Regulatory T cells in Human Allergic Disease

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Abstract

Allergic diseases are frequent and rising in prevalence, and result from activation of T-helper (Th) 2 cells by common environmental antigens (allergens). CD4+CD25+ regulatory T cells prevent pathology in many animal models of disease and human CD4+CD25+ regulatory T cells suppress T cell activation *in vitro*. However, at the commencement of this project there were few reports of suppression of Th2 responses by CD4+CD25+ regulatory T cells. This thesis therefore investigated the suppression of Th2-type T cell responses to allergen by human CD4+CD25+ regulatory T cells *in vitro*. The ability of regulatory T cells from atopic and non-atopic donors, to suppress allergen driven T cell responses was then compared. Finally, the effect of allergen immunotherapy treatment on the suppression of allergen driven T cell responses was investigated.

Results from this thesis are among the first to describe the suppression of allergen driven T cell responses by human CD4+CD25+ regulatory T cells and furthermore were the first to relate suppression by CD4+CD25+ T cells with expression of allergic disease in humans. Such cells from non-atopic donors were able to suppress both proliferation and cytokine production by the allergen stimulated CD4+CD25- T cell subset. Inhibition of proliferation by CD4+CD25+ T cells from atopic donors was significantly reduced. Suppression was further diminished when CD4+CD25+ T cells were isolated from grass pollen allergic patients during the hayfever season. Data from this thesis has also shown that effective allergen immunotherapy treatment is not associated with an increase in the ability of CD4+CD25+ T cells to suppress Th2 responses. However, a separate novel population of IL-10 secreting T cells within the CD4+CD25- subset did suppress Th2

responses more effectively when isolated from atopic donors who had received allergen immunotherapy treatment compared to those from untreated atopic individuals.

The data presented here suggests that allergic disease may result in part from an inbalance between allergen activation of regulatory CD4+CD25+ T cells and effector Th2 cells. Treatment to enhance regulatory T-cell responses, in concert with reducing Th2 cell activation, may be beneficial in the prevention and treatment of allergic disease.

Dedication

This thesis is dedicated to my family. In particular to my parents, for all their support and for the use of their summerhouse as an office for the past few months, and to three little sources of inspiration; Anna, James and "bump" (possibly Bat boy!)

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Abbreviations

A	Atopic
Ab	Antibody
Ag	Antigen
APC	Antigen presenting cell
BCG	Bacille Calmette-Guérin
BSA	Bovine serum albumin
CD	Cluster of differentiation
CI	Confidence interval
CTL	Cytotoxic T cell
CTLA-4	Cytotoxic T lymphocyte antigen-4
Cy	Cychrome
DC	Dendritic cell
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylene dinitrolenotetracetic acid
ELISA	Enzyme-linked immunosorbent assay
Fc	Fragment crystallisable
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GITR	Glucocorticoid-induced TNF receptor
HA	Haemagglutinin
HF	Hayfever
ICOS	Inducible Costimulatory molecule
ICOSL	Inducible Costimulatory molecule ligand
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IT	Immunotherapy
ITAM	Immunoreceptor tyrosine based activation motif
L	Ligand
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
ml	Millilitre
MLR	Mixed lymphocyte reaction
mRNA	Messenger RNA

MW	Molecular weight
µg	Microgram
µl	Microlitre
NA	Non-atopic
ng	Nanogram
NK	Natural killer
OD	Optical density
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
pg	Picogram
Phl PV	*Phleum pratense V*
PPD	*M. tuberculosis* derived purified protein derivative
R	Receptor
RAST	Radioallergosorbent test
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI	Rosewell Park Memorial Institute medium
SD	Standard deviation
SEM	Standard error of the mean
SPT	Skin prick test
SSC	Side scatter
TCR	T cell receptor
TGF	Transforming growth factor
Th1	T helper 1
Th2	T helper 2
TNF	Tumour necrosis factor
TLR	Toll-like receptor
Treg	Regulatory T cell

Chapter 1 - Introduction

1.1. Introduction to the thesis

Atopic allergic diseases are common and increasing in prevalence in the developed world. An international study of asthma and allergy reported that in 2003, Australia, New Zealand and the United Kingdom had the highest prevalence of asthma, with 20% of children aged 13-14 years reporting symptoms [1]. Allergic rhinitis affects approximately 30% of adults and 40% of children in industrialised societies [2]. In the US alone 3.5 million workdays are lost annually [3] and $6 billion spent on prescription medication for treatment of allergic rhinitis [4]. Available medication for allergic disease controls symptoms in the majority of patients, although those with moderate to severe disease remain symptomatic despite treatment [5].

The reason why some individuals mount strong immunoglobulin (Ig)-E mediated Th2-type T cell responses upon exposure to non-pathogenic common aeroallergens such as grass pollen and cat dander, which drive the development of atopic allergic disease, whilst other non-atopic individuals do not, is at present unknown. A better understanding of the suppression of Th2 responses could lead to novel strategies for therapeutic intervention.

Recently, T cells with the ability to suppress immune pathology *in vivo* and T cell responses to activation by polyclonal and antigenic stimuli *in vitro* have been discovered [6]. This thesis will discuss the role of regulatory T cells in the suppression of human allergic disease.

1.2. Human allergic disease

1.2.1. Hypersensitivity reactions

Hypersensitivity reactions are harmful immune responses that produce tissue injury and may cause serious disease. These reactions were classified by Coombs and Gell [7] into four types based on the distinct mechanisms, cells and mediators involved, see Table 1.1. Although this classification scheme has highlighted the differences in mechanisms between various hypersensitivity reactions, it is important to note that the situation is more complex and overlap between the groups exists.

Clemens Von Pirquet originally defined allergy as "an altered capacity of the body to react to a foreign substance." This extremely broad definition, which could encompass almost any immunological reaction, can be further restricted to "disease following a response by the immune system to an otherwise innocuous antigen" [1]. Allergy is often equated with immunoglobulin-E (IgE)-mediated Type I hypersensitivity and will be used in this sense for this thesis.

1.2.2. Type 1 mediated hypersensitivity reactions

Allergic diseases such as rhinitis, asthma and atopic eczema are increasing in prevalence in developed countries [1]. They are among the most common causes of chronic ill health and contribute greatly to national health care costs [4;8].

The majority of humans mount significant IgE responses only as a defence against parasitic infections. Atopic individuals however, mount an enhanced IgE response to certain ubiquitous antigens (allergens) after inhalation, ingestion or skin contact, causing them to have symptoms of one or more atopic disease, such as atopic rhinitis, asthma or eczema [9].

	Type I	Type II	Type III	Type IV		
Ig Isotype / T cell type involved	IgE	IgG	IgG	Th1	Th2	Tc
Antigen	Soluble antigen	Cell or matrix-associated antigen	Soluble antigen	Soluble antigen	Soluble antigen	Cell-associated antigen
Effector mechanism	Mast cell activation	FcR+ cells (phagocytes, NK cells)	FcR+ cells and complement	Macrophage activation	Eosinophil activation	Cytotoxicity
Example of reaction	Allergic rhinitis, asthma, systemic anaphylaxis	Allergy to penicillin	Serum sickness, arthus reaction	Contact dermatitis, tuberculin reaction	Chronic asthma, chronic allergic rhinits	Contact dermatitis

Table 1.1. The four types of hypersensitivity reactions as classified by Coombs and Gell.
Types I-III are antibody mediated and distinguished by the class of antibody involved. Type I is mediated by IgE activation of mast cells and Types II and III are mediated by IgG, which can engage Fc receptor and complement-mediated effector mechanisms depending on the subclass of IgG and nature of allergen involved. Type IV reactions are T cell mediated, and can be further subdivided into three groups. In the first group tissue damage is caused by the activation of macrophages by Th1 cells. In the second, Th2 cells cause inflammatory responses in which eosinophils predominate and in the third tissue damage is caused directly by cytotoxic lymphocytes.

When inhaling common aeroallergens such as dust, pollen and cat dander, most non-atopic individuals will mount an immunological response, producing allergen-specific IgG_1 and IgG_4 but not IgE [9]. T cell cytokine profiles from atopic and non-atopic subjects also differ [10]. Allergen-specific CD4+ T cell clones prepared from peripheral blood of atopic individuals are highly polarised Th2 cells, whereas those derived from non-atopic donor blood show a Th0 or Th1 profile [11;12].

1.2.3. Allergens

The term allergen refers specifically to non-parasitic antigens capable of stimulating IgE-mediated Type I hypersensitivity reactions in atopic individuals. Common allergens are shown in Table 1.2.

Before an allergen can stimulate IgE production, it must access areas that are patrolled by the immune system. The lungs, nose, eyes and gut are prime sites for an allergic reaction. Generally, the more concentrated the allergen, the more likely it is to induce a response, and to come into contact with cells of the immune system it must be soluble [13].

When an allergen is inhaled or ingested it is broken down into smaller particles by enzymes. Chemical fractionation of various allergens has revealed that most of the fractions elicit an IgM or IgG response by peripheral blood mononuclear cells from atopic individuals, and only a small number of fractions, termed "major allergens", will cause an IgE response [14]. The major allergen associated with the cat, for example, is *Feline domesticus* (Fel D) I, which is an acid glycoprotein with a molecular weight of about 18kDa derived from the saliva and the epithelium [15], and the major allergenic proteins in the timothy grass pollen are *Phleum pratense* (Phl p) I, II and V [16].

Source of allergen	Group	Examples
Airborne	Grasses	Rye, couch, wild oat, timothy, bermuda, Kentucky blue, cocksfoot
	Weeds	Ragweed, parietaria, plantain, mugwort
	Trees	Alder, birch, hazel, beech, Cupressae, oak, olive
	Moulds	*Aspergillus spp, Cladosporium spp, Alternaria spp* Basidiospores,
	Cereal grains	Wheat, rye oat
	Animal dander and urine	Cat, dog, horse, rabbit, guinea pig, hamster
	Bird feathers	Budgerigar, parrot, pigeon, duck, chicken
	House dust mite	*Dermatophagoides pteronyssinus, D. farinae, Euroglyphus maynei*
	Insects	Cockroach, fly, locust, midge
Oral	Foods	Seafood, legumes, peanuts, nuts, cereals, dairy products, eggs, fruits, tomatoes, mushrooms, alcoholic beverages, coffee, chocolate
	Drugs	Penicillins, sulphonamides and other antibiotics, sulphasalazine, carbamezapine
Injected	Insects	Bee and wasp stings, ant and mosquito bites
	Drugs	Blood products, sera, vaccines, contrast media, drugs (including anti-asthma drugs and antibiotics)

Table 1.2. Common sources of allergen and examples.
Adapted from Allergy 2nd Edition [13].

1.3. Adaptive immunity

The innate immune system provides a first line of defence against common micro-organisms and is essential for the control of bacterial and viral infections. Innate immune responses depend mainly on phagocytic cells such as neutrophils and macrophages. The adaptive immune response however provides a versatile defence mechanism, which confers increased protection against subsequent reinfection by the same pathogen. The lymphocytes of the adaptive immune system, which possess antigen-binding cell surface receptors, are produced in the bone marrow by haematopoiesis and display the immunological attributes of specificity, diversity, memory and self/non-self recognition. The innate and adaptive immune systems are not exclusive; many of the cells involved in the innate response also participate in the effector mechanisms of adaptive immune responses.

1.3.1. Lymphocytes

The two major populations of lymphocytes are B-lymphocytes (B cells) and T-lymphocytes (T cells). B cells leave the bone marrow expressing cell surface bound antibody with a unique antigen binding specificity. When a naïve B cell encounters for the first time the antigen to which its receptor is specific, the cell divides rapidly and the progeny differentiate into memory B cells or plasma cells. Memory B cells have a relatively long life span and continue to express membrane bound antibody with the same specificity as the parent cell. In contrast plasma cells, live for only a few days and do not express membrane bound antibody, but produce vast quantities of the same specific antibody in a form that can be secreted [17].

During the process of thymic maturation, T cells develop a unique antigen binding receptor on their cell surface membrane called the T cell receptor (TCR).

Although the B cell receptor can recognise antigen alone, the TCR can only recognise antigen that is associated with the membrane protein complex called the major histocompatibility complex (MHC) [18].

1.3.2. Thymic maturation of T cells

Unlike B cells, which mature in the bone marrow, T cell precursors migrate from the bone marrow to the thymus to mature, where the T cell receptor genes are rearranged. There are four TCR gene loci; α, β, γ and δ. Rearrangement of these genes leads to cells bearing either γδ or αβ TCRs. Early in ontegeny γδ T cells predominate, but from birth onwards more than 90% express TCRs encoded by rearranged αβ genes. During αβ T cell development, the generation of a functional β-chain leads to the development of a pre-TCR. This receptor signals the developing cell to proliferate, to express the membrane glycoproteins CD4 and CD8 and eventually to rearrange the α-chain. Rearrangement of the α-chain continues in these CD4/CD8 double positive thymocytes until positive selection allows the maturation of an αβ T cell expressing either CD4 or CD8[19].

Each individual TCR is specific for a combination of MHC molecule and peptide antigen and is thus said to be MHC-restricted. Antigen presenting MHC molecules are encoded by a highly polymorphic set of genes and their variability extends the range of peptides that can be presented to T cells in any one individual. Not all TCRs will be able to recognise self-MHC molecules; in fact the vast majority of thymocytes die at the CD4/CD8 double positive stage because they are not positively selected [20].

Positive selection also determines co-receptor specificity. Experiments with mice transgenic for rearranged TCR genes clearly show that it is the specificity of the

TCR for self-MHC molecules that determines which co-receptor it will express. MHC I molecules are generally expressed on nucleated cells and the expression of MHC II is restricted to lymphoid cells such as T cells, B cells and antigen presenting cells. In mice made transgenic for a TCR that recognises antigen associated with MHC class I molecules, mature T cells that express that TCR are CD8+ cells [21]. Similarly, if the TCR trangenes encode a receptor specific for antigen presented by class II MHC molecules, mature T cells are CD4+ [18]. Negative selection removes those developing thymocytes that react strongly with ubiquitous self-antigens in the thymus by the process of apoptosis, thereby deleting potentially harmful self-reactive T cells [22].

1.3.3. CD4 and CD8 T cells

T cells are identified by the expression of the surface molecule CD3 and can be further categorised into T helper (Th) and T cytotoxic (Tc) subsets by the presence of membrane glycoproteins CD4 and CD8, respectively [23]. CD4+ Th cells provide a critical signal required for the activation of B cells by cell/cell interactions mediated by CD40-CD40L, resulting in increased antibody production [24]. CD4+ T cells also secrete cytokines, which induce the activation of many cell types including other T cells and APCs [25]. In contrast to CD4+ T cells, CD8+ Tc cells exhibit cytotoxic activity [26]. They protect from infection through the direct killing of pathogen-infected cells. Cell killing is mediated through secretion of granulysin and perforin, or by initiating apoptosis of the target cell through FAS/FASL interactions [27].

1.3.4. Antigen presenting cells (APC)

Professional APCs can be divided into two major systems: dendritic cells (DCs) and the monocyte-macrophage system, all of which originate from pluripotent

bone marrow CD34+ haematopoietic progenitor cells (HPCs). During their maturation, DCs are characterised by different phenotypes and functions. Immature DCs have an excellent ability for the surveillance of peripheral tissues and although their capacity to take up and present antigen is high, they are weak at priming T cells. Phenotypically they show low expression of co-stimulatory molecules, activation markers and MHC II. During their maturation, for example with TNFα, they lose their ability to take up and process antigens and they upregulate both co-stimulatory molecules and MHC II. Mature DCs therefore acquire the ability to prime T cells and are the most potent inducers of primary T cell responses. DCs also play a role in promoting T cell tolerance to self-antigens, both in the thymus during the process of negative selection, and in the periphery [28].

At least 2 distinct DC precursor cells have been identified in the blood; the DC1 subset expresses the myeloid surface antigen CD11c. The CD11c-negative population forms the plasmacytoid precursors of lymphoid DCs (DC2). The two types of DC precursor in the blood that differentiate into DC1 and DC2 seem to drive Th1 and Th2 responses, respectively [29].

1.3.5. Processing and presentation of antigens

Foreign proteins must be degraded into small peptides, which form complexes with MHC, before they can be recognised by T cells. Whether the association is with class I or class II MHC molecules is determined by the nature of the antigen. MHC I molecules collect peptides derived from proteins synthesised in the cytosol such as viral proteins and display them on the cell surface [17]. MHC II molecules however, bind and display peptides derived from proteins found in intracellular vesicles such as those from pathogens present in macrophage vesicles or internalised by phagocytes [30].

APCs internalise foreign antigens by phagocytosis and/or endocytosis. Once internalised, antigen is degraded into peptides within the endocytic-processing pathway. The MHC class II molecule and its associated invariant chain are exported from the rough endoplasmic reticulum (rER), through the Golgi apparatus. The acidic environment of the endocytic pathway causes the invariant chain to be degraded and the resulting CLIP (class II-associated invariant chain peptide) fragment is replaced by antigenic peptide. The MHC class II-peptide complex is then exported to the cell surface and MHC II bound peptide is presented to CD4+ Th cells [17;30].

Proteins from endogenous antigen and intracellular pathogens are covalently linked to ubiquitin in an ATP-dependent manner within the cytoplasm of the cell. The ubiquitin-protein complex is degraded by a proteasome complex, which generates small peptides. After transportation across the rER membrane by an ATP-binding transporter, TAP, MHC I assembly is mediated by the chaperone protein calnexin. Binding of antigenic peptide releases calnexin and stabilises MHC class I. The MHC class I-peptide complex is then transported to the cell surface via the Golgi apparatus, where it can be recognised by CD8+ Tc cells [17].

1.3.6. The role of cell-adhesion molecules in the initial T cell/APC interaction

During their migration through the cortical region of the lymph node, naïve T cells transiently bind to each APC they encounter. The initial binding between T cells and APCs is mediated by LFA-1, CD2 and ICAM-3 on the T cell, and ICAM-1, ICAM-2, LFA-3 and DC-SIGN on the APC [31;32]. This transient binding is required to allow the T cell enough time to sample the MHC-peptide complexes on the cell surface of the APC. In the rare cases that a T cell recognises the peptide to which its TCR is specific, signalling through the TCR induces a conformational change in LFA-

1, which greatly increases its affinity for ICAM-1 and ICAM-2, therefore stabilising the association between the antigen-specific T cell and the APC [33]. This association can persist for several days during which time the T cell may divide and/or differentiate and its progeny, which will also adhere to the same APC, differentiate into effector T cells.

1.3.7. T Cell Activation

T cells circulate from the blood stream to the lymphoid organs and back to the blood, sampling the MHC-peptide complexes on the APC. Although activated memory or effector T cells can be further activated by stimulation through the TCR alone [34], antigen specific clonal expansion of naïve T cells requires high density, sustained TCR signalling and must be accompanied by a second signal, which may be a soluble factor such as IL-2 [35] or a co-stimulatory interaction between the APC and the T cell [36]. TCR signalling alone results in anergy and eventual apoptosis of the cell [37].

CD28 is the best-described co-stimulatory molecule, it binds B7.1 (CD80) and B7.2 (CD86), which are members of the immunoglobulin superfamily [38]. CD28 is expressed by CD4+ and CD8+ cells and its level of expression is increased following activation [39]. Ligation of CD28 causes production of cytokines such as IL-1, IL-4, IL-5, TNF and IFN-γ [39;40]. It also induces expression of the T cell growth factor IL-2 [41], which is required for optimal cell cycle progression [42], and up-regulation of the anti-apoptosis gene *bcl*-X_L, which protects from Fas dependant cell death [43].

B7.2 is constitutively expressed by professional APCs whereas B7.1 is only expressed after the APC is activated. It is thought that B7.2/CD28 interactions are required for initial T cell activation whilst B7.1/CD28 interactions, which are

stronger, are needed for a sustained T cell response, perhaps to clear a persistent pathogen [44]. Once a T cell is activated, it expresses a number of proteins that sustain or modify the co-stimulatory signal driving clonal expansion and differentiation [45]. CD40L is one such protein. CD40L interacts with CD40 on APCs and causes further expression of B7 molecules and therefore sustained T cell proliferation [46].

Other co-stimulatory molecules inhibit T cell responses. CTLA-4 (CD152) for example binds B7 molecules 20 times as avidly as CD28 and delivers an inhibitory signal to the T cell, inhibiting IL-2 production and cell cycle progression [47-49]. This causes the progeny of the T cell to become less sensitive to stimulation by the APC. CTLA-4 is therefore essential for suppressing unwanted T cell responses [50]. This is illustrated by the fact that CTLA-4$^{-/-}$ mice suffer from a fatal disorder characterised by massive lymphoproliferation [51 52]. A role for CTLA-4 in the generation and function of regulatory T cells has also been described [6;53;54], and has been discussed further in Chapter 4.

Another CD28-related protein, inducible co-stimulator (ICOS), which is induced on activated T cells [55] induces only low IL-2 production when interacting with its ligand, ICOSL [56] which is expressed on monocytes, B cells and activated DCs [57]. The interaction between ICOS and ICOSL is also important in the development of a subset of regulatory T cells, which secrete IL-10 [58].

1.3.8. T cell signalling

The complex of signalling chains associated with the TCR is collectively termed CD3 [59]. The TCR/CD3 receptor complex is also associated with a homodimer of ζ -chains which signal to the interior of the cell upon antigen binding (Figure 1.1.). The initial events in TCR signalling are implemented by two Src-family kinases Lck and Fyn [60].

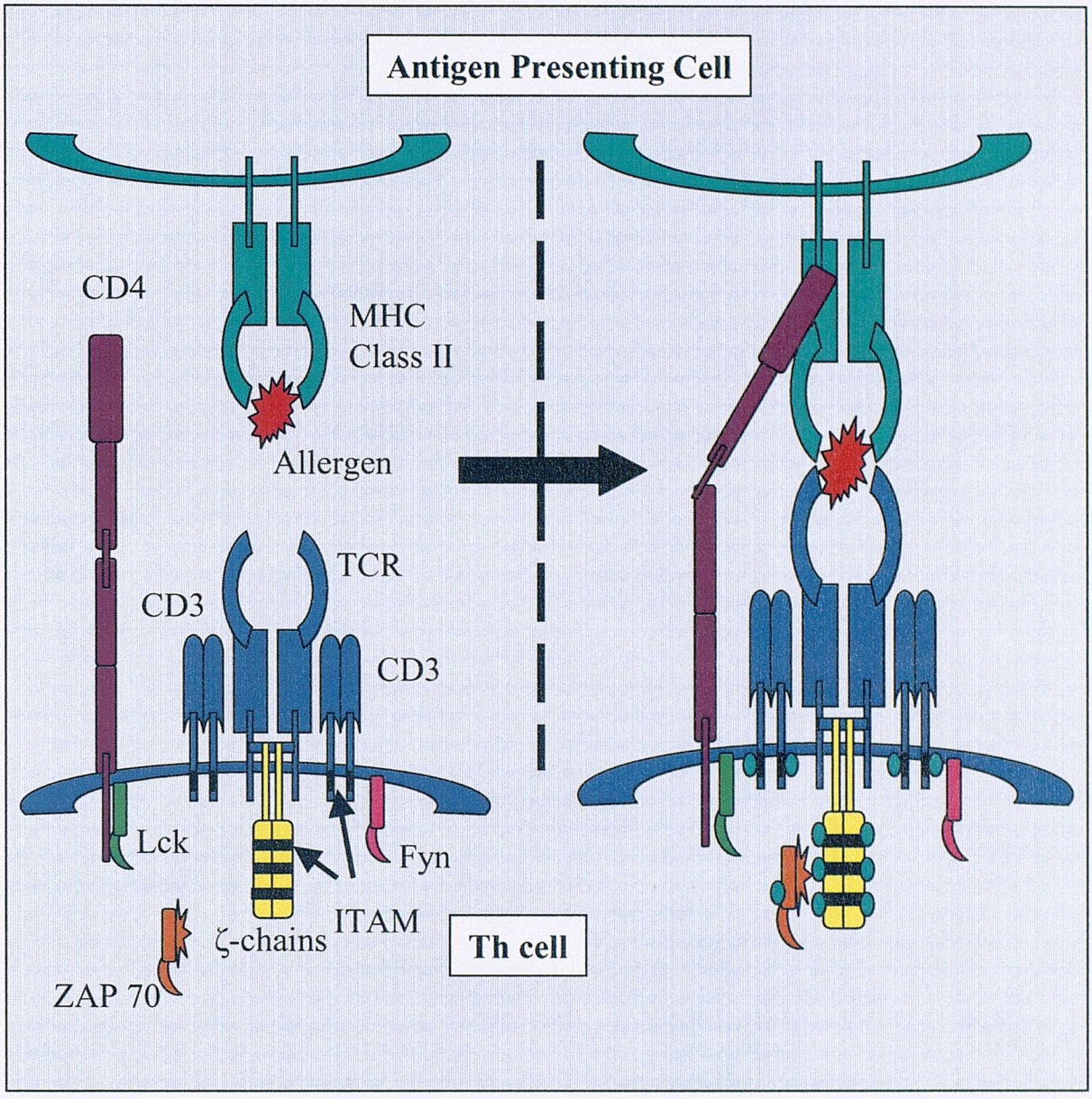


Figure 1.1. Presentation of allergen from an APC to a Th cell. Binding of the TCR/CD3 receptor complex to the MHC-bound allergenic peptide allows Lck and Fyn to induce phosphorylation of specific immunoreceptor tyrosine-based activation motifs (ITAM)s, which are associated with CD3.

