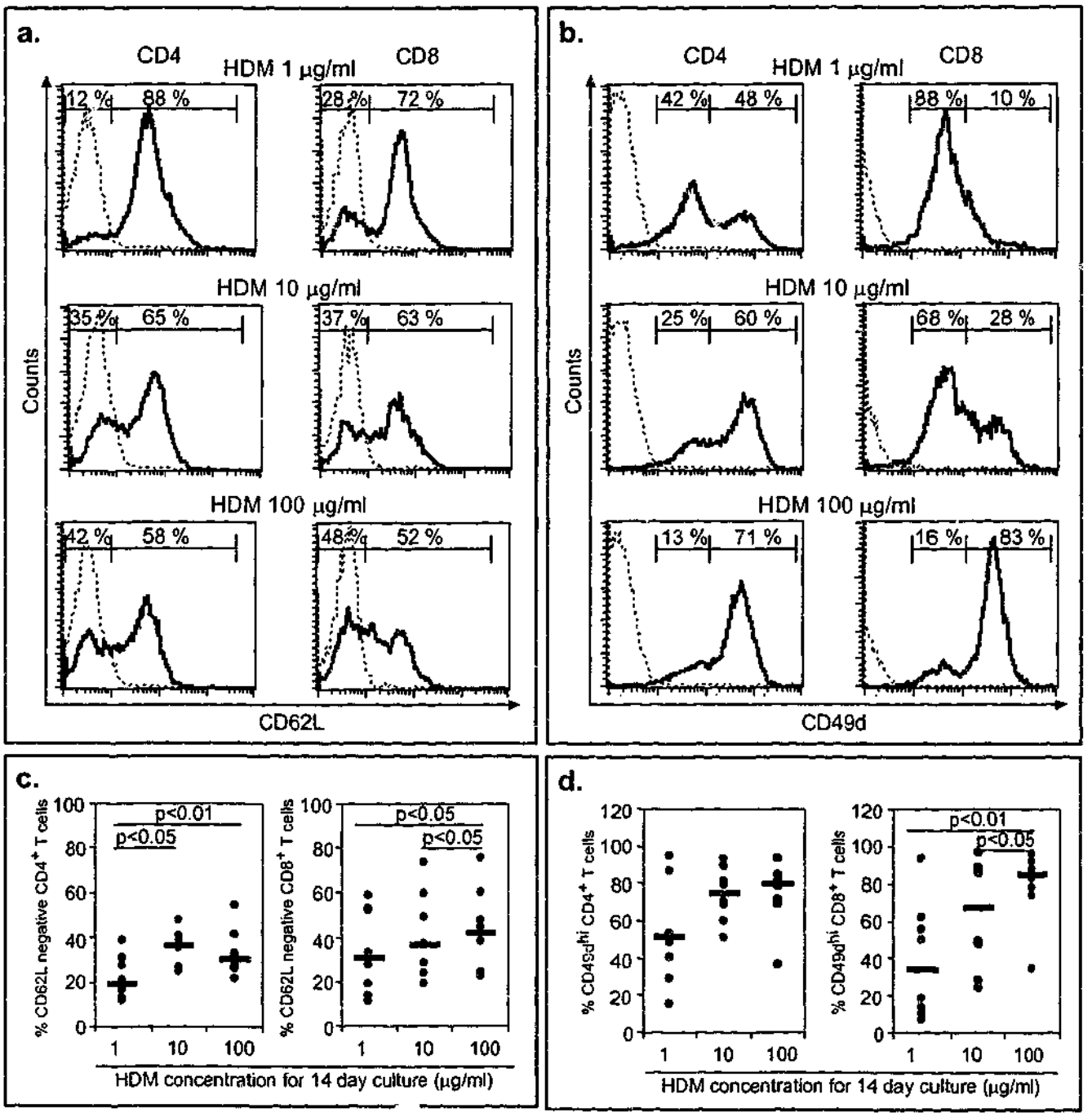

Figure 5.1 Effect of allergen concentration on $CD4^+$ and $CD8^+$ T cell expression of CD62L and CD49d.

PBMC from 8 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-CD62L or anti-CD49d antibodies. The expression of CD62L and CD49d by $CD4^+$ and $CD8^+$ T cells was determined by flow cytometry. Positive staining was determined at the level of intensity where the isotype control histogram (dotted line) crossed the test histogram (solid line). Background staining from the isotype control was then subtracted from the test values. 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 were CD62L negative were greater in the 10 µg/ml ($p<0.05$) and 100 µg/ml ($p<0.01$) HDM extract-stimulated cultures in comparison to 1 µg/ml. There were greater proportions of $CD8^+$ T cells that were CD62L negative in the 100 µg/ml HDM-extract stimulated cultures in comparison to the 1 and 10 µg/ml HDM-extract stimulated cultures ($p<0.05$). **(d)** There was a trend for greater proportions of $CD4^+$ T cells expressing $CD49d^{hi}$ in the 10 µg/ml ($p=0.09$) and 100 µg/ml ($p=0.05$) HDM extract-stimulated cultures in comparison to 1 µg/ml however statistical significance was not reached. Greater proportions of $CD8^+$ T cells expressing $CD49d^{hi}$ were observed in the 100 µg/ml HDM extract-stimulated cultures in comparison to 1 µg/ml ($p<0.01$) and 10 µg/ml ($p<0.05$). In the graphs in Figures 1c and 1d each symbol represents one HDM-allergic donor and the bars represent the median values.

Table 5.2 Median number and ranges in parentheses of T cells expressing CD62L$^-$, CD49d^{hi}, CCR3$^+$ or CCR5$^+$ in HDM-allergic donor PBMC cultures (n=8) stimulated for 14 days with 1, 10 or 100 μg/ml HDM extract.

	CD4$^+$ T cells (x10^{-5})			CD8$^+$ T cells (x10^{-5})		
HDM extract (μg/ml)	1	10	100	1	10	100
CD62L$^-$	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 μg/ml HDM extract; † $p<0.05$ between 10 and 100 μg/ml HDM extract

5.3.2 T Cell Expression of CCR3 and CCR5 in PBMC Cultures Stimulated with Different Allergen Concentrations

Expression of CCR3 and CCR5 by $CD4^+$ and $CD8^+$ T cells in 14-day cultures stimulated with either 1, 10 or 100 μg/ml HDM extract was also determined by flow cytometry. Analysis was again performed at day 14 after repeated allergen stimulation in order to determine dose-dependent changes in chemokine receptor expression by T cells. 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 Figure 5.2a, b and the results for 8 HDM-allergic donors are shown in Figure 5.2c, d. Analysis of CCR3 revealed very low expression by a minor subset of T cells for most donors. No significant differences in the proportions or numbers of $CD4^+$ and $CD8^+$ T cells expressing CCR3 between cultures stimulated with different concentrations of HDM extract were observed (Figure 5.2c; Table 5.2). There was a trend for decreased proportions of $CCR5^+$ $CD4^+$ T cells ($p=0.09$) with a corresponding trend for increased proportions of $CCR5^+$ $CD8^+$ T cells ($p=0.1$) at high allergen concentration in comparison to low however these changes did not reach statistical significance (Figure 5.2d). There were no significant changes in the numbers of $CD4^+CCR5^+$ or $CD8^+CCR5^+$ T cells between cultures stimulated with different concentrations of allergen (Table 5.2).

5.3.3 The Cytokine Profile of $CD4^+CD62L^-$ T Cells is Skewed to IFN-γ Predominant at High Allergen Concentration

To determine if repeated stimulation with different allergen concentrations could lead to alterations in the cytokine phenotype of $CD62L^-$ T cells, HDM allergic donor PBMC were cultured for 14 days with either 1, 10 or 100 μg/ml HDM extract and analysed by

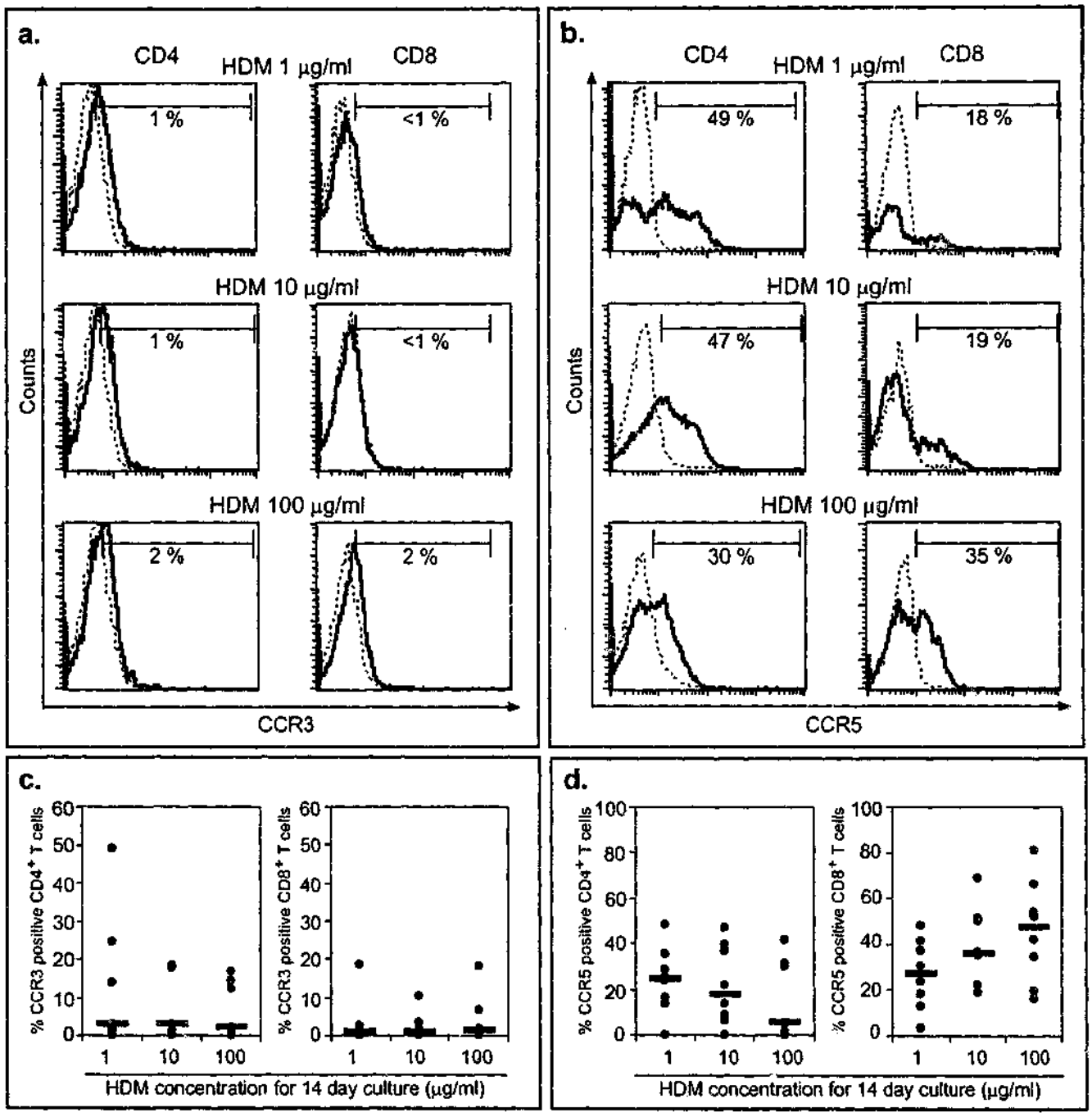

Figure 5.2 The effect of allergen concentration on $CD4^+$ and $CD8^+$ T cell expression of CCR3 and CCR5.

PBMC from 8 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. The expression of CCR3 and CCR5 by $CD4^+$ and $CD8^+$ T cells was determined by flow cytometry. Positive staining was determined at the level of intensity where the isotype control histogram (dotted line) crossed the test histogram (solid line). Background staining from the isotype control was then subtracted from the test values. 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 to 1 µg/ml however statistical significance was not reached. In the graphs in Figures 2c and 2d each symbol represents one HDM-allergic donor and the bars represent the median values.

intracellular cytokine staining and flow cytometry. Representative profiles of CD62L expression by $CD4^+$ and $CD8^+$ T cells and profiles of IL-4 versus IFN-γ for $CD4^+CD62L^-$ and $CD8^+CD62L^-$ T cells in HDM extract-stimulated cultures for one HDM-allergic donor are shown in Figure 5.3a, b. The proportions of cytokine positive $CD4^+CD62L^-$ and $CD8^+CD62L^-$ T cells in HDM extract-stimulated cultures for a total of 10 donors are shown in Figure 5.3c, d. Significantly greater proportions of IFN-γ positive $CD4^+CD62L^-$ T cells were observed in cultures stimulated with 100 μg/ml HDM extract in comparison to 1 and 10 μg/ml ($p<0.01$). The numbers of $CD4^+CD62L^-$ IFN-γ^+ T cells were increased in cultures stimulated with 100 μg/ml HDM extract in comparison to 1 μg/ml ($p<0.01$; Table 5.3). In contrast, significantly lower proportions of $CD4^+CD62L^-$ T cells expressing IL-4 were observed in cultures stimulated with 100 μg/ml HDM extract in comparison to 1 μg/ml ($p<0.01$). No changes in $CD4^+CD62L^-$IL-4^- T cell numbers were observed between cultures (Table 5.3). Although the proportions of IFN-γ positive or IL-4 positive $CD8^+CD62L^-$ T cells did not differ between cultures stimulated with different allergen concentrations, there were significantly greater numbers of $CD8^+CD62L^-$IFN-γ^+ T cells in cultures stimulated with 100 μg/ml HDM extract in comparison to 1 μg/ml ($p<0.05$; Table 5.3). No changes in $CD8^+CD62L^-$IL-4^+ T cell numbers were observed between cultures.

5.3.4 The Cytokine Profile of $CD4^+CD49d^{hi}$ T cells is Skewed to IFN-γ Predominant at High Allergen Concentration

As CD49d is involved in the migration of T cells to peripheral sites of inflammation the cytokine profiles of T cells expressing $CD49d^{hi}$ were analysed in 14-day cultures stimulated with 1, 10 and 100 μg/ml HDM extract. Again this was performed at day 14 to determine if repeated stimulation with different allergen concentrations could lead to

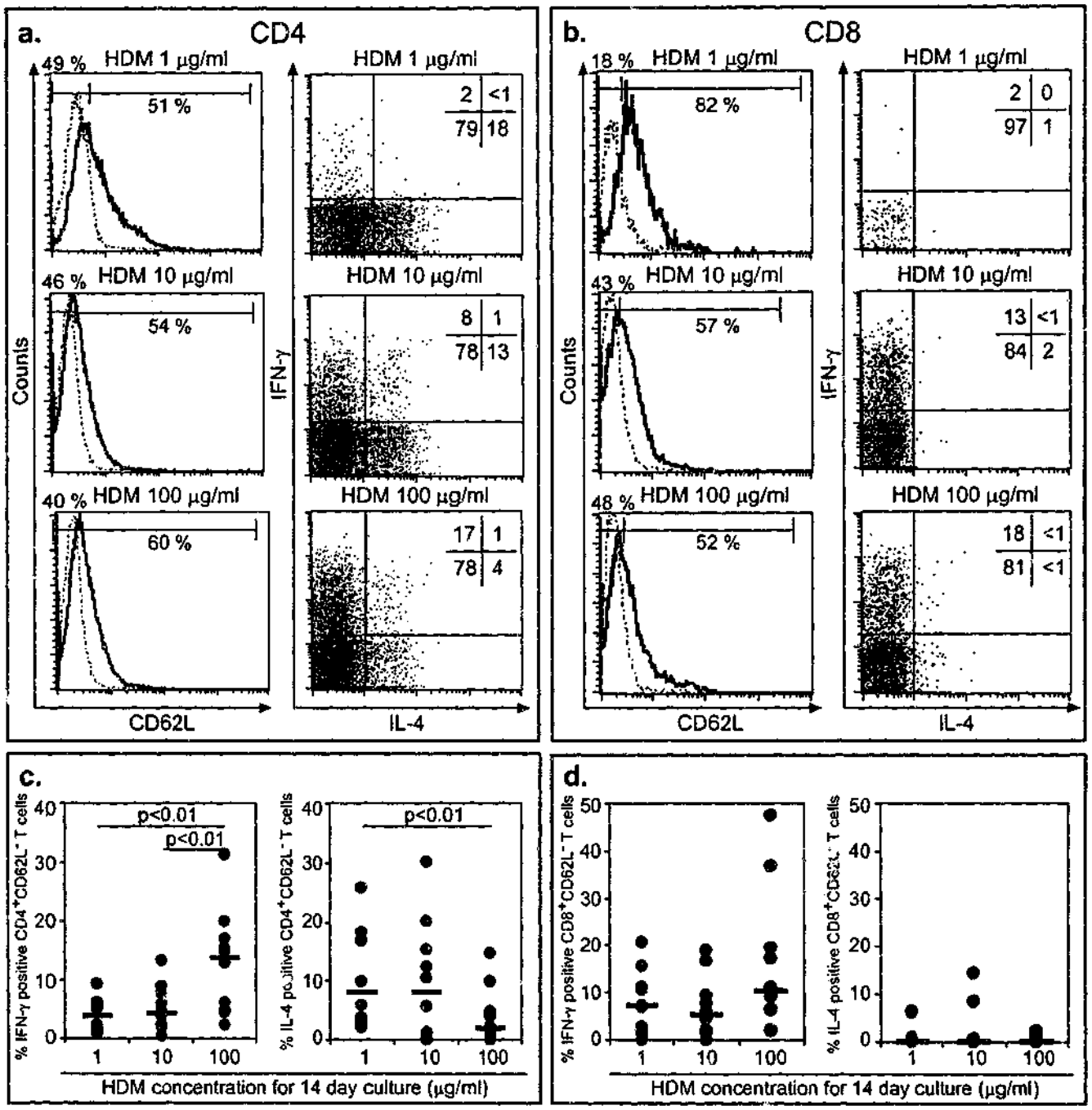

Figure 5.3 Cytokine profile of $CD4^+CD62L^-$ and $CD8^+CD62L^-$ T cells in cultures stimulated with different allergen concentrations.

PBMC from 10 HDM-allergic donors cultured with HDM extract (1, 10 and 100 µg/ml) for 14 days were harvested and stimulated with anti-CD3/IL-2 in the presence of Brefeldin A for 6 hours. Cells were analysed by flow cytometry following labelling with anti-CD4, anti-CD8, anti-CD62L, anti-IL-4 and anti-IFN-γ antibodies. Representative profiles of CD62L expression by $CD4^+$ **(a)** and $CD8^+$ **(b)** T cells in HDM-stimulated cultures for one HDM-allergic donor are shown with the isotype control histogram as the dotted line and the test histogram as the solid line. Corresponding profiles of IL-4 versus IFN-γ for $CD4^+CD62L^-$ **(a)** and $CD8^+CD62L^-$ **(b)** T cells are also shown. **(c)** Proportions of $CD4^+CD62L^-$ T cells expressing IFN-γ were greater in cultures stimulated with 100 µg/ml HDM extract in comparison to 1 and 10 µg/ml ($p<0.01$). Proportions of $CD4^+CD62L^-$ T cells expressing IL-4 were lower in the 100 µg/ml HDM extract-stimulated cultures in comparison to 1 µg/ml ($p<0.01$). **(d)** Proportions of $CD8^+CD62L^-$ T cells that were expressing either IFN-γ or IL-4 did not differ between cultures stimulated with different concentrations of allergen. In the graphs in Figures 3c and 3d each symbol represents one HDM-allergic donor and the bars represent the median values.

Table 5.3 Median number and ranges in parentheses of cytokine positive T cells expressing CD62L$^-$, CD49d^{hi} or CCR5$^+$ in 14-day HDM extract (1, 10 or 100 μg/ml) stimulated cultures (n=10) after 6 hour incubation with anti-CD3 and IL-2 in the presence of Brefeldin A.

	CD4$^+$ T cells (x10^{-5})			CD8$^+$ T cells (x10^{-5})		
HDM extract (μg/ml)	1	10	100	1	10	100
CD62L$^-$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)
CD49d^{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$^-$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 μg/ml HDM extract

changes in the cytokine phenotype of $CD49d^{hi}$ T cells. Representative profiles of CD49d expression by $CD4^+$ and $CD8^+$ T cells and profiles of IL-4 versus IFN-γ for $CD4^+CD49d^{hi}$ and $CD8^+CD49d^{hi}$ T cells in HDM extract-stimulated cultures for one HDM-allergic donor are shown in Figure 5.4a, b. The proportions of cytokine positive $CD4^+CD49d^{hi}$ and $CD8^+CD49d^{hi}$ T cells in HDM extract-stimulated cultures for a total of 10 donors are shown in Figure 5.4c, d. Significantly greater proportions of IFN-γ positive $CD4^+CD49d^{hi}$ T cells were observed in cultures stimulated with 100 μg/ml HDM extract in comparison to 1 μg/ml ($p<0.01$) and 10 μg/ml ($p<0.05$). The numbers of $CD4^+CD49d^{hi}IFN\text{-}\gamma^+$ T cells were increased in cultures stimulated with 100 μg/ml HDM extract in comparison to 1 μg/ml ($p<0.01$; Table 5.3). In contrast, significantly lower proportions of $CD4^+CD49d^{hi}$ T cells expressing IL-4 were observed in cultures stimulated with 100 μg/ml HDM extract in comparison to 1 μg/ml ($p<0.05$). The numbers of $CD4^+CD49d^{hi}IL\text{-}4^+$ T cells did not differ between cultures stimulated with different allergen concentrations (Table 5.3). Although proportions of IFN-γ or IL-4 positive $CD8^+CD49d^{hi}$ T cells did not change between cultures stimulated with different allergen concentrations, there were significantly greater numbers of $CD8^+CD49d^{hi}IFN\text{-}\gamma^+$ T cells in cultures stimulated with 100 μg/ml HDM extract in comparison to 1 μg/ml ($p<0.01$; Table 5.3). No changes in $CD8^+CD49d^{hi}IL\text{-}4^+$ T cell numbers were observed between cultures.

5.3.5 The Cytokine Profile of $CD4^+CCR5^+$ T Cells is Skewed to IFN-γ Predominant at High Allergen Concentration

The effects of repeated stimulation with different allergen concentrations on the cytokine profiles of T cells expressing CCR3 and CCR5 chemokine receptors required for homing to peripheral sites was analysed by surface marker and intracellular cytokine

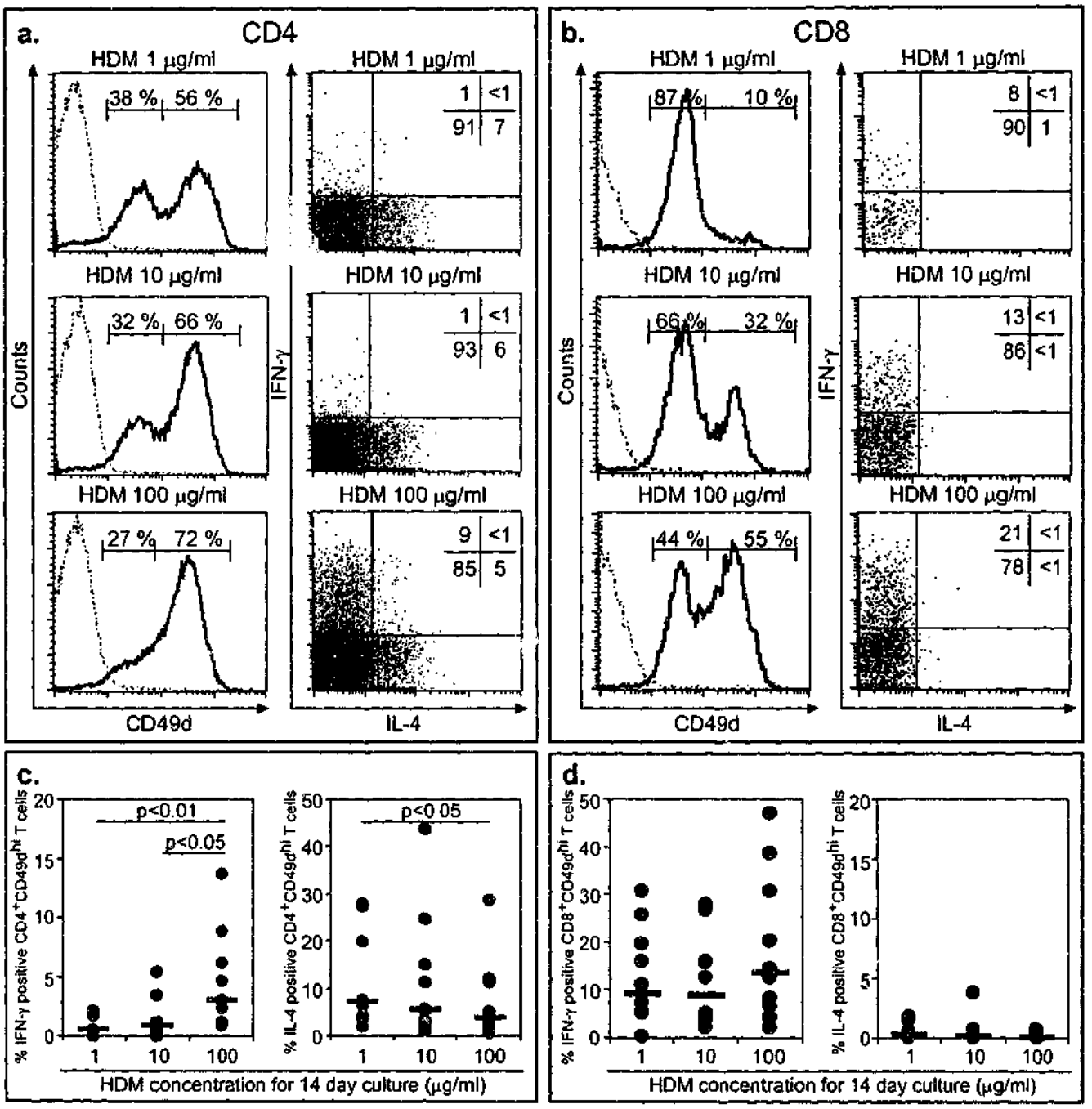

Figure 5.4 Cytokine profile of $CD4^+CD49d^{hi}$ and $CD8^+CD49d^{hi}$ T cells in cultures stimulated with different allergen concentrations.

PBMC from 10 HDM-allergic donors cultured with HDM extract (1, 10 and 100 µg/ml) for 14 days were harvested and stimulated with anti-CD3/IL-2 in the presence of Brefeldin A for 6 hours. Cells were analysed by flow cytometry following labelling with anti-CD4, anti-CD8, anti-CD49d, anti-IL-4 and anti-IFN-γ antibodies. Representative profiles of CD49d expression by $CD4^+$ **(a)** and $CD8^+$ **(b)** T cells in HDM-stimulated cultures for one HDM-allergic donor are shown with the isotype control histogram as the dotted line and the test histogram as the solid line. Corresponding profiles of IL-4 versus IFN-γ for $CD4^+CD49d^{hi}$ **(a)** and $CD8^+CD49d^{hi}$ **(b)** T cells are also shown. **(c)** Proportions of $CD4^+CD49d^{hi}$ T cells expressing IFN-γ were greater in cultures stimulated with 100 µg/ml HDM extract in comparison to 1 µg/ml ($p<0.01$) and 10 µg/ml ($p<0.05$). Proportions of $CD4^+CD49d^{hi}$ T cells expressing IL-4 were lower in the 100 µg/ml HDM extract-stimulated cultures in comparison to 1 µg/ml ($p<0.05$). **(d)** 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 Figures 4c and 4d each symbol represents one HDM-allergic donor and the bars represent the median values.

staining on 14-day HDM extract-stimulated cultures. After the 6-hour anti-CD3/IL-2 stimulation required for the detection of intracellular IL-4 and IFN-γ, expression of CCR3 was down-regulated, so that upon flow cytometric analysis the staining of CCR3 was less than 1 % positive with respect to the isotype control (Figure 5.5). Therefore the cytokine phenotype of $CCR3^+$ $CD4^+$ and $CD8^+$ T cells could not be determined. However CCR5 expression was not affected and so T cells expressing CCR5 were analysed for intracellular IL-4 and IFN-γ. Representative profiles of CCR5 expression by $CD4^+$ and $CD8^+$ T cells and profiles of IL-4 versus IFN-γ for $CD4^+CCR5^+$ and $CD8^+CCR5^+$ T cells in HDM extract-stimulated cultures for one HDM-allergic donor are shown in Figure 5.6a, b. The proportions of cytokine positive $CD4^+CCR5^+$ and $CD8^+CCR5^+$ T cells in HDM extract-stimulated cultures for a total of 10 donors are shown in Figure 5.6c, d. Although only a subset of $CD4^+$ T cells expressed low levels of CCR5, significantly greater proportions of $CD4^+CCR5^+$ T cells expressed IFN-γ in cultures stimulated with 100 μg/ml HDM extract in comparison to 1 μg/ml ($p<0.001$) and 10 μg/ml ($p<0.01$). The numbers of $CD4^+CCR5^+IFN\text{-}\gamma^+$ T cells were increased in cultures stimulated with 100 μg/ml HDM extract in comparison to 1 μg/ml ($p<0.05$; Table 5.3). 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 μg/ml HDM extract in comparison to 1 μg/ml. Although proportions of IFN-γ or IL-4 positive $CD8^+CCR5^+$ T cells did not change between cultures stimulated with different allergen concentrations, there were significantly greater numbers of $CD8^+CCR5^+IFN\text{-}\gamma^+$ T cells in cultures stimulated with 100 μg/ml HDM extract in comparison to 1 μg/ml ($p<0.05$; Table 5.3). No changes in $CD8^+CCR5^+IL\text{-}4^+$ T cell numbers were observed between cultures.