Binding of the TCR/CD3 complex to the MHC-bound antigenic peptide allows Lck and Fyn to induce phosphorylation of specific immunoreceptor tyrosine-based activation motifs (ITAMs) [61], which are associated with CD3 and the TCR. Lck is associated with the cytoplasmic tails of CD4 and CD8 and these co-receptors are crucial regulators of the initial stage of T cell activation [62].

Phosphorylation of ITAMs initiates the recruitment of the protein tyrosine kinase ζ -chain associated protein (ZAP)-70[63]. ZAP-70 is then activated through phosphorylation by Lck and in turn phosphorylates linker of activation (LAT) and Src homology 2 (SH2)–domain-containing leukocyte protein (SLP-76). This initiates a chain of events (Figure 1.2.) which results in induction of the transcription factors NFκB, NFAT and AP-1, leading to cell proliferation and differentiation [64].

1.4. T cell classification

1.4.1. CD4+ T cell classification

Mosmann and Coffman first described the presence of two functionally distinct CD4+ helper T cell subsets in 1986 [65]. Th1 clones were characterised by the production of interferon- (IFN) γ, whereas Th2 clones secreted interleukin- (IL) 4. Th0 clones, which produced the cytokines of both clones, and Thp clones, which appeared to serve as precursors to Th clones, were also identified [66]. It was subsequently discovered that some of these cytokines not only induced the proliferation and cytokine production of one clone, but also suppressed both proliferation and cytokine production of the other [67]. These cross-regulatory properties were soon linked to observations made more than 30 years ago (summarised by Parish, [68]), indicating that an inverse relationship existed between cell-mediated immunity and humoral immunity in response to antigenic stimuli. Murine Th1 clones

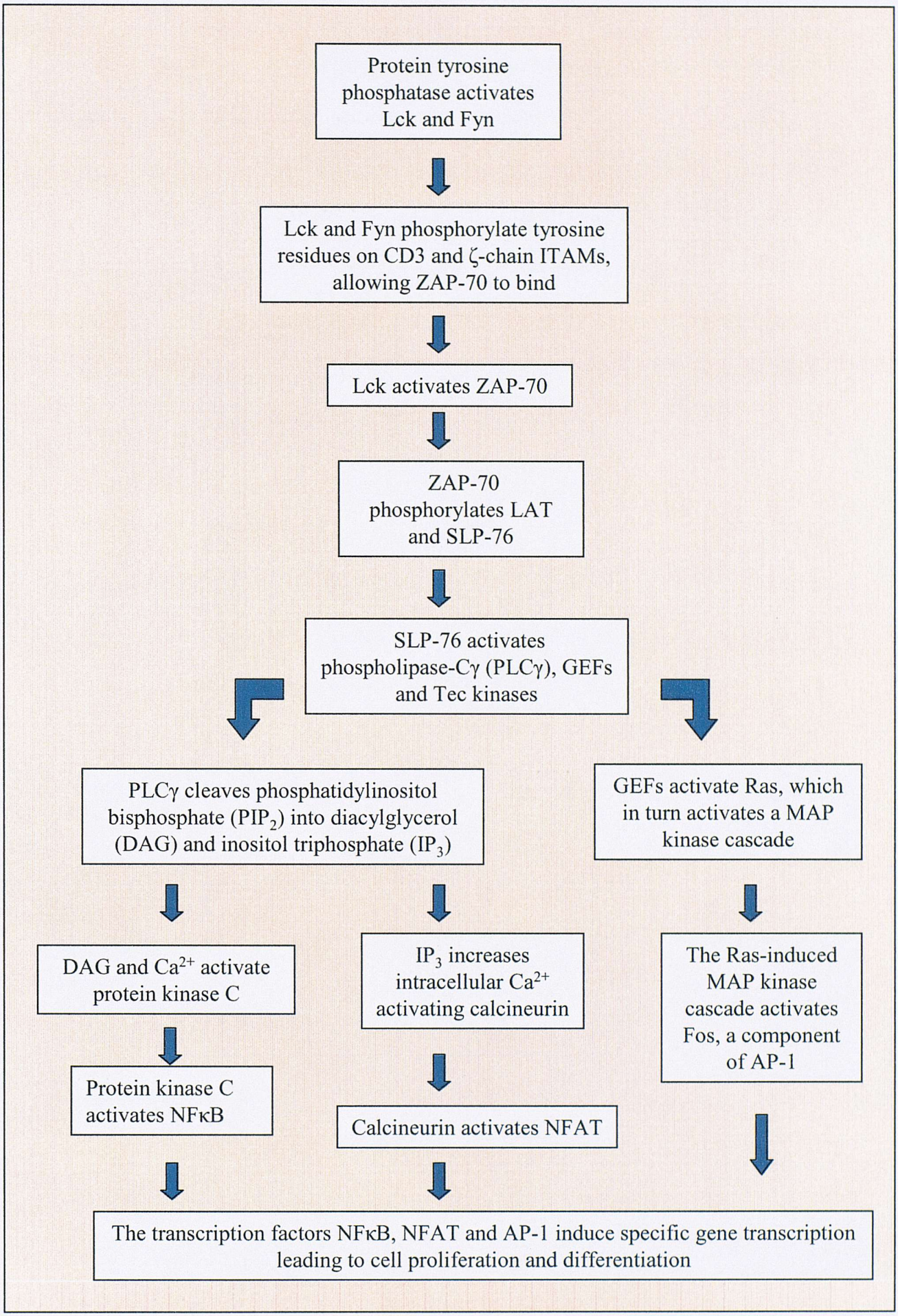


Figure 1.2. The signalling cascade resulting from TCR activation.

provided better help for cell mediated immunity, whereas Th2 clones were more important for B cell development and antibody production [69].

Although the Th1/Th2 polarisation is clear cut in artificially immunised murine models, it is usually less restricted in human Th cell-mediated responses and evidence of human Th1 and Th2 clones are only found in individuals with certain disease states, particularly chronic disease [70]. This illustrates the fact that Th1 and Th2 cells are not two distinct subsets, they simply represent polarised forms of the highly heterogeneous CD4+ Th-mediated immune response.

1.4.2. Th cell phenotype

T helper (Th) 1 cells produce interferon (IFN)-γ and lymphotoxin and play a central role in cell-mediated immunity [65;71]. Th1 cells are implicated in animal models of organ-specific autoimmune diseases, as well as chronic inflammatory diseases including inflammatory bowel disease [72-74]. Th1 cells are also associated with the expression of the chemokine receptors CCR1, CCR5, and CXCR3, [75;76] and receptors for IL-18 [77] and IL-12 [78]

Th2 cells produce IL-4, IL-5, and IL-13 and are important in helminth immunity, as well as playing a major role in allergic and atopic manifestations [79]. They also express CCR3, CCR4 [75;80;81] and the orphan receptor T1/ST2 [82-84]

1.4.3. Th cell development

Th1 and Th2 subsets develop from the same precursor, which is a mature naïve CD4+ T cell, producing mainly IL-2 upon antigenic stimulation [85;86]. The most clearly defined factors determining the differentiation of Th1 and Th2 cells from this precursor are cytokines present in the immediate miroenvironment [87]. For example,

bacterial stimuli activate macrophages and subsequently natural killer (NK) cells to produce IL-12 and IFN-γ respectively, which drive the development of Th1 cells. This type of immune response is appropriate for the eradication of microbial pathogens. Conversely, production of IL-4 early in an immune response directs the development of a Th2 humoral response from naïve precursors.

The source of IL-4 at the beginning of an immune response is currently unknown. It is possible that Th2 priming occurs either as a default pathway in the absence of stimulation by bacteria and viruses through Toll-like receptors (TLRs) or through currently unidentified Th2-type activating receptors [88]. Recent studies suggest that the effect of antigen dose and the state of maturation of the DC together with the stimulation of the DC by pathogen-associated microbial patterns (PAMPs) can influence the development of a Th1 or Th2 response [89;90].

Early interaction of IL12 with the IL12R on naïve Th cells induces STAT4 activation. This transcription factor is implicated in Th1 cell development as mice deficient for this factor lack Th1 cells [91]. Another transcription factor involved in Th1 lineage and IFN-γ gene expression is protein T-box expressed in T cells (Tbet). Transduction of T-bet into fully polarised Th2 cells converts them into Th1 cells and simultaneously represses the cytokines IL-4 and IL-5 [92].

Binding of IL-4 with its receptor IL-4R on naïve Th cells induces STAT6 [93]. The proto-oncogene c-maf is also specifically expressed by Th2 cells and binds to a maf response element within the IL-4 proximal promoter[94]. Although c-maf is critical for high levels of IL-4, an even more important transcription factor is required for initiation of IL-4 production. GATA-3 inhibits the production of IFN-γ, increases the transactivation of the IL-4 promoter and directly regulates IL-5 and IL-13 expression

[88;95]. Therefore Th1/Th2 polarisation involves a balance between the Th1-specific T-bet and the Th2-specific GATA-3 transcription factors.

1.4.4. Th cell function

The cytokine secretion pattern of Th1 and Th2 cell subsets defines their differential functions [67]. A combination of IL-12 and IFN-γ production by Th1 cells causes delayed-type hypersensitivity reactions and IFN–γ induces activation of macrophages and results in the killing of intracellular pathogens. Th1 cells also promote complement fixing [96] and the production of opsonising antibodies [97].

Th2 cells provide optimal B cell help and induce immunoglobulin class switching to IgG1, IgA and IgE antibody production [98]. Th2 responses are therefore important in allergen and parasitic responses, through IgE induced mast cell degranulation and in mucosal defence through induction of IgA secretion. Th2-associated cytokines have multiple effects on cells such as eosinophils, mast cells, B cells, T cells and APC, which are described in detail in Table 1.3.

In addition to being a stable cell marker on Th2 effector cells, T1/ST2 is important in Th2 effector function. Treatment of mice with antibody to T1/ST2 enhances Th1 cell responses in mice [99], and its neutralization inhibits allergic airway inflammation [100]. Furthermore, T1/ST2 is an inhibitor of interleukin 1 receptor and Toll-like receptor 4 signalling, and maintains endotoxin tolerance [101].

Cytokine	Cell type produced by	Action of cytokine in atopic disease
IL-4	Th2 cells, eosinophils, mast cells, basophils	B cell switch to IgE synthesis, mast cell development, eosinophil and basophil activation, mucus secretion, Th2 cytokine production, increases endothelial cell VCAM expression
IL-5	Th2 cells, eosinophils, mast cells, basophils	Eosinophil and basophil differentiation, maturation and activation
IL-6	Th1 cells, Th2 cells, macrophages, endothelial cells	T and B cell growth factor, co-factor for IgE synthesis
IL-9	Th2 cells, eosinophils, mast cells, basophils	Mast cell and eosinophil development, airway hyperresposiveness, mucus secretion
IL-13	Th2 cells, eosinophils, mast cells, basophils	Mast cell development, B cell switch to IgE production, eosinphilia, airway hyperresponsiveness, mucus hypersecretion
IL-25	Th2 cells	Favours Th2 cell development and IL-4, 5 and 13 production

Table 1.3. The role of Th2 cytokines in atopic allergic disease.
Adapted from Larche *et al* [102].

1.4.5. The association of Th1 and Th2 subsets with different diseases

The fine balance between Th1 and Th2 responses is very important, especially as the two populations are counter-regulatory [67]. Certain pathological conditions are associated with strongly polarised type 1 or 2 immune responses.

The clinical importance of Th1 cells has been confirmed in humans by the demonstration that specific genetic defects in the Th1 pathway result in extreme susceptibility to microorganisms such as Mycobacteria and Salmonella [103;104]. Th1 cells have also been implicated in animal models of organ-specific autoimmune diseases such as multiple sclerosis and insulin-dependent diabetes, as well as chronic inflammatory diseases including inflammatory bowel disease [72-74].

Clinical conditions that are characterised by increased IgE levels, which suggest an increase in the ratio of IL-4 or IL-13 to IFN-γ, and blood or tissue eosinophilia, which suggests increased levels of IL-5, indicate type-2 cytokine predominant diseases. Examples of these conditions include Ommen's syndrome, hypereosinophilic syndrome (HES), Kimura's disease, Job's syndrome, Wells' syndrome, atopic conditions and helminthic parasite infections [105].

Understanding immune responses from the perspective of type 1 and type 2 cytokine regulation and dysregulation is of great importance and could afford insights into the pathogenesis and therapy of many human diseases.

1.4.6. Th2 cells and allergic disease

The high levels of Th2 cytokines that are found at sites of allergic inflammation [106-111] are consistent with a Th2-based aetiology of atopic disease. The cytokine secretion profile of PBMCs in *in vitro* allergen-driven cell culture system also differs between atopic and non-atopic donors. PBMCs from atopic donors

produce relatively high levels of the Th2 cytokines IL-5 and IL-13 compared to the Th1 cytokine IFN-γ than those from non-atopic donors [112;113]. When PBMCs are stimulated with an antigen that tends to induce a Th1 cytokine profile, such as *M. tuberculosis* derived purified protein derivative (PPD), there is no difference in cytokine production between atopic and non-atopic donors [112].

In mouse models of allergic asthma, airway inflammation is dependant on CD4+ T cells, and more specifically is seen when TCR transgenic Th2, but not Th1 cells are adoptively transferred [114-117]. The main role of IL-4 is during the initial priming of Th2 effector cells [116;118-121], whereas IL-5 and IL-13 are more directly involved in characteristic atopic pathology such as eosinophilia and mucus hypersecretion [121-138]

1.4.7. CD8+ T cells and allergic disease

CD8+ T cells are also further categorised into Tc1 and Tc2 subsets, which secrete type 1 and type 2 cytokines, respectively and have similar effects to Th1 and Th2 cells during an active immune response [26;139;140]. CD8+ T cells have a strong bias towards differentiation into IFN-γ secreting cells and during intial priming require a higher dose of IL-4, than do CD4+ T cells, in order to differentiate into IL-4-producing cells. Bystander B cell help by CD8+ Tc2 cells may also occur *in vivo* due to their expression of CD40L [140] or through the secretion of type-2 cytokines.

CD8+ T cells may also play a role in the pathogenesis of human allergic disease. Increased frequencies of cutaneous lymphocyte associated antigen (CLA)+ CD8+ T cells producing Th2 cytokines are present in the peripheral blood of individuals with atopic dermatitis [141-143], and up to 30% of the T cell infiltrate within the skin of such patients is of a CD8+ phenotype [144]. Along with CD4+ T cells, there

is also an accumulation of CD8+ T cells in the lungs of individuals with asthma after allergen provocation [145;146] and during episodes of acute asthma [147]. Furthermore, in both mice and rats, ovalbumin-specific CD8+ T cells can modify IgE production [148-152] and in some systems CD8+ T cells contribute to murine airway eosinophilia and hyperresponsiveness [153-155].

1.5. The cellular basis of atopic allergic reactions

When an atopic individual is exposed to an allergen to which they have previously been sensitised and to which they have specific IgE, they will immediately mount an immune reaction which, (depending on the site of exposure) will result in either a cutaneous wheal and flare reaction, sneezing, runny nose or wheezing. This is known as the early phase reaction, and may be followed some six to nine hours later by an edamatous, red swelling of the skin, sustained blockage of the nose or further wheezing in the lung. This reaction is dependent on the concentration of allergen encountered and is known as the late phase reaction [156] (Figure 1.3).

1.5.1. The Early Phase Reaction

Molecules such as histamine, tryptase, leukotrienes, prostaglandins and platelet activating factor are released by mast cells and basophils when an allergen interacts with membrane-bound IgE, causing immediate hypersensitivity. This is the basis of acute allergic reactions such as anaphylaxis, rhinoconjunctovitis and urticaria. The IgE is bound to mast cells and basophils by the alpha chain of the high affinity IgE receptor (FcεR1-α) [157].

Histamine is the primary effector mediator secreted by mast cells. Extra cellular histamine has a wide variety of effects, mediated through specific H1, H2 or

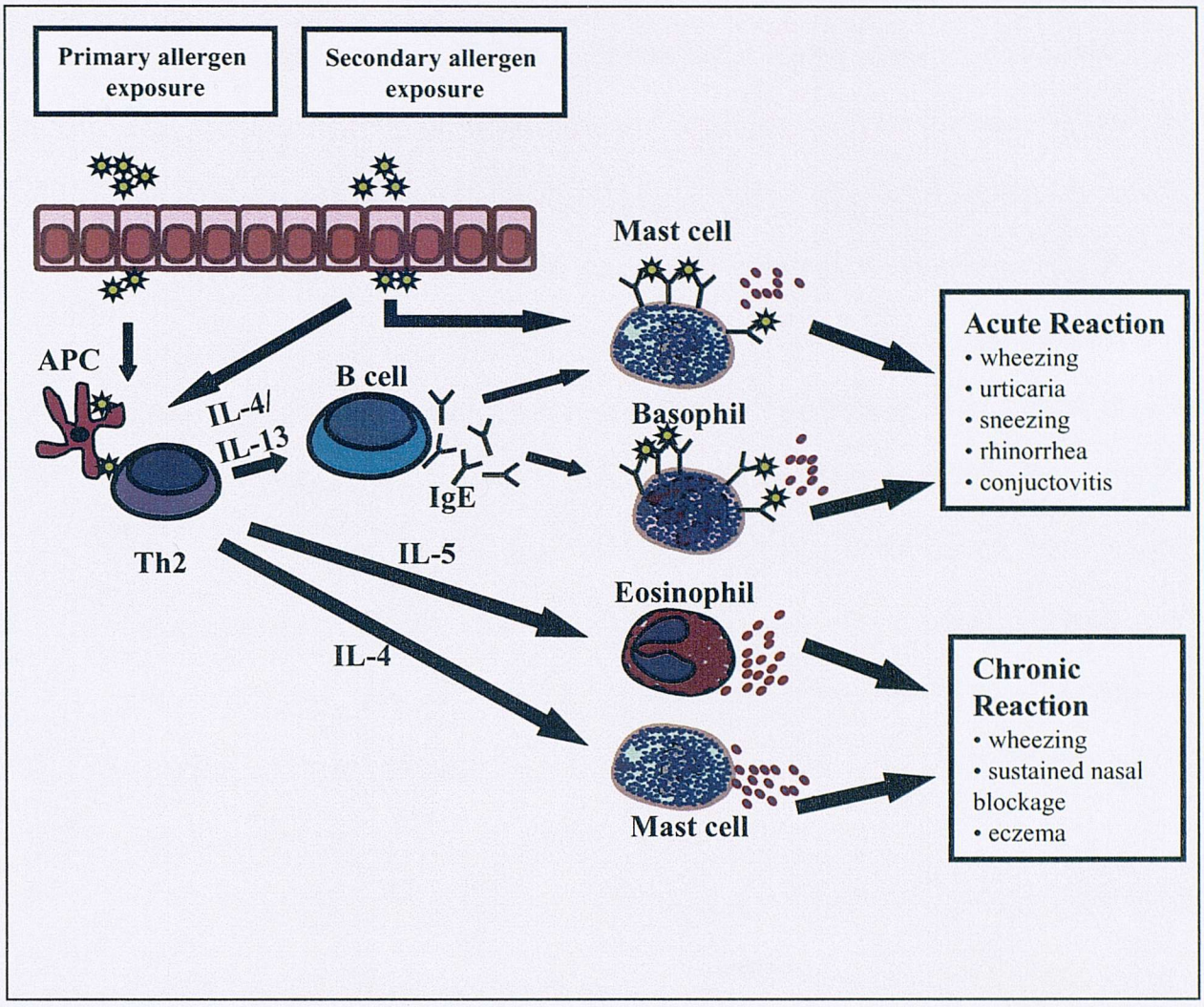


Figure 1.3. The cellular basis of acute and chronic allergic reactions. After exposure to allergen, APCs present allergen to Th2 cells. The production of IL-4 and IL-13 by Th2 cells causes B cells to secrete IgE. Allergen interacting with membrane-bound IgE on the surface of mast cells and basophils, induces secretion of histamine, lipid mediators and cytokines, which contribute to acute allergic reactions. The Th2 cytokines IL-5 and IL-4 recruit an inflammatory infiltrate consisting of eosinophils, mast cells and T cells which drive chronic allergic reactions.

H3 receptors. In asthma, histamine has been implicated in bronchoconstriction,vasodilation, oedema, stimulation of bronchoconstrictor reflexes and mucus secretion. In rhinitis, histamine is responsible for rhinorrhea and in the skin, it plays a pivotal role in the wheal and flare response and in urticaria [13].

The most abundant prostaglandin released by the mast cell is prostaglandin D_2 (PGD_2). In the lung, PGD_2 acts as a potent bronchoconstrictor agent. It also contributes to nasal blockage in rhinitis and has vasodilator properties in the skin [13]. LTC_4 and LTD_4, which are leukotrienes released by mast cells, are also highly potent bronchoconstrictor agents. Furthermore, leukotrienes cause increased permeability of post-capillary venules, as illustrated by prolonged wheal responses to intradermal injection, and they have potent vasoconstrictor properties, which may be important in systemic anaphylaxis [158].

Mast cells also release tryptase, a four-chain neutral protease that activates the protease-activated receptors on endothelial and epithelial cells. This causes the up-regulation of adhesion molecules, which in turn selectively recruit eosinophils, basophils and other inflammatory cells to the area of inflammation. Finally, platelet activating factor is another lipid-derived mediator released by mast cells that may play a role in bronchial hyperresponsiveness [159].

1.5.2. The Late Phase Reaction

Late phase reactions can be provoked by the activation of mast cells or T cells. In the cutaneous late phase reaction, eosinophils and neutrophils accumulate, followed by an infiltration of CD4+ T cells and basophils [160]. Antigen presenting cells such as DCs and langerhans cells are critical in both initiating and controlling allergic inflammation. They present antigen to CD4+ Th2 cells in an MHC II-restricted

fashion. When alveolar macrophages from atopic asthmatic subjects present allergen to CD4+ T cells they stimulate secretion of Th2-type cytokines, whereas alveolar macrophages from non-atopic subjects do not [161]. IL-4, IL-5, IL-9 and IL-13 are the classic Th2 type cytokines produced by allergen stimulated cells from atopic subjects [156].