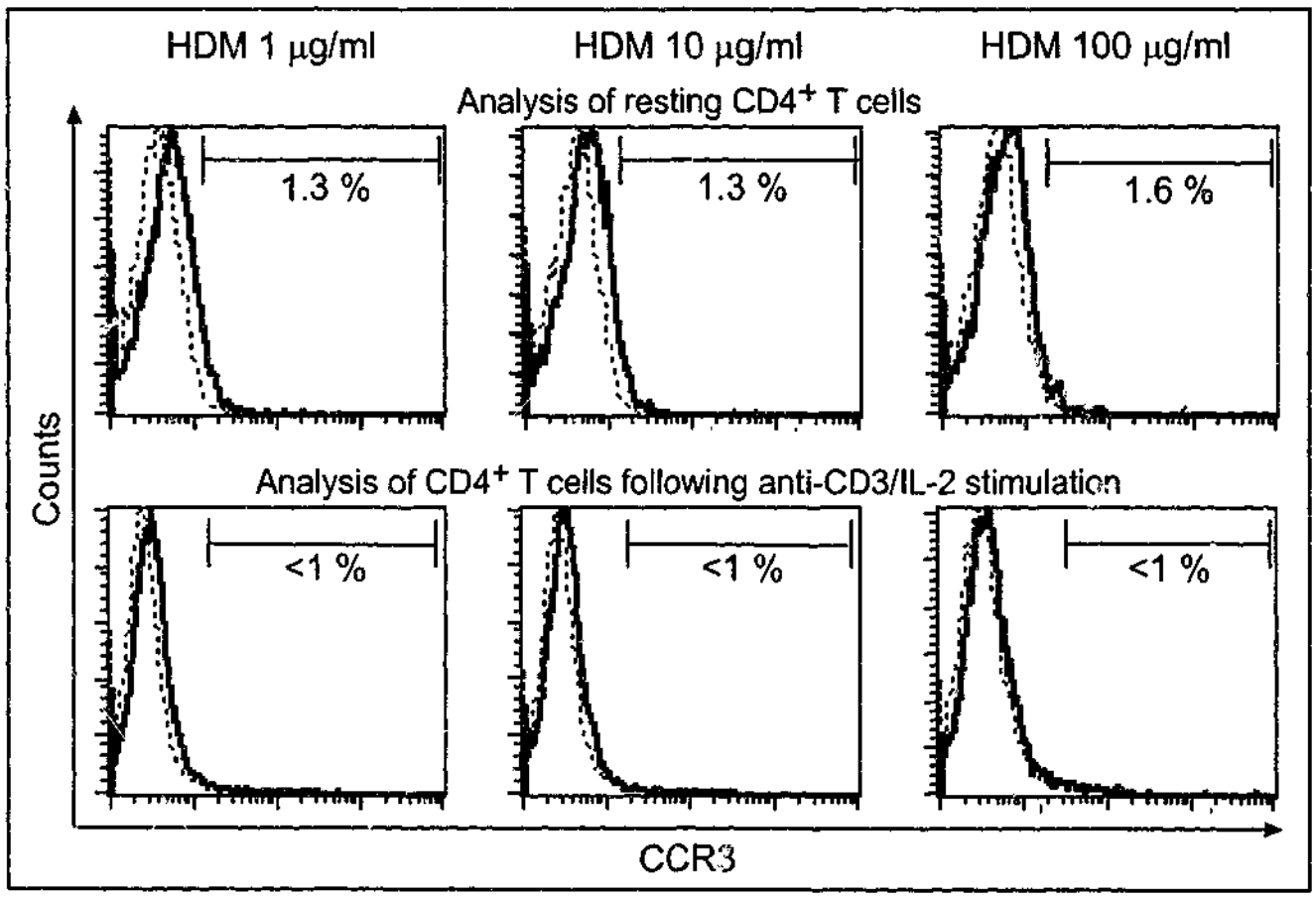

Figure 5.5 Downregulation of CCR3 expression on $CD4^+$ T cells during anti-CD3/IL-2 stimulation.

HDM-allergic donor PBMC were cultured with HDM extract (1, 10 and 100 µg/ml) for 14 days. The cultured cells were then labelled with anti-CD4 antibody and either anti-CCR3 antibody or the appropriately labelled isotype control immediately after harvesting or following a 6 hour stimulation with anti-CD3/IL-2 in the presence of Brefeldin A required for intracellular cytokine detection. $CD4^+$ T cell histograms of CCR3 staining (solid line) and isotype control staining (dotted line) for each HDM extract concentration culture are illustrated. Markers and percentages shown on these histograms indicate positive staining and were determined according to minimal (<1%) isotype control staining.

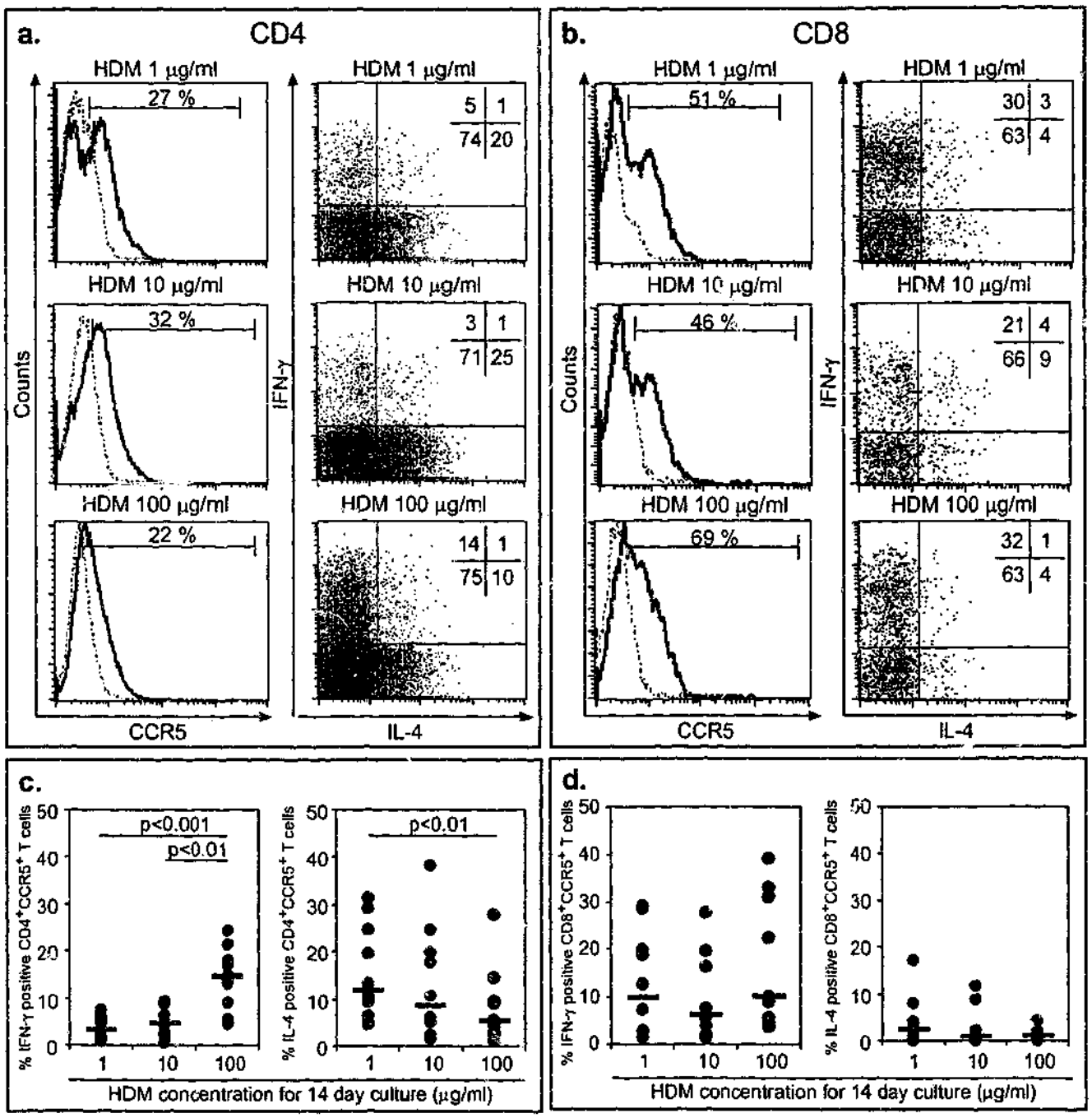

Figure 5.6 Cytokine profile of $CD4^+CCR5^+$ and $CD8^+CCR5^+$ T cells in cultures stimulated with different allergen concentrations.

PBMC from 10 HDM-allergic donors cultured with HDM extract (1, 10 and 100 µg/ml) for 14 days were harvested and stimulated with anti-CD3/IL-2 in the presence of Brefeldin A for 6 hours. 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 CCR5 expression by $CD4^+$ **(a)** and $CD8^+$ **(b)** T cells in HDM-stimulated cultures for one HDM-allergic donor are shown with the isotype control histogram as the dotted line and the test histogram as the solid line. Corresponding profiles of IL-4 versus IFN-γ for $CD4^+CCR5^+$ **(a)** and $CD8^+CCR5^+$ **(b)** T cells are also shown. **(c)** Proportions of $CD4^+CCR5^+$ T cells expressing IFN-γ were greater in cultures stimulated with 100 µg/ml HDM extract in comparison to 1 µg/ml ($p<0.001$) and 10 µg/ml ($p<0.01$). Proportions of $CD4^+CCR5^+$ T cells expressing IL-4 were lower in the 100 µg/ml HDM extract-stimulated cultures in comparison to 1 µg/ml ($p<0.01$). **(d)** 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 Figures 5c and 5d each symbol represents one HDM-allergic donor and the bars represent the median values.

5.4 DISCUSSION

During clinically successful SIT many studies have observed immune deviation from dominant secretion of IL-4 and IL-5 from allergen-specific T cells to predominant secretion of IFN-γ (McHugh *et al.*, 1995; Ebner *et al.*, 1997; Eusebius *et al.*, 2002), with increased IFN-γ^+ cell numbers at sites of allergen challenge (Varney *et al.*, 1993; Durham *et al.*, 1996; Wachholz *et al.*, 2002). Whether the high dose administration of allergen during SIT promotes trafficking of IFN-γ^+ T cells to sites of allergen encounter is unknown. Therefore this study aimed to determine whether high allergen concentration in comparison to low could lead to alterations in T cell expression of adhesion molecules and chemokine receptors. Furthermore dose-dependent changes in cytokine production by T cells expressing surface markers for peripheral tissue trafficking which included $CD62L^-$, $CD49d^{hi}$ and $CCR5^+$ were analysed. Culturing HDM-allergic donor PBMC for 14 days with high allergen concentration in comparison to low resulted in increased proportions of T cells expressing $CD62L^-$ or $CD49d^{hi}$ trafficking phenotypes. In addition high allergen concentration altered the cytokine profile of "peripheral tissue trafficking" T cells, with increased numbers of T cells expressing IFN-γ. These results suggest that repeated stimulation with high allergen concentration can induce increased T cell trafficking and in particular enhanced trafficking of IFN-γ-producing T cells.

In this study, the induction of changes in the production of cytokines by "peripheral tissue trafficking" T cells was consistently observed at an allergen concentration greater than 10 μg/ml. Relating the allergen concentration in the high dose *in vitro* cultures (100 μg/ml) to that delivered to T cells *in vivo* during SIT is difficult. However allergen doses administered for SIT (approximately 10 μg/ml) are likely to result in substantially

higher concentrations than the picogram/L air concentrations estimated to be encountered naturally (Tovey *et al.*, 1981; Bousquet *et al.*, 1998a). Hence the changes in T cell responses upon stimulation with either low or high allergen concentration were assessed. However a limitation of this research was that analysis of T cell responses at concentrations of allergen lower than 1 μg/ml could not be undertaken because T cell survival in cultures at lower concentrations could not be maintained. Nevertheless, the results obtained using the concentration range in this current study argue strongly for improved efficacy of SIT using high doses of allergen.

In allergic individuals, local expression of IL-4 and IL-5 by cells in the tissue is well recognised as a feature of nasal late-phase responses to allergens in allergic rhinitis. In nasal biopsies 24 hours after allergen challenge increased numbers of IL-4 and IL-5 mRNA$^+$ cells have been observed, with no change in IFN-γ mRNA$^+$ numbers (Durham *et al.*, 1992). As T cells are the major source of IL-4 and IL-5 in these biopsies (Ying *et al.*, 1993; Ying *et al.*, 1994) and increased CD4$^+$ T cell numbers have been observed in tissues after allergen challenge (Varney *et al.*, 1992), it is possible that allergen-specific T cells traffic into the nasal mucosa upon allergen exposure. During SIT, changes in peripheral tissue cytokine expression have been observed with increased numbers of IFN-γ mRNA$^+$ cells detected in allergen-challenged nasal mucosa and skin after grass pollen SIT (Varney *et al.*, 1993; Durham *et al.*, 1996; Wachholz *et al.*, 2002). These results indicate that an alteration in the cytokine phenotype of T cells trafficking to peripheral sites of allergen encounter may accompany successful SIT. The results from the current study suggest that allergen concentration may be a key factor in promoting these changes.

In studies on grass pollen immunotherapy, increases in IFN-γ mRNA$^+$ cell numbers at sites of allergen challenge were associated with reduced allergen-induced late-phase responses and decreased infiltration of T cells and activated eosinophils (Durham *et al.*, 1996). As mentioned in Chapter 4, IFN-γ can inhibit IL-4-induced expression of the germline epsilon transcript in human B cells, thereby preventing IgE production (Venkataraman *et al.*, 1999). Consequently, increase local production of IFN-γ by SIT could inhibit IgE-mediated inflammatory cell activation and in turn reduce local allergic inflammation. Furthermore, in a murine model of asthma, activated IFN-γ$^+$ T cells have also been shown to inhibit lung eosinophilia and airway epithelial mucus production, common features of human airway inflammation (Cohn *et al.*, 1999). Thus the production of IFN-γ by both CD4$^+$ and CD8$^+$ T cells expressing a "peripheral tissue trafficking" phenotype by stimulation at high allergen concentration could have beneficial effects in the airways of allergic patients. Such immune deviation coupled with IL-10 production, as has been described in bee venom (Akdis *et al.*, 1998b) and grass pollen (Francis *et al.*, 2003) SIT, would down-regulate allergen-induced inflammation (Levings *et al.*, 2002).

That increased numbers of CD8$^+$IFN-γ$^+$ T cells with a "peripheral tissue trafficking" phenotype were found at high allergen concentration was a surprising observation. Very few studies have looked at changes in cytokine production by CD8$^-$ T cells during SIT. In those that have, the CD8$^+$ T cell population was found to be minor and to have decreased or unchanged IFN-γ production after SIT (Majori *et al.*, 2000; O'Brien *et al.*, 2000). However as shown in Chapter 4 expansion of CD8$^+$IFN-γ$^+$ T cells in allergen-stimulated cultures can occur at sufficiently high doses. The results in this chapter indicate that the generation of allergen-specific CD8$^+$IFN-γ$^+$ T cells with a "peripheral

tissue trafficking" phenotype is also dose-related, again highlighting the advantage of using high dose allergen administration in SIT to achieve immune deviation in peripheral tissues.

The activation of T cells by antigen results in the shedding of CD62L and an up-regulation of CD49d (Chao *et al.*, 1997; Hamann *et al.*, 2000). T cells expressing $CD62L^{-}$ $CD49d^{hi}$ are described as having an effector phenotype and have been shown to traffic to different locations than their naïve counterparts, with reduced recirculation through lymphoid tissues and increased migration to peripheral tissues such as the lung (Hamann *et al.*, 2000). In agreement with the results in this current study, Pacheco and colleagues observed CD49d up-regulation on human T cells after allergen stimulation *in vitro* (Pacheco *et al.*, 1998). They also demonstrated that T cells derived from bronchoalveolar lavage of allergic subjects express a density of CD49d three times higher than those of T cells obtained from bronchoalveolar lavage of normal subjects. In a number of animal models the importance of CD49d in T cell migration into sites of peripheral allergen challenge has been confirmed using *in vivo* blocking experiments (Nakajima *et al.*, 1994; Richards *et al.*, 1996; Abraham *et al.*, 2000). By examining the cytokine profiles of $CD49d^{hi}$ $CD4^{+}$ and $CD8^{+}$ T cells, cytokine skewing to IFN-γ predominant was observed at high concentration. Thus 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 expression of CD49d for binding to VCAM-1 on endothelial cells enabling migration into peripheral tissues such as the lung.

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. Increased production of the chemokine, eotaxin, has been observed after allergen challenge in the bronchoalveolar lavage fluid from atopic asthmatics (Lilly *et al.*, 2001). Allergen-specific T cell expression of CCR3, the receptor for eotaxin, was one chemokine receptor analysed in this current study. As CCR3 expression is upregulated in the presence of IL-4 (Jinquan *et al.*, 1999), a CCR3 signal on T cells stimulated in the presence of HDM extract due to allergen-induced IL-4 production was expected. However CCR3 expression was not altered in the allergen-stimulated T cell cultures and only a very small proportion of T cells in these cultures expressed this marker. Thus CCR3 may be only playing a limited role in determining the T cell contribution to the cytokine milieu at peripheral sites of allergen encounter. Recent studies suggest other chemokine receptors may be important for trafficking of Th2-type cells. For example, the role of CCR4 in T cell migration in allergy has been investigated. CCR4 mRNA$^+$ cell numbers in atopic asthmatic lungs were found to be increased after allergen challenge (Nouri-Aria *et al.*, 2002). In addition, increased numbers of T cells expressing the CCR4 protein after allergen challenge in bronchial biopsies from atopic asthmatics has also been observed (Panina-Bordignon *et al.*, 2001). A subset of these CCR4$^+$ T cells were double positive for CCR8, suggesting that CCR8 may also be involved in the recruitment of T cells to the lung in allergic inflammation (Panina-Bordignon *et al.*, 2001). 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.

CCR5 was also examined in this study. In contrast to previous findings that CCR5 is expressed by only Th1 cells (Odum *et al.*, 1999; Yamamoto *et al.*, 2000), but in agreement with Sallusto and colleagues (1998) expression of CCR5 by both IFN-γ^+ and

IL-4$^+$ T cells was observed. As the chemokine ligands for CCR5, i.e. RANTES and MIP1-α, are produced after allergen challenge in the bronchoalveolar lavage fluid from atopic asthmatics (Cruikshank *et al.*, 1995; Holgate *et al.*, 1997), T cells expressing CCR5 potentially could 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 (Campbell *et al.*, 2001). In the current 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 IFN-γ$^+$ CD4$^+$ T cells to sites of allergic inflammation, such as in the lung.

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 trafficking of T cells to peripheral tissues. 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.

CHAPTER 6

ANALYSIS OF THE T CELL RESPONSE DURING HDM IMMUNOTHERAPY[1]

6.1 INTRODUCTION

A randomised controlled clinical trial of conventional HDM immunotherapy using the new standardised Alustal® *Dermatophagoides pteronyssinus* extract (Stallergenes, France) was carried out at the Asthma and Allergy Clinic, Alfred Hospital, Melbourne, Australia. This clinical study provided an opportunity to evaluate the effects of *in vivo* treatment with high concentrations of allergen on allergen-specific T cell function using the same assays as used for the *in vitro* studies presented earlier in this thesis. Efficacy of immunotherapy was determined by monitoring allergen-induced late phase responses and symptom and medication scores throughout the 9-month treatment period.

The immunological study involved the analysis of allergen-induced T cell cytokine production, adhesion molecule and chemokine receptor expression and proliferation before and following immunotherapy. With respect to cytokine production, $CD4^+$ and $CD8^+$ T cell production of IL-4 and IFN-γ were analysed. As IL-10 has been shown to play a role in down-regulating allergen-specific T cell cytokine production and proliferation during SIT (Bellinghausen *et al.*, 1997; Akdis *et al.*, 1998b; Jutel *et al.*, 2003), T cell synthesis of this cytokine was also examined. Recently, T cells producing IL-10 following allergen immunotherapy have been identified as a subset of

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[1] This chapter is based on the publication: **Leanne M. Gardner**, Frank C. Thien, Jo A. Douglass, Jennifer M. Rolland and Robyn E. O'Hehir (2003) Induction of T regulatory cells by standardised house dust mite immunotherapy: an increase in $CD4^+IL\text{-}10^+$ T cells expressing peripheral tissue trafficking surface markers. Clinical and Experimental Allergy (submitted).

$CD4^+CD25^+$ regulatory T cells (Francis *et al.*, 2003; Jutel *et al.*, 2003). The IL-10 positive $CD4^+$ T cells detected in the current study were therefore further characterised to determine whether they exhibited a regulatory cell phenotype.

As discussed in Chapter 5, SIT can also alter cytokine production in allergen-challenged peripheral tissues. Increased IFN-γ^+ cell numbers have been identified in peripheral tissues following grass pollen immunotherapy (Varney *et al.*, 1993; Durham *et al.*, 1996; Nasser *et al.*, 2001; Wachholz *et al.*, 2002). Hence the present study determined whether SIT could alter expression by circulating IL-4- and IFN-γ-positive T cells of surface molecules that can influence trafficking to peripheral tissues (CD62L, CD49d, CCR3 and CCR5). Following wasp venom immunotherapy, increased numbers of IL-10^+ cells have also been identified in allergen-challenged tissues (Nasser *et al.*, 2001). As trafficking of IL-10-producing T cells into peripheral target tissues upon allergen re-exposure could lead to a down-regulation of pathogenic allergen-specific T cell responses (Akdis *et al.*, 1998b), IL-10 synthesis by T cells expressing a peripheral trafficking phenotype was also assessed.

6.2 EXPERIMENTAL PROCEDURES

6.2.1 Patients and Immunotherapy Protocol

Twelve HDM-allergic patients were recruited at the Asthma and Allergy Clinic, Alfred Hospital, according to the selection criteria detailed in Section 2.2. The patients participated in a randomised open-label parallel group study in which they were randomised at a 2:1 ratio to either receive SIT with Alustal® *D. pteronyssinus* extract, or to observation with standard pharmacotherapy and clinical monitoring (non-SIT group). The clinical characteristics of the 12 patients selected for this study are shown in Table 6.1. For SIT, patients received injections of the Alustal preparation according to a standard protocol, beginning at a dose of 0.1 ml of 0.1 IC/ml and increasing at weekly intervals for 13 weeks until a maintenance dose of 0.4 ml of 10 IC/ml was reached, which was continued monthly. For patients randomised to observation, persistent rhinitis was managed with pharmacotherapy including intranasal steroids and oral antihistamines. Intranasal steroids were kept at the same dose during the study and antihistamines were used as required. Topical antihistamines were not allowed.

6.2.2 Clinical Assessment of the Efficacy of HDM Immunotherapy

Efficacy of HDM SIT was assessed by allergen-induced cutaneous late phase response and symptom and medication scores and quality of life scores (QOL) were recorded before and at 9-months of treatment.

6.2.2.1 Allergen-Induced Cutaneous Late Phase Responses

The cutaneous late phase response to HDM allergen was assessed by the intradermal injection of 0.03 ml of 100 BAU/ml of aqueous house dust mite extract (Allpyral, Australia) into the flexor surface of the non-dominant forearm before treatment and at

Table 6.1 Characterisitics of donors used in this study.

Patient	Sex	Age	Rhinitis	Asthma	HDM skin test reactivity (wheal mm)	HDM EAST score (AEU/ml)
A7	F	40	Y	Y	13	...
A30	F	31	Y	N	14 x 9	...
A31	F	27	Y	Y	15	...
A32	F	22	Y	Y	12	...
A33	M	57	Y	Y	8 x 6.5	...
A34	M	36	Y	N	6	...
A35	M	43	Y	N	16 x 12	2
A36	F	29	Y	N	12	3 (9.5)
A37	F	45	Y	N	10	...
A38	M	46	Y	Y	11 x 10	...
A39	M	39	Y	Y	12	3 (5.3)
A40	F	30	Y	N	15	...

A, atopic; F, female; M, male; ..., not available; Y, yes; N, no

9-months of treatment. The wheal diameter was measured and recorded at 15 minutes. Patients returned in 24 hours for measurement of the late phase response with the outline of induration traced onto transparency paper. Subsequently, the tracings were digitally photographed and imported into a computer for analysis of the surface area of the late phase response by the Alfred/Medseed Wound Imaging System (AMWIS©) software (Bayside Health and Medseed Pty Ltd, Melbourne, Australia).

6.2.2.2 Rhinoconjunctivitis symptom score and medication requirements

A diary symptom and medication chart was maintained daily 2 weeks prior to treatment and prior to the 9-month visit by recording chest, nose, eyes and throat symptoms, as well as medication usage. Each symptom was scored from 0 (the symptom is absent) to 3 (the symptom is disabling and causes discomfort). Scores for each symptom were then added together to reach a 'total symptom score'. Rhinitis medications were also recorded, and daily dose requirements of antihistamines for symptom relief were added to give a 'total relief medication score'.

6.2.2.3 Rhinoconjunctivitis quality of life questionnaire

A disease specific Juniper Rhinoconjunctivitis Quality of Life Questionnaire (Juniper and Guyatt, 1991) was administered before treatment and at the 9-month visit. Patients were asked to recall their experiences during the previous week and to give their responses on a 7-point scale.

6.2.3 Immunological Studies

The methods required for the analysis of changes in T cell cytokine production, expression of adhesion molecules and chemokine receptors, and proliferation during immunotherapy are detailed in Sections 2.5 and 2.6. Briefly, peripheral blood was taken from patients before and at 3- and 9-months of treatment. Separated PBMC were cultured in the presence of HDM extract (25 μg/ml) for 14 days. On day 14, $CD4^+$ and $CD8^+$ T cells were stimulated in the presence of anti-CD3/IL-2 and analysed by flow cytometry for the detection of intracellular IL-4, IFN-γ and IL-10. $CD4^+$ and $CD8^+$ T cell expression of CD62L, CD49d, CCR3 and CCR5 and coexpression of these markers with cytokines was also analysed by flow cytometry. The proliferative response of cultured T cells to HDM extract was assessed using T cell proliferation assays.

6.2.4 Statistical Analysis

Data were analysed with the aid of the Instat 2.0 statistical package. For clinical and immunological analyses, statistical significance of differences between results obtained at various time points of SIT was assessed using a Wilcoxon Signed Rank test. Statistical significance between groups was analysed using a Mann Whitney test. The Spearman Rank test was used to assess the correlations between clinical observations and immunological results. A p-value of 0.05 was considered statistically significant.

6.3 RESULTS

6.3.1 Clinical Efficacy of SIT

The mean (±SD) total symptom score in SIT patients at baseline was 103.7 ± 69.2, and decreasing to 63.7 ± 57.8 (p=0.012) at 9-months, while relief medication requirement scores did not significantly change (7.4 ± 6.2 at baseline compared with 5.9 ± 7.5 at 9-months; p=0.41). The rhinitis QOL scores showed significant improvement going from 75.9 ± 23.1 at baseline to 47.6 ± 35.3 at 9-months (p=0.015). None of these scores in the non-SIT group showed any significant changes although the subject numbers were small (n=3). The mean surface area (± SD) of the late phase response to HDM in the SIT group at baseline was 60 ± 37 cm^2 and reduced significantly at 9-months to 28 ± 19 cm^2 (p=0.004; Figure 6.1). There was no significant change in late phase response surface area of the non-SIT group.

6.3.2 IL-4 and IFN-γ Production by $CD4^+$ and $CD8^+$ T Cells Pre- and Post-SIT

IL-4 and IFN-γ production by $CD4^+$ and $CD8^+$ T cells in 14-day HDM extract-stimulated cultures generated from PBMC from patients receiving SIT were analysed before and during treatment. Representative intracellular IL-4 and IFN-γ profiles of $CD4^+$ and $CD8^+$ T cells for one patient receiving SIT are shown in Figure 6.2a. The proportions of IL-4 positive $CD4^+$ and $CD8^+$ T cells were significantly decreased at 9-months of SIT in comparison to pre-SIT ($CD4^+$ T cells p=0.039; $CD8^+$ T cells p=0.05; Figure 6.2b, c). No significant changes in the proportions of IL-4 positive $CD4^+$ and $CD8^+$ T cells between observed pre-SIT and 3-months of SIT, and between 3-months and 9-months of SIT. SIT had no effect on the absolute numbers of $CD4^+IL\text{-}4^+$ and $CD8^+IL\text{-}4^+$ T cell numbers (Table 6.2). There was a trend for decreased proportions IFN-γ positive $CD4^+$ T cells at 9-months of SIT in comparison to pre-SIT levels

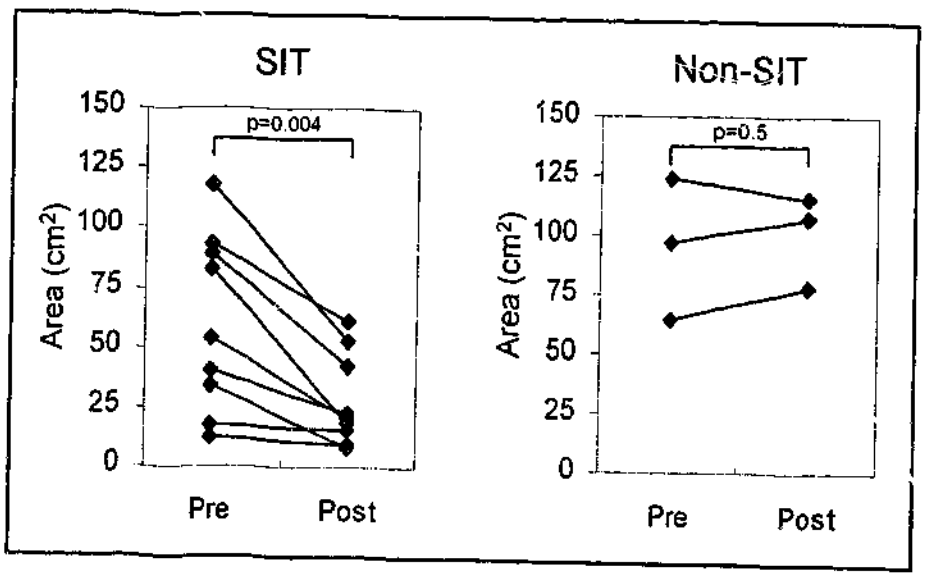


Figure 6.1 Surface area of the cutaneous late phase response to HDM allergen before and at 9-months of treatment in the SIT group and the Non-SIT group.
The late phase response to HDM allergen was assessed by intradermal injection of 0.03 mL of 100 BAU/ml of aqueous HDM extract into the flexor surface of the non-dominant forearm. Patients returned in 24 hours for measurement of the late phase response with the outline of induration traced onto transparency paper from which the surface area of the late phase response was subsequently determined by Alfred/Medseed Wound Imaging System software. Each symbol in the graphs in represents one subject.