Infiltration of eosinophils into the bronchial wall and lumen is a prominent pathological feature of asthma. Skin diseases such as atopic dermatitis, chronic urticaria and episodic angiodema are also associated with eosinophilia. IL-5 causes release of both mature and immature eosinophils from the bone marrow [162]. The eosinophil, which is a tissue inflammatory cell that matures in the bone marrow, is transported in the bloodstream to sites of inflammation. There, it releases mediators which may play an important role in the later inflammatory stages of asthma, rhinitis, conjuctovitis and cutaneous allergies [156].

1.5.3. The treatment of atopic allergic disease

Allergen avoidance

Decreasing exposure to the allergen that triggers the allergic reaction is usually successful, especially if the allergy is to a particular food, drug or animal. It is more difficult however, to avoid house dust mites or seasonal pollens and moulds as these have a widespread airbourne distribution [163].

Drug treatment

For the relief of symptoms, α-stimulants (decongestants) that are vasoconstrictors are widely used among hayfever sufferers as both topical preparations and as tablets. They possess α-adrenoreceptor stimulant properties,

which cause contraction of the smooth muscle, increasing reactive hyperaemia. Anti-cholinergic drugs are used in the control of watery rhinorrhea and virus-induced rhinitis. Many drugs with anti-histamine activity have been introduced since the first histamine antagonists were developed 70 years ago and these drugs are widely used to treat the symptoms of many allergic diseases [164].

To suppress allergic inflammation, topical glucocorticoids are efficacious and recommended therapy in current guidelines for asthma, rhninitis and allergic skin disease [165-167]. These drugs act by complexing to intracytoplasmic receptors, and directly increase or repress gene transcription, or interact with transcription factors, inhibiting T cell activation and production of Th2 cytokines *in vitro* [168], and have been shown to reduce expression of Th2 cytokines in the airway mucosa of asthmatics *in vivo* [169;170]. IL-10 producing regulatory T cells can be induced by a combination of corticosteroids and Vitamin D3 [171]. Furthermore, there is a defect in corticosteroid-induced IL-10 production by T cells from corticosteroid resistant asthmatic patients [172].

Specific allergen immunotherapy

Specific allergen immunotherapy involves administering increasing concentrations of allergen extracts subcutaneously over a long period of time. This is an extremely effective treatment for patients suffering from seasonal allergic rhinitis and to a lesser extent those with perennial rhinitis. The effects are long lasting, especially if the treatment is continued for several years [173]. There is a potentially fatal side effect of specific immunotherapy however, which is a risk of anaphylaxis, particularly during the "up-dosing" phase of treatment [163].

The mechanism by which specific allergen immunotherapy works is extremely complex: IgG antibodies compete with IgE for allergen [174] and immunotherapy also inhibits the release of inflammatory mediators from mast cells and basophils [175]. The influence of immunotherapy on T cells is central to its inhibition of the late phase response. Immunotherapy promotes a switch from the Th2-type cytokine profile, to a Th1 profile. After several months or years of treatment the intensity of the early phase reaction and total serum IgE levels are decreased[174]. Increased secretion of IL-10 by *in vitro* allergen-stimulated PBMCs from immunotherapy treated donors [176;177] and expression of CD25 by T cells [178], suggests a role for regulatory T cells in the suppression of atopic disease. The role of regulatory T cells in the mechanism of specific allergen immunotherapy treatment will be further discussed in Chapter 5.

1.5.4. Susceptibility to atopic allergic disease

Genetic links

Atopy is hereditary, however it is difficult to conduct linkage studies of atopic diseases as they are multi-factorial in nature, but such studies have been carried out and several loci have been linked to atopy. The genes for the β-chain of the high affinity IgE receptor (FcεR1-β) [179] and the IL-4 family of cytokines that are located on chromosome 5 are linked to atopy [180]. Positional cloning has also suggested a link between specific regions on chromosomes 2q, 6q, 12q and 13q and atopy and asthma [181]. Many of these regions have also been linked to other immune disorders such as rheumatoid arthritis (chromosome 2) and IBD (chromosomes 2 and 12) [181].

The hygiene hypothesis

Over the past few decades there has been a marked increase in the prevalence of allergic diseases in Western Europe, the United States of America and Australasia, but there has been no such increase in developing countries. It has been suggested that a Western lifestyle could account for this increase in prevalence. In developed countries the immune system is deprived of microbial antigens that could tip the balance to a type 1 response during immune system development. We live in a relatively clean and germ-free environment and there is widespread use of antibiotics for childhood diseases [182]. There is much evidence to support this, the "hygiene" hypothesis. For example, atopy and allergic asthma are less frequent in populations exposed to *Heliobacter pylori*, *Toxoplasma gondii* and hepatitis A virus [183]. Other factors that are associated with protection against the development of a Th2 responses to allergens include having contact with other children (either younger siblings [184], or at day care centres [185]) and living on farms in a rural environment [186]. It has also been suggested that being born when the pollen count is high may protect against the development of hayfever [181]. All these studies indicate that repeated immune system stimulation in the first few years of life may be crucial for protection from atopic allergic disease.

1.6. Regulatory T cells

1.6.1 Regulation of immune responses

Though crucial for immunity to infection, over-zealous or inappropriate T cell responses can cause damage to the host, which is termed immunopathology. The inflammatory conditions of allergy and autoimmune disease are also caused by exaggerated immune responses, in these cases directed towards innocuous

environmental antigens (allergens) and self-antigens respectively. The fine balancing act between protection from infection on one hand and protection from immunopathology on the other requires careful regulation.

It is important to differentiate between the various mechanisms that can lead to T cell tolerance. Firstly, clonal deletion of self-reactive T cells occurs in the thymus during negative selection [22], and lymphocytes are also deleted by apoptosis immediately after encountering soluble antigen or after persistent activation and proliferation, (known as activation-induced cell death, AICD)[187;188]. Secondly, lymphocytes that are partially activated by encounter with antigen in the absence of sufficient co-stimulatory signals acquire a state of inactivation (anergy), in which they are partially or totally unresponsive to classical antigen-induced proliferation [189 190]. Thirdly, lymphocytes which are naturally occurring or which have been induced in the periphery, actively suppress autoaggressive effector T cells and are termed regulatory T cells [191].

The existence of regulatory T cells was first proposed in the 1960s, when it was hypothesised that T cells could act to suppress the immune response as well as to augment it [192]. It was presumed that the suppression was due to the antigen-specific production of soluble mediators by the cells, but when no specific mediator could be isolated, the idea of suppressor cells was abandoned. The discovery of different populations of T helper cells, also led people to believe that there was no specific suppressor population, but that instead immune suppression was caused by counter regulatory cytokines produced by Th1 and Th2 cell subsets.

1.6.2. Naturally occurring CD4+CD25+ regulatory T cells

Experiments using animal models of autoimmunity showed that thymectomy of mice on day 3 of life caused a wide range of organ specific autoimmune diseases such as oophoritis, thyroiditis and gastritis, depending on the strain of mouse used [193-196]. The disease was T cell mediated and if thymectomy was delayed until day 7, they did not develop autoimmunity. Disease progression could be suppressed by injection of CD4+ T cells from adult mice or by thymus transplant before day 14 [197]. Experiments such as these suggested that there was a population of cells that developed in the thymus of mice between days 3 and 7 of life that could suppress T cell mediated autoimmune disease[12;178;198]. Using adoptive transfer techniques to identify the subset of CD4+ T cells responsible for the suppression of disease, the regulatory population was further analysed [74;199-202] and found to be CD25+ [203-206].

In general CD25, which is the IL-2 receptor α-chain (IL-2Rα), cannot be detected on resting, non-regulatory CD4+ T cells, and its expression is upregulated after exposure to antigen. There are differences however between CD4+CD25+ regulatory T cells and activated T cells, with respect to phenotypic characteristics and expression of CD25. Mouse and human CD4+ T cells with the most potent regulatory properties express high and sustained levels of CD25 [207] [208] in contrast to recently activated T cells which express CD25 only transiently and at a lower level.

The expression of the IL-2Rα chain does not seem to be directly associated with the regulatory function itself as T cells with suppressive abilities are found within both the CD25+ and CD25- subsets [209]. Recent studies have illustrated a role for IL-2 in the development of CD4+CD25+ regulatory T cells in the thymus [210;211]. When they undergo homeostatic expansion *in vivo*, these cells lose expression of

CD25 and hence are no longer responsive to IL-2; nevertheless, they retain potent suppressive activity when isolated and tested *in vitro* [212].

1.6.3. Suppression by CD4+CD25+ T cells in vitro

To analyse the suppressive function of CD4+CD25+ regulatory T cells further, a simple *in vitro* assay was established in which CD4+CD25+ T cells were cocultured with CD4+CD25- T cells, and the degree of suppression assessed by measuring the proliferation of cocultured cells upon antigenic stimulation in the presence of APCs [201;213;214]. The CD4+CD25+ population of cells was hyporesponsive and was able to suppress activation of T cells to polyclonal stimulation *in vitro* [201;213;214].

Suppression *in vitro* requires stimulation of the CD4+CD25+ T cell through the TCR, it is cell contact dependant and is accompanied by cell cycle arrest [201;213;214]. The activation of CD4+CD25+ T cells is antigen specific but once activated they can suppress T cell responses in an antigen independent manner [215]. The effects of regulatory T cells on other T cells, either directly or indirectly through APCs [216], can result in inhibition of cytokine production, down-regulation of expression of co-stimulatory molecules and/or adhesion molecules, inhibition of proliferation, induction of anergy [217] or elimination of the effector population by promoting cell death [218]. Regulatory T cells can also convert the effector cell to a regulatory T cell phenotype, a process known as infectious tolerance [219 220;221].

1.6.4. Human CD4+CD25+ regulatory T cells

The CD4+CD25+ regulatory population isolated from human peripheral blood and thymus has now been extensively characterised *in vitro* [208;222-227]. It is anergic to polyclonal stimulation, for example by cross-linking CD3 and CD28, and it

can suppress T cell responses to polyclonal stimulation and to antigens such as cows milk antigen (CMA), tetanus toxoid (TT) and human heatshock protein 60 (hHSP60)[228]. The CD4+CD25+ T cell population also suppresses CD8+ T cell responses [229-231] and mixed lymphocyte reactions (MLR) [232].

CD4+CD25+ T cells constitutively express CTLA-4 [53;54], they also express high levels of the glucocorticoid-induced tumour necrosis factor (TNF) receptor (GITR)[233-235] and the transcription factor FOXP3 [236-238]. The phenotype of this naturally occurring population of regulatory T cells is further described in Chapter 3.

1.6.5. The mechanism of suppression by regulatory T cells

The precise molecular mechanism by which CD4+CD25+ regulatory T cells suppress the activation and proliferation of other T cells is currently controversial. If this mechanism could be elucidated, it could be manipulated and either enhanced or blocked depending on the desirable therapeutic outcome. Several cytokines, cell surface receptors and a transcription factor have been implicated in the mechanism of suppression, but as yet no definitive molecule has been found to mediate suppression of all T cell responses both *in vitro* and *in vivo* by regulatory T cells.

Animal models of autoimmune diseases suggested a role for the cytokines IL-10 and TGF-β in the suppressive activity of CD4+CD25+ T cells. IL-10 has been implicated in the suppression of murine models of inflammatory bowel disease [239], transplantation tolerance, graft versus host disease, chronic parasitic infection and autoimmunity [240], and anti-TGF-β treatment abrogates the suppression of IBD [241] and thyroiditis [242].

The use of gene deficient mice however has shown that the mechanisms of suppression are dependent on the suppression assay studied and the strain of mice

used. IL-10 deficient T cells, for example, effectively prevent autoimmune disease [198;239;243]. Neutralisation of TGF-β1 with either monoclonal antibody (mAb) or soluble TGF-βRII-Fc does not reverse *in vitro* suppression mediated by resting or activated CD4+CD25+ T cells. Responder T cells from Smad3 gene deficient or dominant-negative TGF-β type RII transgenic (DNRIITg) mice, which are both unresponsive to TGF-beta1-induced growth arrest, are as susceptible to CD4+CD25+ T cell-mediated suppression as T cells from wild-type mice. Furthermore, CD4+CD25+ T cells from neonatal TGF-$\beta1^{-/-}$ mice are as suppressive as CD4+CD25+ from TGF-$\beta1^{+/+}$ mice [244].

The suppression of T cell responses by human and mouse CD4+CD25+ regulatory T cells *in vitro* is independent of soluble cytokines. *In vitro*, suppression requires contact between the regulatory T cell and the target cell [201;213;214]. This *in vitro* suppressive cell contact was not due to killing of the responder population via a Fas/FasL or TNF/TNF receptor dependent pathway [213]. Roles for CTLA-4 [53;54;245], GITR [233-235] and cell membrane bound TFG-β have been proposed [246]. Regulatory CD4+CD25+ T cells express both GITR and CTLA-4 and suppression is partially reversed in some models by addition of blocking antibodies to these receptors [53;54;233-235;245]. Although blocking reagents to CTLA-4 and GITR reverses suppression of *in vitro* T cell responses by mouse CD4+CD25+ T cells, there have been no such reports showing such reversal of suppression by human CD4+CD25+ T cells.

Regulatory T cells may also affect the functional activity of other T cells indirectly by modifying the function of the APC. There is evidence from *in vitro* studies that regulatory T cells downregulate the expression of co-stimulatory molecules such as B7.1 and B7.2 on APCs, thereby rendering the APC less equipped to trigger T cell activation [247].

Finally the transcription factor FOXP 3, which is expressed by CD4+CD25+ but not CD4+CD25- T cells, has recently been implicated in suppression by this regulatory T cell subset [236]. *FoxP3* $^{-/-}$ mice suffer from lymphoproliferative disease, and adoptive transfer of CD4+CD25+ T cells from wild type mice suppresses this disease [248;249]. Mutations of the human gene FOXP3, the ortholog of the murine FoxP3, cause immune dysregulation, polyendocrinopathy, enteropathy, and in an X-linked syndrome (IPEX) [250-252] which is associated with autoimmune disease in multiple endocrine organs, IBD, atopic dermatitis, and fatal infections [253]. Ectopic expression of the FoxP3 gene also converts CD4+CD25- T cells into cells with phenotypic and functional properties similar to those of naturally occurring CD4+CD25+ T cells [236-238].

The mechanism of suppression by regulatory T cells is discussed in more detail in Chapter 3.

1.6.6. The identification of other regulatory T cell populations

As well as naturally occurring CD4+CD25+ T cells, a spectrum of other types of regulatory T cells have been induced *in vivo* and *in vitro*. Many are induced in response to antigen presented in a "tolerogenic form", for example as excess soluble antigen or as an altered peptide ligand with concomitant costimulatory blockade or with IL-4 and/or IL-10, or by a "tolerogenic route" for example mucosal administration [254]. At least some subsets of CD4+CD25+ regulatory T cells can be induced in the periphery, [255;256] so the exact distinction between the "naturally occurring" and "experimentally induced" subsets is at present unclear and some overlap between groups is likely.

At present, regulatory T cells are defined primarily by their function, as no definitive marker has been identified. Regulatory T cells are therefore defined operationally as T cells that protect against pathological or clinically undesirable inflammatory immune responses. Two properties that are shared by many known regulatory T cell populations are an impaired capacity to respond to proliferative signals *in vitro* and the ability to inhibit immune cell functions either directly through cell contact or through secretion of anti-inflammatory mediators.

In vitro, the hyporesponsive and suppressive qualities of regulatory T cells are shared by many anergic T cells, [213] [212] and the relationship between anergic and regulatory T cell populations is also as yet unknown. *In vivo*, the suppressive nature of regulatory T cells is demonstrated most convincingly by their ability to suppress auto-immune disease and transplant rejection in animal models. An important issue to consider with these experiments however, is that in most transfer experiments, the hosts are lymphopenic and therefore regulatory functions can not be easily distinguished from proliferation and clonal expansion of the transferred cells [217;257].

Tr1 cells

Repeated stimulation of CD4+ T cells in the presence of IL-10 *in vitro* results in the differentiation of a subset of T cells, defined as T regulatory type 1 (Tr1) cells with a profile of cytokine production distinct from that of Th1 or Th2 cells. Tr1 cells which have been generated from human [258] and murine [259] T cells, produce high levels of IL-10, significant amounts of IFN-γ, IL-5 and TGF-β but little or no IL-4 or IL-2, and suppress the proliferation and cytokine production of other T cells *in vitro*. Furthermore, *in vivo* Tr1 cells prevent the induction of IBD in SCID mice when transferred with pathogenic $CD45RB^{hi}$ CD4+ T cells [259]. Tr1 cells may also play a role

in the prevention of allergic disorders, as they prevent airway Th2 cell activation and eosinophilia in a murine model of inhaled allergen challenge [260].

Similar to CD4+CD25+ regulatory T cells, Tr1 cells must be activated through the TCR to exert their regulatory effects *in vivo* and the effector function is not antigen-specific, as they can inhibit the function of T cells responding to unknown intestinal antigen, a process known as "bystander suppression" [259].

In vitro, Tr1-mediated suppression is still observed when the regulatory and responder cells are separated using a transwell. Addition of IL-10 and TFG-β neutralising antibodies partially restores the antigen-specific proliferative response of naïve CD4+ T cells [259], suggesting that the regulatory effects of Tr1 cells are at least partially mediated by soluble factors [261]. The presence of IL-10 has also resulted in the clonal expansion of CD4+CD25+ regulatory T cells without loss of function [222]. It is therefore possible that Tr1 cells and CD4+CD25+ T cells are derived from the same precursor cell [261].

Th3 cells

Th3 cells arise after oral administration of antigen and produce high concentrations of TGF-β. They were originally isolated from mice and protect from development of immune pathology in several animal models [262-264]. Th3 cells express CD4 and promote IgA production, the major immunoglobulin class in the mucosa.

TGF-β-secreting Th3 cells require IL-4 for their *in vitro* generation. Although early induction of TGF-β is promoted by IL-4, once secretion of TGF-β has occurred, TGF-β itself further enhances its own production [265]. This population of cells is not just an *in vitro* generated cell population as it has also has also been isolated from the

peripheral blood of patients with multiple sclerosis [266], implying a role for this subset of cells in the suppression of human auto-immune disease.

IL-10 producing T cells

Another phenotypic population of regulatory T cells is generated *in vitro* with a combination of the immunosuppressive drugs 1α, 25-dihydroxyvitamin D3 (VitD3) and dexamethasone, and concurrent inhibition of Th1 and Th2-associated cytokines. This population of cells produces high levels of IL-10 and no typical Th1 or Th2 cytokines[171]. The regulatory function of these *in vitro* generated IL-10-producing T cells is demonstrated by their ability to prevent central nervous system inflammation when targeted to the site of inflammation, in an IL-10 dependent manner.

Repeated injection of mice with antigen [267] or peptide [268] also results in the generation of IL-10 producing cells. These cells fail to proliferate in response to antigen, showed markedly reduced secretion of IL-2, IL-4 and IFN-gamma but produce high levels of IL-10 [269]. A similar population was generated from human naïve CD4+ T cells after repeated stimulation with allogeneic immature dendritic cells [270].

Allergen immunotherapy treatment involves repeated stimulation with increasing concentration of allergen (or peptide fragments) and is an effective treatment for human allergic disease, (described further in Chapter 5). IL-10 is implicated as a mechanism by which specific allergen immunotherapy treatment is effective. PBMCs from donors who have received allergen immunotherapy produce more IL-10 and proliferate less when stimulated *in vitro* with allergen, than those from atopic donors who have not received treatment [176;177]. This suggests the

induction of an IL-10 producing regulatory T cell population capable of suppressing allergic disease.

1.6.7. Other cell types with the ability to suppress immune responses

CD8+ Tr cells

CD8+ T cells act not only as effector cells after intracellular infection, but also as regulatory T cells as demonstrated by the fact that CD8-/- mice develop experimental allergic encephalomyelitis (EAE) in a more severe and chronic way, than do wild type mice [271]. In *Leishmania major* infection, CD8+ IFN-γ producing T cells mediate protective immunity generated by vaccination [272;273]. CD8+ T cells residing in the gut, also play an important role in the suppression of intestinal immune responses [274] and antigen specific CD8+ cells inhibit IgE responses and IL-4 production in a murine ovalbumin challenge model [149].

TCR γδ+ T cells

Mucosal sites contain a large number of TCR γδ+ T cells. These cells play an important role in both the induction and regulation of mucosal immunity [217;275]. TCR γδ+ murine intraepithelial lymphocytes for example, play an active role in the tolerance induced by orally administered sheep red blood cells [276]. TCR γδ+ T cells can also produce immunoregulatory cytokines, and elimination of TGF-β and IL-10 producing TCR γδ+ T cells from mice with tumours augments CTL and natural killer (NK) cell activities leading to tumour regression [277]. Such cells also regulate cutaneous inflammation [278]. Finally, administration of an insulin aerosol to NOD mice

protects them from the onset of diabetes, protection which can be transferred by CD8αα+γδ T cells [279].

NKT cells

NKT cells are defined as thymocytes that express αβ TCR, are negative for both CD4 and CD8 coreceptors and express the NK cell receptors. Although the role of NKT cells is at present unclear, they primarily display immune stimulatory functions [280]. CD1d-restricted NKT cells however, are required for the induction of systemic tolerance by anterior chamber-associated immune deviation (ACAID). NKT cells cannot transfer tolerance however, suggesting that they are required for the differentiation of another population of regulatory T cells [281].

NKT cells are also required for induction of tolerance against xenogenic islet grafts in mice treated with anti-CD4 monoclonal antibody [282]. The mechanism by which NKT cells exert their regulatory function is unclear, although roles for LFA-1/ICAM-1 or CD28/B7 blockade have been proposed [283]. TGF-β may also be involved in their suppressive activity, as NKT cells secrete this cytokine and it induces CD8+ T cells to develop inhibitory activity and reduces CD4+ T helper cell activity [284].

1.6.8. The role of the CD4+CD25+ regulatory T cell in human disease

A population of T cells that can suppress an immune response has obvious clinical relevance. Enhancing suppression of immune responses could assist the treatment of transplant rejection, autoimmune disease and allergy, and decreasing suppression (and therefore increasing immune activitity) could help in cancer therapy or treatment of persistent infections.

Transplantation tolerance

A role for regulatory T cells in mouse models of transplantation tolerance was identified in 1975 when suppressor T cells were identified in mice made unresponsive to skin allografts [285]. *In vivo*, alloantigen specific regulatory T cells prevent rejection initiated by CD4+ T cells in both organ [286 287 288] and bone marrow transplantation [289 203;290]. CD4+CD25+ regulatory T cells also suppress rejection mediated by allo-antigen specific CD8+ TCR-transgenic T cells, [229 230;231] and tolerogenic dendritic cells induced by Vitamin D3 receptor ligands induce regulatory T cells and inhibit allograft rejection [291].

There have also been studies investigating the role of human regulatory T cells in transplant tolerance. A novel human to mouse *trans vivo* model identified donor reactive cell mediated immune responses in kidney and liver transplant patients. The results showed that the donor reactive delayed type hypersensitivity reaction was weaker in tolerant patients and that suppression of the response was dependent on either IL-10 or TGF-β [292]. Furthermore, kidney transplant patients with no history of acute rejection demonstrated regulation to the mismatched HLA-DR allopeptides compared to those that had suffered episodes of acute rejection, and that this regulation was mediated by CD4+CD25+ T cells [293].