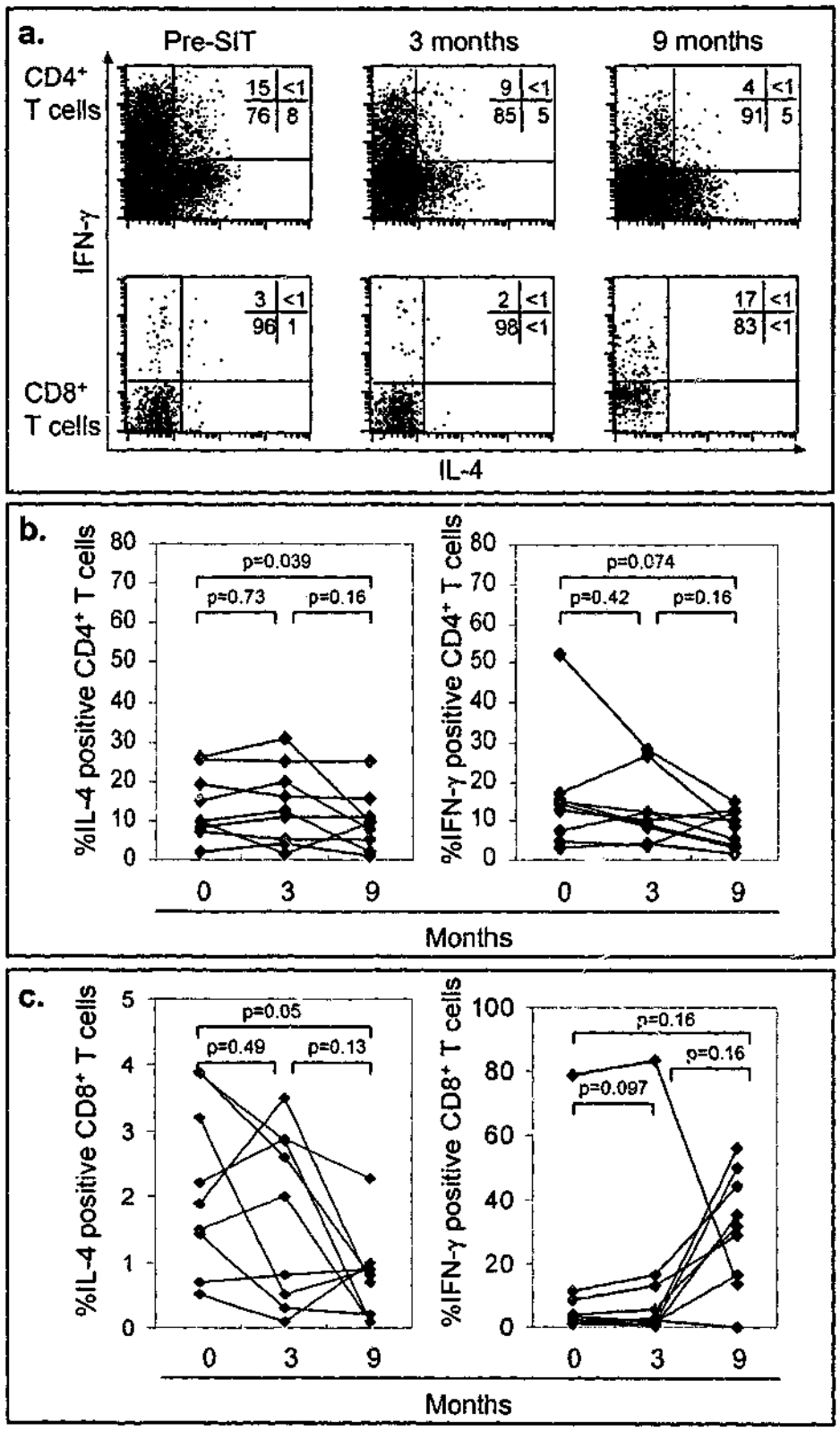


Figure 6.2 IL-4 and IFN-γ production by CD4⁺ and CD8⁺ T cells before and at 3- and 9-months of SIT.

PBMC, from the 9 subjects receiving SIT, were cultured with HDM extract (25 μg/ml) for 14 days. Cells were then harvested and stimulated with anti-CD3/IL-2 for 6 hours in the presence of Brefeldin A. Following stimulation, cells were labelled with anti-CD4, anti-CD8, anti-IL-4 and anti-IFN-γ antibodies and analysed by flow cytometry. **(a)** Representative intracellular IL-4 and IFN-γ staining of CD4⁺ and CD8⁺ T cells pre-SIT and at 3- and 9-months of SIT are shown. Isotype controls were used to set quadrant markers and quadrant percentages are shown. **(b)** Percentages of IL-4 positive CD4⁺ T cells were significantly lower at 9-months of SIT in comparison to pre-SIT (p=0.039). The proportions of IFN-γ positive CD4⁺ T cells remained unchanged at 3- and 9-months of SIT in comparison to before SIT. **(c)** Percentages of IL-4 positive CD8⁺ T cells were lower at 9-months of SIT in comparison to pre-SIT (p=0.05). The proportions of IFN-γ positive CD8⁺ T cells remained unchanged at 3- and 9-months of SIT in comparison to before SIT. Each symbol in the graphs in represents one subject receiving SIT.

Table 6.2 Numbers of $CD4^+$ and $CD8^+$ T cells expressing IL-4 and IFN-γ before and during SIT (n = 9).

	$CD4^+$ T cells						$CD8^+$ T cells					
	$IL\text{-}4^+$ cell number (10^5)			IFN-γ^+ cell number (10^5)			$IL\text{-}4^+$ cell number (10^5)			IFN-γ^+ cell number (10^5)		
Subject	**Pre-SIT**	**3 mths**	**9 mths**	**Pre-SIT**	**3 mths**	**9 mths**	**Pre-SIT**	**3 mths**	**9 mths**	**Pre-SIT**	**3 mths**	**9 mths**
A30	47.8	9.7	16.9	96.8	16.9	11.3	0.3	0.1	0.01	0.8	0.3	1.2
A33	17.2	18.1	12.0	13.1	17.6	57.8	1.4	3.2	0.7	1.0	2.4	57.1
A34	159.1	3.7	31.2	844.6	59.3	50.0	17.1	0.3	0.4	46.2	8.7	12.2
A35	20.2	73.5	77.4	11.4	25.1	9.8	0.5	1.7	0.7	0.4	1.5	0.03
A36	68.0	94.1	12.6	78.0	125.2	13.7	0.2	0.1	0.1	33.8	45.1	0.9
A37	6.5	10.0	4.7	41.7	29.0	20.4	0.9	1.0	0.03	2.4	3.0	11.1
A38	17.6	24.8	60.7	27.9	22.0	69.3	0.2	0.3	0.6	2.6	6.1	29.3
A39	26.7	14.8	9.2	4.2	4.0	1.0	1.3	0.5	0.01	0.4	0.1	0.5
A40	72.3	131.4	82.0	13.4	15.9	101.2	1.1	4.5	0.1	0.3	2.1	60.6
Mean	48.4	42.2	34.1	125.7	35.0	37.2	2.6	1.3	0.3	9.8	7.7	19.2

PBMC were cultured for 14 days in the presence of 25 μg/ml HDM extract. Cells were stimulated with anti-CD3/IL-2 for 6 hours, labelled with anti-CD4 and anti-CD8 antibodies and stained for intracellular IL-4 and IFN-γ. $CD4^+$ and $CD8^+$ T cell expression of IL-4 or IFN-γ was determined by flow cytometry.

however statistical significance was not reached (p=0.074; Figure 6.2b). No changes in the proportions of IFN-γ positive $CD4^+$ T cells were observed between pre-SIT and 3-months of SIT. Although the proportions of IFN-γ positive $CD8^+$ T cells were high at 9-months of SIT, no significant differences were seen between pre-SIT and at 3- and 9-months of SIT (Figure 6.2c). SIT had no effect on the absolute numbers of $CD4^+IFN\text{-}\gamma^+$ and $CD8^+IFN\text{-}\gamma^+$ T cell numbers (Table 6.2).

6.3.3 IL-10 Production by $CD4^+$ and $CD8^+$ T Cells Pre- and Post-SIT

The production of IL-10 by $CD4^+$ and $CD8^+$ T cells in 14-day HDM extract-stimulated cultures generated from PBMC from patients before and during SIT was analysed by intracellular cytokine staining. Representative IL-10 staining of $CD4^+$ and $CD8^+$ T cells for one patient receiving SIT is shown in Figure 6.3a. The proportions of IL-10 positive $CD4^+$ T cells and the absolute numbers of $CD4^+IL\text{-}10^+$ T cells were greater at 9-months of SIT in comparison to pre-SIT (% p=0.008; number p=0.039) and to 3-months of SIT (% and number p=0.008; Figure 6.3b). No differences in proportions of IL-10 positive $CD4^+$ T cells and the absolute numbers of $CD4^+IL\text{-}10^+$ T cells were seen between pre-SIT and at 3-months of SIT. There was an inverse correlation between the change in allergen-induced late phase responses and the change in $CD4^+IL\text{-}10^+$ T cell numbers following SIT (r=0.75; p=0.026). The proportions of IL-10 positive $CD8^+$ T cells and the absolute numbers of $CD8^+IL\text{-}10^+$ T cells were not affected by SIT (Figure 6.3b).

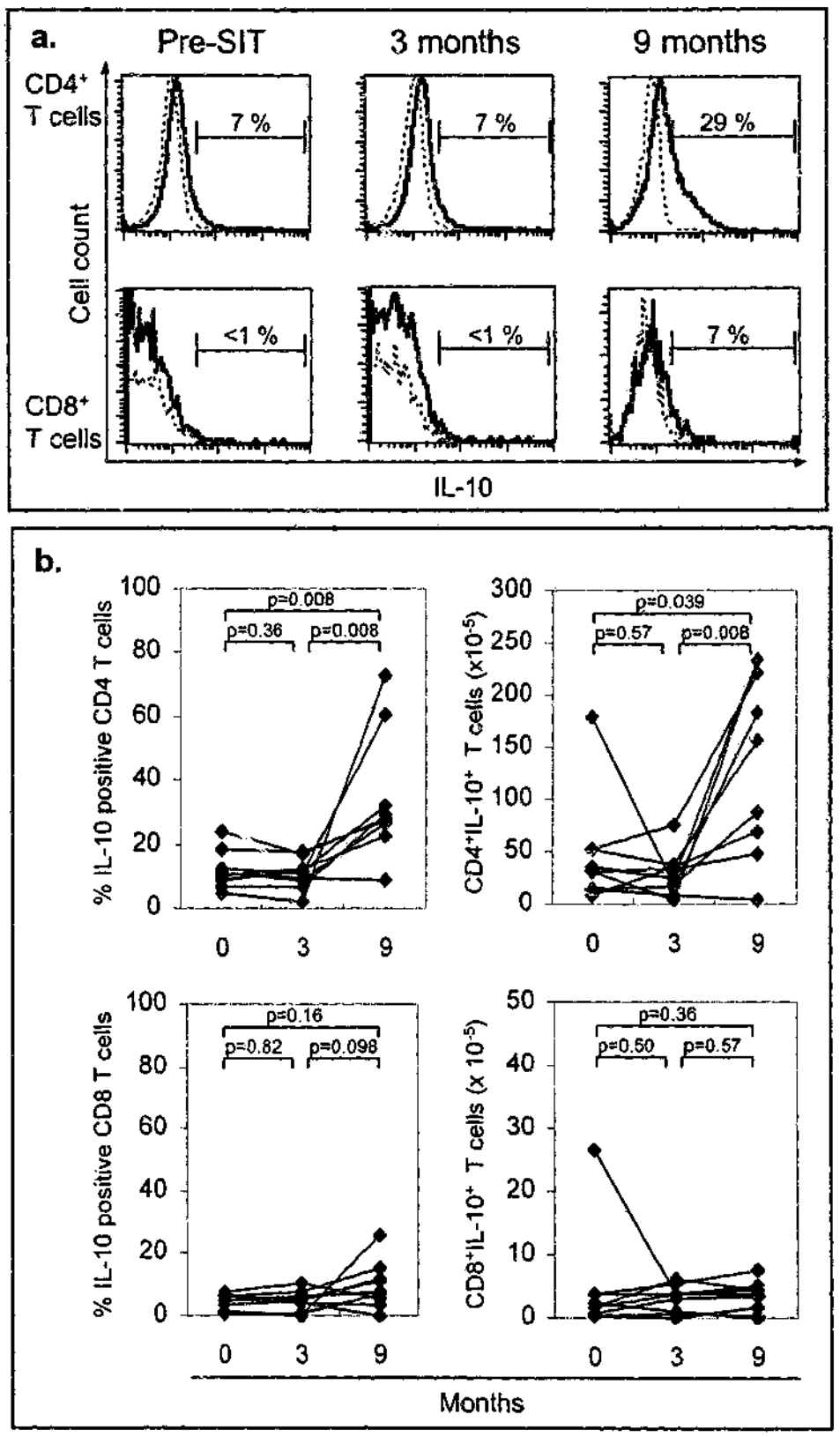


Figure 6.3 IL-10 production by CD4⁺ and CD8⁺ T cells before and at 3- and 9-months of SIT.

PBMC, from the 9 subjects receiving SIT, were cultured with HDM extract for 14 days and stimulated with anti-CD3/IL-2 for 20 hours with Brefeldin A added for the last 4 hours. Cells were labelled with anti-CD4, anti-CD8 and anti-IL-10 antibodies and analysed by flow cytometry. **(a)** Representative IL-10 profiles for CD4⁺ and CD8⁺ T cells pre-SIT and at 3- and 9-months of SIT are shown. Positive staining of the test histogram (solid line) was determined at the level of intensity where isotype control histogram (dotted line) intensity was less than 1 % positive. **(b)** Percentages of IL-10 positive CD4⁺ T cells and the absolute numbers of CD4⁺IL-10⁺ T cells were significantly greater at 9-months of SIT in comparison to pre-SIT (% p=0.008; number p=0.039) and at 3-months of SIT (% and number p=0.008). No significant differences in the percentages of IL-10 positive CD8⁺ T cells and the absolute numbers of CD8⁺IL-10⁺ T cells were observed between pre-SIT and at 3- and 9-months of SIT. Each symbol in the graphs in represents one subject receiving SIT.

6.3.4 Intracellular IL-10 Staining Co-localises to $CD4^+CD25^+$ T Cells at 9-months of SIT

T cells producing IL-10 following SIT have been identified as a subset of $CD4^+CD25^-$ regulatory T cells (Francis *et al.*, 2003; Jutel *et al.*, 2003). Hence IL-10-producing $CD4^+$ T cells were double labelled with anti-CD25 antibody in 14-day HDM extract-stimulated cultures from three patients who had received 9-months of SIT (Figure 6.4). When the $CD4^+CD25^-$ and $CD4^+CD25^+$ T cell subsets were examined, the majority of $CD4^+$ T cell IL-10 staining co-localised to $CD4^+CD25^+$ T cells.

6.3.5 T Cell Expression of Surface Markers Involved in Peripheral T Cell Trafficking Pre- and Post-SIT

The effect of SIT on the expression of adhesion molecules, CD62L and CD49d, and chemokine receptors, CCR3 and CCR5, by allergen-specific $CD4^+$ T cells was analysed by staining 14-day HDM extract-stimulated cultures and flow cytometry. No differences in the proportions or absolute numbers of $CD4^+$ or $CD8^+$ T cells expressing CD62L negative or $CD49d^{hi}$ were observed between pre-SIT and at 3- or 9-months of SIT (Table 6.3). Similarly, no differences in the proportions or absolute numbers of $CD4^+$ or $CD8^+$ T cells expressing CCR3 or CCR5 were observed between pre-SIT and at 3- or 9-months of SIT (Table 6.3).

6.3.6 IL-4 and IFN-γ Production by $CD4^+$ and $CD8^+$ T Cells Expressing a Peripheral Tissue Trafficking Phenotype Pre- and Post-SIT

The IL-4 and IFN-γ phenotype of allergen-specific $CD62L^-$, $CD49d^{hi}$, $CCR3^+$ or $CCR5^+$ T cells in 14-day HDM extract-simulated cultures generated from PBMC from patients receiving SIT was determined. After the 6-hour anti-CD3 stimulation required for the

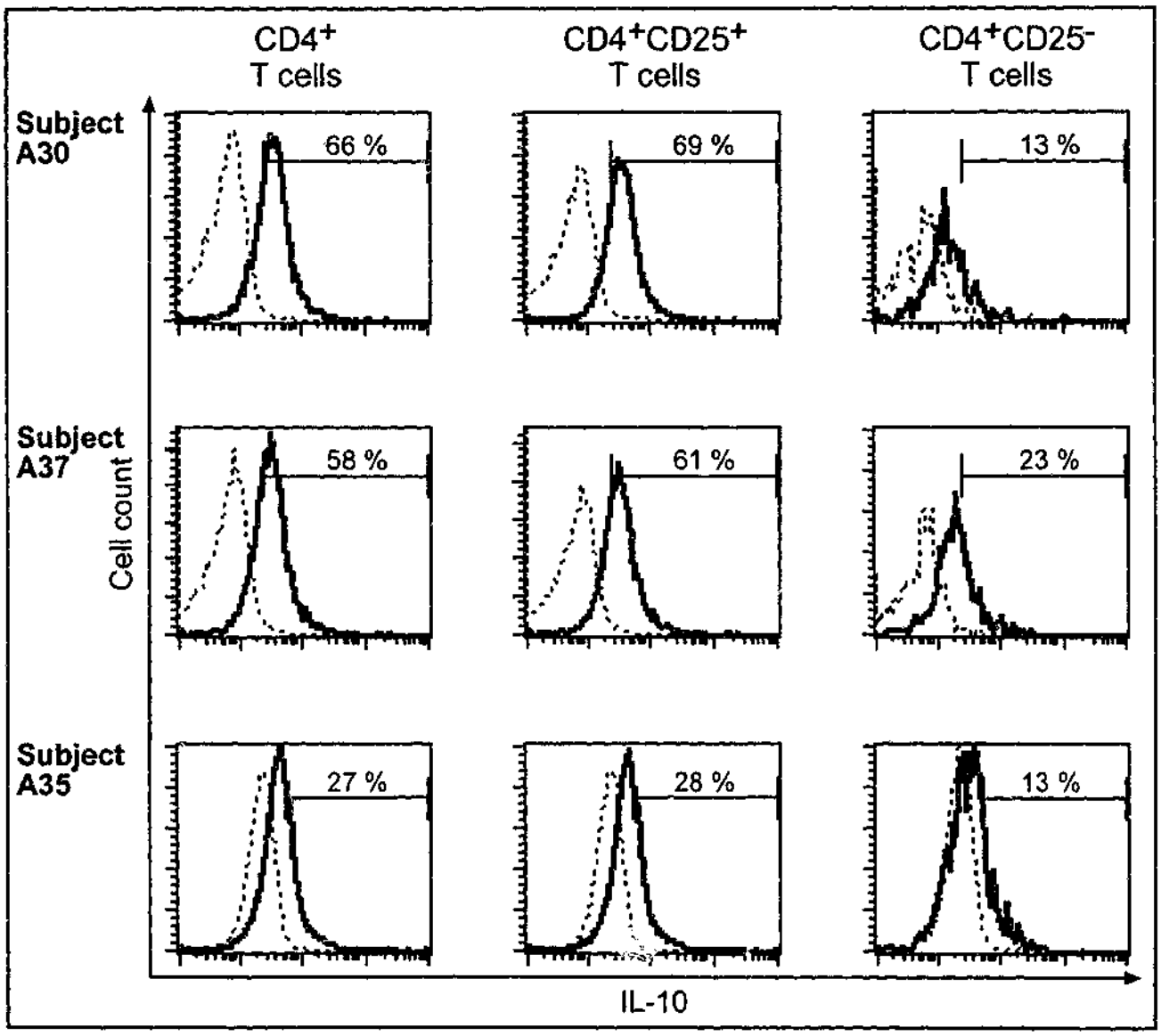


Figure 6.4 Histograms of intracellular IL-10 staining of $CD4^+$ T cells, $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells at 9-months of SIT.

PBMC from three subjects (A30, A37 and A35) receiving SIT were cultured with HDM extract (25 µg/ml) for 14 days, stimulated with anti-CD3/IL-2 for 20 hours with Brefeldin A added for the last 4 hours. Following stimulation, cells were labelled with anti-CD4, anti-CD25 and anti-IL-10 antibodies and analysed by flow cytometry. Positive staining of the test histogram (solid line) was determined at the level of intensity where isotype control histogram (dotted line) intensity was less than 1 % positive.

Table 6.3 Proportions and numbers of CD62L$^-$, CD49dhi, CCR3 and CCR5 positive CD4$^+$ and CD8$^+$ T cells before and during SIT (n = 9).

	Mean % positive T cells (± SD)			Mean number (x10^{-5}) of positive T cells (± SD)		
Surface marker	**Pre-SIT**	**3 mths**	**9 mths**	**Pre-SIT**	**3 mths**	**9 mths**
CD4$^+$CD62L$^-$	29.2 (± 12.4)	26.1 (± 15.2)	23.7 (± 13.0)	127.6 (± 141.1)	75.7 (± 55.9)	95.5 (± 81.5)
CD4$^+$CD49d^{hi}	77.0 (± 10.4)	69.2 (± 26.5)	82.9 (± 11.2)	322.4 (± 315.4)	195.2 (± 115.2)	320.5 (± 194.9)
CD4$^+$CCR3$^+$	16.6 (± 13.3)	10.0 (± 14.5)	22.7 (± 13.0)	99.5 (± 147.0)	21.4 (± 31.3)	92.7 (± 83.2)
CD4$^+$CCR5$^+$	26.9 (± 21.9)	22.9 (± 21.4)	24.4 (± 17.9)	77.7 (± 75.2)	62.5 (± 61.7)	102.1 (± 110.1)
CD8$^+$CD62L$^-$	40.5 (± 11.4)	40.3 (± 10.6)	36.7 (± 15.3)	75.3 (± 174.4)	25.2 (± 14.8)	32.7 (± 29.4)
CD8$^+$CD49d^{hi}	55.0 (± 28.6)	45.7 (± 30.1)	67.6 (± 25.5)	130.0 (± 325.3)	35.6 (± 38.7)	65.4 (± 56.8)
CD8$^+$CCR3$^+$	6.7 (± 9.7)	6.5 (± 8.9)	17.2 (± 11.4)	5.7 (± 7.1)	4.2 (± 6.1)	11.5 (± 13.3)
CD8$^+$CCR5$^+$	38.3 (± 22.1)	40.0 (± 24.1)	30.0 (± 26.3)	36.5 (± 66.4)	22.6 (± 17.3)	34.2 (± 41.5)

PBMC were cultured for 14 days with 25 µg/ml HDM extract. Cells were then labelled with anti-CD4, anti-CD8, anti-CD62L, anti-CD49d, anti-CCR3 and anti-CCR5 antibodies and analysed by flow cytometry.

detection of intracellular IL-4 and IFN-γ, expression of CCR3 was down-regulated, so that upon flow cytometric analysis the staining intensity of CCR3 was equal to or less than the isotype control (data not shown). Thus the cytokine phenotype of $CD4^+CCR3^+$ and $CD8^+CCR3^+$ T cells could not be determined (see Figure 5.5). Representative histograms of intracellular IL-4 and IFN-γ staining of $CD4^+CD62L^-$, $CD4^+CD49d^{hi}$ and $CD4^+CCR5^+$ T cells are shown for one patient receiving immunotherapy in Figure 6.5.

When comparing the results from the 9 patients receiving SIT, there were no significant differences in the percentages of $CD4^+CD62L^-$, $CD4^+CD49d^{hi}$ or $CD4^+CCR5^+$ T cells expressing IL-4 or IFN-γ when comparing pre-SIT to 3- or 9-months of SIT (Table 6.4). SIT had no effect on the absolute numbers of $CD4^+CD62L^-IL\text{-}4^+$, $CD4^+CD49d^{hi}IL\text{-}4^+$, $CD4^+CCR5^+IL\text{-}4^+$, $CD4^+CD62L^-IFN\text{-}\gamma^+$, $CD4^+CD49d^{hi}IFN\text{-}\gamma^+$ or $CD4^+CCR5^+IFN\text{-}\gamma^+$ T cells (Table 6.4). In addition, there were no differences in the percentages or absolute numbers of $CD8^+CD62L^-$, $CD8^+CD49d^{hi}$ or $CD8^+CCR5^+$T cells expressing IL-4 or IFN-γ during SIT (Table 6.5).

6.3.7 IL-10 Production by $CD4^+$ and $CD8^+$ T Cells Expressing a Peripheral Tissue Trafficking Phenotype Pre- and Post-SIT

Production of IL-10 by allergen-specific $CD62L^-$, $CD49d^{hi}$, $CCR3^+$ and $CCR5^+$ T cells in 14-day HDM extract-stimulated cultures generated from PBMC from patients before and during SIT was determined by surface marker and intracellular cytokine staining. After the 20-hour anti-CD3/IL-2 stimulation required for the detection of intracellular IL-10, T cell expression of CCR3 was down-regulated and thus the cytokine phenotype of $CCR3^+$ cells could not be determined (see Figure 5.5). Representative histograms of

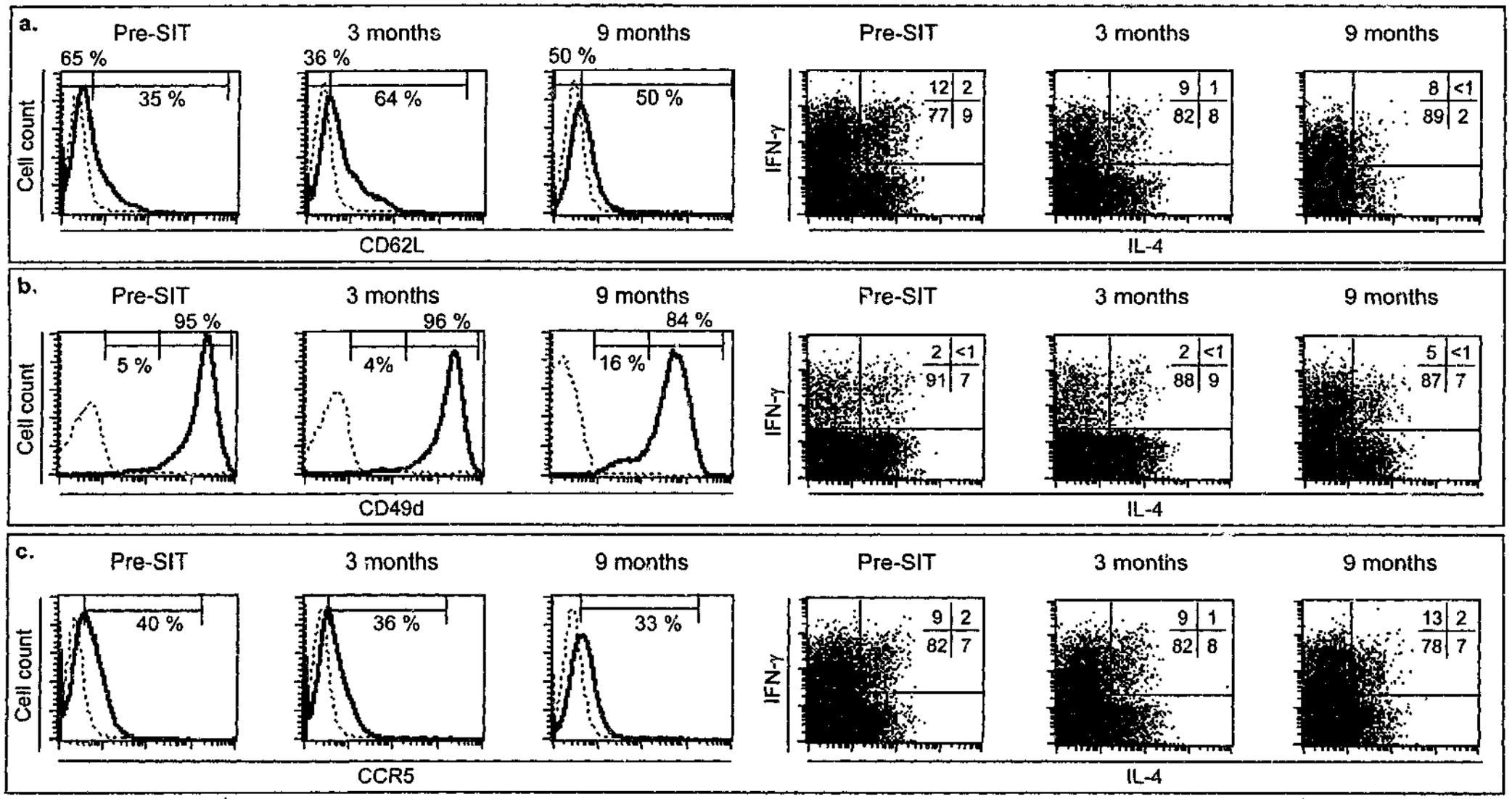


Figure 6.5 Representative $CD4^+$ T cell expression of CD62L, CD49d, and CCR5 and the co-expression of intracellular IL-4 and IFN-γ by $CD4^+CD62L^-$, $CD4^+CD49d^{hi}$ and $CD4^+CCR5^+$ T cells at pre-SIT and at 3- and 9-months of SIT.