Tumour immunity

Naturally occurring CD4+CD25+ regulatory T cells engaged in the maintenance of self-tolerance may impede the generation and activation of tumour-effector T cells recognising autologous tumour cells [6]. Preliminary studies show that antibody depletion of CD25+ T cells in rodents facilitates tumour immunity [294;295]. Interestingly, a combination of CD25 depletion and CTLA-4 blockade was found to

be more effective than either treatment alone. There is also evidence for suppression of anti-tumour responses by CD4+CD25+ T cells infiltrating lung cancers in humans [296].

Autoimmune disease

Although there is clear evidence that clonal deletion of T and B cells that recognise self-antigens at immature developmental stages is a major mechanism of self-tolerance, potentially hazardous self-reactive lymphocytes are still present in the periphery of normal individuals [297]. Regulatory T cells suppress both the activation and expansion of self reactive T cells [191].

The relevance of regulatory T cells to the inhibition of autoimmune diseases in animal models is now well established. Studies in a number of experimental models of organ-specific autoimmune disease provide convincing evidence that regulatory T cells capable of controlling autoimmunity are an integral part of the T cell repertoire. Organ-specific autoimmune manifestations including gastritis, oophoritis, orchitis and thyroiditis, caused by neonatal thymectomy of mice can be inhibited by the transfer of $CD4^+CD25^+$ T cells [203;206]. Similarly, the development of autoimmune thyroiditis or diabetes in rats subjected to adult thymectomy followed by fractionated doses of sublethal irradiation (TxX) is inhibited by the transfer of $CD4^+CD45RC^-$ T cells from normal rats [242;298].

Transfer of wildtype $CD4^+$ T cells can also inhibit the spontaneous development of autoimmune EAE in mice transgenic for a myelin basic protein (MBP)-specific TCR [299;300] and the development of spontaneous autoimmune diabetes in a genetically susceptible strain [301], indicating that immune suppression by regulatory T cells is not restricted to experimentally induced disease.

A recent study has shown a significant decrease in the effector function and frequency of CD4+CD25hi regulatory T cells from peripheral blood of patients with multiple sclerosis as compared with healthy donors [302]. Further studies in humans have also shown that CD4+CD25+ regulatory T cells can be isolated from the target organs of patients with rheumatoid arthritis, [303] suggesting a role for these cells in regulation of autoimmune disease in humans.

Infectious diseases

CD4+CD25+ regulatory T cells also regulate virus-specific primary and memory T cell responses [304]. A study evaluating the role of regulatory T cells in viral immunopathological lesion stromal keratitis a frequent cause of human blindness resulting from a T cell-mediated inflammatory response to HSV in the corneal stroma, found that lesions were significantly more severe in mice depleted of regulatory T cells. The mechanism of control of stromal keratitis involved suppression of antiviral immunity and impaired T cell migration to lesion sites [305].

In humans, human cytomegalovirus (HCMV) persistence is associated with a higher frequency of CD4+CD25+ regulatory T cells [306] and CD4+ CD25+ regulatory T cells control T-cell responses to both human immunodeficiency virus (HIV) and cytomegalovirus (CMV) antigens [307], indicating that chronic viral infections may induce regulatory T cells that inhibit antiviral immune responses.

The role of regulatory T cells in immunity to infection is not restricted to viral pathogens. CD4+ and CD8+ regulatory T cells have also been implicated in the perpetuation of chronic infectious diseases such as leprosy [308] and *Leishmania major* infection [309;310].

Th2 mediated diseases

Data regarding CD4+CD25+ T cell regulation of Th2-type responses in allergic disease is sparse. Animal models of allergic airways sensitisation are useful for defining potential immunological mechanisms in asthma and allergic disease [311] and Tr1 cells have been shown to prevent airway Th2 cell activation and eosinophilia in a murine model of inhaled allergen challenge [260]. Mature pulmonary dendritic cells in the bronchial lymph nodes of mice exposed to respiratory allergen also induced the development of IL-10 producing regulatory T cells, a process which required T-cell costimulation via the ICOS-ligand pathway [58].

Although murine CD4+CD25+ T cells suppress *in vivo* IgE production [312], depletion of these cells actually reduced airway eosinophilia, numbers of T cells and IgE levels in a mouse model of asthma [313]. This may be because CD25 is also a marker of recently activated T cells or memory effector cells, so both regulators and effectors are removed. However, co-transfer of *ex vivo* expanded CD4+CD25+ ovalbumin (OVA)-specific T cells together with Th2 cells had no effect on subsequent inhaled OVA challenge [314], and OVA-specific airway CD4+CD25+ T cells reduced airway inflammation but not airway hyper-responsiveness in response to inhaled challenge [315].

Possible roles for regulatory T cells in the suppression of human allergic disease are shown in Figure 1.4.

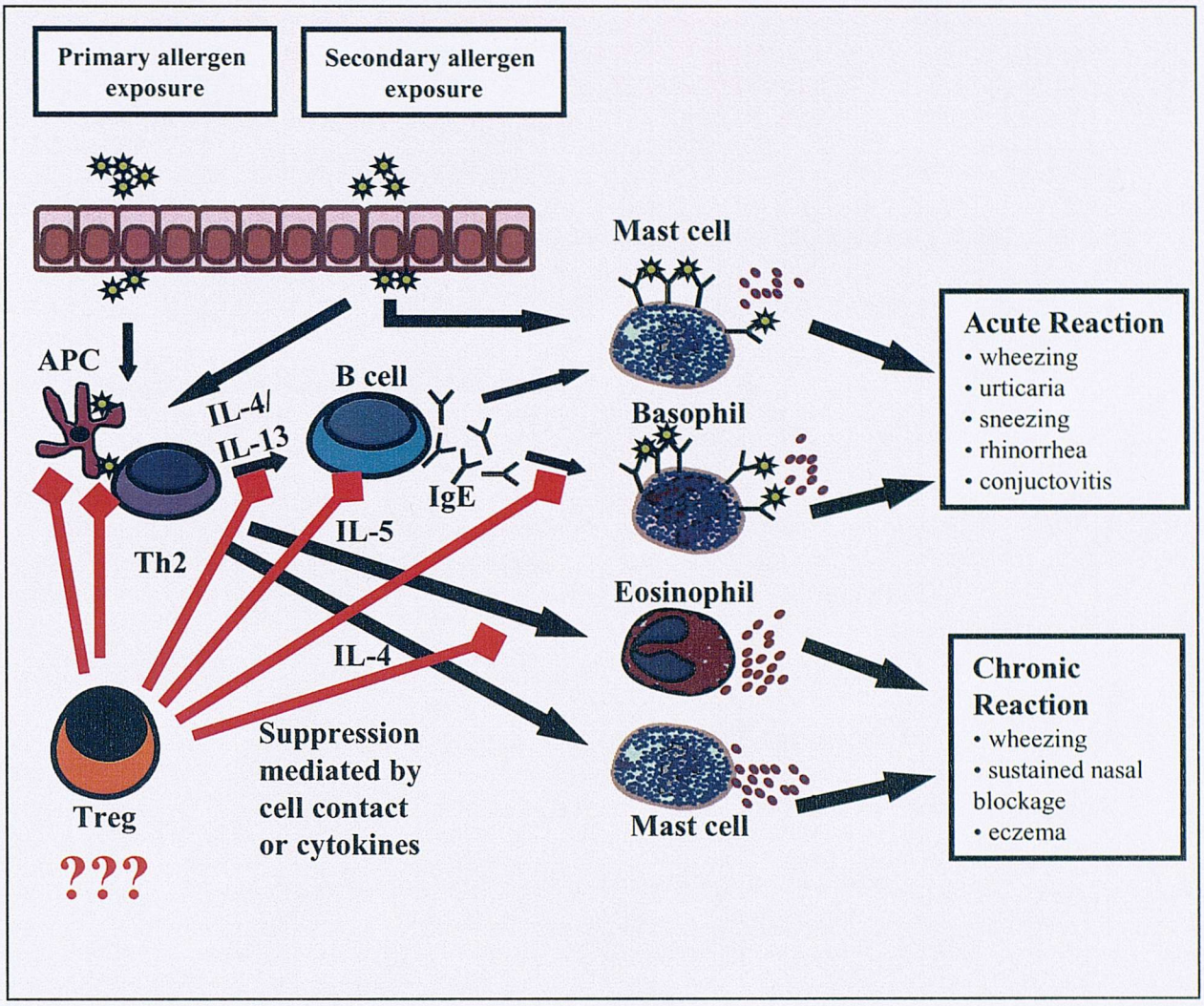


Figure 1.4. The role of regulatory T cells in suppression of Th2 responses to allergen. The role of regulatory T cells (Treg) in the suppression of Th2 responses to allergen is at present unclear. Tregs may suppress Th2 cytokine production or expansion, or may act directly on the APC or B cell. Suppression may be mediated through cell contact or soluble mediators.

1.7. Hypotheses

Human CD4+CD25+ regulatory T cells have been reported to suppress responses to some antigens, but at the commencement of this project there was no data illustrating their suppression of T cell responses to aeroallergens. This thesis therefore tested the following hypotheses:

1) **CD4+CD25+ regulatory T cells from non-atopic donors suppress Th2-type T cell responses to aeroallergens.**

2) **The suppression of Th2-type T cell responses to aeroallergens by CD4+CD25+ regulatory T cells from atopic donors is not as effective as that by those from non-atopic donors.**

3) **Regulatory T cells are involved in the protection elicited by conventional allergen immunotherapy treatment.**

1.8. Aims of the project

This thesis investigated the role of CD4+CD25+ regulatory T cells in the suppression of allergen driven Th2-type responses. The aims of this project were threefold:

1) To develop an *in vitro* assay in which the suppression of allergen driven T cell responses by CD4+CD25+ regulatory T cells could be investigated.

2) To examine the relationship between expression of atopic allergic disease and suppression of allergen driven T cell responses *in vitro* by CD4+CD25+ regulatory T cells.

3) To determine the effect of specific allergen immunotherapy treatment on the suppression of allergen driven T cell responses by regulatory T cells from both the CD4+CD25+ and CD4+CD25- subset.

Chapter 2 Materials and Methods

Unless otherwise stated, all chemicals were purchased from Sigma, Poole, Dorset, UK.

2.1. Ethical statement

Local ethics committees of the Royal Brompton NHS Trust or St. Mary's Hospital London approved the studies described in this thesis and all volunteers involved gave written informed consent. There were no adverse effects from blood donation by any of the volunteers recruited for the studies described herein.

2.2. Study population

2.2.1. Donor recruitment

Non-atopic volunteers were recruited by advertisement and from staff of Imperial College, London. Atopic volunteers with seasonal allergic rhinitis to grass pollen (hayfever) or allergy to cat were recruited from the allergy clinics of the Royal Brompton Hospital and St Mary's Hospital London or by advertisement. Subjects undergoing allergen immunotherapy treatment had received grass pollen allergen immunotherapy treatment for at least 18 months at either the Royal Brompton or St Mary's Hospital, London.

2.2.2. Diagnosis of atopy

All volunteers completed a standard questionnaire about allergic symptoms and history of allergic disease. To establish presence or absence of atopy, skin prick tests (SPTs) to common aeroallergens such as cat dander, house dustmite, grass pollen

and aspergillus (ALK, Horsholm, Denmark) were performed. Serum specific IgE was detected by Radioallergosorbent test (RAST) (Pharmacia, Milton Keynes, UK) in the hospital haematology laboratory. Atopic donors were defined on the basis of a positive IgE (> 0.35 IU/L) or SPT (as defined below), and a history of allergic symptoms, whereas non-atopic donors had no history of allergic symptoms, negative SPTs, and normal amounts of serum IgE. Hayfever patients had a positive SPT to grass pollen allergen extract but not other allergens. These donors were studied between June and August when the grass pollen count was high and they had symptoms of allergic disease, unless stated otherwise.

2.2.3. Skin prick tests (SPTs)

A soluble extract of the allergen (ALK) was placed on the skin of the forearm and then pricked beneath the epidermis with a needle. The diameter of the wheal and flare reaction was measured after 15 minutes. A positive (histamine 10mg/ml) and negative (diluent) were always used and a positive reaction was regarded as being 2 mm greater than the negative control.

2.2.4. Immunotherapy protocol

Grass pollen allergen immunotherapy treated donors had received subcutaneous injections of Alutard® SQ *Phleum pratense* (ALK, Horsholm, Denmark) according to a standard protocol (see Chapter 5). Patients had originally received 13-15 injections of increasing doses of allergen to reach the "maintenance dose" within 7-8 weeks of starting treatment. Once this dose had been reached injections were given every six weeks. Volunteers involved in this study had been receiving "maintenance dose" injections for at least 18 months.

2.3. *Allergen stocks*

Cat dander and grass pollen allergen extracts were a kind gift from Dr. Gitte Nordskov Hansen (ALK). These extracts were reconstituted in sterile AB media (see section 2.4.) and frozen in aliquots at –20 °C until needed. Recombinant Phl p V was a kind gift from Dr. Oliver Cromwell (Allergopharma, Reinbek Germany) and was also reconstituted in sterile AB media and frozen in aliquots at –20 °C.

2.4. *Cell culture*

2.4.1. Composition of AB media

The AB media in which human PBMCs and T cells were cultured consisted of RPMI 1640 medium with 5% human AB serum, 100 μg/ml penicillin/streptomycin (Invitrogen Life Technologies, California, USA), and 2 mM L-glutamine (Invitrogen Life Technologies).

2.4.2. Isolation of peripheral blood mononuclear cells (PBMC)s

150 ml (unless otherwise stated) of human peripheral blood was obtained into three 50 ml syringes (BD, Biosciences, Belgium) each containing 0.5 ml heparin (Leo Laboratories, Buckinghamshire, UK) and then diluted in an equal volume of phosphate-buffered saline solution (PBS). 25 ml of diluted blood was then layered over 20 ml Ficoll paque (Amersham, Buckinghamshire, UK) in 12 x 50 ml tubes (Corning, New York, USA). PBMC were isolated at room temperature by density centrifugation at 16,000 rpm in a Rotina 46R centrifuge (Wolf Laboratories, Germany) for 20 minutes with the brake off. On completion of PBMC isolation, cells were washed with PBS and centrifuged at 4°C for 10 minutes at 16,000 rpm. Cells were resuspended in AB media, a 90 μl aliquot was removed and stained with 10 μl

trypan blue. The number of viable cells recovered was calculated using a haemocytometer.

2.4.3. Separation Buffer

The buffer used for the CD4+, CD25+ and IL-10 producing cell isolation experiments consisted of PBS with 0.5% bovine serum albumin (BSA) and 2 mM ethylenediaminetetraacetic acid (EDTA), at pH 7.2.

2.4.4. Isolation of CD4+CD25+ and CD4+CD25- T cells

PBMC were washed in degassed separation buffer. CD4+ T cells were then isolated using the MACs CD4 negative selection kit, (Miltenyi Biotech, Bisley, UK) according to the manufacturers instructions. Briefly, the cell pellet was resuspended in separation buffer in a total volume of 80 µl per 10^7 total cells and incubated with 20 µl hapten-antibody cocktail (containing monoclonal hapten-conjugated CD8, CD11b, CD16, CD19, CD36, and CD56 antibodies) per 10^7 cells for 10 minutes at 6-12 °C. The cells were then washed twice with separation buffer, resuspended in a total volume of 80 µl per 10^7 total cells and incubated with 20 µl MACS anti-hapten microbeads per 10^7 cells for 15 minutes at 6-12 °C. After a further washing step, cells were resuspended in 500 µl separation buffer per 10^8 cells and applied to an LS+ MACs column and the effluent collected. The column was then rinsed with 12 ml separation buffer and the effluent collected as the enriched CD4+ Th cell fraction.

This CD4+ enriched population was then further divided into CD4+CD25+ and CD4+CD25- enriched subsets using MACs anti-CD25 microbeads, (Miltenyi Biotech) according to the manufacturers instructions. Briefly, CD4+ cells were counted, resuspended in 90 µl separation buffer per 10^7 cells and incubated with 10 µl

CD25 microbeads per 10^7 cells for 15 minutes at 6-12 °C. The cells were then washed with separation buffer, resuspended in 500 µl separation buffer per 10^8 cells and applied to an MS+ MACs column. The column was rinsed with 3 ml separation buffer. The effluent was collected as the CD4+CD25- fraction and the cells eluted after removing the column from the magnetic field were the CD4+CD25+ subset. The purity of the separation was assessed by flow cytometry with a FACscaliber (BD Biosciences, Belgium). Unless otherwise stated cells were used immediately for flow cytometry and proliferation assays.

2.4.5. Isolation of CD4+CD25- IL-10 producing cells

An IL-10 secretion assay cell enrichment and detection kit (Miltenyi Biotech) was used to isolate CD4+CD25- cells that spontaneously produced IL-10, according to the manufacturers instructions. Freshly isolated CD4+CD25- T cells were cultured in AB media at 37 °C, 5 % CO_2 overnight, washed with cold separation buffer and resuspended in 80 µl cold AB media per 10^7 cells. 20 µl IL-10-catch reagent per 10^7 cells was then added and the cells were incubated for 5 minutes on ice. This catch reagent is a bi-specific antibody, which binds to CD45 on the cell surface and captures IL-10. After this incubation period, 10 ml warm AB media was added per 10^7 cells and incubated for 45 minutes at 37 °C under slow continuous rotation using a MACSmix. The cells were then put on ice for 5 minutes and washed twice with cold separation buffer. They were then resuspended in 80 µl buffer per 10^7 cells and incubated with 20 µl IL-10 detection antibody-PE per 10^7 cells for 10 minutes on ice. After this incubation the cells were washed with separation buffer and resuspended in 80 µl buffer per 10^7 cells and 20 µl anti-PE microbeads per 10^7 cells for 15 minutes at 4-8 °C. Finally the cells were washed in cold separation buffer, resuspended in 500 µl

buffer and applied to an MS+ MACs column. The column was washed with 3 ml cold separation buffer. Both the effluent containing the unlabelled cells and the magnetically labelled CD4+CD25- IL-10 secreting cells were collected and resuspended in AB media. The cells were used immediately for allergen stimulated proliferation assays.

2.4.6. Irradiation of PBMC

Irradiated PBMCs were used as APC in some cultures. 8 x 10^6 c/ml diluted in AB media were irradiated for 4 minutes at 8000 rads using a Gamma cell 3000 Elan irradiator (Nordian International, Vancouver, Canada). Cells were then washed in PBS and resuspended in AB media.

2.4.7. Freeze/thawing of cells

For some experiments PBMC or CD4+CD25- T cells were frozen at –70 °C until needed. Cells were diluted to 10 x 10^6 c/ml in AB media and 1 ml aliquots placed in freezing vials (Sarstedt, Germany) on ice. An equal volume of freezing media (90% foetal calf serum (FCS) (Invitrogen Life Technologies) 10% dimethyl sulfoxide (DMSO)) was added and the cells were transferred to –70 °C. After a minimum of 24 hours, the cells were transferred to a liquid nitrogen storage facility. When needed for an experiment, the cells were thawed quickly in a 37 °C waterbath and then immediately washed and resuspended in AB media.

2.5. Proliferation assays

2.5.1. Stimulation of cells with anti-CD3/anti-CD28

In initial experiments to confirm that the CD4+CD25+ T cells isolated had suppressive function, mixing experiments were performed with a polyclonal stimulus of anti-CD3/anti-CD28 as has previously been described [208;222-227]. CD4+CD25+ and CD4+CD25- cells were cultured either alone at 0.4 x 10^6 c/ml or at a 1:2 ratio (0.8:0.4 x 10^6 c/ml), for 72 hours at 37 °C and 5% CO_2 in flat-bottomed 96 well plates (Corning) with plate bound anti-CD3 antibody (BD Pharmingen, Oxford, UK) at 5 μg/ml, and soluble anti-CD28 antibody (BD Pharmingen) at 10 μg/ml.

2.5.2. Stimulation of cells with allergen or purified protein derivative (PPD)

PBMCs, CD4+CD25+ or CD4+CD25- were incubated in triplicate at 2 x 10^6 c/ml in AB media in flat-bottomed 96 well plates (Corning) for 6 days (unless stated otherwise) at 37 °C, 5 % CO_2. This cell density and time of culture had previously been shown to be optimal for proliferation and cytokine production to allergen (see Chapter 3). For the mixing experiments CD4+CD25- cells were at a final concentration of 2 x 10^6 c/ml to which CD4+CD25+ cells were added at a final concentration of 1 x 10^6 c/ml (unless stated otherwise). A cell number control contained 3 x 10^6 CD4+CD25- c/ml. Each well also contained 2 x 10^6 c/ml of irradiated autologous PBMCs and 100 μg/ml (unless otherwise stated) of cat or grass pollen allergen extract, 25 μg/ml PPD (Evans vaccines, Liverpool, UK) or as a negative control, AB media alone. The cells were incubated at 37 °C and 5% CO_2 for 6 days. On day six, 100 μl of supernatant was removed from each well and frozen at – 20 °C for later cytokine analysis.

2.5.3. Quantification of cellular proliferation by thymidine incorporation

The allergen-specific or polyclonal proliferation of the cells was measured by thymidine incorporation. During the last 8 hours of culture, 0.0038 MBq thymidine (ICN Pharmaceuticals Ltd, UK) was added to each well, the plate was then frozen at -20 °C until needed for analysis. After defrosting, the cells were then harvested onto filters (Perkin Elmer, UK), air dried, sealed in plastic bags (Perkin Elmer) and 2.5ml scintillation fluid added. Thymidine incorporation was measured in counts per minute (CPM) using a microbeta liquid scintillation counter (Perkin Elmer). Allergen specific proliferation was calculated as proliferation to allergen minus proliferation to media alone.

2.5.4. Transwell experiments

In some experiments CD4+CD25+ and CD4+CD25- cells were separated by a transwell insert (Corning) with 0.4 μM pores. In these experiments CD4+CD25- T cells were cultured in 24 well plates (Corning) at 2 x 10^6 c/ml in the presence of irradiated PBMC at 2 x 10^6 c/ml and allergen extract at 100 μg/ml or media alone. In some wells, CD4+CD25+ T cells were added at a ratio of two CD4+CD25- T cells to one CD4+CD25+ T cell. The CD4+CD25+ T cells were either added directly to the well with the CD4+CD25- T cells, or were separated from the CD4+CD25- cells by the transwell insert. The cells were cultured for six days and proliferation measured by thymidine incorporation as before.

2.5.5. Addition of reagents to block cytokines /cell surface molecules

In some experiments, blocking antibodies to cytokines or cell surface receptors were added to the mixed cultures. In at least three separate experiments for each

antibody, IL-10 (R&D Systems, Abingdon, UK), IL-10R (BD Pharmingen), TGF–β (R&D Systems), CTLA-4 (Ancell, Maryland, USA), glucocorticoid-induced TNF receptor, (GITR) (R&D Systems) or isotype matched control antibodies (R&D Systems) were added at a concentration of 10 μg/ml (as suggested by the suppliers) to mixed cultures stimulated with allergen extract as described above.

2.6. Cytokine analysis

2.6.1. Enzyme-linked immunosorbant assay (ELISA)

In early experiments, IL-5 and IFN-γ from allergen driven PBMC culture supernatants were quantified using OptEIA kits (BD Pharmingen). Briefly, 96 well microtiter plates (Nunc, Roskilde, Denmark) were coated with 100 μl of capture antibody diluted in the recommended buffer overnight at 4 ^{0}C. After 5 washes with PBS containing 0.5 % Tween-20, plates were blocked with 200 μl PBS containing 10 % FCS and left for 1 hour at room temperature. Samples and standards (diluted in PBS + 10 % FCS) were then incubated for a further 2 hours at room temperature. After 5 washes, bound cytokine was detected using biotinylated antibodies pre-mixed with avidin-HRP followed by tetramethylbenzidine (TMB) and hydrogen peroxidase. Optical densities were read at 450 nm. The mean optical density of wells containing no cytokine was subtracted from the results obtained for samples and standards. The concentration of cytokine in each sample was calculated from a standard curve.

2.6.2. Luminex analysis

Supernatants were taken from allergen driven cultures at day 6 of culture and frozen at –20 °C until needed for analysis. Cytokine anlaysis of these samples for IL-2, IL-4, IL-5, IL-10, IL-12, IL-13 and IFN-γ was carried out using the Luminex bead

system (Luminex, Riverside, California, USA) using labelled cytokine capture antibody pairs (Biosource International, Camarillo, California, USA) according to the manufacturers instructions. In brief, analyte specific antibody beads were added to a 96 well filter plate and then washed. Next, 100 µl of pre-mixed cytokine standard or sample was added to each well, the plate was covered and put on a plate shaker for 2 hours in the dark. The solution was then removed by suction and 100 µl of biotinylated detection antibody was added to every well, the plate was again covered and put on a plate shaker for an hour in the dark. This solution was removed by suction and 100 µl of Streptavidin-RPE was added to each well, the plate was shaken in the dark for a further 30 minutes after which the solution was removed, the cells were washed and 100 µl of wash solution was added, it was then loaded onto the Luminex platform for analysis. In each experiment there were negative control wells which contained media only to give a background value which was then subtracted from all standard and sample values. A standard curve was drawn from the standard values and the cytokine concentration from the samples was extrapolated from this curve. All consumables used were contained in the Biosource kit.

2.7. Flow cytometric analysis

2.7.1. Cell Surface Antigens

CD4+CD25+ and CD4+CD25- cells were tested for purity by flow cytometry using a FACscaliber (BD Biosciences). CD3-FITC conjugated mouse anti-human antibody, CD4-Cychrome conjugated mouse anti-human antibody, CD25-PE conjugated mouse anti-human antibody and CD69-FITC conjugated mouse anti-human antibody, were purchased from BD Pharmingen (Heidelberg, Germany).

Freshly isolated cells were washed with Cell Wash (BD Pharmingen). 200,000 cells were then incubated with 5 µl of the appropriate antibody or matched isotype control in a total volume of 100 µl and incubated for 30 minutes at 6-12 °C in the dark. After 30 minutes, the cells were washed with Cell Wash, and resuspended in 500 µl Cell Wash. Data was then acquired immediately and 30,000 lymphocyte events analysed with CellQuest Pro software (BD Biosciences). In some cases after washing, cells were fixed for 20 minutes at room temperature with Cellfix (BD Pharmingen) and the data acquired the following day.

2.7.2. Intracellular Staining

To detect expression of intracellular CTLA-4 on CD4+CD25+ T cells, cell surface staining for CD4 and CD25 was carried out, as above, and then the cells were permeabilised using a saponin buffer (1 x PBS, 2% BSA, 0.5% Saponin). CTLA-4 antibody (BD Pharmingen) or matched isotype control antibody (BD Pharmingen) were added to the cells for a further 30 minutes on ice in the dark. The cells were then washed with Cell Wash and data acquired immediately using the FACscaliber.

2.8. Real time polymerase chain reaction (RT-PCR)

2.8.1. RNA extraction

RNA was extracted from PBMC, CD4+CD25+ and CD4+CD25- T cells, which had been pelletted, the supernatant removed and the cells "snap frozen" in liquid nitrogen. These cell pellets were then stored at –70 °C until needed for future processing. RNA was then extracted using the Absolutely RNA RT-PCR Miniprep kit from Stratagene (La Jolla, USA) according to the manufacturers instructions. All reagents were provided with the kit. Cells were first homogenised using the lysis

buffer/β-mecaptoethanol provided. Following cell lysis, the sample was then pre-filtered in a spin cup to remove particles and reduce the amount of DNA. The filtrate was then transferred to a spin cup with a silica-based fibre matrix to which the RNA bound. Treatment with a low-salt wash buffer and digestion with DNase removed any remaining DNA and a series of washes removed the DNase and other proteins. Pure RNA was then eluted from the fibre matrix with a small volume of low-ionic-strength buffer and captured in a microcentrifuge tube.

2.8.2. Quantitating RNA yield

To quantitate the RNA, a small sample was removed, diluted in elution buffer and mixed well. The spectrophotometer was set to read at an optical density (OD) of 260 nm and blanked with elution buffer. The OD of the sample was then measured at 260 nm. To calculate the concentration of RNA in the sample the following equation was used:

Concentration = A_{260} x dilution factor x 0.04 μg/μl

The spectrophotometer was then blanked at OD 280 nm with elution buffer and the OD of the sample measured at 280 nm. The ratio of the 260 nm to the 280 nm measurements indicates the purity of the sample. Ratios of 1.8 to 2.1 indicate pure samples; lower ratios indicate possible protein contamination.

2.8.3. Purification of RNA using Lithium Chloride

Lithium chloride precipitation offers major advantages over other RNA precipitation methods in that it does not efficiently precipitate DNA, protein or carbohydrate. To precipitate RNA using lithium chloride (Ambion, Texas, USA), the final concentration of lithium chloride in the RNA solution was brought to 2.5 M, the

solution was chilled for 30 minutes at –20 °C, and centrifuged at top speed in a microcentrifuge for 15 minutes. The supernatant was then discarded and the pellet washed with ice cold 70% ethanol to remove residual salt. Finally the pellet was resuspended in nuclease-free H_20.

2.8.4. Generation of cDNA

RNA was then reverse transcribed using the superscript first-strand synthesis system for RT-PCR (Invitrogen Life Technologies), which is optimised for quantitative, real time PCR and uses both oligo-dT and random hexamer primers. The reactions contained the following (all reagents were contained in the Life Technologies kit):

$Oligo(dT)_{12-18}$	0.5 µl
Random Hexamer	0.5 µl
RNA sample (up to 1 µg total RNA)	x µl
10x Buffer	2 µl
25 mM $MgCl_2$	4 µl
10 mM dNTP	1 µl
0.1 M DTT	2 µl
RNaseOUT	1 µl
Superscript II (50 U/µl)	1 µl
Rnase/DNase-free distilled water	20 – (12 + x) µl

The reactions were then incubated at 25 °C for 10 minutes, 42 °C for 50 minutes and 70 °C for 15 minutes. On completion of the assay, the product was diluted 1 in 5 in Rnase/DNase-free distilled water.

2.8.5. TaqMan PCR reactions

25 µl PCR procedures were carried out in triplicate (ABI Prism 7700 sequence detection system; Applied Biosystems, Foster City, CA, USA) with TaqMan universal PCR mastermix (Applied Biosystems), FOXP3-specific primers, and 6-carboxyfluorescein-labelled TaqMan probe (Applied BioSystems). Samples were standardised to the ribosomal RNA component 18S (Applied Biosystems) and levels of FOXP3 compared to that of the "house keeping" gene hypoxanthine phosphoribosyl transferase (HPRT). Primer and probe mixtures were purchased using an "assay on demand" service from Applied Biosystems.

For each well the following mix was prepared:

Primer and probe mix	1.25 µl
Master mix	12.5 µl
Water	8.75 µl

22.5 µl of FOXP3, 18S or HPRT mix were added to each well. 2.5 µl of cDNA was then added in triplicate and the plate sealed. The plate was centrifuged at 2000 rpm for 2 minutes to ensure that there were no air bubbles. If the plate was not analysed immediately, it was wrapped in foil, stored at 4 °C and analysed within 24 hours.

An ABI Prism 7700 sequence detection system was used, with the following cycler conditions:

50 °C for 2 minutes

95 °C for 10 minutes

95 °C for 15 seconds
60 °C for 1 minute
} 40 cycles

2.8.6. Analysis of data from TaqMan PCR

As the controls and the gene of interest had similar amplification efficiencies, FOXP3 message was quantified with the comparative threshold for detection method, http://appliedbiosystems.com. The amount of starting material was normalised to that of 18S and levels of mRNA were then compared to that of HPRT.

2.9. Statistics

The results are expressed as scatterplots, or median + 95% confidence interval. Statistical significance was determined by non-parametric analysis, using the Mann Whitney U test. When data was from paired samples, the Wilcoxon matched pairs test was performed. All statistical analysis was completed using Prism software (version 3; Graph Pad, San Diego, CA, USA). Statistical significance was accepted when $p < 0.05$.

Chapter 3 – The suppression of *in vitro* allergen stimulated T cell responses by human CD4+CD25+ regulatory T cells.

3.1. Introduction

This chapter will introduce the *in vitro* assay, which was used to investigate the suppression of allergen driven peripheral blood T cell responses by CD4+CD25+ regulatory T cells. First, the proliferation and cytokine production of PBMC and CD4+CD25+ and CD4+CD25- T cell subsets from non-atopic donors to allergen stimulation was examined. The ability of CD4+CD25+ T cells from non-atopic donors to suppress Th2 responses to allergen was then determined. Finally the mechanism of suppression of allergen driven T cell responses by CD4+CD25+ T cells was investigated using neutralising antibodies to soluble mediators and cell surface receptors.

3.1.1. T cell responses following stimulation with allergen in vitro

PBMCs from both atopic and non-atopic individuals proliferate and produce cytokines when stimulated *in vitro* with allergen [316;317]. Proliferative responses to protein from the tetanus toxoid used in tetanus vaccination are completely absent in T cells from infants but present in those from adults, whereas inhalant allergens cause T cell proliferation in cells from nearly 100% of adults and children and 60% of infants [316]. These findings suggest that T cell responses to allergen are initiated early in life after the initial antigen exposure [318].

The cytokine secretion profile of PBMCs in such an *in vitro* allergen-driven cell culture system differs between atopic and non-atopic donors. PBMCs from atopic donors produce relatively higher levels of the Th2 cytokines IL-5 and IL-13 compared

to the Th1 cytokine IFN-γ, than do those from non-atopic donors [112;113]. When PBMCs are stimulated with an antigen that induces a Th1 cytokine profile, such as *M. tuberculosis* derived purified protein derivative (PPD), there is no difference in cytokine production between atopic and non-atopic donors [112]. The reason for the difference in allergen stimulated T cell cytokine profile between atopic and non-atopic donors is unknown. One possibility is that Th2-type responses are actively suppressed in non-atopic individuals. Understanding the mechanism of this suppression may provide a clue to the cause of allergic disease and assist in the discovery of novel therapeutic strategies.

3.1.2. The phenotype of CD4+CD25+ regulatory T cells

Regulatory CD4+CD25+ T cells were characterised in mice as cells that prevented the development of autoimmune disease *in vivo* and that suppressed the activation of CD4+CD25- T cells to polyclonal or antigenic stimulation *in vitro* [319]. Other populations of T cells with regulatory properties have also been isolated, some naturally occurring and some experimentally induced [254], and these have been described in Chapter 1. The regulatory cells discussed in this chapter are of the "naturally occurring" CD4+CD25+ phenotype.

The CD4+CD25+ regulatory T cell population in both mouse and humans has now been extensively characterised *in vitro* [208;222;224-228;320]. When compared *in vitro* to CD4+CD25- cells and unseparated PBMC, human CD4+CD25+ cells are hyporesponsive to polyclonal stimulation, alloantigens and antigens such as cows milk antigen, tetanus toxoid and human heatshock protein 60 (hHSP60) [228]. Furthermore, this population of cells can suppress the proliferative response of the CD4+CD25- subset activated by these stimuli *in vitro* [208;222;224-228;320]. They also

suppress mixed lymphocyte reactions [232]. Suppression *in vitro* requires stimulation of the CD4+CD25+ T cell through the TCR, it is cell contact dependent, and is accompanied by cell cycle arrest [201;213;214]. The suppression of disease in animal models is discussed in Chapter 1.

CD4+CD25+ T cells are the only lymphocyte population in both mouse and humans that constitutively express CTLA-4, which is a negative co-stimulatory molecule upregulated late during T cell activation [48;54]. They also express high levels of the glucocorticoid-induced tumour necrosis factor (TNF) receptor (GITR) [233;235] and the transcription factor FOXP3 [236;237;321]. A summary of the phenotype of human CD4+CD25+ regulatory T cells is shown in Table 3.1.

The high activation and differentiation state of the CD4+CD25+ regulatory T cell population is confirmed by the expression of molecules that are required for migration to areas of inflammation. L-selectin [322;323], which is required for entry into lymph nodes is present on CD4+CD25+ cells, as are the inflammatory chemokine receptors CCR2, CCR4, CCR5 and CCR8 and adhesion molecules CD54, LFA-1 and $\alpha_E\beta_7$ [203;324-326].

3.1.3 Suppression of Th2 responses by CD4+CD25+ T cells

Most published data on the activity of regulatory T cells relates to inhibition of Th1 responses, and the manipulation of this cell subset in various disease models is discussed further in Chapter 1. There are few reports of regulation of Th2 responses by regulatory T cells. Human CD4+CD25+ T cells have been shown to suppress T cell responses *in vitro* to Nickel [327] and cows milk antigen [328], and a recent report

Isolated from:	Cell surface phenotype	References
Thymus	CD122+	226
	CTLA-4+	
Tonsil	CD45RBlow	224
	CD45RO+	
	CD69+/-	
	CD28+	
	CTLA-4+	
	CD95high	
	Bcl-2low	
Peripheral Blood	CD45RBlow	224
	CD69-	224;227
	CD62L+/-	208;222;224;227
	CD38+/-	224
	CD122+	222;224;225
	CD134+	208;224
	CTLA-4	222-225;227
	GITR+	329
	CD45RO+	208;222-225;227;228
	CD154-	222 227 ,
	MHCIIlow	208;222;223;225
	CD71+	208
	TCRαβ	223
	CD95high	224
	Bcl-2low	224
	CD28+	224
	CCR4+	326
	CCR8+	326
	FOXP3+	321

Table 3.1. The phenotype of human CD4+CD25+ regulatory T cells.

demonstrates that CD4+CD25+ T cells can suppress Th2 cell development and associated eosinophilia in a mouse model of pulmonary inflammation [314]. IL-10 secreting Tr1 cells, prevent Th2 cell activation and eosinophilia in a murine model of inhaled allergen challenge [260], and mature bronchial dendritic cells exposed to allergen induce IL-10 producing regulatory T cells capable of blocking the development of airway hyperresponsiveness [330]. *In vivo* administration of mycobacterium vaccine prior to antigenic sensitisation via inhaled allergen challenge also reduces the cellular inflammation and airway hyperresponsiveness after further allergen challenge, an effect attributed to induction of an IL-10 producing regulatory T cell population [331].

Other studies have shown no such role for regulatory T cells in the suppression of Th2-type animal models of allergic disease. Although murine regulatory T cells suppress *in vivo* IgE production [312], depletion of CD4+CD25+ cells reduces airway eosinophilia, T cell numbers and IgE in a mouse model of asthma [313]. This may be because removing CD25+ T cells will deplete both regulatory and effector T cells. Furthermore, co-transfer of *ex vivo* expanded CD4+CD25+ ovalbumin (OVA)-specific T cells together with Th2 cells had no effect on responses to subsequent inhaled OVA challenge [314]. Moreover in another model OVA-specific airway CD4+CD25+ T cells reduced airway inflammation but not airway hyper-responsiveness in response to inhaled challenge [315].

3.1.4. Mechanisms of suppression by CD4+CD25+ regulatory T cells

If the mechanisms by which CD4+CD25+ T cells suppress immune responses can be dissected, it may be possible to manipulate this pathway to either enhance or dampen immune responses in various immune diseases. To date however, the

mechanisms by which regulatory T cells mediate suppression have not been conclusively elucidated. Potential mechanisms of suppression of T cell responses by regulatory T cells from humans and mice are shown in Table 3.2. Different cytokines, cell surface receptors and transcription factors have all been implicated depending on the nature of the assay. The suppressive mechanism seems to vary depending on the animal model studied, and whether the suppression is studied *in vivo* or *in vitro.*

i) The role of suppressor cytokines

Several suppressor cytokines have been implicated in the suppression of animal models of autoimmune disease by CD4+CD25+ cells. These have been established using cytokine deficient animals or by the administration of neutralising anti-cytokine antibodies in adoptive transfer studies [198;239;332].

Autoimmune gastritis in the nu/nu mouse is suppressed by $CD4^+CD25^+$ T cells deficient in IL-4 or IL-10 and, in this model TGF-β neutralisation also has no effect on suppression of disease [239]. In contrast however, suppression of inflammatory bowel disease (IBD) in SCID mice is dependent upon both IL-10 and TGF-β [332].

Most *in vitro* studies using CD4+CD25+ T cells have failed to show a role for soluble suppressor cytokines. Addition of neutralising antibodies to IL-4, IL-10 or TGF-β[201;213;214;244] do not reverse suppression of proliferation of CD4+CD25- cells *in vitro,* and CD4+CD25+ T cells from IL-4 and IL-10 deficient mice are not

Production of immunosuppressive cytokines by CD4+CD25+ cells			
Mouse *(in vitro* studies)		**Human *(in vitro* studies)**	
Cytokine	**Refs**	**Cytokine**	**Refs.**
IL-10	214;246;255;333	IL-10	225;226
TGF-β	246;255	TGF-β (membrane bound)	246
Reversal of CD4+CD25+ T cell suppression			
Mouse *(in vitro* studies)		**Human *(in vitro* studies)**	
Suppression reversed by:	**Refs**	**Suppression reversed by:**	**Refs**
IL-2	213;214;334;335	IL-2 (partial reversal)	208;223-227
Anti-IL10 sR	255	IL-4 (partial reversal	223
Anti-TGF-β sR	255	IL-15 (partial reversal	225
Anti-TGF-β	246	Anti-PD-L1 (partial reversal)	208
Anti-CTLA-4	255;335	Anti-TGF-β	246
Anti-CD28	213;214;334;336		
GITR signalling	233;235		
Reversal of CD4+CD25+ T cell suppression			
Mouse *(in vivo* studies)			
Suppression reversed by:	**Refs**		
Anti-IL-10R	287		
IL-10-/-	337;338		
Anti-TGF-β	53		
Anti-CTLA-4	53;54;286;339		
TRANCE/RANK block	340		
GITR signalling	233;235		
FoxP3-/-			
No reversal of suppression by CD4+CD25+ cells			
Mouse *(in vitro* studies)		**Human (*in vitro* studies)**	
Suppression not reversed by:	**Refs**	**Suppression not reversed by:**	**Refs**
Anti-IL-10	213;214;227;255	Anti-IL-10	222-227
Anti-TGF-β	214;227;244;255	Anti-TGF-β	222-227
Anti-IL-4	213;214	Anti-IL-4	224;226
Anti-IL-2	214	Anti-CD28	208;223
Anti-IFN-γ	214	GITR signalling	329
Anti-CTLA-4	214 336,	Anti-CTLA-4	208;222;223
Anti-FAS-L	213		
Anti-TNF	213		

Table 3.2. The mechanisms of suppression of T cell responses by mouse and human CD4+CD25+ regulatory T cells.

impaired in their ability to suppress this response [198;239;243]. Furthermore, *in vitro* assays using transwell inserts to separate the regulatory and responder cells show that regulation is dependent upon cell contact [201;213;214]. The contradictory nature of these results highlights how much care must be taken when comparing data from *in vitro* and *in vivo* studies. However, it may also reflect a role for innate signalling in regulatory T cell function *in vivo.*

One *in vitro* study suggests that latent cell surface bound TGF-β may be delivered directly to the responder cell by cell-cell contact [246]. This latent molecule would then be converted to active TGF-β and exert its suppressive effect. Other studies however have failed to find such a role for TGF-β. Responder cells from a transgenic mouse expressing a dominant negative form of the receptor (TGF-βRII) that canot respond to TGF-β, were suppressed by CD4+CD25+ cells [341] and CD4+CD25+ cells from TGF-β deficient mice were not impaired in their suppressive ability [244].

ii) *The role of CTLA-4*

CD4+CD25+ T cells constitutively express CTLA-4 [54], but there is considerable controversy over whether this expression reflects that these cells are part of the activated/memory population of leukocytes, or whether CTLA-4 has an important functional role in suppression. T cells stimulated by CTLA-4 produce a large amount of TGF-β [262], which may contribute to their suppressive properties. Anti-CTLA-4 antibody or fragment of antibody (FAB) reverses suppression of proliferation *in vitro* [54] and suppression of inflammatory bowel disease *in vivo* [53]. However, anti-CTLA-4 antibody or FAB does not reverse suppression by CD4+CD25+ cells *in vitro* [201;213;214;244].

iii) *The role of GITR*

Members of the TNF receptor superfamily are logical candidates for the mechanism by which CD4+CD25+ T cells mediate suppression, as engagement of ligands of some TNF receptors leads to inhibition of cytokine production and cell growth similar to suppression by regulatory T cells. GITR was identified by two different groups simultaneously as being highly expressed on CD4+CD25+ regulatory T cells [233;235] and cross-linking this receptor abrogates suppression of T cell responses by murine CD4+CD25+ T cells both *in vitro* and *in vivo* [233;235].

iv) *The role of FOXP3*

The genetic disorder IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) is a rare monogenic disease of male children that is accompanied by autoimmune disease, IBD and severe allergy [253], similar to disease symptoms produced in mice with the "scurfy" phenotype [251]. Both conditions are associated with a mutation in the gene *FOXP3* (*Foxp3* in mice) which encodes a transcription repressor [248;250-252] and is expressed by CD4+CD25+ T cells in the thymus and periphery [237;238;240]. Ectopic expression of the *Foxp3* gene converts murine naive T cells to cells with phenotypic and functional properties similar to those of naturally occurring CD4+CD25+ T cells [321]. Furthermore, transfer of CD4+CD25+ T cells from normal mice prevents autoimmune disease in *Foxp3*-defective mice [237], indicating that FOXP3 may play an important role in both the development and suppressive function of CD4+CD25+ regulatory T cells.

v) *The role of the APC*

CD4+CD25+ regulatory T cells either suppress the activation of responder cells directly through cell contact dependent mechanisms, or through production of soluble mediators, or indirectly through modulation of APCs. CD4+CD25+ T cells have been shown to suppress OVA-specific CD8+ T cell responses both in the presence and absence of APCs [231]. APCs may however have a role in the development of regulatory T cells, as mature pulmonary dendritic cells can induce the development of IL-10 producing regulatory T cells *in vivo* [58].

3.1.5. Hypothesis

Human CD4+CD25+ T cells have been reported to suppress responses to some antigens, but there is little published data showing suppression of human T cell responses to aeroallergens. The hypothesis of this chapter is that **CD4+CD25+ regulatory T cells from non-atopic donors suppress Th2-type T cell responses to aeroallergens**. The mechanism of this suppression was also investigated.

3.2 Results

3.2.1. In vitro proliferation and cytokine production of PBMCs from non-atopic donors after allergen extract stimulation in vitro.

In order to investigate the ability of human CD4+CD25+ regulatory T cells to suppress Th2 responses to common aeroallergens, an *in vitro* assay was devised in which allergen stimulated T cells proliferated and produced cytokines to maximum levels. PBMCs were isolated from human peripheral blood of non-atopic donors and stimulated *in vitro* with allergen extracts. These donors had no history of atopic disease, had low allergen-specific IgE and were skin prick test negative to grass pollen allergen (Table 3.3.). As previously described [113], allergen stimulation caused PBMCs to proliferate at higher levels than seen with addition of media alone (Fig. 3.1). A concentration of 100 - 200 μg/ml of both grass pollen and cat dander allergen extract gave maximum PBMC proliferation, (Fig. 3.1a and b); a concentration used in previous studies [112]. PBMCs from non-atopic donors also proliferated to stimulation with purified recombinant Phl PV, a major grass pollen allergenic protein [16]. The optimum proliferation of PBMCs stimulated with Phl PV was at a concentration of 7.5 - 12.5 μg/ml (Fig. 3.1c).