PBMC from a subject receiving SIT was cultured with HDM extract (25 μg/ml) for 14 days and stimulated with anti-CD3/IL-2 for 6 hours in the presence of Brefeldin A. Following stimulation, cells were labelled with anti-CD4, anti-CD62L, anti-CD49d, anti-CCR5, anti-IL-4 and anti-IFN-γ antibodies and analysed by flow cytometry. Representative histogram plots of the expression of CD62L **(a)**, CD49d **(b)** and CCR5 **(c)** by $CD4^+$ T cells and corresponding dot plots of IL-4 versus IFN-γ staining of $CD4^+CD62L^-$ T cells **(a)**, $CD4^+CD49d^{hi}$ T cells **(b)** and $CD4^+CCR5^+$ T cells **(c)** at Pre-SIT and 3- and 9-months of SIT are shown. Test histograms are shown by solid lines and appropriately labelled isotype control histograms are shown as dotted lines. Positive staining of the test histogram was determined at the level of intensity where the test histogram crossed the isotype control histogram. Background values from the isotype control histograms were then subtracted. Isotype controls were used to set quadrant markers in the dot plots. Quadrant percentages are shown in each plot.

Table 6.4 Proportions and numbers of IL-4 and IFN-γ positive $CD4^+$ T cells expressing $CD62L^-$, $CD49d^{hi}$ or $CCR5^+$ before and during SIT (n = 9).

Surface marker and cytokine	Mean % cytokine positive T cells (± SD)			Mean number ($x10^{-5}$) of cytokine positive T cells (± SD)		
	Pre-SIT	3 mths	9 mths	Pre-SIT	3 mths	9 mths
$CD62L^- IL\text{-}4^+$	12.5 (± 9.8)	10.6 (± 12.2)	8.2 (± 10.3)	35.1 (± 44.6)	29.5 (± 41.5)	21.2 (± 27.9)
$CD49d^{hi} IL\text{-}4^+$	12.0 (± 8.3)	11.4 (± 10.4)	7.1 (± 8.0)	36.3 (± 32.7)	32.3 (± 40.5)	25.8 (± 28.0)
$CCR5^+ IL\text{-}4^+$	18.2 (± 11.2)	17.2 (± 11.7)	14.9 (± 12.6)	23.6 (± 17.5)	21.6 (± 27.3)	19.0 (± 18.0)
$CD62L^- IFN\text{-}\gamma^+$	8.9 (± 7.9)	12.0 (± 10.6)	9.8 (± 9.3)	67.6 (± 156.4)	22.8 (± 37.1)	31.8 (± 40.0)
$CD49d^{hi} IFN\text{-}\gamma^+$	4.2 (± 7.1)	5.2 (± 6.4)	4.8 (± 6.0)	32.8 (± 80.8)	9.6 (± 12.8)	17.6 (± 17.0)
$CCR5^+ IFN\text{-}\gamma^+$	10.9 (± 11.7)	12.0 (± 10.8)	10.7 (± 9.6)	28.8 (± 46.7)	20.8 (± 33.7)	25.6 (± 31.1)

PBMC were cultured for 14 days in the presence of 25 μg/ml HDM extract. Cells were stimulated with anti-CD3/IL-2 for 6 hours, labelled with anti-CD4, anti-CCR5, anti-CD62L and anti-CD49d antibodies and stained for intracellular IL-4 and IFN-γ. Percentages of $CD4^+CCR5^+$, $CD4^+CD62L^-$ and $CD4^+CD49d^{hi}$ T cells expressing IL-4 or IFN-γ were determined by flow cytometry.

Table 6.5 Proportions and numbers of IL-4 and IFN-γ positive $CD8^+$ T cells expressing $CD62L^-$, $CD49d^{hi}$ or $CCR5^+$ before and during SIT (n = 9).

Surface marker and cytokine	Mean % cytokine positive T cells (± SD)			Mean number ($x10^{-5}$) of cytokine positive T cells (± SD)		
	Pre-SIT	3 mths	9 mths	Pre-SIT	3 mths	9 mths
$CD62L^- IL\text{-}4^+$	0.8 (± 1.1)	1.2 (± 1.3)	1.0 (± 1.5)	1.4 (± 3.0)	0.6 (± 1.2)	0.2 (± 0.5)
$CD49d^{hi} IL\text{-}4^+$	0.3 (± 0.7)	0.3 (± 0.4)	0.5 (± .7)	0.1 (± 0.2)	0.1 (± 0.1)	0.1 (± 0.2)
$CCR5^+ IL\text{-}4^+$	3.4 (± 3.0)	3.2 (± 2.9)	6.2 (± 5.9)	1.7 (± 4.0)	0.9 (± 1.4)	0.5 (± 0.6)
$CD62L^- IFN\text{-}\gamma^+$	6.0 (± 6.8)	11.6 (± 9.4)	10.7 (± 10.0)	18.9 (± 51.1)	8.2 (± 9.2)	9.7 (± 14.0)
$CD49d^{hi} IFN\text{-}\gamma^+$	12.9 (± 13.6)	17.7 (± 13.0)	17.2 (± 10.1)	35.3 (± 96.5)	9.8 (± 9.4)	13.1 (± 14.5)
$CCR5^+ IFN\text{-}\gamma^+$	6.2 (± 7.0)	11.4 (± 10.3)	12.6 (± 10.9)	14.5 (± 40.9)	4.0 (± 4.5)	6.5 (± 9.4)

PBMC were cultured for 14 days in the presence of 25 µg/ml HDM extract. Cells were stimulated with anti-CD3/IL-2 for 6 hours, labelled with anti-CD8, anti-CCR5, anti-CD62L and anti-CD49d antibodies and stained for intracellular IL-4 and IFN-γ. Percentages of $CD8^+CCR5^+$, $CD8^+CD62L^-$ and $CD8^+CD49d^{hi}$ T cells expressing IL-4 or IFN-γ was determined by flow cytometry.

intracellular IL-10 staining of $CD4^+CD62L^-$, $CD4^+CD49d^{hi}$ and $CD4^+CCR5^+$ T cells are shown for one patient receiving immunotherapy in Figure 6.6.

When comparing results for the 9 patients receiving SIT, significant increases in the proportions of $CD4^+CD62L^-$ T cells expressing IL-10 at 9-months of SIT in comparison to pre-SIT ($p=0.019$) and to 3-months of SIT ($p=0.004$; Figure 6.7a) were seen. The absolute numbers of $CD4^+CD62L^-IL\text{-}10^+$ T cells were increased at 9-months of SIT in comparison to 3-months ($p=0.019$; Figure 6.7a). There was a trend for increased numbers of $CD4^+CD62L^-IL\text{-}10^+$ T cells at 9-months of SIT in comparison to pre-SIT however statistical significance was not reached ($p=0.074$). The proportions of $CD4^+CD49d^{hi}$ T cells expressing IL-10 and the absolute numbers of $CD4^+CD49d^{hi}IL\text{-}10^+$ T cells were significantly greater at 9-months of SIT in comparison to pre-SIT (% and number $p=0.039$) and 3-months of SIT (% and number $p=0.004$; Figure 6.7b). No differences in percentage of $CD4^+CD49d^{hi}$ T cells expressing IL-10 and the absolute numbers of $CD4^+CD49d^{hi}IL\text{-}10^+$ T cells were seen when comparing pre-SIT to 3-months of SIT (Figure 6.7b). Analysis of IL-10 expression by $CD62L^+$ and $CD49d^{lo}$ $CD4^+$ T cells revealed that the majority of IL-10 staining co-localised with $CD4^+$ T cells expressing an activated phenotype ($CD62L^-$ and $CD49d^{hi}$; data not shown). Significant increases in the proportions of $CD4^+CCR5^+$ T cells expressing IL-10 at 9-months of SIT in comparison to pre-SIT ($p=0.027$) and to 3-months of SIT ($p=0.004$; Figure 6.7c) were also observed. The absolute number of $CD4^+CCR5^+IL\text{-}10^+$ T cells were also increased at 9-months of SIT in comparison to 3-months ($p=0.004$) however were no differences between pre-SIT and 3- or 9-months of SIT (Figure 6.7c).

Within the $CD8^+$ T cell population, significant increases in the proportions of $CD8^+CD62L^-$ T cells expressing IL-10 were observed at 9-months of SIT in comparison

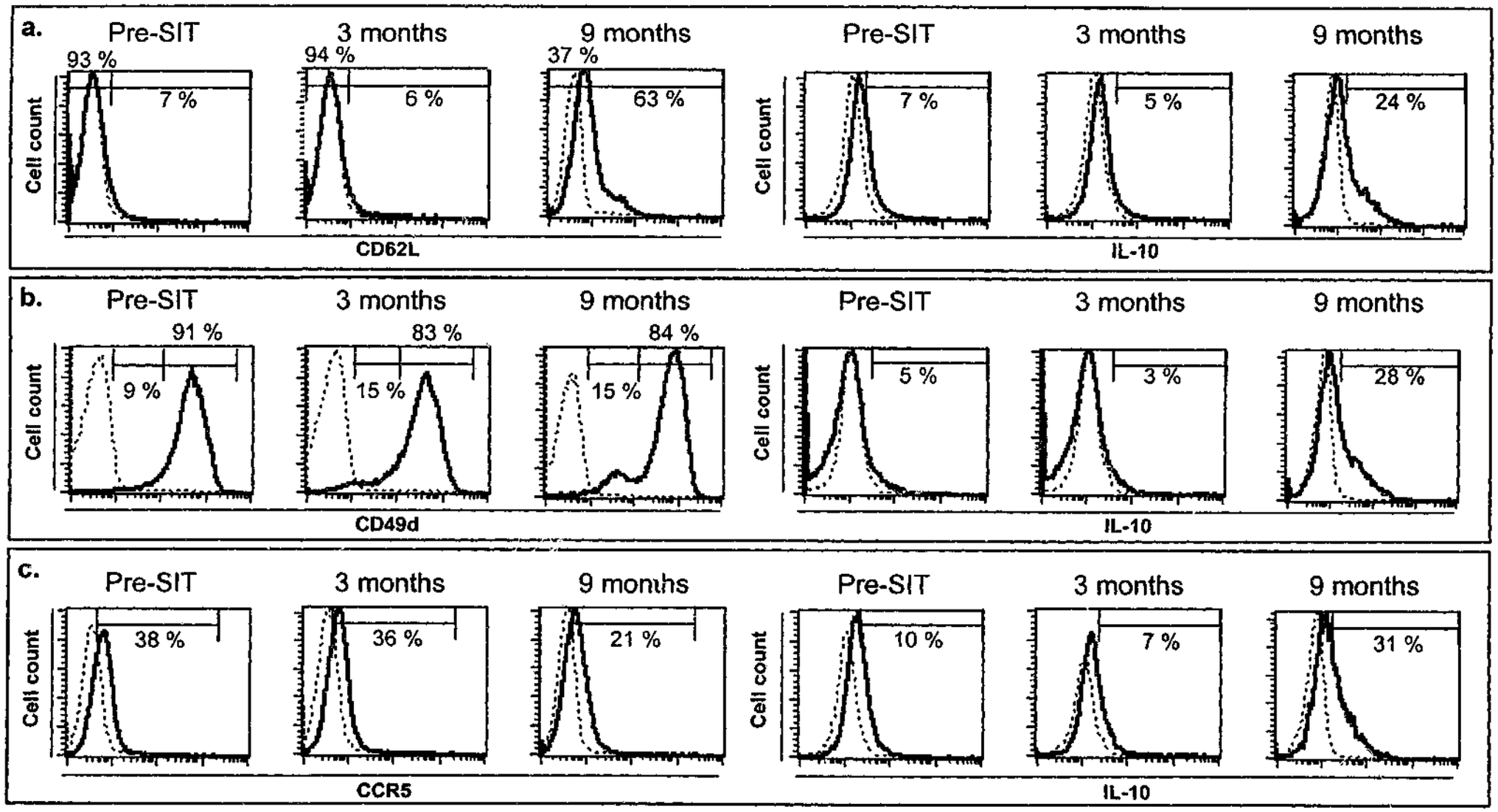


Figure 6.6 Representative CD4⁺ T cell expression of CD62L, CD49d, and CCR5 and co-expression of intracellular IL-10 by CD4⁺CD62L⁻, CD4⁺CD49d^{hi} and CD4⁺CCR5⁺ T cells at pre-SIT and at 3- and 9-months of SIT.

PBMC from a subject receiving SIT was cultured with HDM extract (25 μg/ml) for 14 days and stimulated with anti-CD3/IL-2 for 20 hours with Brefeldin A added for the last 4 hours. Following stimulation, cells were labelled with anti-CD4, anti-CD62L, anti-CD49d, anti-CCR5 and anti-IL-10 antibodies and analysed by flow cytometry. Representative histogram plots of the expression of CD62L **(a)**, CD49d **(b)** and CCR5 **(c)** by CD4⁺ T cells and the corresponding IL-10 histograms of CD4⁺CD62L⁻ T cells **(a)**, CD4⁺CD49d^{hi} T cells **(b)** and CD4⁺CCR5⁺ T cells **(c)** at pre-SIT and 3- and 9-months of SIT are shown. Test histograms are shown by solid lines and appropriately labelled isotype control histograms are shown as dotted lines. Positive staining of the test histogram was determined at the level of intensity where the test histogram crossed the isotype control histogram. Background values from the isotype control histograms were then subtracted.

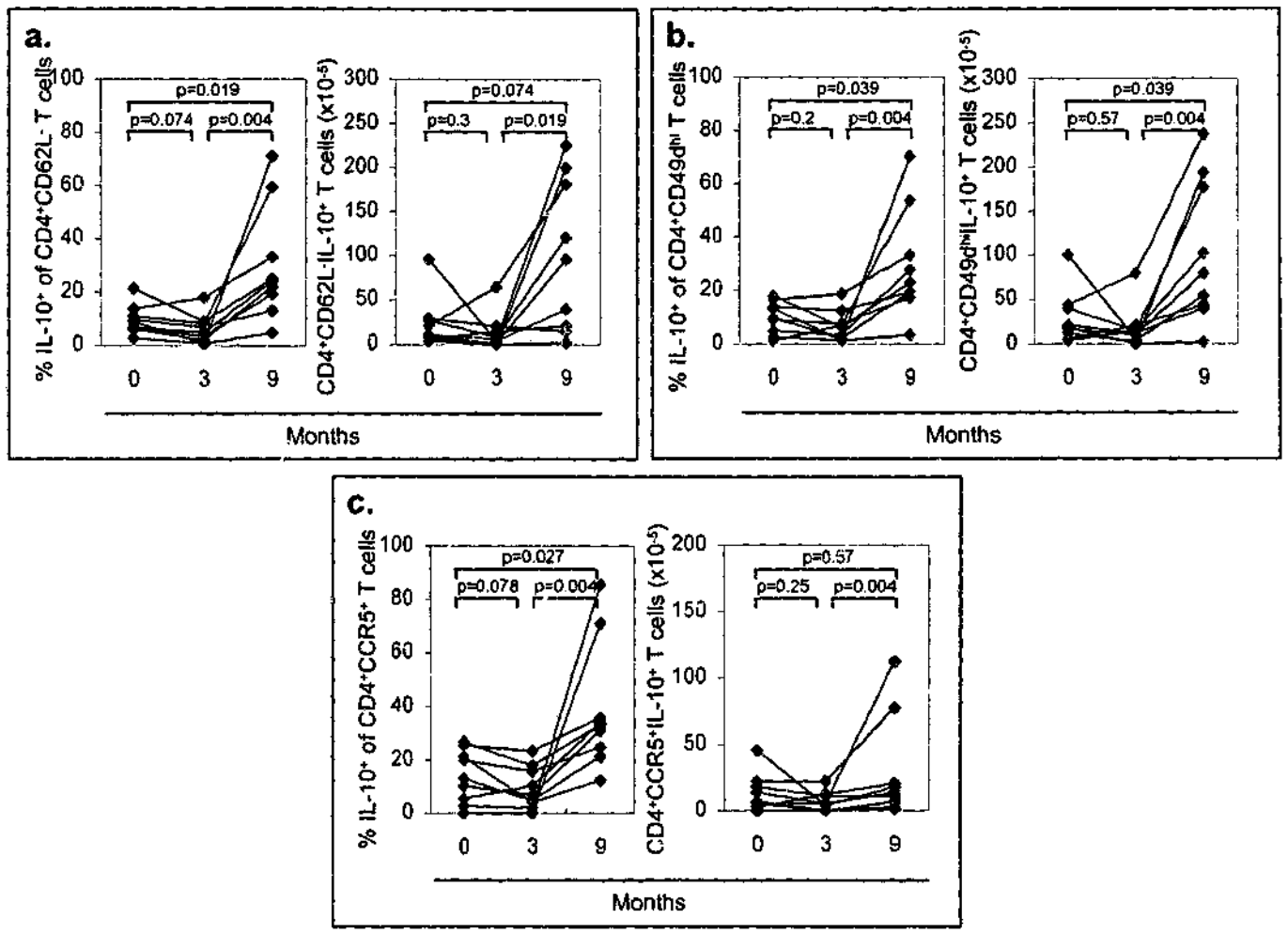


Figure 6.7 IL-10 production by $CD4^+CD62L^-$ T cells, $CD4^+CD49d^{hi}$ T cells and $CD4^+CCR5^+$ T cells at pre-SIT and at 3- and 9-months of SIT.

PBMC from the 9 subjects receiving SIT were cultured with HDM extract (25 μg/ml) for 14 days and stimulated with anti-CD3/IL-2 for 20 hours with Brefeldin A added for the last 4 hours. Cells were labelled with anti-CD4, anti-CD62L, anti-CD49d, anti-CCR5 and anti-IL-10 antibodies and analysed by flow cytometry. **(a)** Significant increases in the proportions of $CD4^+CD62L^-$ T cells expressing IL-10 at 9-months of SIT in comparison to pre-SIT ($p=0.019$) and to 3-months of SIT ($p=0.004$) were seen. $CD4^+CD62L^-IL\text{-}10^+$ T cell numbers were increased at 9-months of SIT in comparison to 3-months ($p=0.019$). There was a trend for increased $CD4^+CD62L^-IL\text{-}10^+$ T cell numbers at 9-months of SIT in comparison to pre-SIT ($p=0.074$). **(b)** The percentage of $CD4^+CD49^{hi}$ T cells expressing IL-10 and $CD4^+CD49^{hi}IL\text{-}10^+$ T cell numbers were significantly greater at 9-months of SIT in comparison to pre-SIT (% and number $p=0.039$) and to 3-months of SIT (% and number $p=0.004$). **(c)** Significant increases in the proportions of $CD4^+CCR5^+$ T cells expressing IL-10 at 9-months of SIT in comparison to pre-SIT ($p=0.027$) and to 3-months of SIT ($p=0.004$) were observed. $CD4^+CCR5^+IL\text{-}10^+$ T cell numbers were increased at 9-months of SIT in comparison to 3-months ($p=0.004$), with no differences seen when comparing pre-SIT with 3-months of SIT and with 9-months of SIT. Each symbol in the graphs represents one subject receiving SIT.

to pre-SIT (p=0.018) and at 3-months of SIT (p=0.039; Table 6.6). The absolute numbers of $CD8^+CD62L^-IL\text{-}10^+$ T cells were unaffected during treatment. No differences in the proportions of $CD8^+CD49d^{hi}$ T cells expressing IL-10 and the absolute numbers of $CD8^+CD49d^{hi}IL\text{-}10^+$ T cells were seen when comparing pre-SIT to 3- or 9- months of SIT (Table 6.6). The proportions of $CD8^+CCR5^+$ T cells expressing IL-10 and the absolute numbers of $CD8^+CCR5^+IL\text{-}10^+$ T cells were also unaffected by HDM-SIT (Table 6.6).

6.3.8 Allergen-Specific T Cell Proliferation Pre- and Post-SIT

The 14-day HDM extract-stimulated cultures, generated from PBMC obtained from patients receiving SIT, were tested for T cell reactivity to HDM extract by proliferation assay. T cell proliferation to HDM extract for one patient before and at 3- and 9-months of SIT is shown in Figure 6.8. When comparing the results for the 9 patients, before treatment all showed T cell proliferative responses to HDM extract (Table 6.7). At 9 months of SIT in comparison to pre-SIT, the proliferative response to HDM extract was decreased in 5/9 patients. Of these 5 patients, 4 also showed decreased proliferation at 3 months in comparison to pre-SIT with only one showing increased proliferation. However as the other 4 patients showed increased proliferative responses to HDM extract at 9-months of SIT and had variable responses at 3-months of SIT, overall there was no significant change in allergen-induced proliferation following immunotherapy.

For one patient (subject A34) who displayed reduced allergen-specific T cell proliferation at 9-months of SIT, whether the addition of IL-2 could restore the T cell proliferative response thus indicating reversal of anergy or inhibition of T regulatory cell-mediated suppression was assessed (Akdis and Blaser, 1999; Schwartz, 2003).

Table 6.6 Proportions and numbers of IL-10 positive $CD8^+$ T cells expressing $CD62L^-$, $CD49d^{hi}$ or $CCR5^+$ before and during SIT (n = 9).

	Mean % IL-10 positive T cells (± SD)			Mean number ($x10^{-5}$) of IL-10 positive T cells (± SD)		
Surface marker	**Pre-SIT**	**3 mths**	**9 mths**	**Pre-SIT**	**3 mths**	**9 mths**
$CD62L^-$	1.5 (± 1.5)	1.4 (± 2.2)	7.6*† (± 8.5)	2.1 (± 5.4)	0.7 (± 1.1)	4.4 (± 6.5)
$CD49d^{hi}$	3.0 (± 2.8)	2.3 (± 1.9)	5.6 (± 5.8)	1.4 (± 2.3)	0.8 (± 1.0)	2.9 (± 2.9)
$CCR5^+$	5.4 (± 4.8)	5.3 (± 7.1)	25.4 (± 34.0)	4.0 (± 11.0)	0.7 (± 0.8)	1.6 (± 2.0)

* $p < 0.05$ between Pre-SIT and 9-months of SIT

† $p < 0.05$ between 3- and 9-months of SIT

PBMC were cultured for 14 days in the presence of 25 µg/ml HDM extract. Cells were stimulated with anti-CD3/IL-2 for 20 hours, labelled with anti-CD8, anti-CCR5, anti-CD62L and anti-CD49d antibodies and stained for intracellular IL-10. Percentages of $CD8^+CCR5^+$, $CD8^+CD62L^-$ and $CD8^+CD49d^{hi}$ $CD8^+$ T cells expressing IL-10 was determined by flow cytometry.

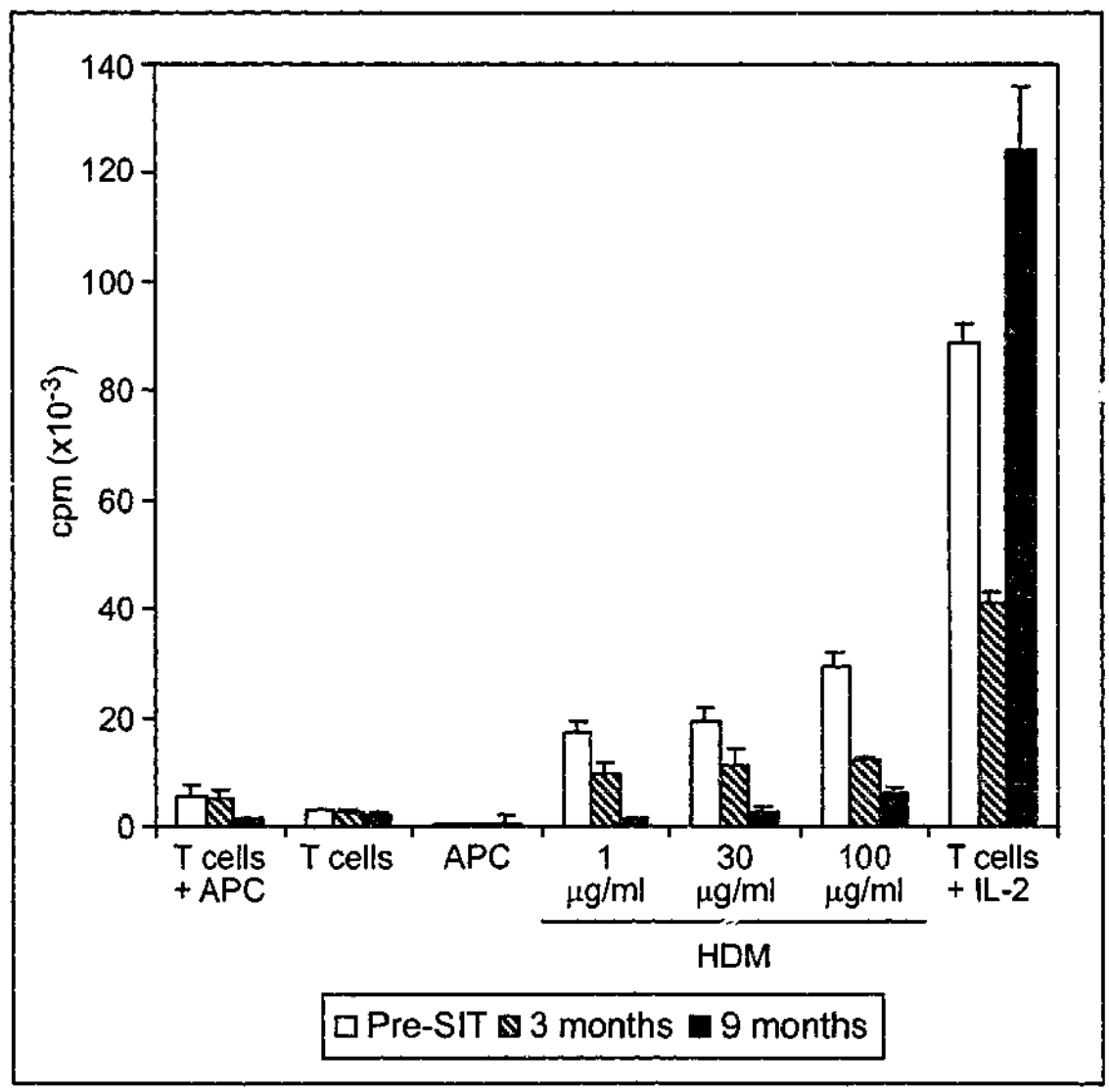


Figure 6.8 Representative T cell proliferation to HDM extract before and at 3- and 9-months of SIT.

PBMC taken before and at 3- and 9-months of SIT from one subject were cultured with HDM extract (25 µg/ml) for 14 days. Cultured cells were incubated in the presence of an equal number of irradiated PBMC and HDM extract (10, 30 and 100 µg/ml) in round-bottom 96-well plates.Cultures were performed in triplicate. Positive control cultures were T cells with IL-2 (100 U/ml) and negative control cultures were T cells alone, APC alone and T cells with APC without antigen. After 48 hours, cultures were pulsed with 1 µCi ^{3}H-thymidine and harvested 16 hours later. Proliferation as correlated with ^{3}H-thymidine incorporation was measured by liquid scintillation spectroscopy and results expressed as average cpm and standard deviation.

Table 6.7 T cell proliferation to HDM extract before and during SIT (n = 9).

	Proliferation (Δ cpm)		
Subject	**Pre-SIT**	**3 mths**	**9 mths**
A30	31761	5491	611
A33	8007	6904	27893
A34	138335	25174	23051
A35	43982	73779	48067
A36	47459	33962	41110
A37	31722	35477	15967
A38	10827	28669	46745
A39	23957	6776	4820
A40	26251	11531	34776
Mean	42006	27029	26033

Maximal proliferative responses of 14-day HDM extract-stimulated cultures generated from PBMC of subjects receiving SIT expressed as Δ cpm.

Stimulating patient A34's 14-day HDM extract culture with HDM extract (100 μg/ml) in the presence of IL-2 resulted in increased proliferation from 24,193 Δ cpm (without IL-2) to 68,577 Δ cpm.

6.4 DISCUSSION

HDM-specific immunotherapy is an effective treatment for severely affected rhinitis patients or mild persistent asthmatics. However to increase the use of this treatment, particularly in severe asthmatic patients, improved efficacy and safety of SIT is required. In the design of allergen preparations for SIT, extract quality is important. Recently, standardised allergen preparations have been developed with known allergen content and less batch-to-batch variation. A clinical trial of HDM immunotherapy was performed at the Alfred Hospital Asthma and Allergy Clinic to assess the efficacy of the standardised Alustal® *D. pteronyssinus* extract from Stallergenes, France. This preparation was demonstrated to be efficacious with significant improvement in total and rhinitis symptom scores. Although the relief medication requirements remained unchanged, significant reductions in cutaneous late phase responses to HDM allergen in the SIT-treated group were observed. There were no significant differences in the cutaneous late phase responses in the non-SIT group indicating that reductions in the late phase response for the SIT-group were not due to the degradation or inactivation of the allergen skin test reagent.

This chapter describes the detailed analysis of the phenotype and function of allergen-stimulated $CD4^+$ and $CD8^+$ T cells before and following 3- and 9-months of SIT. These experiments were performed on peripheral blood obtained from the patients receiving the Alustal® *D. pteronyssinus* preparation. Although the pivotal role for $CD4^+$ T cells in controlling the allergic immune response is well recognised, recent evidence suggests a role for $CD8^+$ T cells in allergy (Seneviratne *et al.*, 2002). A significant decrease in the production of IL-4 by both $CD4^+$ and $CD8^+$ T cell subsets accompanied clinically effective desensitisation. Reduced IL-4 production by $CD4^+$ T cells during HDM

immunotherapy has previously been demonstrated using intracellular cytokine staining (Majori *et al.*, 2000; Fu *et al.*, 2003). In contrast to the results presented in this thesis, Majori *et al.* (Majori *et al.*, 2000) observed no change in $CD8^+$ T cell IL-4 production following HDM SIT. However Majori and colleagues analysed T cell cytokine production in PBMC stimulated with PMA and ionomycin whereas in the present study T cells in 14-day HDM extract-stimulated cultures were analysed. Consistent with the data presented here, decreased $CD8^+$ T cell IL-4 production in HDM T cell lines generated from asthmatic children following HDM SIT was recently demonstrated (Fu *et al.*, 2003). In studies analysing changes in IFN-γ production following HDM immunotherapy results have been variable, with different investigators reporting increased (Lack *et al.*, 1997; Majori *et al.*, 2000) or decreased (Fu *et al.*, 2003) IFN-γ production by $CD4^+$ T cells, and either unchanged (Lack *et al.*, 1997; Majori *et al.*, 2000; Fu *et al.*, 2003) or reduced (O'Br[illegible] *et al.*, 2000) production by $CD8^+$ T cells. Although a trend towards decreased production of IFN-γ by $CD4^+$ T cells and increased production of IFN-γ by $CD8^+$ T cells was observed, these changes were not significant. Thus the current results are consistent with HDM immunotherapy inducing suppression of Th1- and Th2-type cytokine production rather than immune deviation from Th2- to Th1-predominant cytokine production as shown previously in some reports of aeroallergen immunotherapy (Jutel *et al.*, 1995a; Ebner *et al.*, 1997; Eusebius *et al.*, 2002).

In the present study, the increase in $CD4^+$ T cell IL-10 production following HDM immunotherapy may have lead to a suppression of IL-4 production and to a lesser extent IFN-γ production by T cells. Studies analysing T cell cytokine changes during bee venom immunotherapy have shown that neutralisation of IL-10 post-SIT can reverse

SIT-induced suppression of T cell cytokine production and proliferation (Bellinghausen *et al.*, 1997; Akdis *et al.*, 1998b). In the present study, significant reductions in allergen-specific T cell proliferation following SIT were not seen. This observation has also been reported by Francis *et al.* following grass pollen SIT (Francis *et al.*, 2003). They showed that addition of exogenous IL-10 could inhibit allergen-specific T cell proliferation when added to *in vitro* cultures at concentrations greater than those induced by SIT. In contrast, increased IL-10 following semi-rush HDM SIT can result in decreased allergen-specific T cell proliferation and reduced production of IFN-γ, IL-5 and IL-13 (Jutel *et al.*, 2003). Hence the quantities of IL-10 induced during SIT may be dependent upon the allergen preparation used and the type of protocol administered.

In addition to altered peripheral blood T cell cytokine production, SIT can induce changes in peripheral tissue cytokine expression with increased numbers of IFN-γ $mRNA^-$ cells detected in allergen-challenged nasal mucosa and skin after grass pollen SIT (Varney *et al.*, 1993; Durham *et al.*, 1996; Wachholz *et al.*, 2002). Increased IL-10^+ cell numbers and decreased IL-4^+ cell numbers have also been observed in skin biopsies of allergen-induced cutaneous late phase responses following wasp venom SIT (Nasser *et al.*, 2001). As T cell numbers have been shown to increase in tissues after allergen provocation (Varney *et al.*, 1992), it is possible that accompanying successful SIT is an alteration in the cytokine phenotype of those T cells trafficking to sites of allergen encounter in the periphery. Hence the expression of a number of adhesion molecules and chemokine receptors involved in peripheral tissue trafficking of T cells was analysed and then correlated with cytokine production following SIT. No significant differences in allergen stimulated $CD4^+$ or $CD8^+$ T cell expression of CD62L, CD49d, CCR3 and CCR5 were observed following treatment. Moreover there was no difference

in IL-4 and IFN-γ profiles of allergen-stimulated $CD4^+$ or $CD8^+$ T cells expressing peripheral tissue trafficking phenotypes i.e. $CD62L^-$, $CD49d^{hi}$, or $CCR5^+$ before and following SIT. These results differ from those presented in Chapter 5, in which the stimulation of atopic donor PBMC *in vitro* with high allergen concentration resulted in increased numbers of IFN-γ positive T cells expressing a peripheral tissue trafficking phenotype. This difference may be due to the timing of blood collection with respect to the timing of allergen injection. Alternatively the enhanced level of IL-10 synthesis following immunotherapy resulting in decreased IFN-γ production by "peripheral tissue trafficking" T cells. Neutralisation of IL-10 using an anti-IL-10 mAb in HDM extract-stimulated cultures could address this issue. Failure to account for the changes in allergen-induced cytokine production in peripheral tissues following SIT as observed by others (Varney *et al.*, 1993; Durham *et al.*, 1996; Wachholz *et al.*, 2002), could also be due to the involvement of other adhesion molecules and chemokine receptors in allergen-specific T cell peripheral tissue trafficking. For example, increased CCR4 positive T cells have been identified in atopic asthmatic bronchial biopsies after allergen challenge (Panina-Bordignon *et al.*, 2001), indicating that CCR4 may play an important role in T cell migration to the lungs in allergy.

Of great interest was the observation of increased expression of IL-10 by $CD4^+CD62L^-$, $CD4^+CD49d^{hi}$ and $CD4^+CCR5^+$ T cells following immunotherapy. Recently IL-10-producing T cells expressing a $CD4^+CD25^+$ phenotype have been identified following aeroallergen SIT (Francis *et al.*, 2003; Jutel *et al.*, 2003). Here IL-10 positive staining co-localised with $CD4^+CD25^+$ T cells indicating that the $CD4^+$IL-10^+ T cells detected following SIT could be regulatory T cells. These $CD4^+$IL-10^+ T cells were shown to express surface markers for peripheral tissue trafficking. As such these cells may be

responsible for reduced allergen-induced late phase responses observed for individuals receiving HDM-SIT in the present study. This result could also explain increased IL-10 production in allergen-induced late phase responses following wasp venom immunotherapy (Nasser *et al.*, 2001). IL-10, produced by T cells during the late phase response, could switch off allergic inflammation by inhibiting T cell proliferation and production of IL-4, IL-5 and IFN-γ (Del Prete *et al.*, 1993; Schandene *et al.*, 1994; Akdis and Blaser, 2001). Indeed reduced allergen-specific T cell proliferation was observed in 5/9 patients and in the presence of IL-2, T cell responsiveness could be restored. Through this mechanism IL-10 could decrease T cell-mediated induction and perpetuation of the allergen-induced late phase response. IL-10 also influences the functions of effector cells in allergic inflammation. IgE-induced release of cytokines and histamine from human mast cells is inhibited by IL-10 (Royer *et al.*, 2001). IL-10 can also inhibit eosinophil cytokine production (Takanaski *et al.*, 1994) and enhance apoptosis of these cells through the down-regulation of CD40 expression (Ohkawara *et al.*, 1996). Therefore as IL-10 can abate allergen-specific T cell responses and subsequent activation of effector cells, the efficacy of immunotherapy could be improved by enhancing SIT-induced $CD4^+CD25^+$ regulatory T cell activity and production of IL-10.

In this study the standardised Alustal® *D. pteronyssinus* preparation reduced clinical symptoms of allergy in a cohort of patients with perennial rhinitis and caused altered allergen-specific T cell responses. The mechanisms involved in effective desensitisation of allergic individuals included decreased production of allergen-specific Th2-type cytokines and increased $CD4^+$ T cell production of the immunoregulatory cytokine IL-10. By investigating changes in cytokine production by T cells expressing adhesion molecules and chemokine receptors required for peripheral T cell trafficking, SIT was

shown to induce IL-10 production by T cells expressing a peripheral tissue trafficking phenotype. Enhancing IL-10-mediated immune suppression during SIT, through the development of standardised allergen preparations and new administration regimens, will lead to improved efficacy of this potentially curative treatment.

CHAPTER 7

GENERAL DISCUSSION

7.1 INTRODUCTION

In this project mechanisms for down-regulating allergen-specific Th2-type responses by increased allergen concentration were investigated with a view to improving efficacy of allergen-specific immunotherapy. An *in vitro* culture system and HDM-allergic donor PBMC were used. The effects of high dose allergen stimulation on T cell cytokine production, cell division, apoptosis and the expression of adhesion molecules and chemokine receptors involved in peripheral tissue trafficking were analysed at the single cell level by flow cytometry. As a comparison for the *in vitro* findings, modulation of T cell responses during clinical HDM-immunotherapy was also analysed. This chapter discusses the findings from these studies in relation to the current state of knowledge in T cell immunology and considers how the data form a platform for development of new allergen preparations and regimens for clinically effective SIT.

7.2 ANTIGEN-INDUCED MODULATION OF T CELL CYTOKINE RESPONSES

Upon exposure to antigen naïve T cells receive signals from APC through MHC and costimulatory molecule interactions and from cytokines to undergo clonal expansion resulting in increased numbers and frequencies of antigen-specific T cells. During expansion, Th cells acquire functional effector Th1- and Th2-phenotypes. This process of differentiation is normally regulated tightly so that it leads to protective immunity rather than harmful immunopathology. Antigen concentration and form, APC-type,

costimulatory molecule interactions, and the cytokine milieu of maturing T cells can all influence Th cell activation and differentiation. In turn, T cell responses are modulated by apoptosis, anergy of a particular Th cell subset or by the induction of regulatory T cell function. The focus of the present study was allergen concentration. An increased proportion of IFN-γ-producing T cells in cultures of allergic donor PBMC stimulated with high allergen concentration compared with low concentration was associated with expansion of IFN-γ positive $CD4^+$ and $CD8^+$ T cells, enhanced apoptosis of IL-4-producing $CD4^+$ T cells and induction of antigen-specific T cell unresponsiveness. In some cultures, expansion of an IL-10-producing $CD4^+$ T cell population was also observed. It is these mechanisms that may play important roles *in vivo* upon high allergen dose administration during SIT to down-regulate pathogenic Th2-type responses.

The different profiles of Th cell cytokine production observed following stimulation with different concentrations of allergen are possibly due to activation of different intracellular signalling pathways within T cells. When naïve Th cells are stimulated, TCR signalling in the presence of IL-4 results in the activation of GATA-3 via STAT6 (Takeda *et al.*, 1996). GATA-3 then regulates the expression of IL-4, IL-5 and IL-13 (Ouyang *et al.*, 2000). In committed Th2-type cells expression of IL-4 can also involve c-MAF (Ho *et al.*, 1996). In contrast, STAT1 and T-bet activation are important for IFN-γ expression (Szabo *et al.*, 2000; Afkarian *et al.*, 2002). In committed Th1-type cells activation of NFAT during TCR signalling or IL-12 activation of STAT-4 can result in IFN-γ production (Yang *et al.*, 1999; Afkarian *et al.*, 2002). Studies have shown that the level of TCR signalling between APC and T cells can influence which intracellular pathway is activated leading to either dominant Th cell IL-4 or IFN-γ

production (Constant and Bottomly, 1997). Using APL with different binding affinities, Brogdon and colleagues found that weak TCR interactions lead to increased activation of NFATc which localises to the nucleus and increases IL-4 expression (Brogdon *et al.*, 2002). In contrast, strong interactions lead to the activation of NFATp, which causes decreased GATA-3 activity thereby inhibiting expression of IL-4. Increased IFN-γ production following strong TCR interactions has been demonstrated to be due to increased activity of Th1-inducing MAP kinases including ERK, JNK and p38 (Badou *et al.*, 2001). Hence in the present study dominant T cell IFN-γ production observed at high allergen concentration in comparison to low is presumably due to strong TCR signalling resulting from increased ligand density and the numbers of engaging TCR. To elucidate whether differences in allergen concentration do affect intracellular pathways involved in cytokine production, levels of the transcription factors and kinases mentioned above could be measured in allergen-stimulated Th cells. If increased ligand density was responsible for increased IFN-γ production at high allergen concentration in this system, strategies for increased allergen presentation by APC could be employed to enhance allergen-specific Th1-type cell differentiation during SIT. For example administration of allergen peptides using VLPs allows for the delivery of up to 300 copies of an allergen epitope per VLP to APC (Roth, 2000). Potentially this could result in increased APC expression of MHC molecules containing allergen peptides.

The observation that high allergen concentration stimulation of allergic donor PBMC also resulted in the expansion of a population of IFN-γ-producing $CD8^+$ T cells was unexpected as $CD8^+$ T cell responses are usually directed against endogenous antigens such as viruses and intracellular bacteria. However exogenous antigen taken up by APC at high concentration may enter the endogenous pathway for processing and

presentation of peptides by MHC class I (Heath and Carbone, 2001). This process is known as cross-priming. Maecker and colleagues have investigated the ability of human DC to cross-prime $CD8^+$ T cells and the effect antigen concentration has on this process (Maecker *et al.*, 2001b). Stimulation of whole blood from cytomegalovirus (CMV)-seropositive patients with DC in the presence of different concentrations of exogenous CMV antigens resulted in increased $CD8^+IFN\text{-}\gamma^+$ T cells at high concentration in comparison to low indicating that high antigen dose may aid cross-priming. In this thesis the relative numbers of $CD8^+$ T cells present in high allergen concentration cultures at day 14 were low in comparison to $CD4^+$ T cell numbers. Nevertheless the observation that the $CD8^+$ T cells produced IFN-γ after fewer cell divisions than the $CD4^+$ T cells may reflect a role for $CD8^+$ T cell responses in the increased Th1-type differentiation observed at high allergen concentration. It would be of great interest to study novel approaches to enhance this type of allergen-specific $CD8^+$ T cell response during SIT. For example modified viruses such as vaccinia can be used to deliver genes encoding exogenous antigens such as allergen epitopes into APC (Di Nicola *et al.*, 1998). Virus infected APC can then present endogenously synthesised viral antigens, including the sequences of interest, *in vivo* to $CD8^+$ T cells via direct presentation (Norbury *et al.*, 2002). The use of this delivery system in humans has been demonstrated to be safe with no severe or serious adverse events in subjects receiving a modified vaccinia virus Ankara malaria vaccine (Moorthy *et al.*, 2003). Another strategy could employ the use of an inactive Shiga-like toxin 1 from *Escherichia coli* to carry allergen peptide epitopes into the MHC class I pathway in APC for improved presentation to $CD8^+$ T cells (Noakes *et al.*, 1999).

$CD4^+$ and $CD8^+$ T cell responses to foreign and self-antigens can be down-regulated by peripheral T cell deletion. An immune response to a foreign antigen usually terminates

with antigen removal from the body resulting in the elimination of effector T cells by apoptosis (Seder and Ahmed, 2003). Studies focussing on induction of oral tolerance in murine models of autoimmunity, have shown that oral administration of high-dose, soluble antigen *in vivo* can induce deletion of antigen-specific T cells (Chen *et al.*, 1995; Harrison and Hafler, 2000). Usually this procedure results in both Th1- and Th2-type cell apoptosis. In the current project there was a higher level of Th2-type cell apoptosis after stimulation with high allergen concentrations in comparison to low, with no observed differences in Th1-type cell apoptosis. The mechanism for the selective apoptosis of these Th2-type cells is not known. As mentioned in Chapter 4, Fas/FasL interactions could be playing a role, however Th1-type cells have been reported to be more susceptible to Fas-mediated apoptosis (Janssen *et al.*, 2000c). Other receptor/ligand interactions may also be involved, such as TNF/TNFR-I (Rathmell and Thompson, 2002). Nevertheless the administration of high allergen concentrations during SIT to induce Th2-type cell apoptosis could lead to a down-regulation of allergen-induced immunopathology and enhanced treatment efficacy. Studies in oral tolerance (Chen *et al.*, 1995; Harrison and Hafler, 2000), indicate that this may be achieved by the oral delivery of high allergen doses during SIT. However in the clinical setting, oral allergen administration, in which allergen is immediately swallowed, has had mixed results with efficacy of this form of SIT only being marginal (Moller *et al.*, 1986; Mosbech *et al.*, 1987; Taudorf *et al.*, 1987; Giovane *et al.*, 1994; Oppenheimer *et al.*, 1994; Litwin *et al.*, 1997; Van Deusen *et al.*, 1997). In contrast, controlled clinical trials of sublingual allergen immunotherapy have demonstrated efficacy in rhinitis and asthma (Clavel *et al.*, 1998; Passalacqua *et al.*, 1998; Bousquet *et al.*, 1999; Passalacqua *et al.*, 1999; Pradalier *et al.*, 1999; Purello-D'Ambrosio *et al.*, 1999; Pajno *et al.*, 2000; Bahceciler *et al.*, 2001). Sublingual immunotherapy involves the administration of allergen in droplet or soluble tablet form underneath the tongue where it is held for 1-2

with antigen removal from the body resulting in the elimination of effector T cells by apoptosis (Seder and Ahmed, 2003). Studies focussing on induction of oral tolerance in murine models of autoimmunity, have shown that oral administration of high-dose, soluble antigen i*n vivo* can induce deletion of antigen-specific T cells (Chen *et al.*, 1995; Harrison and Hafler, 2000). Usually this procedure results in both Th1- and Th2-type cell apoptosis. In the current project there was a higher level of Th2-type cell apoptosis after stimulation with high allergen concentrations in comparison to low, with no observed differences in Th1-type cell apoptosis. The mechanism for the selective apoptosis of these Th2-type cells is not known. As mentioned in Chapter 4, Fas/FasL interactions could be playing a role, however Th1-type cells have been reported to be more susceptible to Fas-mediated apoptosis (Janssen *et al.*, 2000c). Other receptor/ligand interactions may also be involved, such as TNF/TNFR-1 (Rathmell and Thompson, 2002). Nevertheless the administration of high allergen concentrations during SIT to induce Th2-type cell apoptosis could lead to a down-regulation of allergen-induced immunopathology and enhanced treatment efficacy. Studies in oral tolerance (Chen *et al.*, 1995; Harrison and Hafler, 2000), indicate that this may be achieved by the oral delivery of high allergen doses during SIT. However in the clinical setting, oral allergen administration, in which allergen is immediately swallowed, has had mixed results with efficacy of this form of SIT only being marginal (Moller *et al.*, 1986: Mosbech *et al.*, 1987; Taudorf *et al.*, 1987; Giovane *et al.*, 1994; Oppenheimer *et al.*, 1994; Litwin *et al.*, 1997; Van Deusen *et al.*, 1997). In contrast, controlled clinical trials of sublingual allergen immunotherapy have demonstrated efficacy in rhinitis and asthma (Clavel *et al.*, 1998; Passalacqua *et al.*, 1998; Bousquet *et al.*, 1999; Passalacqua *et al.*, 1999; Pradalier *et al.*, 1999; Purello-D'Ambrosio *et al.*, 1999; Pajno *et al.*, 2000; Bahceciler *et al.*, 2001). Sublingual immunotherapy involves the administration of allergen in droplet or soluble tablet form underneath the tongue where it is held for 1-2

minutes and then swallowed or spat out. At the T cell level, grass pollen sublingual SIT has been found to cause a reduction in allergen-specific T cell proliferation (Fanta *et al.*, 1999). This result is consistent with the induction of allergen-specific T cell deletion and/or anergy. Although this route of allergen administration for SIT is showing therapeutic benefit, more research is required to define the precise immunological mechanisms and long-term efficacy of sublingual SIT. In addition dose-ranging studies are required to determine the optimal allergen dose for effective sublingual allergen immunotherapy.

7.3 REGULATORY T CELLS IN ALLERGEN-SPECIFIC IMMUNOTHERAPY

Studies in mouse models of autoimmune disease and oral tolerance have demonstrated that T-cell-mediated suppression is another mechanism in addition to deletion and anergy involved in the regulation of peripheral effector T cell responses (Maloy and Powrie, 2001; Weiner, 2001). Although a number of T regulatory cell subsets have been identified, information concerning their origin and their precise mechanisms for mediating suppression requires further examination. However evidence is accumulating for the therapeutic benefit for allergy of the enhancement of T regulatory cell function.

$CD4^{+}CD25^{+}$ regulatory T cells are involved in the prevention of autoimmune disease and allograft rejection (McHugh and Shevach, 2002). In mice and human studies, these cells have been shown to display an anergy phenotype, require IL-2 for growth and can inhibit the proliferation of peripheral T cells (Sakaguchi *et al.*, 1995; Suri-Payer *et al.*, 1998; Stephens *et al.*, 2001; Taams *et al.*, 2001). Although *in vitro* $CD4^{+}CD25^{+}$ T cells mediate suppression through cell to cell contact, the exact mechanism *in vivo* is

controversial. In some cases the involvement of signalling via CTLA-4, IL-10 or TGF-β has been demonstrated (Maloy and Powrie, 2001; McHugh and Shevach, 2002). The inhibitory function of $CD4^+CD25^+$ T cells, *in vitro*, can be overcome in the presence of IL-2 (Takahashi *et al.*, 1998). The two other regulatory T cell populations described include Tr1 cells producing large amounts of IL-10 and moderate amounts of TGF-β, and Th3 cells primarily producing high amounts of TGF-β. Tr1 cells can protect against Th1-mediated colitis induced by transfer of naïve $CD45RB^{hi}$ cells into SCID mice and inhibit Th2 immunopathology in immediate hypersensitivity (Groux *et al.*, 1997; Cottrez *et al.*, 2000). Th3 cells are involved in the development of oral tolerance in autoimmune disease models (Weiner, 2001).

That increased IL-10 production by $CD4^+$ T cells expressing CD25 has been observed following SIT here and by others (Akdis *et al.*, 1998b; Francis *et al.*, 2003; Jutel *et al.*, 2003), leads to the question of whether these cells are functioning as Tr1 cells or $CD4^+CD25^+$ T regulatory cells. As the production of IL-10 has been shown to mediate the induction of allergen-specific T cell anergy and reduced cytokine production following immunotherapy (Akdis *et al.*, 1998b; Jutel *et al.*, 2003), these cells are possibly a Tr1 cell population. A recent report demonstrated that $CD4^+CD25^+$ T regulatory cells are present in most atopic individuals (Bellinghausen *et al.*, 2003). They are capable of suppressing T cell proliferation and production of IL-4, IL-5 and IFN-γ. However suppression in this case was not mediated by IL-10, TGF-β or CTLA-4. The cell population was also observed in non-atopic controls, suggesting that these cells alone may not be sufficient for suppression of the clinical symptoms of allergy. Nonetheless the induction of Tr1 cell or $CD4^+CD25^+$ T cell activity during SIT could improve this treatment for allergic disease. Interestingly, vitamin D3 and

dexamethasone have been shown to induce differentiation of human naive $CD4^+$ T cells into IL-10-producing regulatory T cells (Barrat *et al.*, 2002). Therefore perhaps combining these immunosuppressive drugs with high dose allergen for administration during SIT could lead to improved treatment efficacy. Recently, interactions between ICOS, expressed by T cells, with ICOSL expressed by APC have been reported to promote T cell IL-10 production (Witsch *et al.*, 2002). Furthermore, ICOS/ICOSL interactions between DC and T cells in the presence of IL-10 can induce the differentiation of Tr1 cells (Akbari *et al.*, 2002). Hence a future treatment strategy could involve the *in vitro* manipulation of autologous DC to express ICOSL and IL-10. These DC could then be pulsed with allergen and infused back into the allergic patient. The aim of this strategy would be to induce an allergen-specific T regulatory cell population *in vivo* that could suppress ongoing pathogenic allergen-specific Th2-responses.

7.4 T CELL TRAFFICKING IN ALLERGEN-SPECIFIC IMMUNOTHERAPY

Allergen immunotherapy also needs to promote the trafficking of allergen-specific Th1-type cells and T regulatory cells to sites of allergen encounter within the periphery where they can 'switch off' allergic inflammation. Information on the surface molecules involved and the movement of T cells throughout the body following antigen administration *in vivo* can be gained from experiments in mouse models.

A recent report describes the trafficking of TCR transgenic T cells specific for viral haemagglutinin transferred into normal BALB/c mice following intranasal administration of influenza virus (Roman *et al.*, 2002). The naïve $CD4^+$ T cell response was found to be initiated in the draining lymph nodes and result in the expansion of effector $CD4^+$ T cells. A subset of the dividing Th cells that expressed low levels of

CD62L, high levels of CD49d and transient expression of CCR5 were subsequently recruited to the lung. These cells produced predominantly IFN-γ. In the current study increased numbers of $CD4^+$ T cells expressing either $CD62L^-$, $CD49d^{hi}$ or $CCR5^+$ were found to produce IFN-γ following stimulation with high allergen concentration in comparison to low concentration. These results indicate that Th1-type cells generated at high allergen concentration have the potential to traffic into peripheral tissues. Consistent with this notion are studies that have demonstrated increased numbers of IFN-γ $mRNA^+$ cells in nasal and skin biopsies following allergen-specific immunotherapy (Varney *et al.*, 1993; Durham *et al.*, 1996; Wachholz *et al.*, 2002). In the present study however, the increases in IFN-γ-producing T cell numbers expressing "peripheral tissue trafficking phenotype" induced at high allergen concentration *in vitro*, were not observed in patients receiving HDM immunotherapy. That this discrepancy was simply due to the timing of blood samples with respect to allergen injection should be examined. Another possible reason for this discrepancy is that the increase in T cell production of IL-10 following SIT may have down-regulated IFN-γ production by circulating Th1-type cells expressing a peripheral tissue trafficking phenotype.

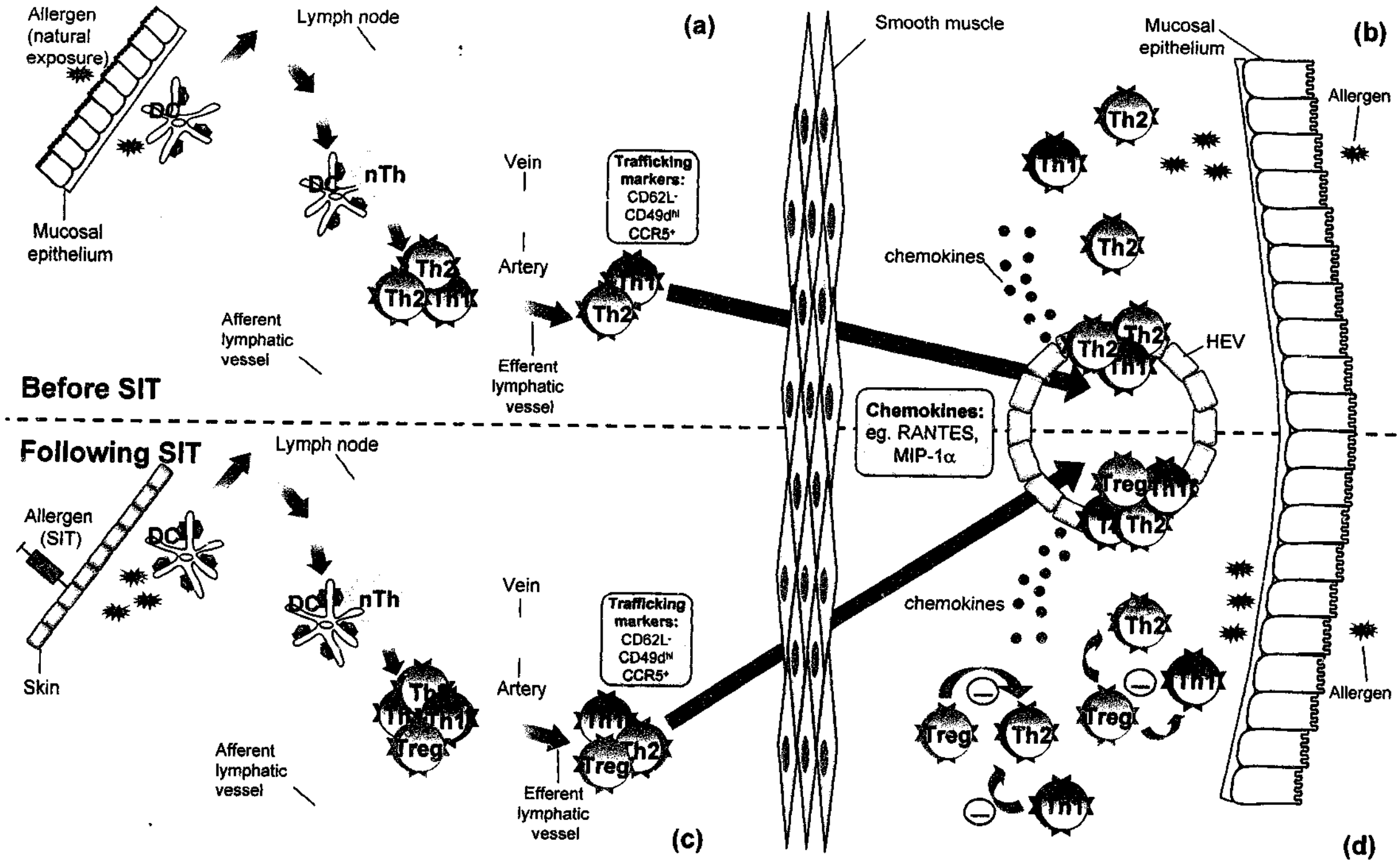
In the present study the IL-10-producing T cells induced during SIT were also shown to express a peripheral tissue trafficking phenotype i.e. $CD62L^-$, $CD49d^{hi}$ or $CCR5^+$. Thus these cells could potentially migrate to sites of allergen encounter in the periphery where the IL-10 production could down-regulate both Th2-induced allergic inflammation and Th1-induced pathology resulting from excess IFN-γ production (Del Prete *et al.*, 1993; Pierkes *et al.*, 1999; Cottrez *et al.*, 2000; Akdis and Blaser, 2001; Royer *et al.*, 2001). In support of $IL\text{-}10^+$ T cell migration to peripheral tissues during SIT, is the observation of increased numbers of IL-10 positive cells in allergen-induced

cutaneous late phase responses following wasp venom immunotherapy (Nasser *et al.*, 2001). Mucosal Tr1 cells identified in mice infected with *Bordetella pertussis* have been shown to express CCR5 (McGuirk *et al.*, 2002). It would be of great interest to further assess the homing and chemotactic migratory ability of T regulatory cells *in vivo* to determine how best to induce trafficking of these cells to sites of allergic inflammation.

Integrating the findings from these studies, a hypothetical model of SIT alteration of T cell trafficking is proposed in Figure 7.1. Upon natural allergen exposure at low concentrations, DC take up allergen and migrate to draining lymph nodes (Vermaelen *et al.*, 2001). Here DC initiate the activation and differentiation of predominantly IL-4-producing allergen-specific Th cells that express surface markers including $CD62L^{-}$, $CD49d^{hi}$ or $CCR5^{-}$ for recruitment into peripheral tissues. These T cells then enter the blood stream where they can be attracted to sites of allergen encounter by chemokines produced in the tissues. Eotaxin, RANTES, MIP-1α can be secreted in the tissues following allergen provocation (Cruikshank *et al.*, 1995; Holgate *et al.*, 1997; Lilly *et al.*, 2001). After entry into the tissues, these IL-4-producing T cells can mediate Th2-induced allergic inflammation (Figure 1.1). In contrast, upon subcutaneous administration of high allergen doses, DC migrate to draining lymph nodes and induce expansion of allergen-specific IFN-γ- and IL-10-producing T cells that express 'peripheral tissue trafficking' surface markers. These cells can then similarly be recruited into peripheral tissues and through either IFN-γ or IL-10 production down-regulate Th2-mediated inflammation (Pene *et al.*, 1988; Akdis and Blaser, 1999; Cohn *et al.*, 1999; Pierkes *et al.*, 1999; Akdis and Blaser, 2001; Huang *et al.*, 2001; Royer *et al.*, 2001; Elser *et al.*, 2002).