When investigating the time course of stimulation, 7 days of culture with cat dander (Fig. 3.2. a and b) or grass pollen (Fig. 3.2. b and c) allergen extract at a cell density of 2 x 10^6 PBMCs per ml gave optimal proliferation. Allergen stimulation of PBMCs with either cat dander of grass pollen extract also induced cytokine production, which peaked at 7 days post-stimulation (Fig. 3.3).

Donor	Gender	Age	IgE to cat dander IU/ml	IgE to grass pollen IU/ml	Total IgE IU/ml	SPT results (mm)	Allergic disease	Allergen extract used in culture
1	F	29	< 0.34	< 0.34	16	0	N	Grass pollen
2	F	43	< 0.34	< 0.34	4	0	N	Cat dander
3	M	26	< 0.34	< 0.34	34	0	N	Grass pollen
4	F	30	< 0.34	< 0.34	5	0	N	Grass pollen
5	M	29	< 0.34	< 0.34	3	0	N	Grass pollen
6	M	25	< 0.34	< 0.34	91	0	N	Grass pollen
7	M	31	< 0.34	< 0.34	61	0	N	Cat dander
8	M	35	< 0.34	< 0.34	111	0	N	Cat dander
9	M	24	< 0.34	< 0.34	40	0	N	Cat dander
Summary	6 = M 3 = F	29 (24 – 43)	< 0.34	< 0.34	34 (3 – 111)	0	9 = N 0 = Y	5 = Grass 4 = Cat

Table 3.3. Clinical details of non-atopic donors
Gender; F = female, M = male. IgE levels measured by RAST test in IU/ml. Allergen specific skin prick test (SPT); wheal diameter (mm). Allergic disease; Y = history of allergic disease, N = no history of allergic disease. Summary is median and range, or summary of data.

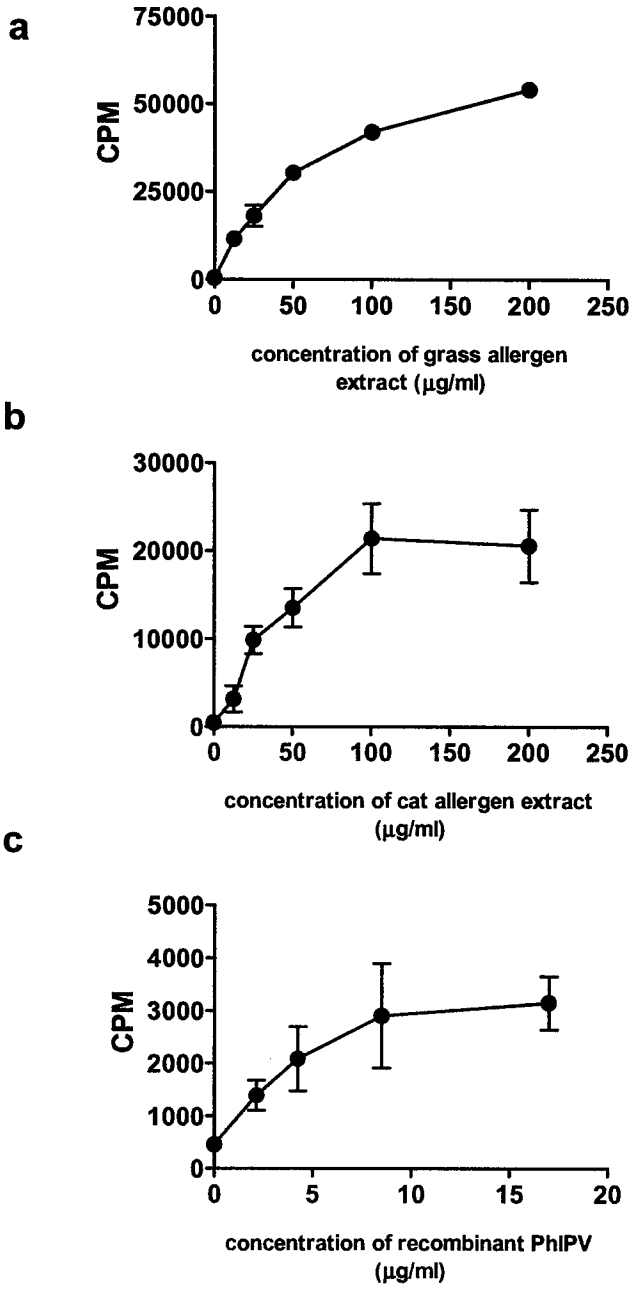


Figure 3.1. Dose response curve of PBMC proliferation to stimulation with allergen extract. PBMCs were isolated from human peripheral blood and stimulated with grass pollen allergen extract (a), cat dander extract (b) or PhlPV (c) at varying concentrations. Proliferation was measured by tritiated thymidine incorporation on day 7. Cells were cultured at 2 x 10^6 c/ml. Representative mean ± SE of triplicate cultures from one of two experiments is shown.

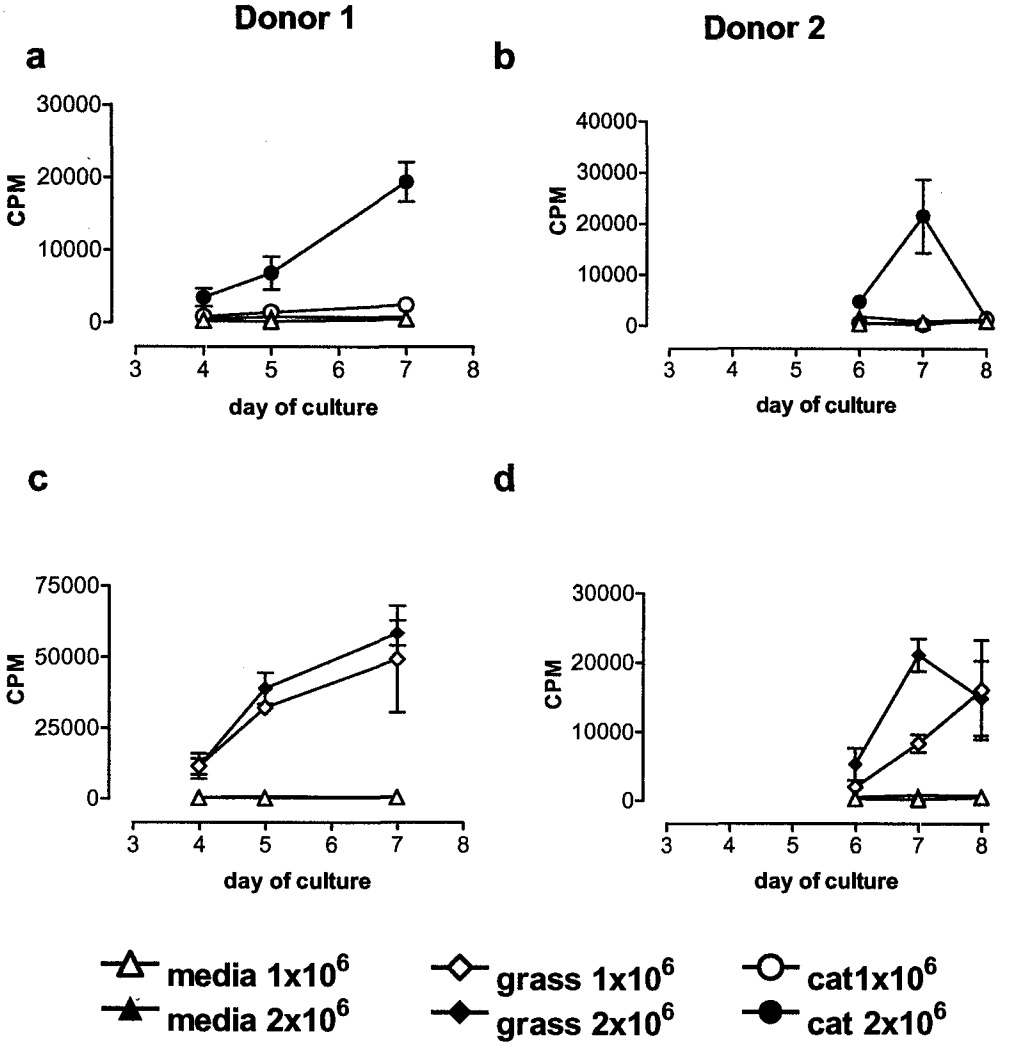


Figure 3.2. Time course of PBMC proliferation to stimulation with allergen extract. PBMCs were isolated from human peripheral blood and stimulated *in vitro* with media alone (triangles) or allergen extract. Cat dander (circles, a + b) or grass pollen (diamonds, c + d) allergen extract was added at 100 μg/ml. Proliferation was measured by tritiated thymidine incorporation on the days shown. Cells were cultured at 1 x 10^6 c/ml (open symbols) or 2 x10^6 c/ml (closed symbols). Means ± SE of triplicate cultures from two representative donors are shown.

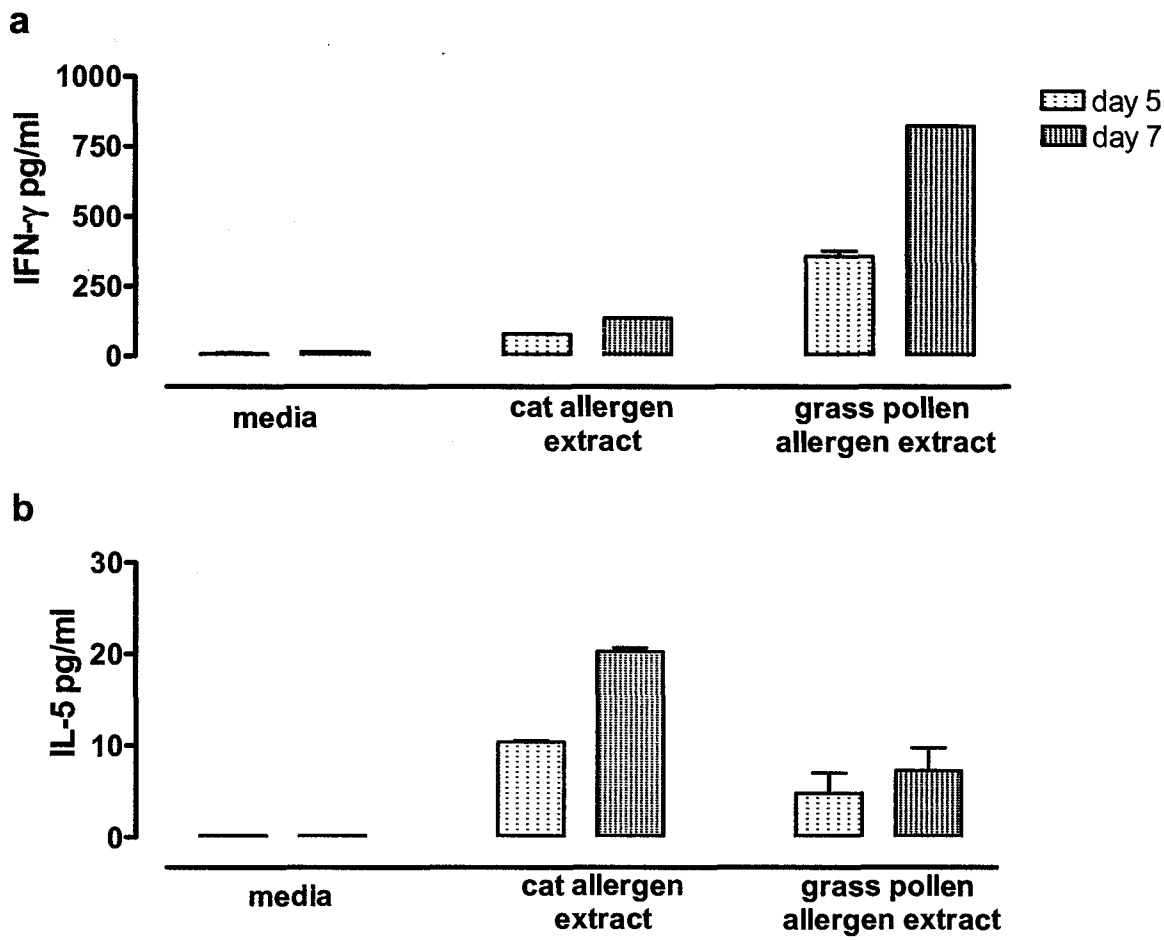


Figure 3.3. Cytokine production by PBMCs stimulated with allergen extract at day 5 and 7 of culture. PBMCs were isolated from human peripheral blood and stimulated *in vitro* at 2 x 10^6 c/ml with media alone or allergen extract. Cat dander and grass pollen extracts were added at 100 μg/ml. Concentration of IFN-γ (a) and IL-5 (b) in the day 5 (light bars) and day 7 (dark bars) culture supernatant was measured by ELISA. Mean + SE of triplicate cultures from one of two experiments is shown.

3.2 2. Isolation of CD4+CD25+ regulatory T cells from human peripheral blood.

Using flow cytometry it was ascertained that 9.8 % (range 4.2 - 19.8) of PBMCs from non-atopic donors were CD4+CD25+ (Fig 3.4b and Table 3.4). CD4+ cells were then isolated from PBMCs by negative selection using immunomagnetic beads and CD4+CD25+ cells were isolated by positive selection. From 150ml blood, 4.3 x 10^6 CD4+CD25+ (range 2.8 – 8.5 x 10^6) and 109.4 x 10^6 CD4+CD25- (range 29 –118.3 x 10^6) cells were recovered. The purity of the CD4+ separation was 95.1% (range 73.9 – 97.8) and the CD25+ separation 84.5% (range 62.2 - 95.8) (Figs 3.4 c, d and e, Table 3.4).

The regulatory activity of the CD4+CD25+ T cell subset was confirmed by adapting an *in vitro* culture system similar to that used by other investigators studying the suppression by CD4+CD25+ T cells of T cell responses to antigenic and polyclonal stimuli [208;222;224-228;320] (Fig. 3.5). When activated with a polyclonal stimulus of anti-CD3 and anti-CD28, PBMCs and CD4+CD25- cells proliferated vigorously, whereas CD4+CD25+ cells were hyporesponsive. When added to the CD4+CD25- subset, the CD4+CD25+ cells inhibited the proliferation of the CD4+CD25- cells (Fig. 3.5).

3.2.3. CD4+CD25- but not CD4+CD25+ cells proliferate and produce cytokines when stimulated in vitro with allergen.

The allergen specific proliferation and cytokine production by the different isolated cell subsets was then investigated. Proliferation (Fig. 3.6) and IL-5, IL-13 and IFN-γ production by CD4+CD25- T cells was significantly higher than that by PBMC or CD4+CD25+ T cells. (Fig. 3.7 a-c). CD4+CD25+ T cells were unresponsive, with no proliferative or cytokine response (Fig. 3.6 and Fig. 3.7 a-d) except for a small

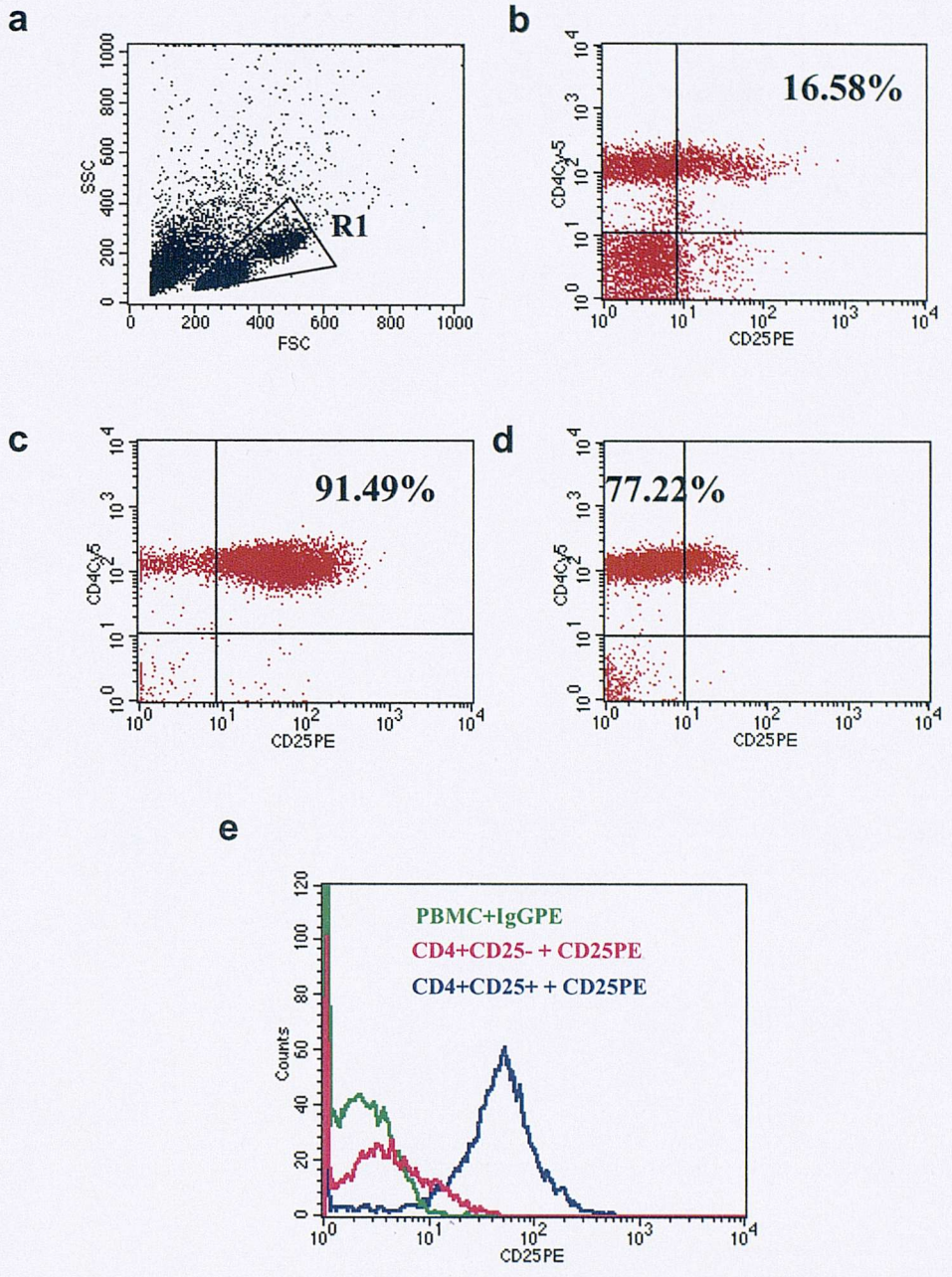


Figure 3.4 Separation of CD4+CD25+ T cells from human peripheral blood. FACs plot, (a) to show the forward scatter (FSC) and side scatter (SSC) properties of PBMCs from a representative non-atopic donor. Dotplot, (b) to show the % CD4+CD25+ cells in PBMCs in the R1 gated region, from a non-atopic donor. Dotplots to show the purity of isolated CD4+CD25+ (c) and CD4+CD25- (d) T cell subsets after separation using MACs immunomagnetic beads. The quadrants have been positioned after determining the expression of a matched isotype control antibody. Histogram (e) to show the expression of CD25 on isolated CD4+CD25+ and CD4+CD25- T cells. Data are representative of 9 donors.

Donor	Total no. of PBMC ($x10^6$)	% CD4+ CD25+ in PBMC	Total no. of CD4+ cells ($x10^6$)	Total no. of CD4+ CD25+ cells ($x10^6$)	Total no. of CD4+ CD25- cells ($x10^6$)	% CD4+ CD25+ recovered from the CD4+ subset	Purity of CD4+ cells	Purity of CD4+ CD25+ cells	Purity of CD4+ CD25- cells
1	335.0	9.8	135.8	5.3	91.0	3.9	95.5	67.7	94.2
2	291.9	5.9	54.4	4.3	43.8	7.9	87.5	70.3	63.5
3	240.9	16.58	80.0	5.9	116.8	7.4	97.8	91.49	77.22
4	440.0	4.2	105.4	8.5	92.9	8.1	73.9	62.2	72.3
5	254.0	8.7	113.4	7.2	118.3	6.3	95.1	72.4	82.8
6	308.8	9.5	126.4	4.0	109.4	3.2	90.9	95.8	54.6
7	232.2	10.5	123.5	3.8	114.1	3.1	95.8	86.1	71.4
8	190.1	19.8	75.9	4.1	29.0	5.4	95.9	88.9	52.1
9	352.8	12.9	106.9	2.8	111.3	2.6	92.9	84.5	75.7
Summary	291.9 (190.1 – 440)	9.8 (4.2 – 19.8)	106.9 (54.4 – 135.8)	4.3 (2.8 – 8.5)	109.4 (29 – 118.3)	5.4 (2.6 -8.1)	95.1 (73.9 - 97.8)	84.5 (62.2 - 95.8)	72.3 (52.1 - 94.2)

Table 3.4. Recovery and purity of cells isolated from the peripheral blood of non-atopic donors.

Total number of cells isolated was calculated by cell count using a haemocytometer. Purity of cells and % CD4+CD25+ within the PBMC subset was calculated using flow cytometry after staining with fluorescent CD4 and CD25 antibodies or isotype-matched controls, see Fig. 3.4. Summary is median and range.

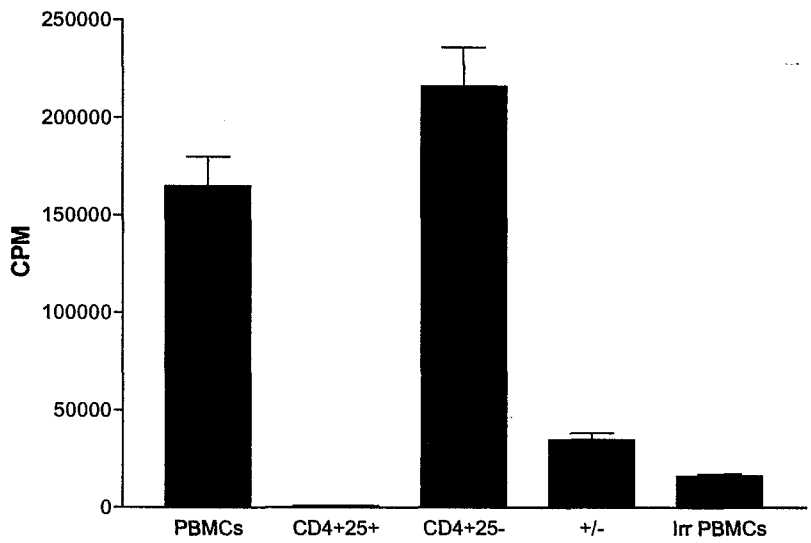


Figure 3.5. Suppression of proliferation of anti-CD3/anti-CD28 activated CD4+CD25- T cells by human CD4+CD25+ regulatory T cells. Proliferation, measured by tritiated thymidine incorporation, of PBMCs, CD4+CD25+ and CD4+CD25- cells after polyclonal stimulation with plate bound anti-CD3 (10 μg/ml) and soluble anti-CD28 (1 μg/ml) Addition of CD4+CD25+ cells to CD4+CD25- cells was at a ratio of 1:2 (labelled +/-). Cells were cultured at 0.4 x 10^6 c/ml (0.6 x 10^6 c/ml in +/- wells) for 3 days. All wells contained irradiated autologous PBMCs. Means + SE of triplicate values are shown from one of five experiments.