Figure 7.1 Migration of allergen-specific T cells in an allergic individual before and following SIT.

(a) Upon natural allergen exposure at low concentrations, DC take up the allergen usually at mucosal surfaces and migrate to draining lymph nodes. Here DC activate and differentiate naïve T cells to become predominantly IL-4-producing allergen-specific Th cells. These Th2-type cells express surface markers including $CD62L^{-}$, $CD49d^{hi}$ and $CCR5^{+}$ for recruitment into peripheral tissues. As such the cells can leave the lymph node via the efferent lymph node and eventually enter the bloodstream. **(b)** The Th2-type cells are then attracted to sites of allergen encounter by chemokines such as RANTES and MIP-1α, which are produced in the tissues following allergen provocation. The Th2-type cells can leave the bloodstream via activated high endothelial venules (HEV) and mediate Th2-induced allergic inflammation. **(c)** Upon subcutaneous administration of high dose allergen during SIT, DC migrate to draining lymph nodes and induce predominate expansion of IFN-γ- and IL-10-producing T cells that express 'peripheral trafficking' surface markers. These Th1-type and T regulatory cells (Treg), can then leave the lymph node via the efferent lymph node and eventually enter the bloodstream. **(d)** In response to chemokines produced in the tissues following allergen-encounter, the Th1-type and T regulatory cells can leave the blood stream via HEV and move into the tissues. Here through the production of IL-10 and IFN-γ, these cells can down-regulate Th2-mediated inflammation. The T regulatory cells can also down-regulate any Th1-mediated immunopathology resulting from excess IFN-γ production.


Allergen (natural exposure)
Mucosal epithelium
Lymph node
DC
nTh
Th2
Th2 Th1
Afferent lymphatic vessel
Before SIT
Following SIT
Allergen (SIT)
Skin
Lymph node
DC
nTh
Th
Th2 Th1
Treg
Afferent lymphatic vessel
(a)
Vein
Artery
Efferent lymphatic vessel
Trafficking markers:
CD62L-
CD49d^hi
CCR5+
Th1
Th2
Vein
Artery
Trafficking markers:
CD62L-
CD49d^N
CCR5+
Th1
Treg
Th2
Efferent lymphatic vessel
(c)
Smooth muscle
Chemokines:
eg. RANTES,
MIP-1α
(b)
Mucosal epithelium
Allergen
Th2
Th1
chemokines
HEV
Treg
Th1
Th2
chemokines
Th2
Th1
Treg
Th2
Th1
Allergen
(d)

In addition to the induction of allergen-specific Th1-type cells and Tr1 cells with peripheral trafficking capabilities, effective SIT needs to generate immunological memory of the response. Two types of memory cells have been identified on the basis of their migratory ability. Central memory T cells are clonally expanded, non-polarised memory cells that express CCR7 and CD62L and as such traffic to lymph nodes (Sallusto *et al.*, 2000). Effector memory T cells, consisting of all memory Th1-type, Th2-type and possibly Tr1 cells, express surface markers for peripheral tissue trafficking so that upon antigen encounter they can migrate quickly to sites of inflammation (Sallusto and Lanzavecchia, 2000). As effector memory T cells do not express CCR7, they can be distinguished from central memory T cells. Analysis of CCR7 expression in the 14-day HDM extract-stimulated cultures used in the present study would provide an indication of the induction of central and effector memory T cells at low and high allergen concentrations. Mechanisms for the induction of human allergen-specific effector memory Th1-type cells and long-lived Tr1 cells warrants significant investigation.

7.5 RATIONAL DESIGN OF HIGH ALLERGEN DOSE REGIMENS FOR EFFECTIVE IMMUNOTHERAPY

The results presented in this thesis indicate that increasing the dose of allergen administered during SIT will more effectively down-regulate allergen-specific Th2-type responses. A number of studies have provided evidence for administration of higher doses of allergen *in vivo* leading to improved efficacy of SIT (Golden *et al.*, 1981; Creticos *et al.*, 1989; Majchel *et al.*, 1992; Haugaard *et al.*, 1993; Ewbank *et al.*, 2003). Using standardised, partially purified HDM extract, Haugaard *et al.* assessed the efficacy of SIT in patients receiving maintenance doses of 0.7 and 7 μg Der p 1

(Haugaard *et al.*, 1993). Although the high dose was associated with a higher rate of side effects, it was more effective at reducing bronchial responsiveness upon inhalation of HDM extract than the lower dose. Increased efficacy of SIT has also been demonstrated with a hymenoptera venom maintenance dose of 100 μg in comparison to 50 μg (Golden *et al.*, 1981). In ragweed immunotherapy studies, high Amb a 1 maintenance doses of 12.4 and 24 μg compared with doses of 0.6 and 2 μg were more effective at reducing histamine release, medication scores and clinical symptoms (Creticos *et al.*, 1989; Majchel *et al.*, 1992). Ewbank *et al.* examined other immunological parameters following subcutaneous standardised cat extract SIT with maintenance doses of 0.6 μg, 3 μg or 15 μg Fel d 1 (Ewbank *et al.*, 2003). Significant dose-dependent decreases in skin reactivity to cat hair extract were seen. Allergen-specific IgG_4 levels increased with increasing Fel d 1 concentration and only at 15 μg Fel d 1 was a significant decrease in $CD4^+IL\text{-}4^+$ T cells detected. These observations point towards the design of allergen preparations with reduced or ablated IgE-binding to permit safe delivery of high allergen concentrations and more effective SIT.

Allergen preparations consisting of peptides encoding for dominant T cell epitopes are an attractive alternative to whole extracts. As small peptides cannot cross-link surface bound IgE on mast cells and basophils, peptide preparations can be given more safely at higher doses than whole extracts (Muller *et al.*, 1998). In some cases, peptide administration has resulted in adverse reactions usually mediated by T cell cytokines, however this can be overcome with careful up-dosing (Oldfield *et al.*, 2002). When designing peptide immunotherapy preparations, an important consideration is the MHC binding-restriction of the epitope peptide chosen. The selection of promiscuous T cell epitopes would allow for peptide presentation by more MHC haplotypes (Sette and

Sidney, 1998; Southwood *et al.*, 1998). Presently, peptide SIT has been shown to be effective in inducing allergen-specific hyporesponsiveness in bee venom and cat allergy (Muller *et al.*, 1998; Oldfield *et al.*, 2002). In addition to peptides, administration of APL may be beneficial in increasing SIT efficacy. APL, encoding T cell epitopes of allergens, can be designed to affect interactions between the MHC and TCR. APL that result in strong TCR-peptide-MHC interactions can induce predominant differentiation of naïve T cells into IFN-γ-producing T cells (Pfeiffer *et al.*, 1995). In addition antagonist APL have been shown to block allergen-specific T cell proliferation (Fasler *et al.*, 1998; Verhoef and Lamb, 2000). However, to date APL have not been assessed in clinically controlled human trials and care should be taken as an APL could potentially activate a T cell population that is cross-reactive with a native allergenic peptide, thus leading to a worsening of allergic disease.

Mutant allergens with ablated IgE-binding sites and retained dominant T cell epitopes could also be given more safely at higher doses than preparations currently administered in conventional SIT. Mutant allergen preparations have an advantage over individual peptides, as they provide numerous T cell epitopes for targeting a greater number of reactive T cells. In addition, mutant allergens with ablated IgE-binding sites are more likely to be taken up phagocytosis or endocytosis by APC instead of by IgE-facilitated uptake. Predominant Th2-type responses have been shown to result from IgE-facilitated uptake of natural allergen by APC (Akdis *et al.*, 1998a).

In addition to altering allergen form for safer SIT preparations, allergens can be incorporated into vehicles for more effective delivery to the immune system. .As mentioned in Chapter 1, liposomes are non-toxic, biodegradable lipid vesicles that can incorporate allergen in such a way that IgE-binding is reduced (Sehra *et al.*, 1998b).

Thus high doses of allergen entrapped in liposomes can be delivered without the induction of IgE-mediated side effects. Recently in a 12 month human trial, a preparation consisting of HDM encapsulated in liposomes was found to be effective and safe for the treatment of allergy-induced asthma (Basomba *et al.*, 2002). In this study T cell changes were not analysed thus further investigation is required to determine the full potential of liposomes in high allergen dose delivery for SIT. However, Li and colleagues have demonstrated that antigenic peptide co-encapsulated with CpG-ODN induced strong IFN-γ production by $CD8^+$ T cells in mice (Li *et al.*, 2003). Thus a vaccine approach incorporating allergen peptide and CpG-ODN into liposomes may also enhance the effectiveness of SIT.

7.6 CONCLUSION

This study has confirmed that high allergen concentration is an important factor that can enhance allergen-specific Th1-type responses. High allergen concentration was found to promote expansion of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells, to enhance programmed cell death of Th2-type cells and to induce anergy. Moreover, high dose administration of allergen *in vivo* was shown to lead to the induction of an IL-10-producing T cell population that exhibits a peripheral trafficking phenotype. These cells were found to co-express CD25 consistent with a regulatory T cell phenotype. The results from the *in vitro* and *in vivo* experiments suggest that SIT leads to immune deviation towards IFN-γ predominant as well as allergen-specific tolerance through the induction of IL-10-producing regulatory T cells. Thus the use of peptide, hypoallergenic mutant or liposome-encapsulated allergen preparations that can be administered safely at higher doses than natural extracts will enhance the efficacy of this treatment.

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

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Abstract

Background: During clinically effective allergen-specific immunotherapy a shift in cytokine dominance from IL-4, IL-5 predominant to IFN-γ 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-γ, proliferation and apoptosis. ***Methods:*** HDM-allergic donor PBMC were cultured for 14 days with different concentrations of HDM extract (1, 10 and 100 μg/ml). T cell intracellular IL-4 and IFN-γ, division (CFSE labelling) and apoptosis (active caspase-3 staining) were analysed by flow cytometry. Proliferation was assessed by ^{3}H-thymidine incorporation. ***Results:*** Increased CD4+IFN-γ+ and CD8+IFN-γ+ 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 favours the expansion of IFN-γ-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-γ 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-γ 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-γ 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-γ 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-γ-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 (2.5×10^6/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 μg/ml) in a final volume 2 ml/well for 7 days at 37°C in a 5% CO_2 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 (1×10^6 cells/ml) were restimulated with HDM extract (1, 10 or 100 μg/ml) in the presence of irradiated (3,000 rad) autologous PBMC (1×10^6/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 (2.5×10^5 cells/well) in a 96-well U bottom plate with immobilised anti-CD3 (OKT3; 10 μg/ml) and rIL-2 (100 U/ml) in a final volume of 200 μl/well for 6 h at 37°C in the presence of 10 μg/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 $IgG_{1,\kappa}$-FITC and $IgG_{1,\kappa}$-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-γ-APC (Pharmingen) or isotype control antibodies (mouse $IgG_{1,\kappa}$-PE and $IgG_{1,\kappa}$-APC; Pharmingen). Percentages of cytokine-positive CD4+ and CD8+ T cells were determined using a FACScaliber flow cytom-

cter (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 μ*M* 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Oregon) to analyse cell division. For this, harvested cells (1×10^7 cells/ml) were incubated with 1 μ*M* 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 (5×10^4/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}H-thymidine (Amersham Biosciences, UK) and harvested 16 h later. Proliferation as correlated with ^{3}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 (5×10^4/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 (5×10^4/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_2 humidified incubator, pulsed for the last 16 h with ^{3}H-thymidine (1 μCi/well) and harvested onto printed glass fibre filters with a 96-well automatic cell harvester. ^{3}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 (7.5×10^5/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_2. 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 10×10^5

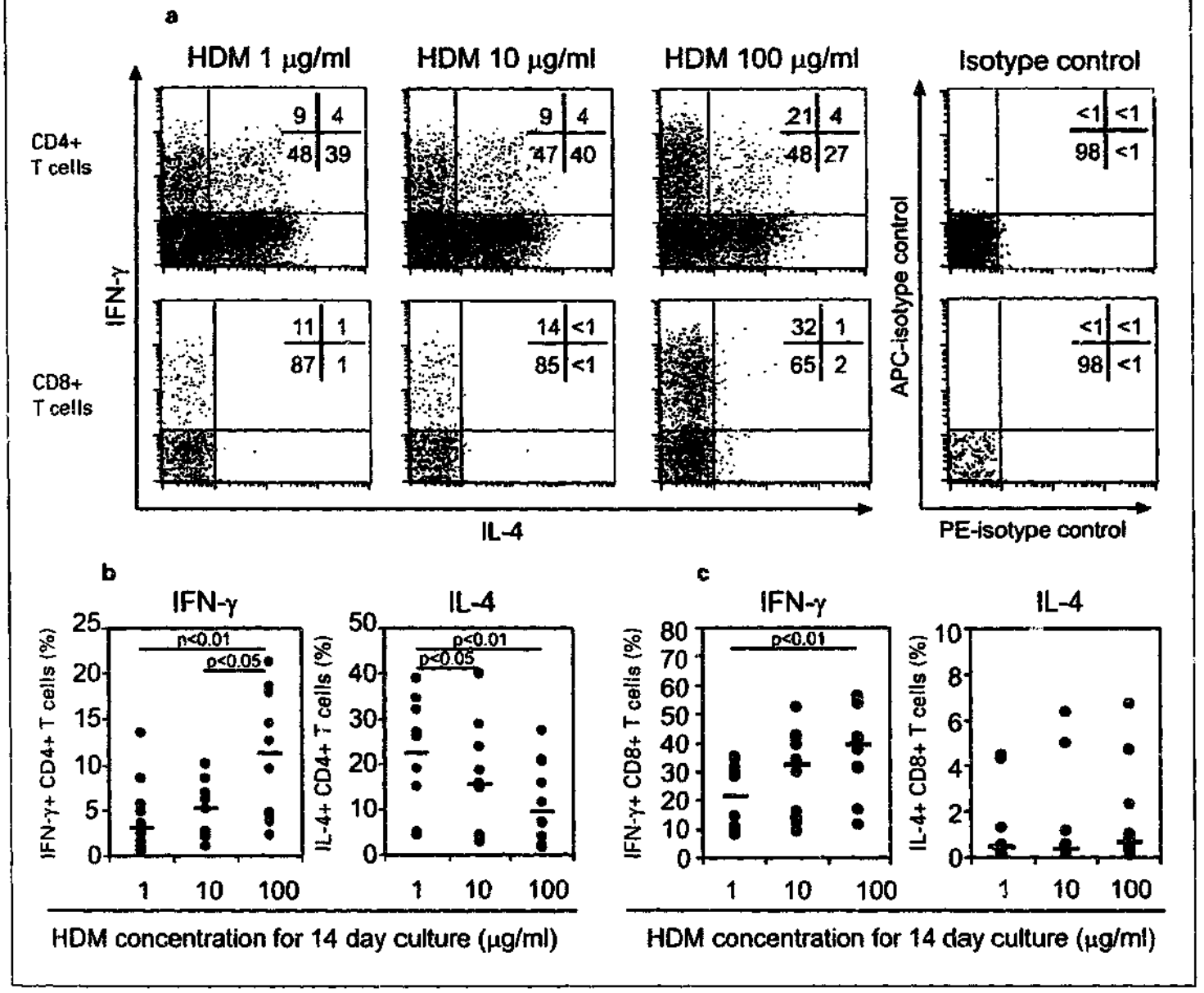

Fig. 1. The effect of allergen concentration on CD4+ and CD8+ T cell cytokine profiles. PBMC from 10 HDM-allergic donors were cultured with HDM extract (1, 10 and 100 µg/ml) for 14 days, stimulated with anti-CD3/IL-2 in the presence of Brefeldin A for 6 h and analysed by flow cytometry following labelling 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 8.4×10^5 respectively) in comparison to 1 μg/ml ($p < 0.05$) (median values 2.7×10^5 and 3.3×10^5, 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 $11–16 \times 10^5$ for CD4+IL-4+ and $0.07–0.1 \times 10^5$ 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 μg/ml HDM extract for 4 days. Latex-specific T cells did not proliferate in the presence of 1, 10 or 100 μg/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-γ 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-γ 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 μg/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-γ Production and Cell Division Number

We next investigated the possible mechanisms involved in skewing cytokine production of CD4+ T cells towards IFN-γ 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-

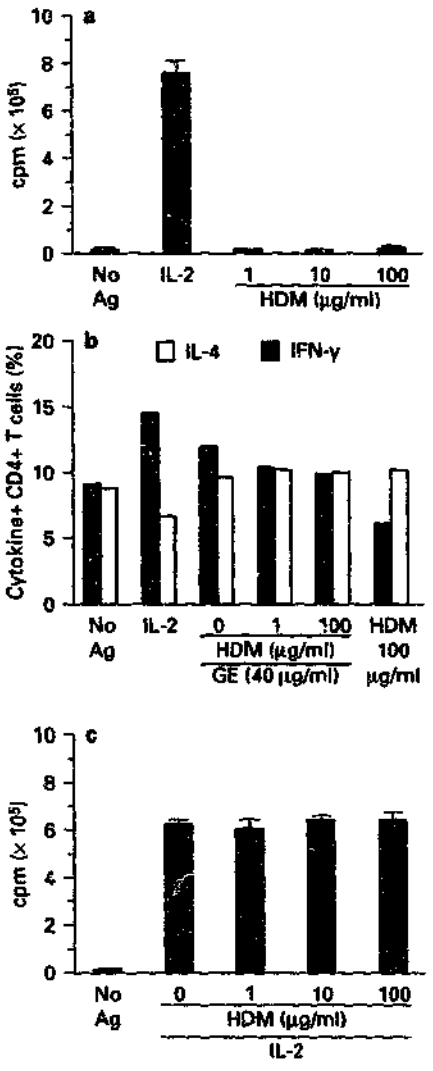

Fig. 2. Assessing HDM extract for toxicity and mitogenicity. Latex-specific T cells were cultured for three weeks in the presence of GE and irradiated PBMC as APC. **a** Mitogenicity of HDM extract was determined by culturing latex-specific T cells with APC and HDM extract at 1, 10 and 100 μg/ml. After 72 h, proliferation as correlated with ^{3}H-thymidine incorporation (cpm) was determined. **b** The ability of HDM extract to nonspecifically induce IL-4 and IFN-γ production was determined by culturing latex-specific T cells 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% CO2. 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 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-γ. **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 μg/ml. After 72 h, proliferation as correlated with ^{3}H-thymidine incorporation (cpm) was determined.

centration cultures (fig. 3c, d; $p < 0.05$ for division 6 in 100 μg/ml HDM extract-stimulated cultures in comparison to 1 μg/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 μg/ml HDM extract altered the relationship between CD4+IFN-γ+ T cell proportion and cell division interval in cultures (fig. 4c). CD4+IFN-γ+ T cell proportions in cultures stimulated with 100 μg/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-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 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-γ in HDM-Stimulated Cultures

To determine whether the relationship between CD8+ T cell division and IFN-γ 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-

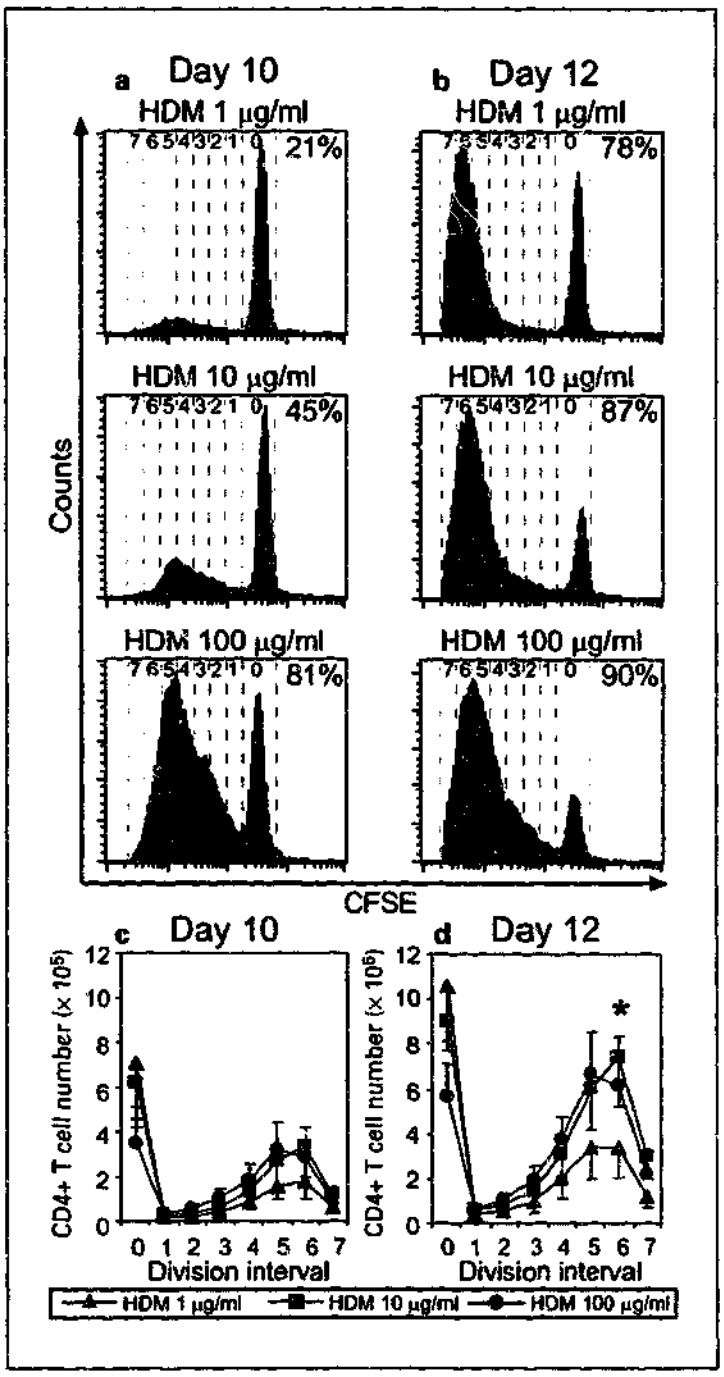

Fig. 3. CD4+ T cell division in allergen stimulated cultures. Seven day HDM extract-stimulated (1, 10 and 100 μg/ml) cultures were labelled with CFSE and restimulated with HDM extract and APC. Histograms of CFSE fluorescence gated on CD4+ T cells at days 10 (**a**) and 12 (**b**) are shown for one donor with the percentage of cells that have undergone 1 or more divisions shown in the top right hand corner of each plot and the dotted lines representing division intervals. These intervals were determined from the anti-CD3 (10 μg/ml) and rIL-2 (100 U/ml) positive control. Performing this experiment on six HDM-allergic donors revealed increased division of CD4+ T cells in the higher HDM concentration cultures in comparison to the 1 μg/ml HDM extract-stimulated cultures at day 10 (**c**) and 12 (**d**). This increase reached statistical significance when the 100 μg/ml HDM extract-stimulated cultures were compared to the 1 μg/ml HDM extract-stimulated cultures at division 6 on day 12 (* $p < 0.05$). The mean number of CD4+ T cells and standard error for six HDM-allergic donors are shown.

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 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

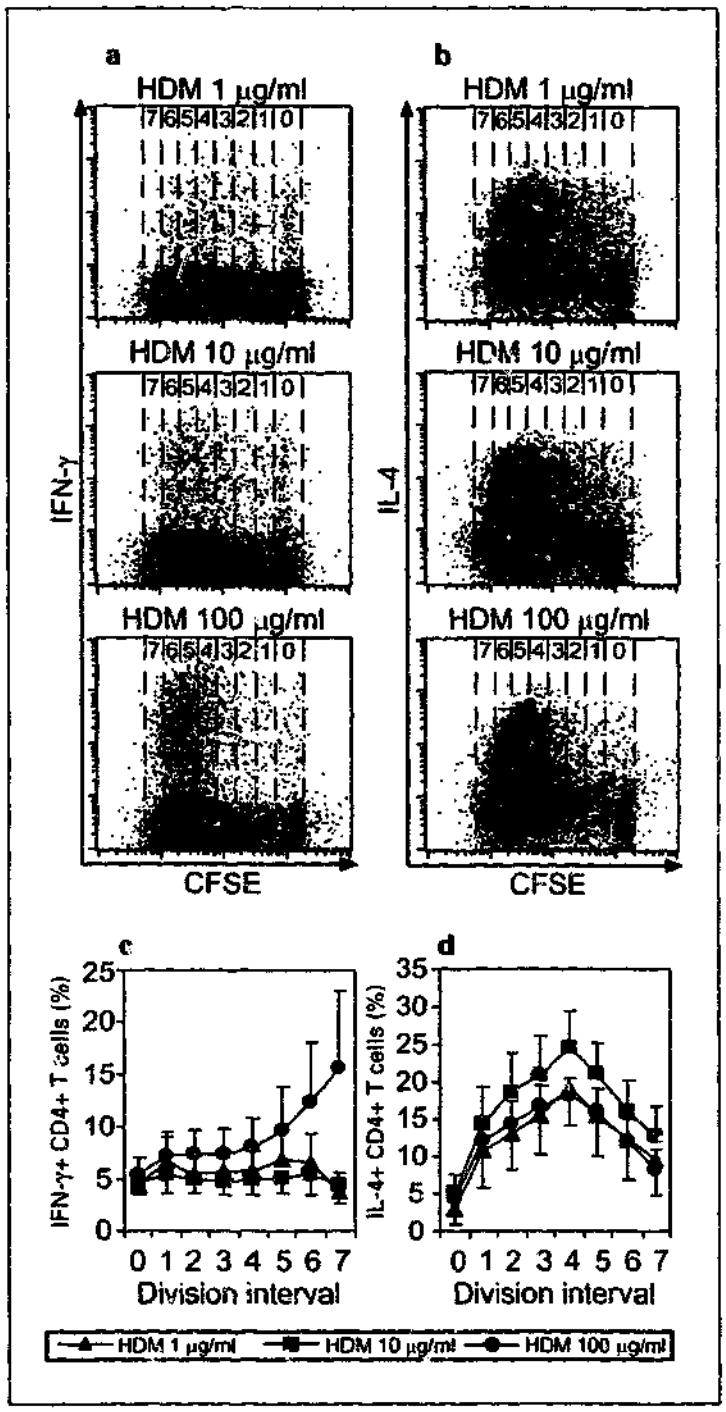

Fig. 4. High allergen concentration alters the relationship between cell division and IFN-γ production but not IL-4 production. Seven day HDM extract-stimulated (1, 10 and 100 μg/ml) cultures were labelled with CFSE and restimulated with HDM extract and APC. On day 12, CFSE labelled cultures were stimulated 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.

did not alter proportions of active caspase-3 positive CD4+IFN-γ+ T cells which were low at all concentrations (fig. 6d). The percentage of active caspase-3 positive CD8+IFN-γ+ T cells was increased in cultures stimulated with HDM extract at 10 μg/ml (median value 10.4 %; $p < 0.05$) and 100 μg/ml (median value 9.9 %; not significant) in comparison to 1 μg/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 μg/ml HDM extract, cells were restimulated with an immunogenic concentration of HDM extract (10 μg/ml) or IL-2 (100 U/ml) (fig. 7). Pretreatment with 100 μg/ml HDM extract resulted in significantly lower proliferative response to 10 μg/ml HDM extract compared to pretreatment with 1 μg/ml ($p < 0.01$) or 10 μg/ml ($p < 0.05$) HDM extract. Cells pretreated with 1, 10 and 100 μg/ml HDM extract showed similar proliferative responses to IL-2.

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

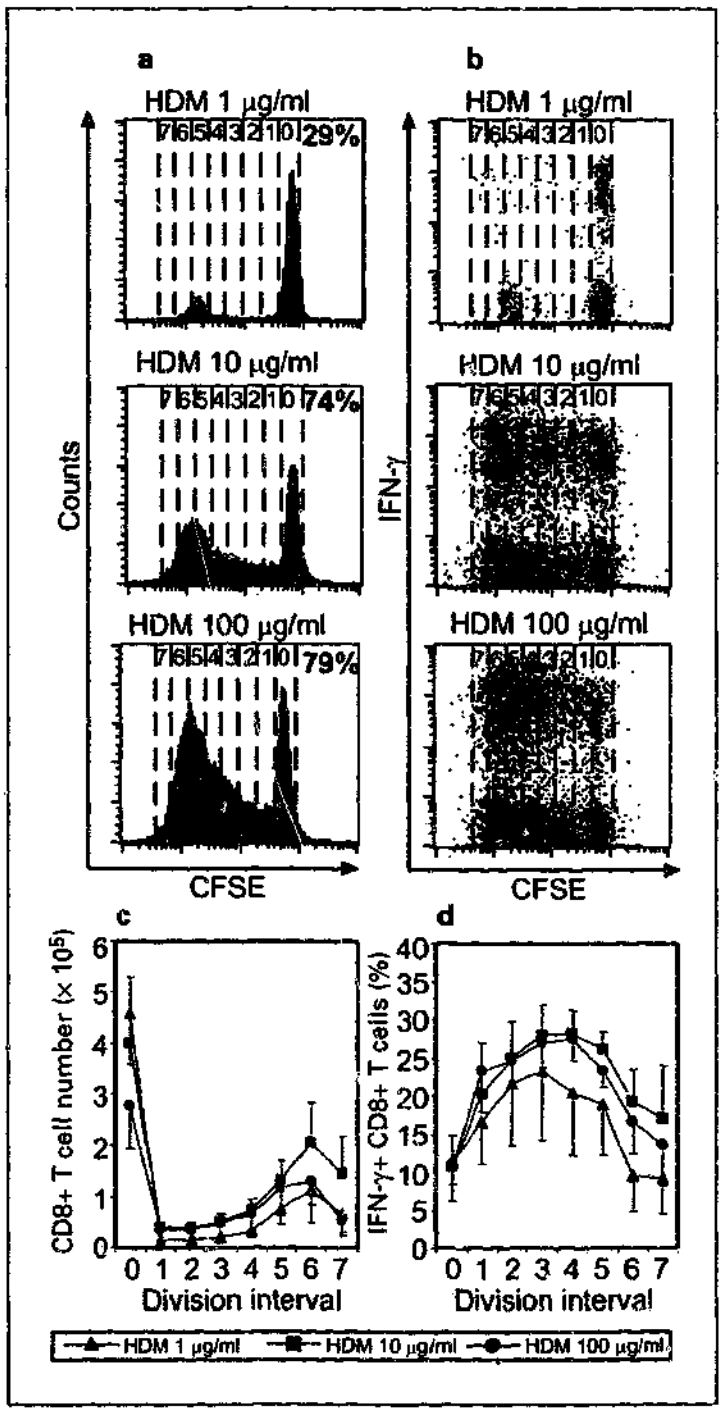

Fig. 5. CD8+ T cells divide and produce IFN-γ in HDM-stimulated cultures. PBMC, cultured with HDM extract (1, 10 and 100 μg/ml) for 7 days, were harvested, labelled with CFSE and restimulated with HDM extract (1, 10 and 100 μg/ml) and APC. **a** Representative histograms of CFSE fluorescence gated on 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-γ were generated by gating on CD8+ T cells from cultured cells labelled with anti-CD8 and anti-IFN-γ 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 μg/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 μg/ml HDM-stimulated cultures in comparison to 1 μg/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-γ positive CD8+ T cells at each division interval in HDM-stimulated cultures for the 4 donors are graphed.

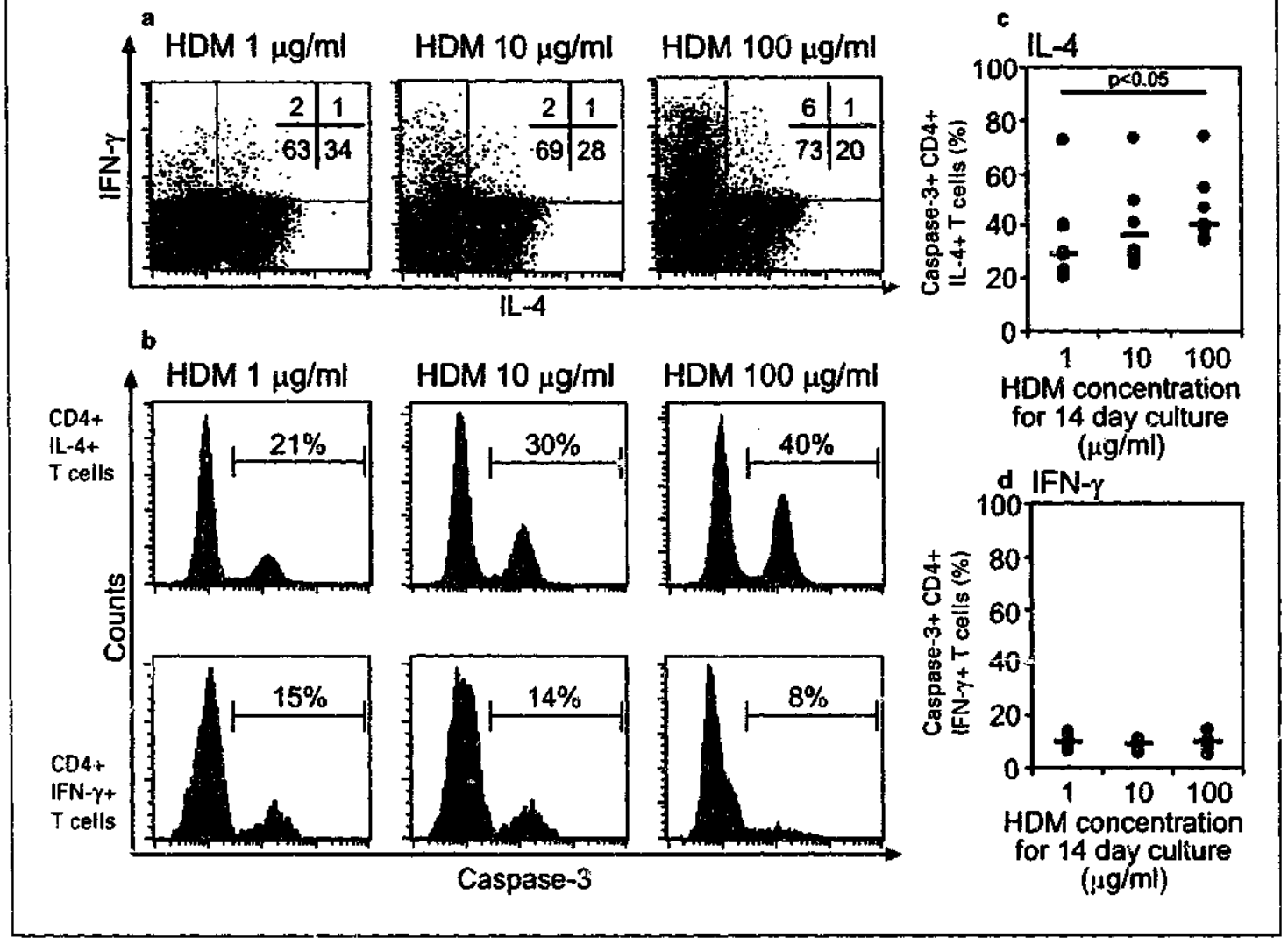

Fig. 6. The effect of allergen concentration on apoptosis of cytokine-positive CD4+ T cells. PBMC from 8 HDM-allergic donors were cultured with HDM extract (1, 10 and 100 µg/ml) for 14 days. Cultured cells were stimulated with anti-CD3/IL-2 in the presence of Brefeldin A for 6 h and analysed by flow cytometry following labelling with anti-CD4, anti-active caspase-3, anti-IL-4 and anti-IFN-γ. **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-γ+ 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 µg/ml HDM extract in comparison to 1 µg/ml HDM extract ($p < 0.05$). **d** The percentages of active caspase-3 positive CD4+IFN-γ+ 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 considerably 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

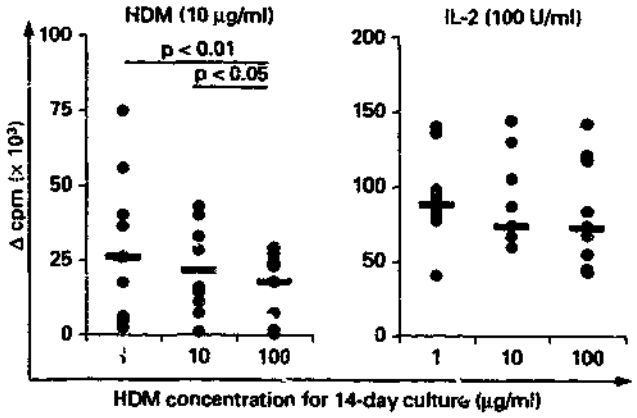

Fig. 7. Decreased proliferation of T cells pretreated with high allergen concentration upon restimulation with an immunogenic allergen concentration. PBMC from 10 HDM-allergic donors were cultured with 1, 10 and 100 µg/ml HDM extract for 14 days and restimulated in the presence of either 10 µg/ml HDM extract or IL-2 (100 U/ml). After 72 h, proliferation as correlated with ^{3}H-thymidine incorporation (Δ cpm) was determined. Cells pretreated with 100 µg/ml HDM extract had significantly decreased proliferation upon restimulation with an immunogenic concentration of allergen in comparison to cells pretreated with 1 µg/ml ($p < 0.01$) and 10 µg/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-γ+ and CD8+IFN-γ+ 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-γ 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-γ by allergen-specific T cells could lead to a reduction in allergen-induced pathology. In human B cells, IFN-γ inhibits IL-4-induced expression of the germline epsilon transcript and consequently prevents IgE production [27]. Via this mechanism IFN-γ, 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-γ reversed bronchial hyperresponsiveness and bronchoalveolar lavage eosinophilia [28]. Increased IFN-γ 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-γ 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-γ 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-α 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-γ 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-γ 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-γ-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-γ 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-γ produced by CD8+ T cells transferred into sensitised rats inhibited allergen-induced eosinophilic inflammation [42]. Of note, we observed IFN-γ production by CD8+ T cells after fewer cell divisions in comparison to CD4+ T cells suggesting that the CD8+ T cell IFN-γ 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-γ production by CD8+ T cells was shown to occur after TCR stimulation independently of cell division indicating that there are different thresholds for IFN-γ 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-γ production at high allergen concentration as seen for the CD4+ T cells. Although CD8+ T cell IFN-γ 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-γ 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-γ 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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T Cell Targeted Allergen Derivatives for Improved Efficacy and Safety of Specific Immunotherapy for Allergic Disease

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Abstract: Allergen-specific T cells play a pivotal role in initiating and regulating the immune response to allergens, with T cell targeted strategies showing promise for improved specific immunomodulation of the adverse immune response in allergic diseases. Atopic allergic individuals respond to allergen stimulation by dominant secretion of IL-4 and IL-5 (Th2-type cytokines) in contrast to non-atopic individuals where there is predominant IFN-γ secretion (Th1-type). Clinically effective, allergen-specific immunotherapy (SIT) is accompanied by altered allergen-specific T-cell response, notably cytokine changes of decreased IL-4 and IL-5 to IFN-γ ratio (Th2/Th1) and enhanced IL-10 secretion. Important contributing factors to these changes are likely to include the allergen concentration and form, adjuvants and antigen presenting cell type.

Current regimens for SIT using high dose unfractionated allergen extracts injected incrementally via the subcutaneous route are limited by IgE-mediated adverse events, especially in asthmatic patients. Allergen derivatives with retained T cell reactivity but abrogated IgE binding should have higher efficacy and safety. Such derivatives include peptides containing dominant T cell epitopes of allergens and chemically-modified or recombinant mutant allergen molecules. Both approaches have been evaluated successfully *in vivo* in animal models and limited clinical trials. Th1-inducing adjuvants including bacterial components or virus-like particles, and DNA vaccines may also promote repolarisation of cytokine secretion from Th2-type to Th1-type but caution is needed as excessive IFN-γ secretion may invoke exuberant pathogenic inflammation. Alternative routes for allergen administration including intranasal, oral and sublingual are also under evaluation. Full elucidation of the mechanisms underlying safer, more effective SIT should facilitate wider clinical application in the treatment of allergic diseases and the availability of reliable laboratory assays for monitoring SIT efficacy based on T cell function.

Keywords: Allergen, immunotherapy, Th1/Th2 polarisation, antigen presenting cell, adjuvant, T cell epitope, peptide, mutant allergen, DNA vaccine

INTRODUCTION

Administration of allergen to treat allergen-induced disease is in retrospect a surprising clinical approach to have been introduced early last century. However, with the growing appreciation of how antigen form and administration can influence the type of immune response, a rational basis for such allergen specific immunotherapy (SIT) is emerging. In the past few decades, well-controlled clinical trials have demonstrated efficacy of SIT in treatment of allergic diseases including rhinoconjunctivitis and asthma, and best practice protocols have been established [1]. Nevertheless, application of this potentially curative treatment is restricted, largely due to the risk of serious adverse events, largely IgE mediated, especially in asthmatics. Moreover, although efficacy of SIT is high for venom-induced allergy, success rates for the more common aeroallergen-induced allergic diseases are lower. Recent major advances in our understanding of regulation of immune responses point to refinements in allergen preparation and delivery for improved efficacy and safety of SIT.

In an allergic individual, allergen exposure, usually via the mucosal route, induces a Th2-polarised response characterised by dominant production of IL-4 and IL-5 cytokines, which drive production of allergen-specific IgE and activation of mast cells, basophils and eosinophils (Fig. 1). The inflammatory mediators released from these activated cells elicit the clinical symptoms of acute and chronic allergic disease. Following successful SIT, allergen-specific T cell proliferative responses are decreased and cytokine profiles shifted to a more Th1-polarised response, similar to that shown by non-allergic individuals [2]. In addition, there is also increased production of the immunoregulatory cytokine IL-10 [3]. These changes are seen early in the course of treatment by SIT and correlate with clinical improvement. In contrast, levels of allergen-specific IgE may decrease but only after some weeks or months of treatment. Thus, new strategies for refining SIT are based on targeting the allergen-specific T cell [2, 4]. By using hypoallergenic preparations that retain T cell reactivity, safety as well as efficacy of SIT will be improved. To achieve the most effective delivery of these preparations, knowledge of factors that influence the cytokine profile of an antigen-stimulated Th cell is required. In this review we discuss the potential role of antigen concentration, antigen

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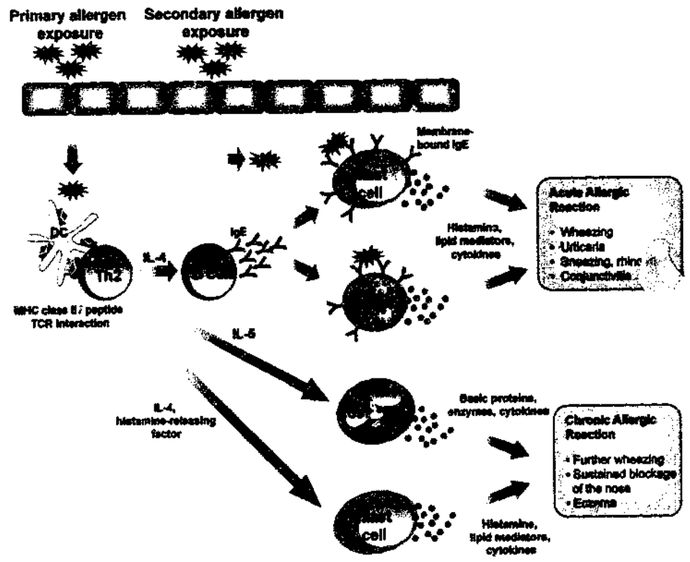

Fig. (1). The allergen-specific T helper cell plays a pivotal role in determining the clinical phenotype resulting from allergen encounter. In allergic individuals, the allergen stimulated T helper cell exhibits a Th2-polarised cytokine response with IL-4 driving IgE class switching by allergen-stimulated B cells and IL-5 promoting eosinophil migration and activation. Cross-linking of mast cell and basophil bound IgE by allergen on subsequent exposure triggers activation of these cells. The inflammatory mediators released from activated mast cells, basophils and eosinophils elicit the symptoms of acute and chronic allergic reactions.

form, adjuvant and antigen presenting cell (APC) type in modulating pathogenic immune responses to allergens, and consider the use of different allergen preparations and adjuvants for more effective, T cell targeted SIT.

1. FACTORS INFLUENCING THE IMMUNE RESPONSE TO ALLERGEN

1.1. Allergen Concentration

The concentration of antigen administered is well known to influence the degree and type of immune response. Therefore, it has been suggested that SIT may exert a beneficial effect on the pathogenic immune response to allergen simply by delivering antigen at a much higher concentration than encountered naturally. In the case of aeroallergens, the levels which are administered in standard subcutaneous SIT are estimated to be several logs higher than those normally inhaled [1, 5]. Several studies have shown that in the case of soluble antigens, a Th2-polarised response is generated by priming with low concentrations of antigen but Th1-polarised responses result at higher antigen concentrations [6]. However, few studies have examined the effect of antigen concentration on Th cell cytokine responses to allergens. In one *in vitro* study, Secrist and colleagues cultured freshly isolated atopic donor CD4+ T cells with different concentrations of rye grass pollen (0.01-10 μg/ml) or house dust mite (HDM; 0.02-20 μg/ml), and observed a dramatic decrease in IL-4 production at higher allergen concentrations [7]. Similarly in an *in vivo* model, when mice were sensitised with 10 μg/ml or 1000 μg/ml ovalbumin (OVA), IL-4 and IL-5, but not IFN-γ, were detected in the bronchoalveolar lavage fluid of low concentration immunised mice, whereas IFN-γ was detected in the fluid of high concentration immunised mice [8].

Carballido and colleagues generated T cell clones (TCC) specific for phospholipase A_2 (PLA_2), the major allergen of bee venom [9]. Analysis of cytokine production following stimulation of the TCC with different concentrations of anti-CD3, led these investigators to conclude that IFN-γ production by Th cells requires a higher threshold of stimulation than IL-4 production. With increased antigen concentration, it would be expected that there is an increased number of MHC-peptide complexes at the surface of the APC leading to multivalent cross-linking and increased TCR aggregation. From a study using a TCRαβ transgenic mouse model specific for a hemoglobin peptide, Grakoui and colleagues concluded that Th1 polarisation of both committed and naive T cells at high antigen concentrations was a result of activation of a critical number of cells to generate sufficient IFN-γ in the culture [10]. It is therefore, likely that the repeated administration of high doses of allergen in maintenance SIT will progressively favour expansion of Th1-type cells by increasing IFN-γ levels in the milieu. Indeed, using an *in vitro* model of SIT we have shown increased numbers of CD4+ IFN-γ+ T cells in 14 day peripheral blood mononuclear cell (PBMC) cultures

stimulated repeatedly with 100 μg/ml HDM extract compared with cultures stimulated with 1 or 10 μg/ml [11]. In addition, we observed development of T cell anergy and increased CD4+IL-4+ T cell apoptosis in these high concentration cultures. Consistent with these results is our previous finding of induction of anergy in HDM-specific TCC pretreated with a supraoptimal concentration of a T cell epitope peptide [12]. Thus, administration of high doses of allergen in SIT could cause a net shift in the allergen-specific Th cytokine profile by a combination of preferential expansion of IFN-γ+ T cells, T cell anergy and deletion of CD4+IL-4+ T cells. The use of non-IgE binding but T cell reactive allergen preparations, such as peptides and modified allergens will enable higher dose and therefore, more effective and safer allergen administration in SIT than currently possible with unfractionated extracts.

1.2. Allergen Form

When designing allergen preparations for more effective SIT, it is important to be aware that antigen form itself can affect Th cell differentiation. In addition by modifying allergen structure, the function of the molecule may be altered with consequent change in the immune response. Interestingly a large number of clinically relevant allergens are biologically active enzymes. The best analysed example is Der p 1, the major allergen of HDM. Der p 1 is a cysteine protease that normally plays a role in mite digestion. It is suggested that this enzyme function plays a role in mucosal epithelium penetration by disrupting tight junctions [13], and importantly it has been shown to promote Th2-type responses by its effects on immune cells. Enzymatically active Der p 1 reduces dendritic cell (DC) IL-12 production by cleaving CD40 [14] and stimulates mast cell and basophil IL-4 production [15] providing the cytokine milieu for Th2 differentiation. It also cleaves the α subunit of the IL-2 receptor (IL-2R, CD25) causing a dramatic reduction in anti-CD3-induced proliferation and IFN-γ secretion [16] but increased IL-4 production [17] by peripheral blood T cells. The enhanced IL-4 production promotes B cell IgE production [18]. Active Der p 1 also cleaves the low affinity IgE Fc receptor (CD23) from B cells further enhancing IgE production [19, 20]. A similar promotion of Th2 responses by enzymatically active Der p 1 was seen in murine studies with increased specific IgE [21] and decreased IFN-γ production [22]. Interestingly the bee venom allergen, PLA_2, also exhibits enzyme activity and has been shown to stimulate mast cell IL-4 production [15, 23].

The structural components of allergens can also influence initiation and exacerbation of allergic immune responses. For example, periodate oxidation of the major Japanese cedar pollen allergen, Cry j 1, to remove carbohydrates resulted in reduced proliferation and production of IL-4 and IL-5 by Cry j 1 stimulated PBMC, T cell lines (TCL) and TCC [24]. Specific IgE reactivity against the carbohydrate moiety has been shown for several glycosylated allergens [25, 26] and glycosylation-dependent T cell recognition of allergens is reported [24, 27, 28]. In fact one study suggests that CD8+ T cells may recognise a glycosylated antigen from *Parietaria judaica* pollen in an MHC-independent manner [28]. However for other allergens, e.g. PLA_2 [29] and Lol p 11 [30] sugar residues show little relevance to IgE binding. Thus it appears that the contribution of the carbohydrate moiety to allergenicity depends on the allergen studied, presumably due to the composition and structure of the sugar residues [30].

Importantly, allergen form can influence the mode of antigen uptake by APC and in this way may determine the APC type. As discussed above, many allergens are glycoproteins, and thus uptake via mannose receptors may occur. Mannose receptor mediated uptake of antigens by DC can result in a 100-fold increase in efficiency of antigen presentation to T cells compared with macropinocytosis (engulfment of large volumes of fluid and solutes) [31]. Increased antigen presentation might well alter subsequent Th cytokine response. Studies have shown that Der p 1 and yeast *Malassezia furfur* allergens can be taken up by APC by the mannose receptor [32, 33], while other allergens may be taken up by other mechanisms including macropinocytosis (soluble allergens) and phagocytosis (particulate allergens) [34, 35]. Whether T cell response to allergen differs depending on mode of uptake has not been assessed. With recent insight into the pivotal role of DC activation status and cytokine profile on Th differentiation, these issues should be resolved for refinement of the most effective SIT allergen preparations.

IgE-facilitated allergen uptake by DC via the low affinity IgE receptor CD23 is another mechanism by which allergen form could influence Th response. Preincubation of Der p 2 with HDM allergic donor serum resulted in proliferation of Der p 2-specific TCC at a 1000-fold lower concentration than Der p 2 without serum using EBV-transformed B cells as APC [36]. This process was dependent on Der p 2 specific IgE in the donor serum. Following SIT for birch pollen allergy, serum IgG was shown to inhibit IgE-mediated allergen presentation resulting in decreased proliferation and IL-4, IL-5, IL-10, and IFN-γ production by allergen-specific T cells [37].

Similarly, the structural integrity of an allergen can affect Th cell cytokine responses. When the tertiary structure of PLA_2 was disrupted by reduction and alkylation, there was preferential stimulation of IFN-γ production in 12-day PBMC cultures by this unrefolded form compared with IL-4 for the refolded form [38]. It was found that PLA_2 in its native form was processed and presented by monocytes and B cells, whereas the denatured form of PLA_2 was processed and presented only by monocytes. There was pronounced IL-12 production by monocytes as APC but undetectable levels for B cells, thus accounting for the observed differences in Th cytokine responses to the two PLA_2 forms.

In addition to being able to divert Th cytokine profiles towards Th1, modified forms of allergens offer the potential for safer SIT if this alteration removes IgE reactivity. An alternative strategy is to use short peptides based on immunodominant T cell epitopes. Both approaches have been tested and are under current evaluation in model systems or clinical trials as will be discussed later.

1.3. Antigen Presenting Cells

The APC can play a pivotal role in determining the T cell response to an antigen. DC are the most effective APC for the activation of naïve T cells. Within the respiratory

epithelium there is a contiguous network of DC, which functions as the 'first line of defence' in surveillance for inhaled antigens [39]. In the periphery, DC are generally in an immature state, specialised for antigen uptake. Interestingly, studies have shown that the state of maturity and location of the DC which presents antigen can influence Th cell differentiation. Stumbles *et al.* showed that when functionally immature rat respiratory tract DC were pulsed with OVA and adoptively transferred to naïve rats, splenic Th cell IL-4 production in response to subsequent OVA challenge was promoted, whereas DC matured by exposure to GM-CSF as APC induced increased T cell production of IFN-γ and undetectable IL-4 [40]. These cytokine differences were associated with altered production of IgG subclasses, with higher levels of IgG_1 OVA-specific antibodies in the case of immature DC and production of OVA-specific antibodies of all IgG subclasses with mature DC. In agreement with these results, Constant and colleagues showed strong IL-5 responses and little IFN-γ production in antigen-stimulated lung tissue cell cultures from mice immunised intranasally, whereas subcutaneous antigen delivery promoted preferential IFN-γ production [41]. Resident lung APC mediating T cell responses exhibited an immature DC surface marker phenotype. Codelivery of lipopolysaccharide with intranasal antigen resulted in a downregulation of IL-5 and IL-13 production with a slight increase in IFN-γ production resulting in a switch in net cytokine balance from Th2- to Th0-type. There was also an upregulation of IL-12 mRNA. The results from both these studies indicate that under steady-state conditions in the respiratory tract the default pathway is Th2 biased and additional signals from the microenvironment are required by the DC to induce Th1 responses.

Several subsets of DC have been described and may differentially regulate Th cell differentiation. Rissoan suggested that myeloid-derived DC, designated DC1, induce Th1 cell differentiation through the production of high levels of IL-12, whereas lymphoid DC, designated DC2, produce low levels of IL-12 and induce Th2 cell differentiation [42]. Human myeloid DC have been further subdivided based on expression of CD1a. CD1a+ monocyte-derived DC were shown to direct Th1 differentiation, whereas CD1a- DC promoted Th2 differentiation [43]. The CD1a- DC lacked IL-12 production even after treatment with the normally Th1-provoking agents anti-CD40, lipopolysaccharide and IFN-γ.

Although, DC are the most efficient APC for naïve T cells, B cells and monocytes may also present allergens to T cells *in vivo*. The conformation and concentration of an antigen can determine which APC will take up an antigen, process and present it to Th cells. As discussed previously, B cells and monocytes present different conformations of PLA_2 and consequently induce different Th cell cytokine profiles [38]. At low antigen concentration, antigen-specific B cells via their surface immunoglobulin antigen receptors are more efficient at antigen uptake than DC or monocytes by phagocytosis or endocytosis [7, 44]. Hence B cells may play an important role in the uptake of allergen *in vivo* upon natural exposure. Human and murine studies have shown that B cells as APC preferentially induce Th2 cell differentiation [7, 38, 45, 46]. In comparison, antigen presentation by monocytes has been shown to induce Th cytokine production preferentially [7, 38, 46].

The mechanisms responsible for B cells and monocytes driving different Th cell differentiation are not fully understood but are likely to involve differential expression of cytokines and costimulatory molecules upon interaction with the T cell. Akdis and colleagues have shown that monocyte induced-Th1 differentiation correlated with high levels of IL-12 production, whereas B cell induced-Th2 differentiation correlated with the inability of B cells to produce IL-12 [38]. Furthermore Skok and colleagues reported that OVA TCR transgenic T cells in spleen cell preparations showed increased numbers of IL-4 producing cells and decreased IFN-γ positive cells when stimulated by preactivated rather than resting B cells [47]. This response was blocked by addition of anti-IL-6 and anti-IL-10 monoclonal antibodies (mAb). Interestingly, the ability of the B cells to induce Th2 cell differentiation through the production of IL-6 and IL-10 was dependent on contact with IL-12 producing DC, suggesting a feedback loop. As discussed in the next section the expression of CD86 by an APC plays an important role in driving development of Th2 responses. B cells cultured with anti-CD86 mAb are unable to stimulate IL-4 production by antigen-specific CD4+ T cells [46]. In addition, B cell induced-IL-4 production by CD4+ T cells is also dependent on the interaction between CD40 on the B cell and CD40L on the T cell [46, 47]. Hence these studies indicate that, although different types of APC induce differential cytokine production from Th cells, there is a level of regulation of APC function that can determine the final outcome of Th cell differentiation.

1.4. Costimulation

Interactions between costimulatory molecules on the APC and Th cell are essential for Th cell activation in addition to specific engagement of the MHC class II-peptide complex with the T cell receptor. Important costimulatory molecules are CD80 (B7.1) and CD86 (B7.2) on the APC, which bind to CD28 on the T cell [48, 49], but other molecules also play a role in strengthening the cell interaction. Interestingly, Th cytokine response may be influenced by which costimulatory molecules are engaged in Th cell activation. Reports suggest that modulation of these interactions could be a strategy for control of allergic diseases.

The importance of B7/CD28 interactions has been studied in a number of murine models of airway hyperresponsiveness. Allergen-sensitised mice treated with CTLA-4Ig, a fusion protein that binds to CD28 and blocks the binding of CD80 and CD86, have reduced levels of airway hyperresponsiveness, decreased eosinophil recruitment, reduction in Th2 cytokine production (IL-4, IL-5 and IL-13) and reduced levels of allergen-specific IgE [50-54]. Similar results have been obtained in CD28 knockout mice [55]. Tsuyuki and colleagues found that blocking CD86 and not CD80 using mAbs gave comparable results to those seen using CTLA-4Ig [54]. These results suggested that CD86/CD28 interactions were essential for the development of Th2 responses. Interestingly Harris and colleagues showed that blockage of CD80 by Y100F-Ig, a CTLA-4-Ig

mutant that does not bind CD86 caused a reduction in eosinophil recruitment to the lungs but did not affect blood eosinophilia or the levels of Th2-dependent antibody [56]. In a more recent study by this group, Y100F-Ig was found to diminish IL-4 and IL-5 production by Th cells after intranasal challenge with antigen but not their ability to migrate and accumulate in the lung [57]. Hence, these T cells could traffic to the lungs upon challenge but once there they could not produce Th2 cytokines to induce eosinophil recruitment and antibody production.

The role of CD80 and CD86 costimulation in human allergic disease has also been studied. Reductions in allergen-specific proliferation and IL-5 and IL-13 production by PBMC from atopic individuals cultured with HDM extract in the presence of CTLA-4Ig and anti-CD86 mAb, but not anti-CD80 mAb, have been observed [58-60]. Allergen-induced proliferation of bronchoalveolar lavage T cells was also inhibited by CD86 mAb and not CD80 mAb [59], but secretion of IL-5 and IL-13 by allergen-stimulated asthmatic bronchial explants was inhibited by both CD80 and CD86 mAbs [60]. Hence, it seems that both CD80 and CD86 costimulation may be important in the development of the allergen-specific Th2-type response in humans, although CD86 is the principal costimulatory molecule in PBMC cultures.