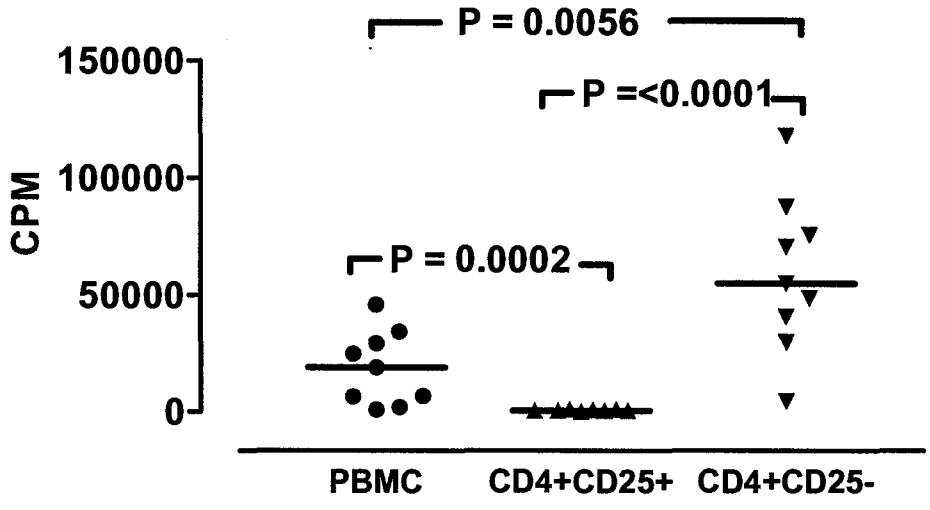


Figure 3.6. Stimulation of human T cell subsets *in vitro* with allergen. Allergen specific proliferation (proliferation to allergen minus proliferation to media) of PBMC, CD4+CD25+ and CD4+CD25- cells from non-atopic donors (n = 9) measured by tritiated thymidine incorporation. Cells were stimulated for 7 days *in vitro* at 2 x 10^6 c/ml with allergen extract (either cat dander or grass pollen) at 100 µg/ml or media alone. All wells contained autologous irradiated PBMC at 2 x 10^6 c/ml. Results from individual donors are shown, a bar represents the median value.

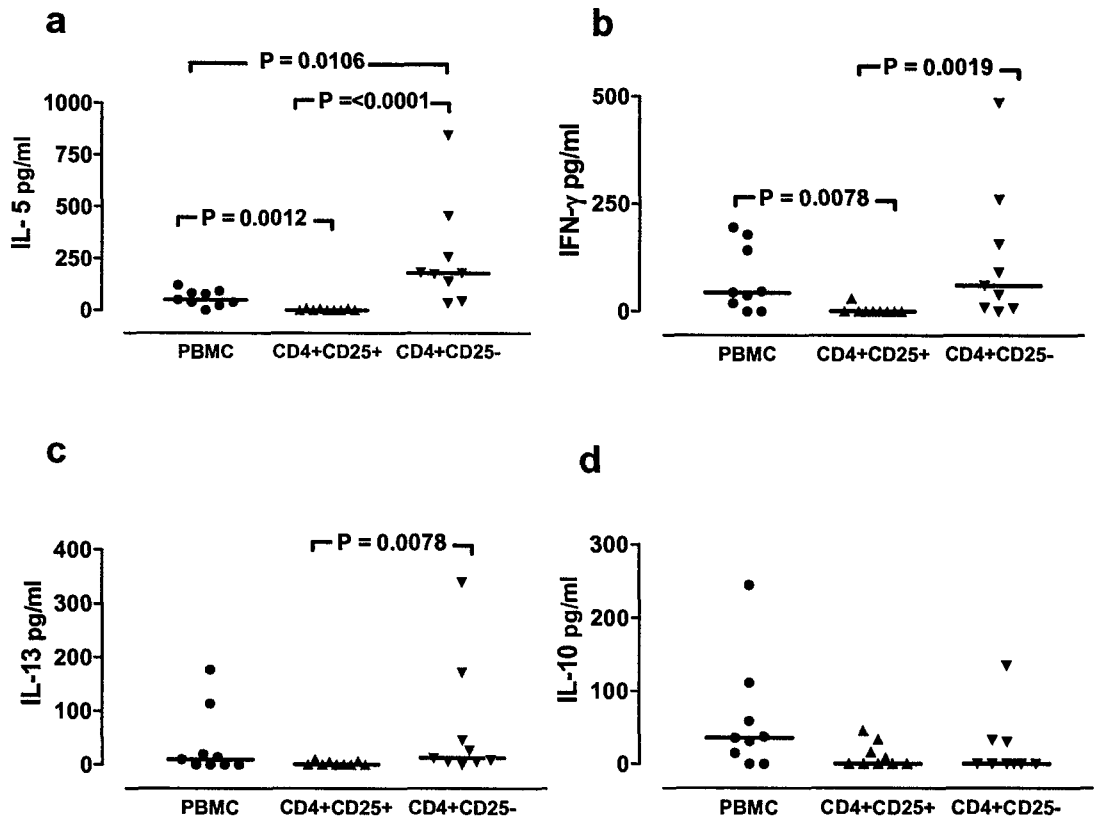


Figure 3.7. Stimulation of human T cell subsets *in vitro* with allergen. Allergen specific IL-5 (a), IFN-γ (b), IL-13 (c) and IL-10 (d) production of PBMC, CD4+CD25+ and CD4+CD25- cells from non-atopic donors (n = 9) measured by Luminex. Cells were stimulated for 7 days *in vitro* at 2 x 10^6 c/ml with allergen extract (either cat dander or grass pollen) at 100 μg/ml or media alone. All wells contained autologous irradiated PBMC at 2 x 10^6 c/ml. Results from individual donors are shown, a bar represents the median value.

amount of IL-10 (Fig. 3.7d). Autologous irradiated PBMCs added as antigen presenting cells did not proliferate or produce cytokines, (data not shown, n = 20 donors). No IL-2, IL-4 or IL-15 was detected in any of the culture supernatants, (data not shown, n = 88 samples). There was no significant difference in the proliferation or cytokine production of PBMC, CD4+CD25+ or CD4+CD25- to grass pollen compared to cat dander allergen extract stimulation, although there was a trend for higher levels of proliferation and cytokine production by CD4+CD25- cells to grass pollen allergen (Fig. 3.8). Stimulation of PBMCs and CD4+CD25- T cells but not CD4+CD25+ T cells with allergen, resulted in higher levels of proliferation and cytokine production than with media alone (Fig. 3.8).

3.2.4. CD4+CD25+ regulatory T cells from non-atopic donors suppress activation of in vitro allergen stimulated CD4+CD25- T cells.

To investigate whether CD4+CD25+ T cells could suppress allergen driven T cell responses, a similar *in vitro* system as those previously used to study the suppression of T cell responses to polyclonal and antigenic stimuli by CD4+CD25+ T cells [208;222;224-228;320], was devised. CD4+CD25- T cells from non-atopic donors were stimulated under conditions that induce optimal allergen-specific proliferation and cytokine production. CD4+CD25+ T cells were added to these cultures to determine whether they could suppress allergen induced proliferation and cytokine production. To verify that suppression of proliferation and cytokine production was not due to an over-crowding effect, CD4+CD25- cells were added in some wells at the same cell concentration as the CD4+CD25+ cells had been in others (Fig. 3.9).

Addition of CD4+CD25+ T cells inhibited allergen specific CD4+CD25- T cell proliferation (Fig. 3.10). The suppression was most effective at a ratio of 1:2

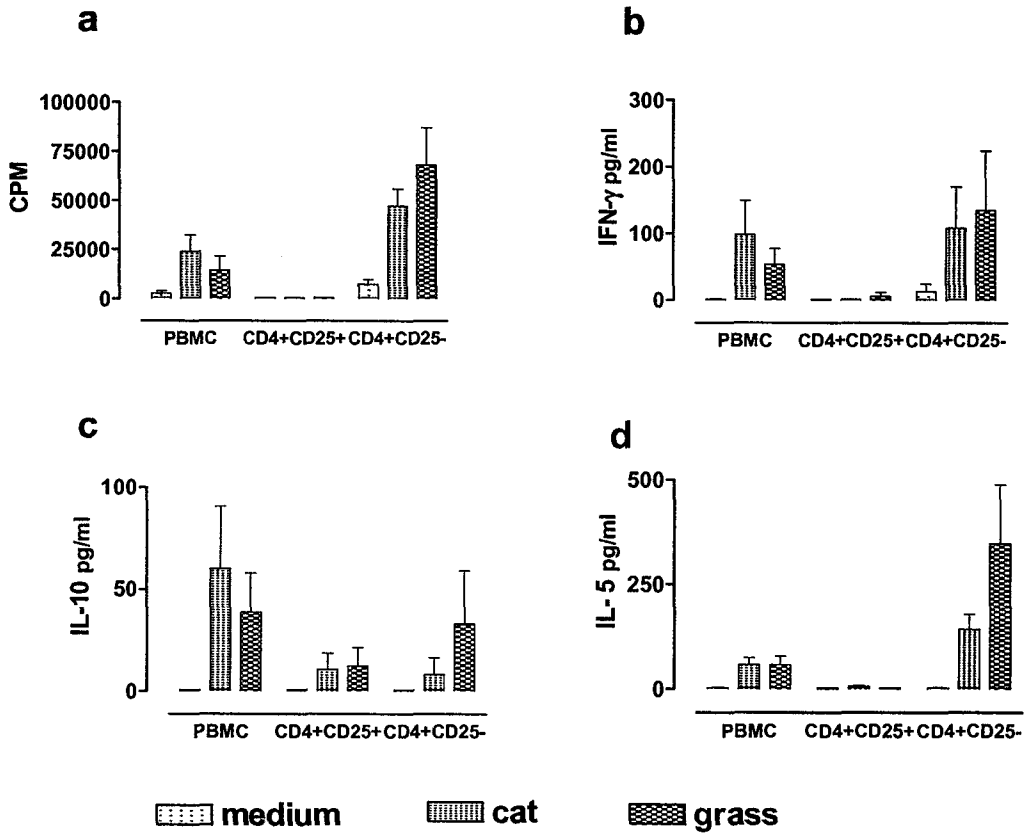


Figure 3.8. Stimulation of human T cell subsets *in vitro* with allergen extract. PBMC, CD4+CD25+ and CD4+CD25- cells isolated from non-atopic donors (n = 9) were stimulated for 7 days *in vitro* at 2 x 10^6 c/ml with cat dander allergen extract (n = 4) or grass pollen allergen extract (n = 5) at 100 μg/ml or media alone. Proliferation (a) was measured by incorporation of tritiated thymidine, and concentration of IFN-γ (b), IL-10 (c) and IL-5 (d) in the culture supernatants by Luminex. All wells contained autologous irradiated PBMC at 2 x 10^6 c/ml . Median values + 95% CI are shown.

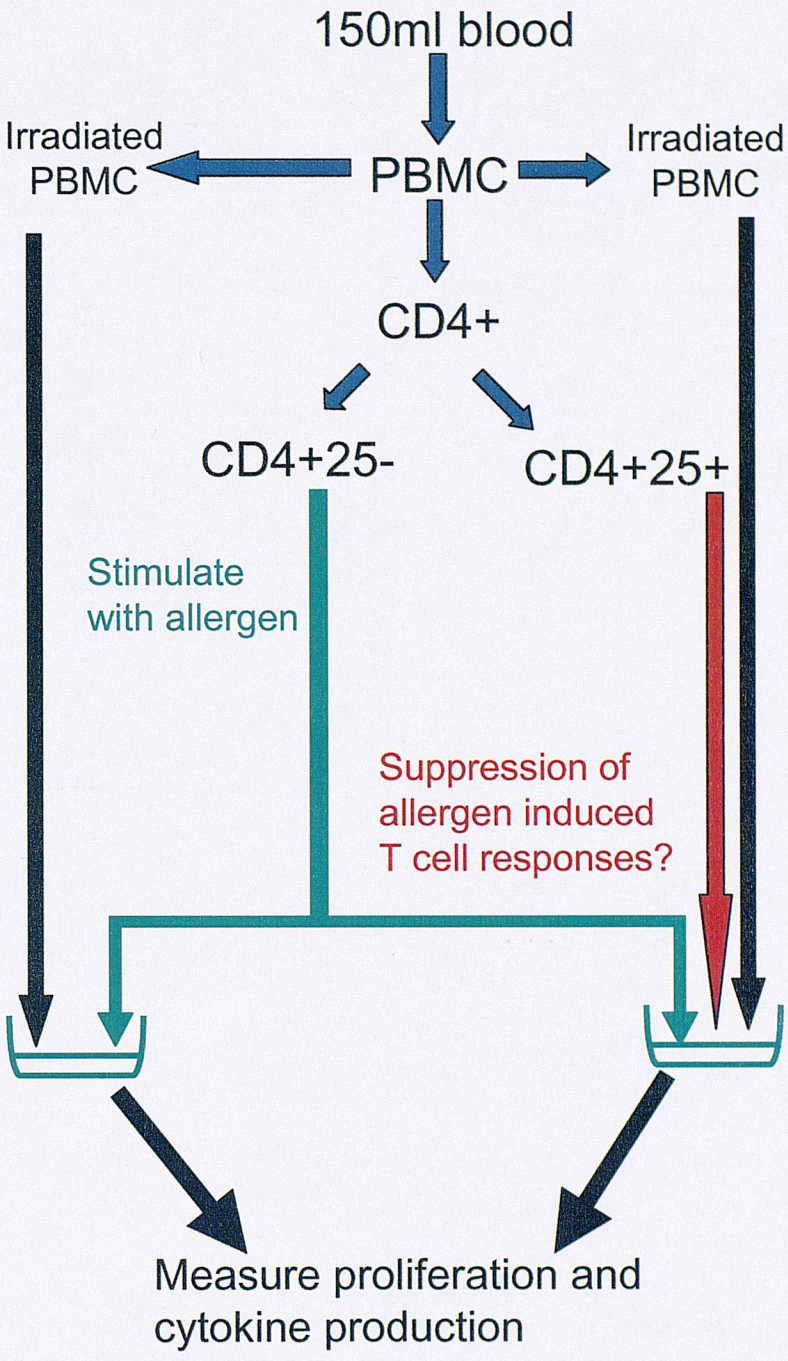
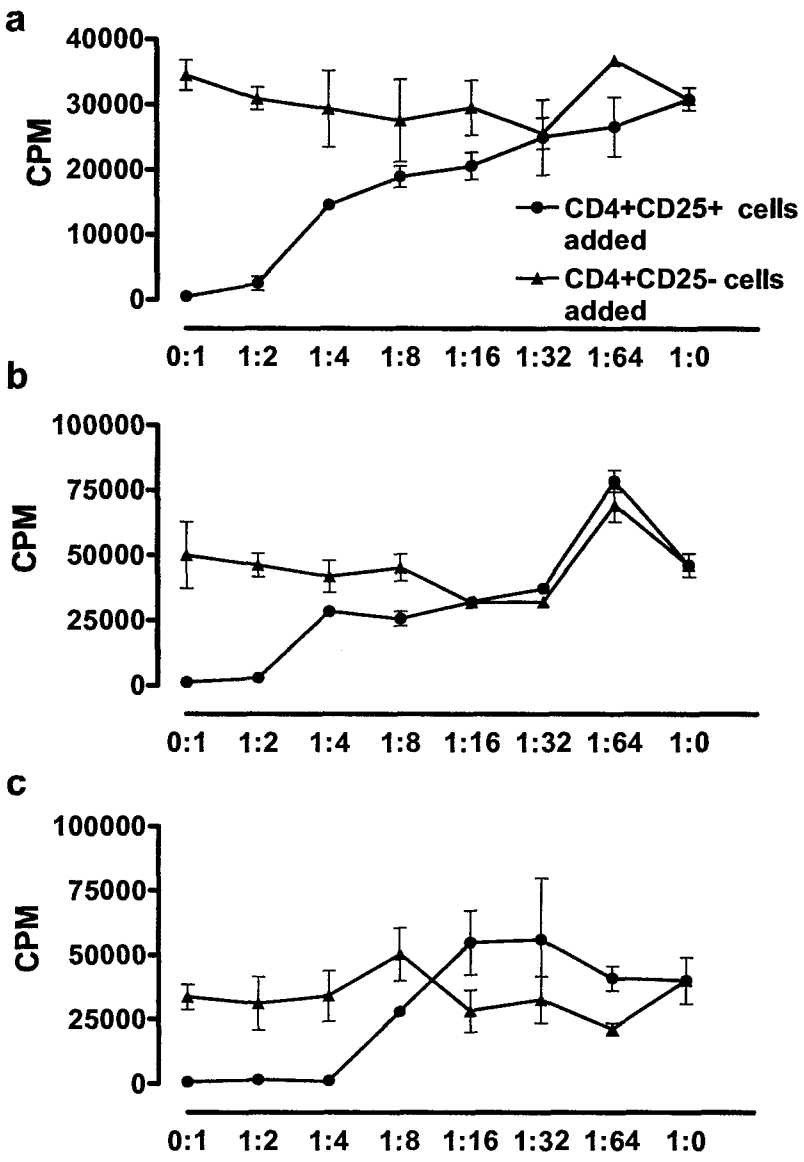


Figure 3.9. Schematic showing details of experiments investigating the ability of human CD4+CD25+ regulatory T cells to suppress allergen-driven T cell responses.



3.10. Suppression of proliferation of allergen stimulated CD4+CD25- T cells by CD4+CD25+ regulatory cells. Allergen specific proliferation, measured by tritiated thymidine incorporation, of CD4+CD25- T cells at 2 x 10^6 c/ml stimulated with allergen extract (either cat dander or grass pollen) at 100 µg/ml. CD4+CD25+ cells (circles) were added to the CD4+CD25- at various ratios. CD4+CD25- cells were added to other cultures as a cell number control (triangles). Mean values ± SE of triplicate cultures from three individual donors are shown (a-c).

(CD25+:CD25- cells), but the effect could still be seen at a 1:4 and in some cases, 1:8 and 1:16 ratio. There was no effect on proliferation when the same number of CD4+CD25- T cells was added (Fig. 3.10).

When studying the time course of suppression of allergen driven T cell responses, IL-5 and IFN-γ production and proliferation peaked at days 6-7 (Fig. 3.11), as seen in cultures of allergen stimulated PBMCs (Fig. 3.2). Suppression of proliferation and IL-5 production was maximal at day 7 (Fig. 3.11a and b, respectively), and suppression of IFN-γ at day 6. (Fig. 3.11d) IL-10 was also suppressed by CD4+CD25+ cells at early time points but was at very low levels in both the CD4+CD25- culture and the CD4+CD25+/- mixed culture by day 6 (Fig 3.11c).

To confirm that the CD4+CD25+ T cells were suppressing T cell activation to allergen and not to any contaminant present in the allergen extract, purified recombinant *Phleum pratense V* (Phl PV) was used as the stimulus. CD4+CD25+ cells suppressed CD4+CD25- cell proliferation following stimulation with this major grass pollen allergenic protein as effectively as following whole allergen exposure (Fig. 3.12). Furthermore, when mouse splenocytes were cultured with these allergen extracts they did not proliferate, suggesting an absence of contamination with agents such as LPS, which may induce non-allergen specific proliferation (data not shown, n = 3).

To verify the reproducibility of the assay, CD4+CD25- and CD4+CD25+ cells were isolated from the same donor on different days. Although the CPM values of allergen specific proliferation were not identical, the values for % suppression of proliferation were reproducible (Fig. 3.13). The formula used for calculating %

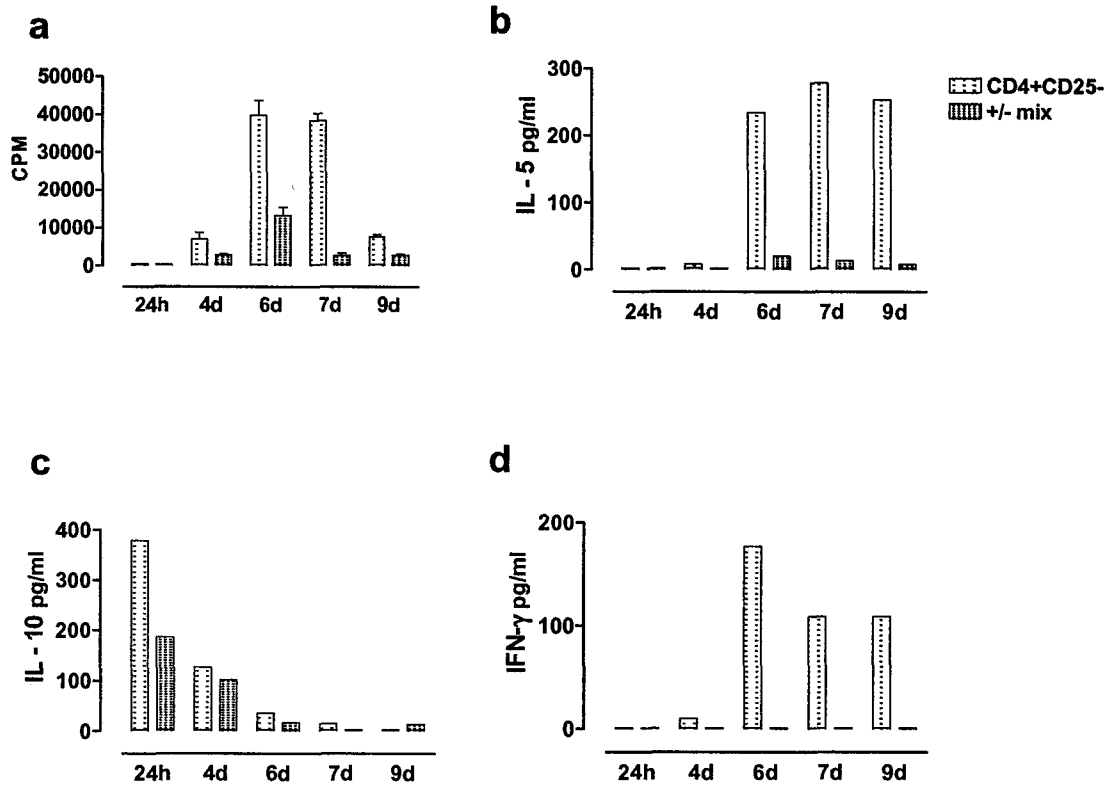


Figure 3.11. Time course of suppression of allergen driven CD4+CD25- T cell responses by CD4+CD25+ regulatory T cells. CD4+CD25- T cells at 2 x 10^6 c/ml were stimulated with allergen extract (either cat dander or grass pollen) at 100 μg/ml or media alone. Either CD4+CD25+ regulatory T cells (dark bars), were added to inhibit the proliferation and cytokine production, or CD4+CD25- T cells (light bars), were added as a cell number control at 1 x 10^6 c/ml. Representative graphs show allergen specific proliferation (a), measured by tritiated thymidine incorporation at day 7, and concentration of IL-5 (b), IL-10 (c) and IFN-γ (d) in the day 6 culture supernatants measured by luminex, from one of two experiments.