Another member of the CD28 family that can also influence Th cell differentiation is ICOS (inducible T-cell co-stimulator). ICOS, expressed by T cells rapidly after TCR stimulation, interacts with ICOSL which is expressed by resting B cells, monocytes and some dendritic cells [61]. Interestingly, ICOS is upregulated on both Th1 and Th2 cells during the initial phase of differentiation but is then downregulated on Th1 cells, while remaining high on Th2 cells [61]. In murine studies of allergic airway disease, ICOS interaction has been shown to play an important role in the regulation of effector Th2 responses [62, 63]. Treatment of mice with ICOS-Ig, to block ICOS costimulation, during sensitisation and challenge resulted in reduced airway inflammation with decreased numbers of eosinophils and lymphocytes in the lung tissue [62]. In this model ICOS blockade did not prevent Th2 cell development, but greatly reduced effector Th2 cell cytokine production. Thus as ICOS costimulation is involved in sustaining Th2 responses during allergic responses, blockade of ICOS may be a useful therapeutic for allergic disease in humans.

Th cell differentiation may be affected by other costimulatory molecule interactions. Two recent reports have demonstrated that LFA-1/ICAM-1 and LFA-1/ICAM-2 interactions inhibit IL-4 and IL-5 production by naïve CD4+ T cells [64, 65]. Blocking either LFA-1/ICAM-1 or LFA-1/ICAM-2 with mAbs lead to a 15- to 40-fold increase in Th2 cytokine production and blocking both ICAM-1 and ICAM-2 lead to a 100- to 1000-fold increase [64]. Interactions between OX40L (a member of the tumor necrosis factor superfamily) on APC and OX40 on T cells can also differentially affect IL-4 and IFN-γ production [66, 67]. Stimulation of naïve CD4+ T cells with anti-OX40 mAb induced preferential IL-4 production and inhibited IL-12-induced IFN-γ production from CD4+ T cells. The above studies suggest that costimulatory molecule interaction with CD28 or OX40 on an allergen-stimulated Th cell promotes Th2 differentiation and suppresses Th1 differentiation, whereas interaction with LFA-1 supports Th1 responses and suppresses Th2 responses. However, recent reports show that these relationships may be tempered by antigen concentration effects. Rogers and colleagues noted that the relationship between LFA-1 engagement and Th1 differentiation only occurred over a certain peptide concentration range and concluded that ligation of cell surface accessory receptors enables low concentrations of antigen to promote responses normally induced only by higher concentrations [68]. Th differentiation is thus, controlled by both the level of expression of multiple accessory molecule pairs and the number and affinity of peptide/MHC complexes. Therefore in the development of new treatment strategies for allergic disease, costimulatory molecules should be seen as important regulatory switches that underlie the effects of antigen concentration and form on modulation of Th cell response.

1.5. Regulatory Cells

The concept of a regulatory T cell subset controlling normal and adverse immune responses has moved from controversy to dogma over the last two decades. Several regulatory cell populations are now described. In the field of allergy research, Rocklin *et al.* were the first to describe the generation of an antigen-specific suppressor cell during desensitisation with ragweed pollen [69]. This was followed by other reports implicating regulatory cells in downregulating the immune response to allergens during SIT [70-72]. The first clear reports of a functional regulatory subset came from animal models of allergy, in which CD8+ T cells were shown to act as suppressor cells by inhibiting allergen-specific IgE production [73-75]. Suppressor function was mediated by IFN-γ production. Evidence for a role of suppressor CD8+ T cells in mediating clinical tolerance to allergens remains controversial [76]. However, we have shown in *in vitro* studies that numbers of CD8+IFN-γ+ cells rise dramatically after culture of allergic donor PBMC with high concentrations of allergen [11], suggesting that if high dose allergen administration can be achieved in SIT using hypoallergenic preparations, benefit from expansion of this regulatory population could be obtained.

Another population of regulatory cells that has been described in mouse and human studies is the CD4+CD25+ T cell subset [77, 78]. These cells are functionally anergic as they do not proliferate or produce IL-2 upon TCR ligation, and they can suppress the proliferation of CD4+ and CD8+ T cells. The mechanism of suppression *in vitro* is considered to be cell-contact dependent, however *in vivo,* production of IL-4, IL-10 and TGF-β may play a role [79]. CD4+CD25+ T cells have been shown to play a pivotal role in the prevention of organ-specific autoimmunity and allograft rejection, but their role in allergic responses has not been defined. Suto and colleagues showed that transfer of OVA-specific TCR transgenic CD4+CD25+ T cell depleted splenocytes into BALB/c RAG-$2^{-/-}$ recipients resulted in decreased antigen-induced eosinophil recruitment into the airways [80]. These results suggest that a regulatory CD4+CD25+ T cell population may in fact promote Th2 cell differentiation in allergic individuals. However, it is also possible that the

administered, starting at 0.1 μg/ml, early and late phase skin reactions to whole cat dander were inhibited without late asthmatic reactions. Patients receiving peptide had significantly reduced PBMC proliferation and production of IL-4, IL-13 and IFN-γ, while IL-10 production was increased. Thus, the use of peptide SIT for cat allergy is promising.

Clinically effective peptide SIT without serious side effects has also been demonstrated for bee venom allergy [121]. The immunotherapy regimen consisted of three immunodominant peptides of PLA_2: PLA_2[46-63], PLA_2[83-93] and PLA_2[114-125]. Peptides were administered subcutaneously in an equimolar mixture to 5 patients starting at 0.1 μg. Successive doses of 1, 3, 6, 12, 25, 50 μg were given weekly until a maintenance dose of 100 μg was reached and continued for an additional two weeks. Post-immunotherapy all patients tolerated subcutaneous provocation with purified PLA_2 without systemic side effects. Clinically successful immunotherapy correlated with the induction of specific anergy of peripheral T cells to PLA_2 and peptides and decreases in both Th2 and Th1 cytokine production. In two patients mild systemic allergic symptoms were observed after bee sting challenge. For both these patients, decreases in specific proliferation and cytokine production were either less pronounced or insignificant. These results may indicate that the peptides used may not represent the immunodominant peptides of PLA_2 for these two individuals. It is important to note that the final dose of peptides injected into these 5 patients corresponded to a 40-times higher amount of PLA_2 than normally given for conventional bee venom immunotherapy. This demonstrates that given in peptide form, higher concentrations of allergen can be tolerated by patients with potentially increased efficacy of SIT.

2.3. Altered Peptide Ligands

An alternative strategy based on dominant T cell epitope peptides is the use of altered peptide ligands (APL) for SIT preparations. The alteration is usually substitution of a single amino acid at a putative T cell contact residue. This substitution results in the formation of peptides that are T cell antagonists or partial agonists. Antagonistic peptides are unable to elicit T cell signaling, and thus inhibit T cell activation whereas partial agonists elicit some but not all the signals required for T cell activation.

Evidence for the downregulation of allergen-specific T cell responses by APL comes from *in vitro* studies. Single amino acid substituted peptides based on T cell epitopes of Cry j 1, Der p 1 and Par j 1 have been found to be antagonistic to specific TCL and TCC [122-124]. These APL inhibited T cell proliferative responses to the native peptide. Furthermore, the peptide analogue of the Par j 1 epitope was able to inhibit proliferation of TCL to the whole Par j 1 molecule providing some evidence for linked suppression [123]. In addition to inhibiting T cell responses, co-cultures of Der p 1-specific TCC and B cells with the antagonistic Der p 1 epitope peptide resulted in an inability of the T cells to provide help for B cell IgE production [124]. This was attributed to the APL inhibiting the induction of CD40L expression on Th cells, which is required for Th cell/B cell interactions.

Partial agonist peptides can also modulate allergen-specific T cell responses. Increased IFN-γ production with no alteration in T cell proliferation and IL-4 production has been demonstrated for APL based on epitopes of Cry j 1, Der p 2 and Der f 1 [122, 125, 126]. Interestingly enhanced IFN-γ production induced by a Der f 1 APL, was associated with increased IL-12 production by the APC. Inhibition of IL-12 with anti-IL-12 antibody inhibited the IFN-γ response [126]. As the increased IL-12 production was not due to enhanced IFN-γ production, it was suggested that T cell/APC interactions mediated through the TCR and APL can directly transmit signals to affect the APC response. A partial agonist based on a PLA_2 epitope has been observed to reduce proliferation of a specific TCC and decrease IL-4 production without affecting IFN-γ production [127]. This APL inhibited IL-4 production by PBMC. In the absence of APC, this APL also induced T cell anergy and reduced IL-4, IL-5 and IL-13 production. Anergic T cells displayed altered TCR signaling due to the lack of tyrosine phosphorylation of ZAP-70. In another study, a Der p 2 epitope APL induced unresponsiveness in a specific TCC at lower concentrations than the native peptide [128]. During induction of tolerance in this model, the APL was 22% more effective at inducing IL-10 production than the native peptide. These results indicate that partial agonist peptides can shift the cytokine profile of allergen-specific T cells from Th2 to Th1 predominant and under the correct conditions induce unresponsiveness to the allergen.

Although *in vitro* studies investigating immune modulation by APL are promising, these antagonistic or partial agonist peptides have not been clinically tested for allergen immunotherapy in humans to date. However, a study by Janssen and colleagues investigated the effect of APL in a murine model of allergic asthma [129]. In this model, BALB/c mice were sensitised and challenged with OVA to induce airway hyperresponsiveness, eosinophilia and OVA-specific IgE production. Cells isolated from the lymph nodes draining the lungs produced Th2 cytokines *in vitro*. Previously defined Th2- and Th1-skewing partial agonist peptides of OVA were used for immunotherapy by subcutaneous injection into sensitised mice. Administration with either the native peptide or the Th2-skewing APL resulted in increased eosinophilia upon OVA challenge. In contrast administration with the Th1-skewing APL resulted in decreased eosinophilia and decreased production of Th2 cytokines by cells isolated from the lymph nodes draining the lungs upon OVA challenge. The results from this study suggest that the use of Th1-skewing APL instead of native peptide may improve the efficacy of peptide SIT.

3. MODIFIED ALLERGENS

An alternative strategy to the use of peptides for T cell targeted SIT is to use modified allergens. These have the advantage of containing the multiple T cell epitopes, which have been identified throughout allergen molecules and they could also be designed to target particular APC by their chemical nature. To overcome the risk of IgE-mediated side effects with current whole allergen preparations, several different approaches have been explored to render allergens hypoallergenic but still effective for SIT.

3.1. Chemically Modified Allergens

An early strategy for the generation of effective but hypoallergenic SIT preparations was to treat the allergen with chemicals, such as glutaraldehyde or formaldehyde. Allergens modified in this way were termed 'allergoids'. In 1970 Marsh and colleagues developed a rye grass pollen group one allergoid by formaldehyde treatment [130], and in 1981 they produced ragweed pollen allergoids by formaldehyde and glutaraldehyde treatment with reduced IgE binding and histamine release from leukocytes [131]. Clinical trials of allergoids of ragweed pollen, mixed grass pollen, Parietaria pollen and dust mite allergens demonstrated that the preparations could be well tolerated and were effective at reducing allergic symptoms [132-139]. Although increased production of IgG_4 blocking antibody, decreased IgE production and reduced histamine release were demonstrated, other immune parameters associated with clinically effective SIT were not analysed in these studies [135, 136, 138]. Modulation of T cell responses by allergoids has however, been investigated *in vitro* [140, 141]. In these studies allergoids induced weaker proliferative responses and reduced production of IL-5 and IFN-γ by specific TCL and TCC in comparison to native allergen. These results suggest that allergoids can downregulate production of both Th2 and Th1 cytokines. Kahlert and colleagues demonstrated that allergoids also affect immune responses at the level of the APC [141]. Grass pollen and recombinant Phl p 5b allergoids were shown to be selectively presented by DC and monocytes and not by B cells. It was suggested that B cell presentation was inhibited as a consequence of destruction of IgE binding epitopes on the allergoid.

Recently, T cell reactivity was addressed for the first time during allergoid SIT [142]. It was found that the treatment did not affect T cell proliferation or production of IL-4, IL-5, IFN-γ and IL-10 by birch pollen specific TCL, but interestingly, nasal secretions showed decreased levels of IL-5 and increased levels of IFN-γ. Although, this suggests a shift from Th2- to Th1-type cytokine production, the cellular source of the cytokines recovered in the nasal secretions was not identified. In this study subjects were only administered seven pre-seasonal injections of birch pollen allergoid, and thus more research is required to determine if and how allergoids can modulate allergen-specific Th cell responses.

3.2. Allergen Isoforms

For some allergens, natural forms with reduced IgE reactivity may exist as isoforms. These usually have only minor sequence differences and would therefore, be expected to exhibit similar T cell reactivity to IgE reactive forms. Studying nine isoforms of the major birch tree pollen allergen Bet v 1, Ferreira and colleagues identified three isoforms, which had low or no IgE-binding activity (Bet v 1, g and l) [143]. Of these isoforms two, Bet v 1d and Bet v 1l, were tested for T cell reactivity and found to induce similar responses in comparison to naturally purified Bet v 1. In addition, the d and l isoforms showed no or very weak cutaneous skin reactions in allergic subjects upon skin prick testing. This research indicates the potential for the use of hypoallergenic isoforms in immunotherapy, however, the extent to which isoforms with reduced IgE binding exist for other allergens is not clear.

3.3. Mutant Allergens

An alternative approach is to use recombinant technology to produce allergen variants with reduced IgE reactivity and retained T cell responses. Site-directed mutagenesis provides a controlled system for disrupting residues important for protein tertiary structure or IgE recognition. For allergen molecules, which contain intramolecular disulphide bonds, mutation of cysteine residues is an obvious strategy for disruption of conformation and thus IgE binding. Cysteine variants prepared for HDM allergens Der p 2, Der f 2 and Lep d 2, cow dander allergen Bos d 2 and Par j 1 showed reduced or abolished IgE binding, retained T cell reactivity and, where tested, reduced skin prick test response [144-148]. Targeting other critical residues for IgE binding by site-directed mutagenesis, hypoallergenic mutants have been prepared for Bet v 1, Lol p 5 and Ara h 1, 2, and 3 [149-152]. For Phl p 5b, deletion mutants have been produced [153]. By making deletions within putative IgE binding regions but outside T cell epitopes, variants were produced with reduced IgE binding, decreased histamine releasing capacity and conserved T cell reactivity. Another recombinant strategy involves the production of hypoallergenic recombinant allergen oligomers [154] or fragments [155, 156]. Overlapping fragments, encoding approximately half of the allergen molecule, produced from Bet v 1 and Bos d 2 have shown reduced IgE binding capacity most likely due to the loss of their native folding within the entire allergen molecule [155, 156]. In addition, the Bet v 1 fragments retained T cell reactivity in comparison to recombinant wildtype Bet v 1. Interestingly these fragments induced differential cytokine production, with the C-terminal fragment inducing Th1-type cytokine production and the N-terminal fragment Th2-type [156]. Using another strategy, mutation of the calcium-binding site of the *Brassica* poilen allergen Bra r 1, produced an allergen variant that also displayed reduced IgE binding capacity [157].

Evaluation of recombinant hypoallergenic preparations as potential immunomodulators in mouse models is limited. Yasue and colleagues showed that administration of a hypoallergenic Der f 2 mutant could suppress immediate allergic reactions in a mouse model of allergic bronchial asthma more effectively than the wild type recombinant Der f 2 [158]. Although preliminary results are encouraging, further studies are required to validate the use of hypoallergenic variants as immunotherapy preparations.

4. ADJUVANTS AND CARRIERS

In parallel with refinement of allergen preparations for SIT are studies on adjuvants or carriers to promote efficacy of SIT. Adjuvants play an important role in targeting the site for immunomodulation and in determining the type of immune response elicited. While alum has been the mainstay adjuvant for allergen vaccines for many years due to its safety and depot effects, it may not be the best adjuvant for down-regulating Th2-mediated disease. Alum is in fact used in many mouse models to drive Th2 responses. Consideration of other adjuvants for promoting Th1-biased responses at mucosal sites is warranted. Improved clinical

efficacy and shorter treatment regimens are additional potential outcomes.

4.1. Bacterial Components

A number of studies have investigated the influence of conjugating allergen to various bacterial components on humoral and cellular immune responses. Jahn-Schmid and colleagues tested the efficiency of crystalline bacterial surface layers to drive Th1 cytokine production by allergen-specific human T cells [159]. TCL from birch pollen allergic donors generated in the presence of surface layers conjugated to recombinant Bet v 1 had higher IFN-γ:IL-4 ratios than TCL generated against the unconjugated recombinant Bet v 1. The Th1-polarised phenotype was associated with increased PBMC IL-12 production upon stimulation with the conjugate. As the size of allergen-surface layer conjugates are 1-5 μm the authors proposed that the potent Th1 shifting ability is due to preferential antigen uptake and subsequent IL-12 production by macrophages. Using another strategy, the dominant T cell epitope Der p 1 [112-140] was inserted within a permissive loop of the bacterial superoxide dismutase and expressed in *Mycobacterium vaccae* [160]. The immunisation of mice with the recombinant bacteria led to the development of a Der p 1 [111-139]-specific Th1 response. Allergen conjugated to the mucosa-binding β subunit of cholera toxin was found by Rask and colleagues to suppress allergen-specific IgE production in sensitised mice [161]. Another potential bacterial component adjuvant, is immunostimulatory monophosphoryl lipid A (MPL) isolated from *Salmonella minnesota* R595. Administration of antigen and MPL leads to systemic immunity characteristic of a Th1 response [162, 163]. Injection of allergen with MPL to allergen-sensitised rats can prevent secondary IgE responses [162]. In humans a new allergy vaccine comprising a tyrosine-adsorbed glutaraldehyde-modified grass pollen extract containing MPL adjuvant was recently trialed in a double-blind, placebo-controlled, randomised clinical study [164]. After only four preseasonal injections this well tolerated vaccine was efficacious, in terms of reduced symptoms and skin prick test scores. However, more research is required to analyse changes in T cell reactivity during SIT using MPL as an adjuvant. Nevertheless, many bacterial components can drive allergen-specific Th1 responses and suppress ongoing allergen-specific IgE responses, and thus their use as adjuvants in SIT is promising.

4.2. Virus-Like Particles

Viral components have also been trialed as immunomodulators. In particular, virus-like particles (VLP) offer promise for allergen immunotherapy. VLP containing the p1 protein of the yeast retrotransposon Ty have been used to present allergen, as peptide, to the immune system and elicit cell mediated responses. VLP containing approximately 300 copies of a fusion protein comprising a relevant dominant T cell epitope peptide linked to the p1 protein have been administered to Der p 1 and Asp f 2 sensitised mice [165, 166]. Reduced IL-5 production was observed for Der p 1 sensitised mice treated with Der p 1 (111-139)-VLP in comparison to native Der p 1 [166]. The Der p 1 (111-139)-VLP treated mice also displayed decreased T cell proliferation to both Der p 1 (111-139) and Der p 1, leading the authors to suggest that VLP peptide administration renders Th2 cells anergic and provides evidence for linked suppression [166]. T cell unresponsiveness was also observed in the mice treated with Asp f 2 peptide-VLP [165] and these mice displayed reduced IgE levels. These results suggest that by enabling delivery of high doses of peptide, VLP could be used to efficiently "turn off" the adverse T cell response to allergen.

4.3. Recombinant Cytokines

Recombinant cytokines are another possibility for SIT adjuvants that can direct the allergen-specific immune response away from Th2 predominant. Immunisation of a recombinant IL-12-OVA fusion protein induced anti-OVA IgG_{2a} antibody and increased levels of OVA-specific IFN-γ production from lymph node cells *in vitro* [167]. Immunisation of a recombinant IL-18-OVA fusion protein induced increased IFN-γ production by OVA-stimulated splenocytes [168]. Both fusion proteins reversed established predominant Th2 OVA responses in mice [167, 168]. In another study, Kumar and colleagues found that mice that received plasmid DNA encoding IL-12 and IFN-γ intramuscularly in addition to subcutaneous injection of allergen produced increased allergen-specific IgG_{2a} antibody and decreased total serum IgE compared with control mice [169]. Allergen-stimulated splenocytes from co-immunised mice produced high levels of IFN-γ and IL-2 and low levels of IL-4 indicating a strong Th1 driven response. These mice also exhibited reduced methacholine-induced airway hyperresponsiveness and abolished lung inflammation in comparison to allergen alone immunised mice. Thus, the use of Th1-promoting cytokines either as recombinant proteins or encoded in DNA plasmids in conjunction with allergen administration is also an interesting alternative adjuvant strategy for SIT.

4.4. Liposomes

Liposomes are another type of adjuvant/carrier that could possibly be used for allergen delivery in SIT. Liposomes are non-toxic, biodegradable lipid vesicles that can incorporate allergen. In 1991, Arora and colleagues demonstrated that liposomes can be used as a non-immunogenic vehicle for antigen presentation *in vivo*. Furthermore, administration of liposome-entrapped allergen into allergen-sensitised mice was shown to reduce specific IgE production [170]. In 1998, Sehra and colleagues demonstrated that immunisation of naïve mice with liposome-entrapped *Artemisia scoparia* pollen induced increased serum levels of IFN-γ and IgG_{2a} compared with mice immunised with free allergen [171]. Upon intravenous allergen challenge, liposome-immunised mice had reduced histamine release and were protected from fatal systemic reactions. Dominant Th1 responses characterised by increased IFN-γ and decreased IL-4 production have also been shown in mice immunised with allergen-entrapped liposomes [172, 173]. Macrophages were shown to be obligatory for processing of the liposome-entrapped allergen [172]. Presumably, IL-12 production from macrophages diverts the response to Th1. By inhibiting IgE binding to allergen, the lipid coating of the liposomes would prevent the Th2-promoting uptake of allergen by B

cells and IgE-bearing dendritic cells. In humans, liposome-allergen delivery has been trialed in a 1 year double-blind, placebo controlled study for HDM allergy [174]. Encouraging results with reduced symptoms and a high level of safety were achieved however, T cell responses were not analysed. Hence the use of liposomes for SIT is promising as this type of adjuvant provides an additional feature of protection of administered allergen from IgE binding, which is beneficial for effective immunomodulation and increased safety.

5. DNA VACCINES

As for the general field of vaccine technology, DNA vaccines have attracted attention for allergen immunotherapy. Of interest was the finding that bacterial DNA itself elicited strong Th1 responses in mice due to the presence of immunostimulatory sequences (ISS) containing CpG motifs [175]. Bacterial DNA has therefore, been evaluated as an immunoregulator as well as an adjuvant for modulation of allergen-specific Th2-type responses. Allergen-encoding DNA vaccines have also been tested for efficacy [176].

5.1. Bacterial DNA as an Immunoregulator and Adjuvant

Administration of bacterial oligodeoxynucleotides containing CpG motifs (CpG-ODN) before allergen sensitisation in mice has been shown to induce predominant allergen-specific Th1 responses for the allergens Bet v 1, Cry j 1, Cry j 2, Aed a 2, Amb a 1 and OVA [177-182]. Increased levels of serum specific-IgG_{2a} and increased production of IFN-γ by *in vitro* allergen-stimulated splenocytes confirmed induction of Th1 responses in these models. Suppression of IgE production [179-181] and decreased production of Th2 cytokines were also observed [177, 179, 182]. A reduction in eosinophilia and airway hyperresponsiveness has been noted in sensitised mice given CpG-ODN before allergen challenge [177, 180, 183, 185]. Using cytokine neutralisation, Broide and colleagues demonstrated that CpG-ODN-mediated reduction in eosinophilia in a murine model of asthma was partially dependent on IL-12, IFN-α/β and IFN-γ [183]. Interestingly in this model, CpG-ODN were found to inhibit bone marrow production of eosinophils. As NK cells have previously been shown to produce high levels of IFN-γ in the presence of CpG-ODN [185], Broide and colleagues investigated the role of NK cells in downregulating eosinophilia [184]. NK cell depletion did not affect reductions in eosinophilia induced by CpG-ODN treatment. However, the ability of CpG-ODN to affect other cells within the immune system may account for these results. CpG-ODN stimulation of B cells results in the upregulation of surface markers including MHC class II, CD40 and CD16/32 and downregulation of CD23 (Fcε receptor) [186]. In macrophages, CpG-ODN induce upregulation of MHC class I, CD40, CD80 and ICAM-1 [186]. Interestingly CpG-ODN-induced IL-12 production from macrophages is responsible for enhanced NK cell production of IFN-γ [185]. More investigation is required to fully understand the Th1 promoting activity of CpG-ODN.

A limited number of studies have investigated the ability of CpG-ODN to modulate human Th2 allergen-specific immune responses. Bohle and colleagues stimulated atopic and non-atopic PBMC with CpG-ODN *in vitro* and observed increased production of IFN-γ for both groups [187]. The IFN-γ production was attributed to NK cells and inhibition experiments indicated that, like in the mouse, this was IL-12 dependent. Further analysis demonstrated that CpG-ODN-treated monocyte-derived DC produce IL-12 and IL-18 and that the production of IL-12 is enhanced in the presence of GM-CSF [188]. Importantly, Marshall and colleagues demonstrated that CpG-ODN can alter established allergen-specific Th responses *in vitro* [189]. Allergic donor PBMC were cultured for 6 days in the presence of Amb a 1 and then cultured with either Amb a 1 alone or Amb a 1 linked to a CpG-ODN. Restimulation with Amb a 1 resulted in a predominant Th2 cytokine profile, whereas restimulation with the CpG-ODN linked Amb a 1 resulted in predominant Th1 cytokine profile. Increased IFN-γ production induced by the CpG-ODN in this study was again shown to be IL-12 dependent. In addition Der p 1 specific TCL treated with CpG-ODN also display predominant Th1 responses [170]. However in this model neutralisation of IL-12 only partially reduced the enhanced IFN-γ production mediated by CpG-ODN. Parronchi and colleagues found that anti-IL-12, anti-IFN-γ and anti-IFN-α antibodies were required to completely block the Th1 shifting effect of the CpG-ODN. Furthermore, ODN lacking CpG motifs could also induce enhanced Th1 cytokine production. Hence, the ISS in DNA required for immune modulation in mouse and humans may differ. Although, further research is required to determine the full capacity of CpG-ODN in downregulating established allergen-specific Th responses, ISS-containing ODN may be useful as adjuvants for peptide or hypoallergenic SIT preparations.

5.2. DNA Vaccines Encoding Allergen Genes

The direct administration of DNA encoding allergen proteins is also a focus of current research. In 1996 Raz and colleagues demonstrated that injection into mice of naked plasmid DNA encoding beta-galactosidase could elicit Th1 responses in comparison to injection of the beta-galactosidase protein, which elicited a Th2 response [191]. The induction of allergen-specific Th1 responses in mice and rats by administration of plasmid DNA encoding the allergen of interest has now been demonstrated for Der p 5, Der f 11, Hev b 5, Bet v 1a, Cry j 1, bovine beta-lactoglobulin and peptides containing T cell epitopes of Der p 1 and PLA_2 [192-201]. In comparison to the native protein, injection of DNA encoding the allergen leads to reduced levels of allergen-specific IgE [192, 194-199, 201]. DNA vaccines may also modulate ongoing allergic responses. In mice sensitised to bee venom allergens, therapeutic treatment with DNA plasmids containing PLA_2 T cell epitope peptides, resulted in a decreased incidence of anaphylaxis, which was associated with increased IFN-γ and IL-10 production and decreased IL-4 production [199]. Furthermore, treatment of Der f 11 sensitised mice with a Der f 11 encoding DNA plasmid resulted in reduced levels of allergen-specific IgE and increased levels of allergen-specific IgG_{2a} [201]. These studies indicate that DNA vaccines have the ability to downregulate the Th2 biased immune response to allergens and therefore, are potential SIT candidates.

More recently a number of new strategies have been employed to increase the effectiveness of DNA vaccines. Toda and colleagues designed two DNA vaccines that encode a T cell epitope of Cry j 1 and an invariant chain (Ii) for the delivery of the epitope peptide to the MHC class II loading pathway [202]. Pretreatment of mice with these vaccines inhibited Cry j 2-specific IgE responses and promoted Cry j 2-specific IgG_{2a} responses in Cry j 2 sensitised mice. In addition, splenocytes from vaccinated mice produced large amounts of IFN-γ when stimulated with Cry j 2 *in vitro*. Roy and colleagues investigated a new delivery system, in which plasmid DNA containing the Ara h 2 gene was complexed to chitosan, a natural biocompatible polysaccharide, to form nanoparticles that can be administered orally [203]. This vaccine reduced the incidence of anaphylaxis in a murine model of peanut allergen-induced hypersensitivity. Injection of the nanoparticles also resulted in increased allergen-specific IgG_{2a} and secretory IgA and decreased IgE. This new strategy offers the use of a non-viral vector for immunotherapy preparations.

CONCLUSIONS

The increasing prevalence of allergic disorders worldwide emphasises the need for improved specific treatment. Elucidation of the underlying immune basis for specific immunomodulation of the adverse Th2-biased response to allergens points to new strategies for improved efficacy and safety of SIT. Currently unavailable are routine laboratory tests for monitoring efficacy of SIT, and T cell based assays now offer this possibility.

A fundamental knowledge of factors influencing the type and magnitude of the immune response to an allergen provides a rational basis for refinement of the form and mode of delivery of allergen in SIT. A variety of different allergen preparations are now being explored for more selective targeting of the allergen-specific T cell including peptides, hypoallergenic allergen forms and DNA vaccines. However, of equal importance is an understanding of how different allergen preparations could target particular APC at different tissue sites in order to modulate responses at sites of allergen encounter. This would also permit development of the more practical delivery of SIT via oral, sublingual or intranasal routes. Finally a note of caution must be expressed for merely skewing responses strongly away from Th2-type towards Th1-type due to the risk of introducing new pathology. Triggering autoimmune responses has even been suggested as a byproduct of such an approach. Of great attraction are strategies for concomitant expansion of a regulatory T cell population, which would inhibit both Th2- and Th1-type inflammatory responses to allergen. Careful evaluation of these new approaches in laboratory and *in vivo* trials will lead to modification of best practice protocols for SIT and a wider application of this potentially curative treatment for allergic diseases including asthma.

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ABBREVIATIONS

APC = Antigen presenting cell

APL = Altered peptide ligand

DC = Dendritic cell

HDM = House dust mite

OVA = Ovalbumin

mAb = Monoclonal antibody

ODN = Oligodeoxynucleotide

PLA_2 = Phospholipase A_2

SIT = Specific immunotherapy

TCC = T cell clone

TCL = T cell line

TCR = T cell receptor

Th = T helper cell

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