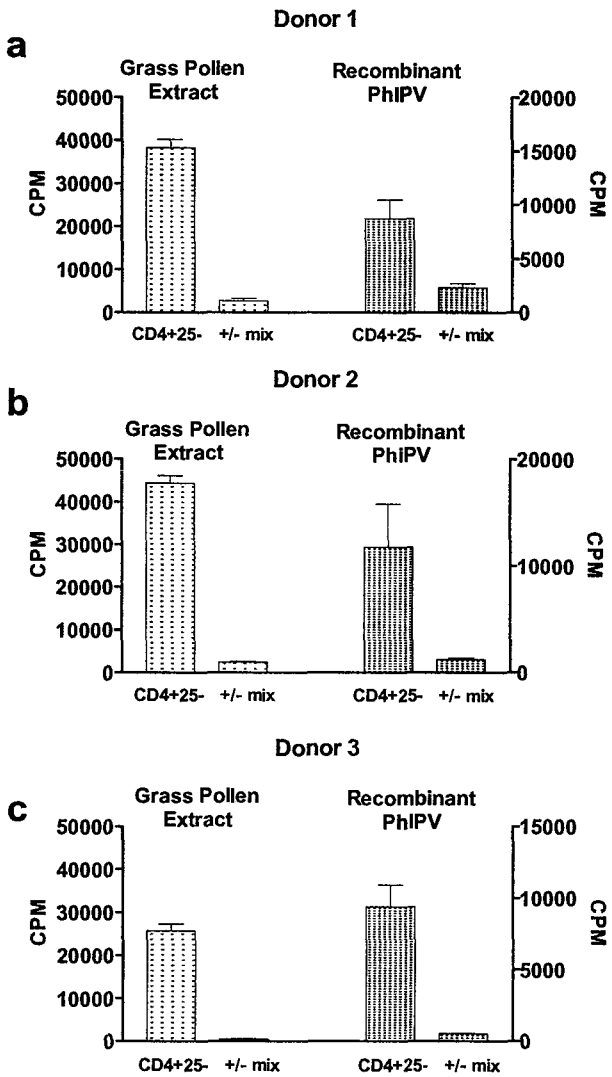


Figure 3.12. Suppression of grass pollen allergen and recombinant PhlPV activated T cell proliferation by human CD4+CD25+ cells. CD4+CD25- T cells at 2 x 10^6 c/ml were stimulated with grass pollen extract at 100 μg/ml (light bars, left axis), PhlPV at 12.5 μg/ml (dark bars, right axis) or media alone. Either CD4+CD25+ regulatory T cells, (marked +/- mix), or CD4+CD25- T cells (marked CD4+CD25-), were added to the cultures at 1 x 10^6 c/ml. All wells contained irradiated autologous PBMC at 2 x 10^6 c/ml. Allergen specific proliferation was measured by tritiated thymidine incorporation at day 7 of cells from 3 representative non-atopic donors (a-c). Mean values + SE of triplicate cultures are shown.

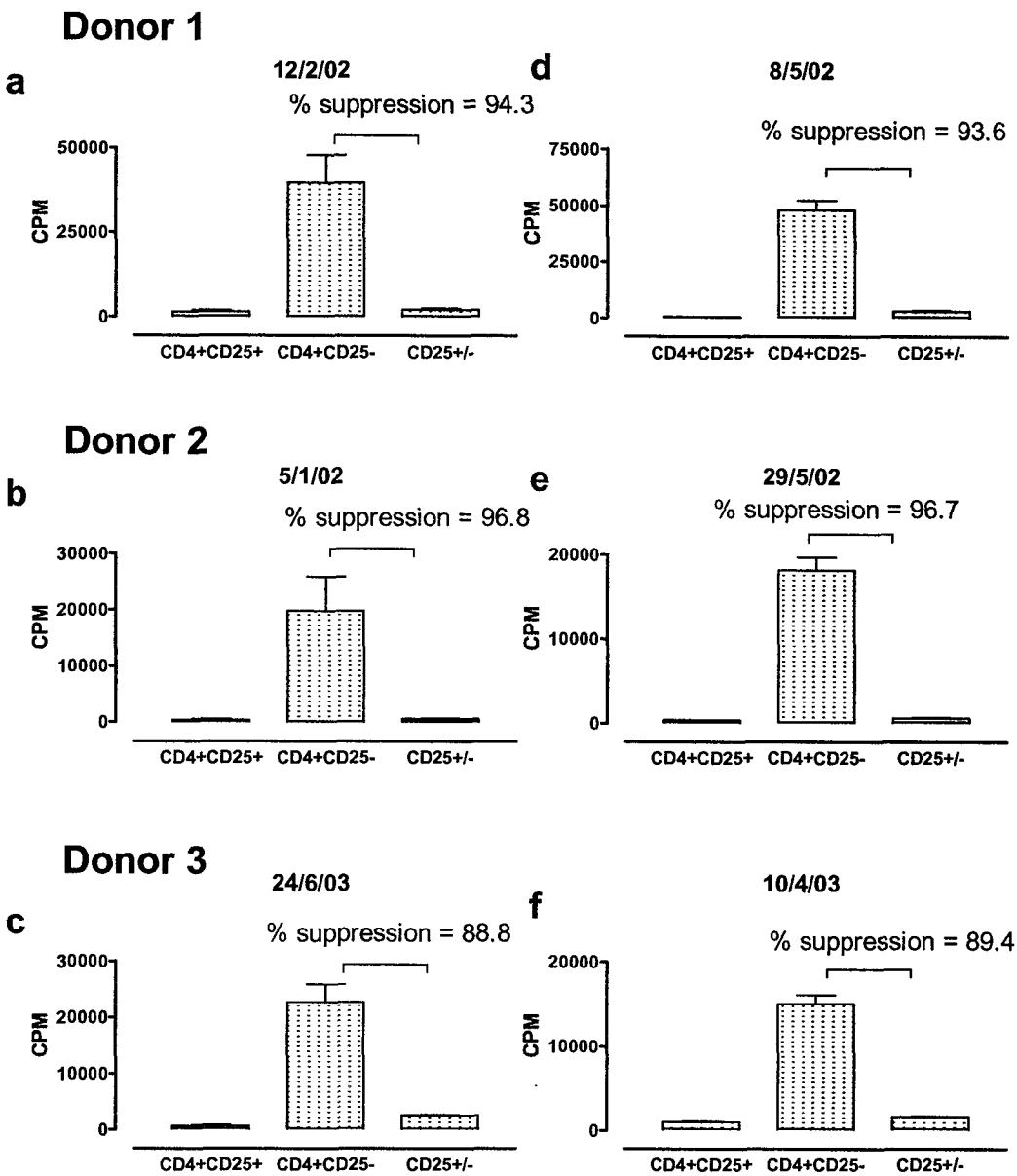


Figure 3.13. Reproducibility of the assay. CD4+CD25+ T cells at 2 x 10^6 c/ml or CD4+CD25- T cells at 3 x 10^6 c/ml were stimulated with grass pollen allergen extract at 100 μg/ml or media alone. CD4+CD25+ regulatory T cells were added to CD4+CD25- T cells at a ratio of 1:2 (marked CD25+/-). All wells contained irradiated autologous PBMC at 2 x 10^6 c/ml. Allergen specific proliferation of cells from 3 representative donors was measured by tritiated thymidine incorporation at day 7 (a-c) and the experiment was repeated at a later date (d-f). Mean values + SE of triplicate cultures are shown. Donors 1 and 2 were non-atopic and donor 3 was atopic.

suppression was executed by inserting values for proliferation or cytokine production into the following equation:

$$\frac{\text{(CD25-/-) minus (CD25+/-)}}{\text{(CD25-/-)}} \times 100 = \text{value for \% suppression}$$

Any % suppression values that were negative values, (ie more proliferation/cytokine production by the mixed CD25+/- cells than the CD25-/- subset), were given values of zero as no suppression had taken place. If there was no proliferation or cytokine produced by the CD25-/- subset then no % suppression value was calculated, as there was nothing to suppress.

This optimised assay was then utilised to study the suppression of allergen driven CD4+CD25- T cell responses by CD4+CD25+ regulatory T cells from 9 non-atopic donors. Allergen-specific proliferation by CD4+CD25- cells was significantly reduced when CD4+CD25+ cells were present (Fig. 3.14a) and % suppression of proliferation by CD4+CD25+ T cells from all 9 donors was more than 90% (Fig. 3.14b). Allergen specific IL-5 and IFN-γ production by CD4+CD25- T cells was also significantly reduced in the presence of CD4+CD25+ cells (Fig. 3.15a and b) and when IL-13 was present in significantly high quantities, the concentration of this cytokine were suppressed also (Fig. 3.15c). The addition of CD4+CD25+ cells did not have any significant effect on the levels of IL-10 in the culture supernatant (Fig. 3.15d). Furthermore, there was no difference in % suppression of proliferation or IL-5 production when the cells were stimulated with cat allergen extract compared with grass pollen allergen extract (Fig. 3.16).

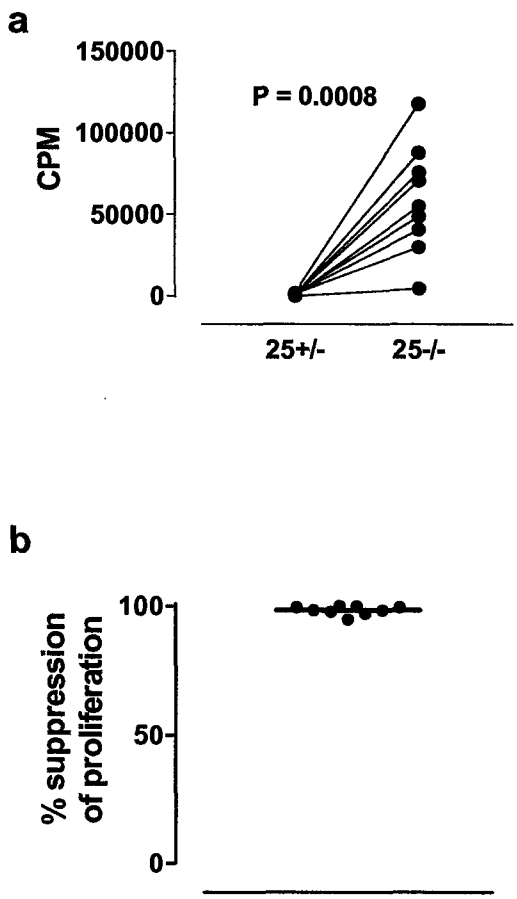


Figure 3.14. Suppression of allergen induced T cell responses by CD4+CD25+ from non-atopic donors CD4+CD25- T cells (marked 25-/-) or CD4+CD25- T cells in the presence of CD4+CD25+ T cells at a ratio of one 25+ to two 25- cells (marked 25+/-), from non-atopic donors (n = 9) were cultured at 3 x 10^6 c/ml in the presence of 100 μg/ml allergen extract (cat or grass), or media alone. Allergen specific proliferation was measured by tritiated thymidine incorporation on day 7. All wells contained irradiated PBMC at 2 x 10^6 c/ml, (a). % suppression of proliferation was calculated by using CPM values in the following equation.

$$\frac{\text{(CD25-/-) minus (CD25+/-)}}{\text{(CD25-/-)}} \times 100$$

Individual % suppression values for suppression of allergen specific CD4+CD25- proliferation by CD4+CD25+ cells from 9 non-atopic donors are shown (b), a bar represents the median value.

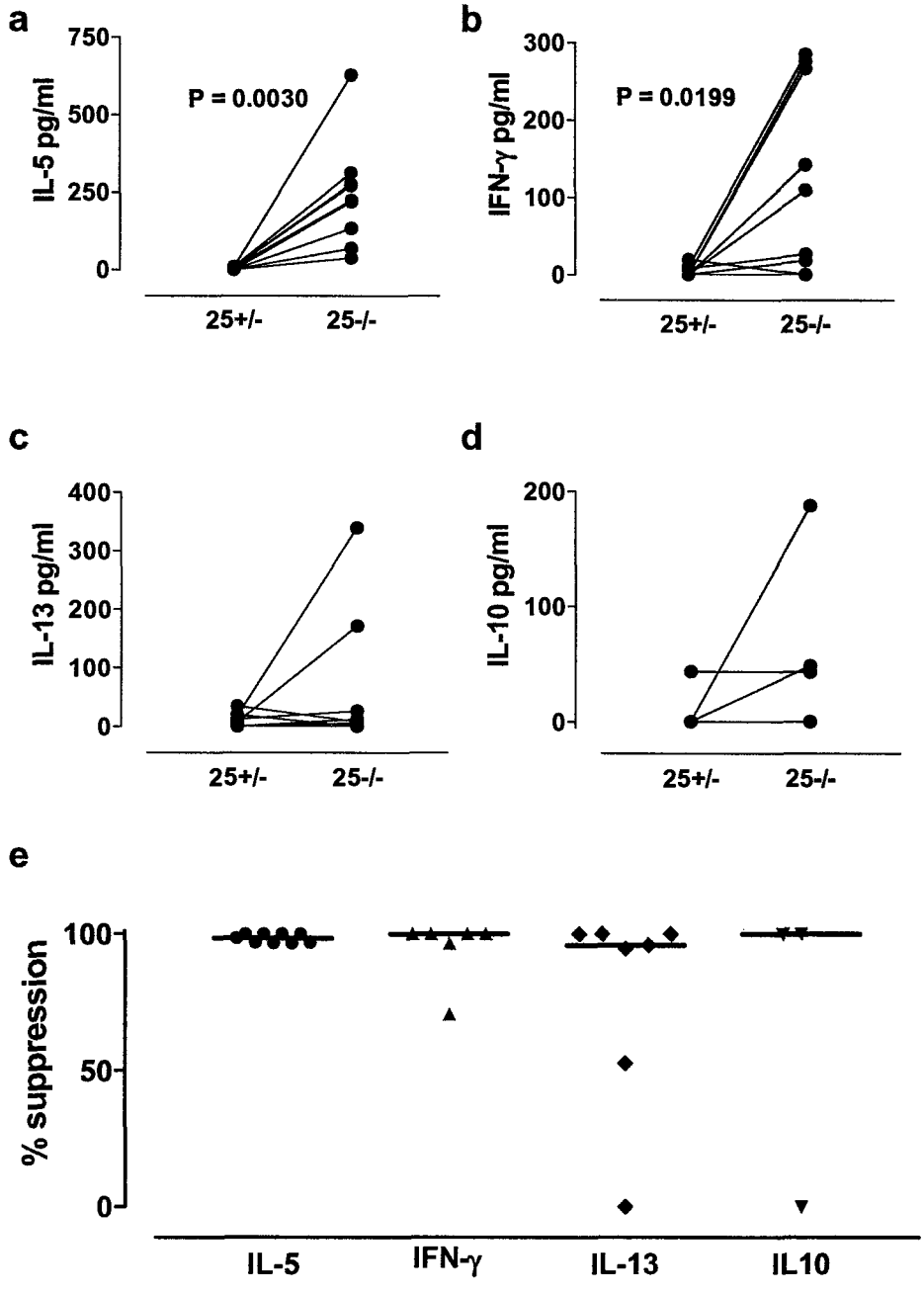


Figure 3.15. Suppression of allergen induced T cell cytokine production by CD4+CD25+ cells from non-atopic donors
CD4+CD25- T cells (marked 25-/-) or CD4+CD25- T cells in the presence of CD4+CD25+ T cells at a ratio of one 25+ to two 25- cells (marked 25+/-), from non-atopic donors (n = 9) were cultured at 3 x 10^6 c/ml in the presence of 100 μg/ml allergen extract (cat or grass) or media alone. Allergen specific IL-5 (a), IFN-γ (b), IL-13 (c) and IL-10 (d) production was measured by Luminex of culture supernatants taken on day 6. All wells contained irradiated PBMC at 2 x 10^6 c/ml. Individual % suppression values for suppression of allergen specific cytokine production by CD4+CD25+ cells from 9 non-atopic donors are shown (e), a bar represents the median value.

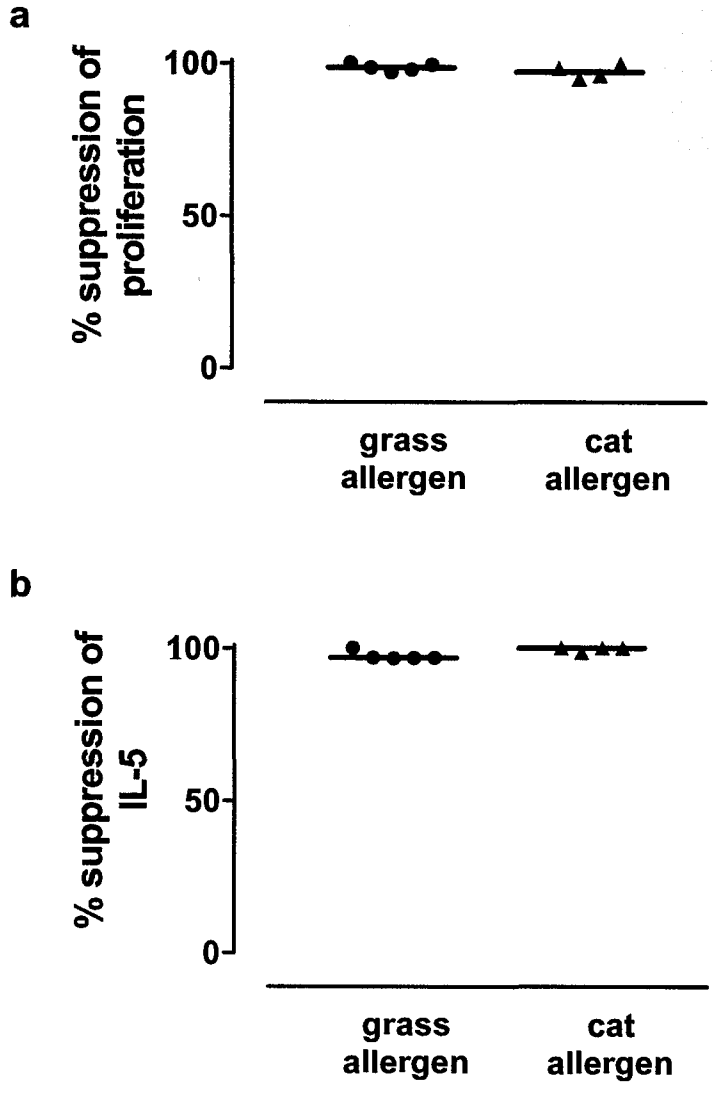


Figure 3.16. Suppression of grass pollen vs cat dander induced T cell responses by CD4+CD25+ cells from non-atopic donors CD4+CD25- T cells or CD4+CD25- T cells in the presence of CD4+CD25+ T cells at a ratio of one 25+ to two 25- cells from non-atopic donors (n = 9) were cultured at 3 x 10^6 c/ml in the presence of 100 μg/ml grass pollen (n = 5, circles), cat dander (n = 4, triangles) allergen extract or media alone. Allergen specific proliferation was measured by tritiated thymidine incorporation on day 7 and cytokine concentration of day 6 supernatants was measured by Luminex. All wells contained irradiated PBMC at 2 x 10^6 c/ml. Individual % suppression values for suppression of allergen specific proliferation (a) and IL-5 production (b) by CD4+CD25+ cells are shown, a bar represents the median value.

3.2.5. An investigation into the mechanism by which human CD4+CD25+ regulatory T cells from non-atopic donors can inhibit allergen specific T cell responses.

Among the factors suggested to be important in the mechanism by which CD4+CD25+ regulatory T cells exert their suppressive effect are the cytokines IL-10 and TGF-β, and cell surface receptors GITR and CTLA-4 [342]. Antibodies that block these molecules, their ligands or their receptors are commercially available and have been used to investigate their role in suppression by CD4+CD25+ regulatory T cells [208;222;224-228;320]. At the time the experiments described here were performed, there had been no reports investigating the role of any of these molecules in the suppression of allergen driven T cell responses by CD4+CD25+ T cells.

The addition of blocking antibody specific to TGF-β 1, 2 and 3 had no reproducible effect on allergen specific proliferation or cytokine production of the CD4+CD25+ or the CD4+CD25- subsets (Fig. 3.17). Anti-TGF-β also had no effect on suppression of proliferation or cytokine production by CD4+CD25+ cells in mixed CD4-CD25+/- cultures (Fig 3.18).

Addition of blocking antibody to IL-10 or IL-10 receptor did not reverse suppression of allergen specific proliferation or cytokine production, (Fig. 3.19 and Fig. 3.20). These antibodies also had no effect on the allergen specific proliferation or cytokine production by CD4+CD25+ cells or CD4+CD25- cells although an increase in IFN-γ production by this latter subset was induced by anti-IL-10/anti-IL-10 receptor antibody (Fig. 3.19 and Fig. 3.20).

The role of CTLA-4 in CD4+CD25+ T cell-mediated suppression was next examined. Anti-CTLA-4 blocking antibody had no effect on proliferation of allergen-stimulated CD4+CD25+ or CD4+CD25- cells or suppression of proliferation by CD4+CD25+ cells (Fig. 3.21). Interestingly, IL-5 production by the CD4+CD25- cells

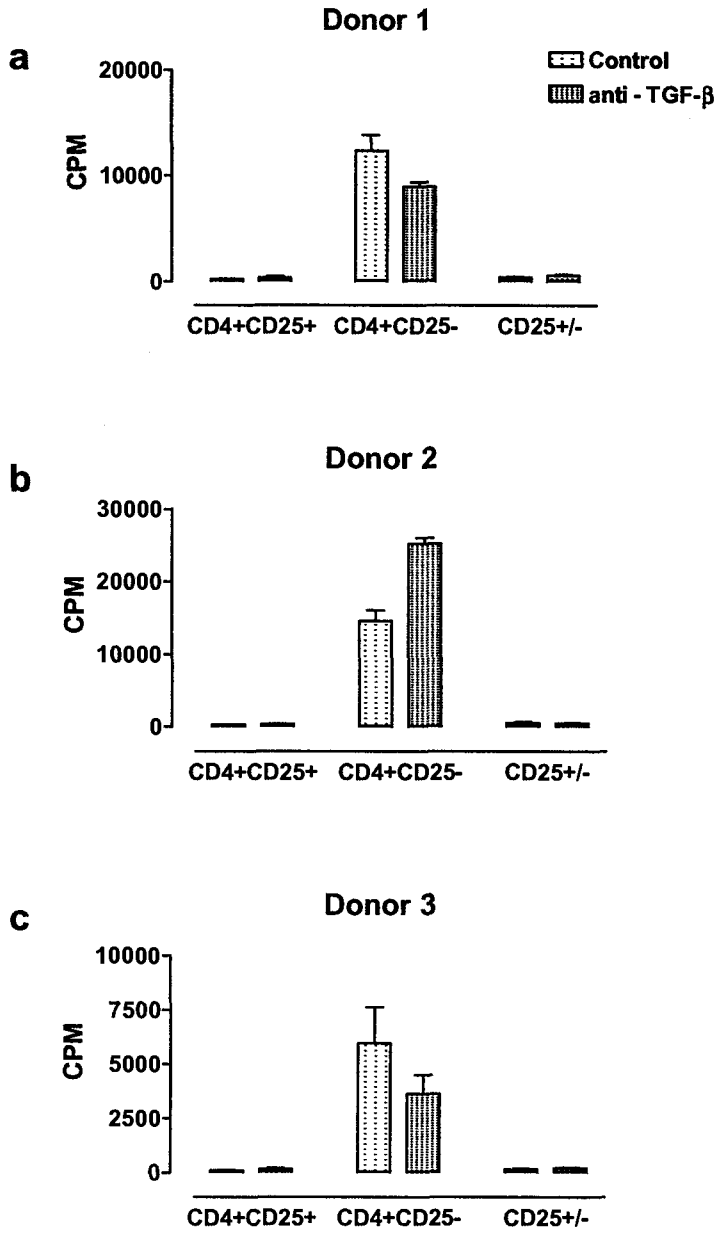


Figure 3.17. Effect of blocking TGF–β on allergen specific proliferation of CD4+CD25+ and CD4+CD25- cells and on the suppression of proliferation by CD4+CD25+ T cells. CD4+CD25+ T cells at 2 x 10^6 c/ml or CD4+CD25- T cells at 3 x 10^6 c/ml were stimulated with grass pollen allergen extract at 100 μg/ml or media alone. CD4+CD25+ regulatory T cells were added to CD4+CD25- T cells at a ratio of 1:2 (marked CD25+/-). All wells contained irradiated autologous PBMC at 2 x 10^6 c/ml. Mouse IgG1 control antibody (light bars) or blocking antibody to human TGF-β 1, 2 and 3 (dark bars) was added at 10 μg/ml. Allergen specific proliferation of cells from 3 representative non-atopic donors was measured by tritiated thymidine incorporation at day 7 (a-c). Mean values + SE of triplicate cultures are shown.

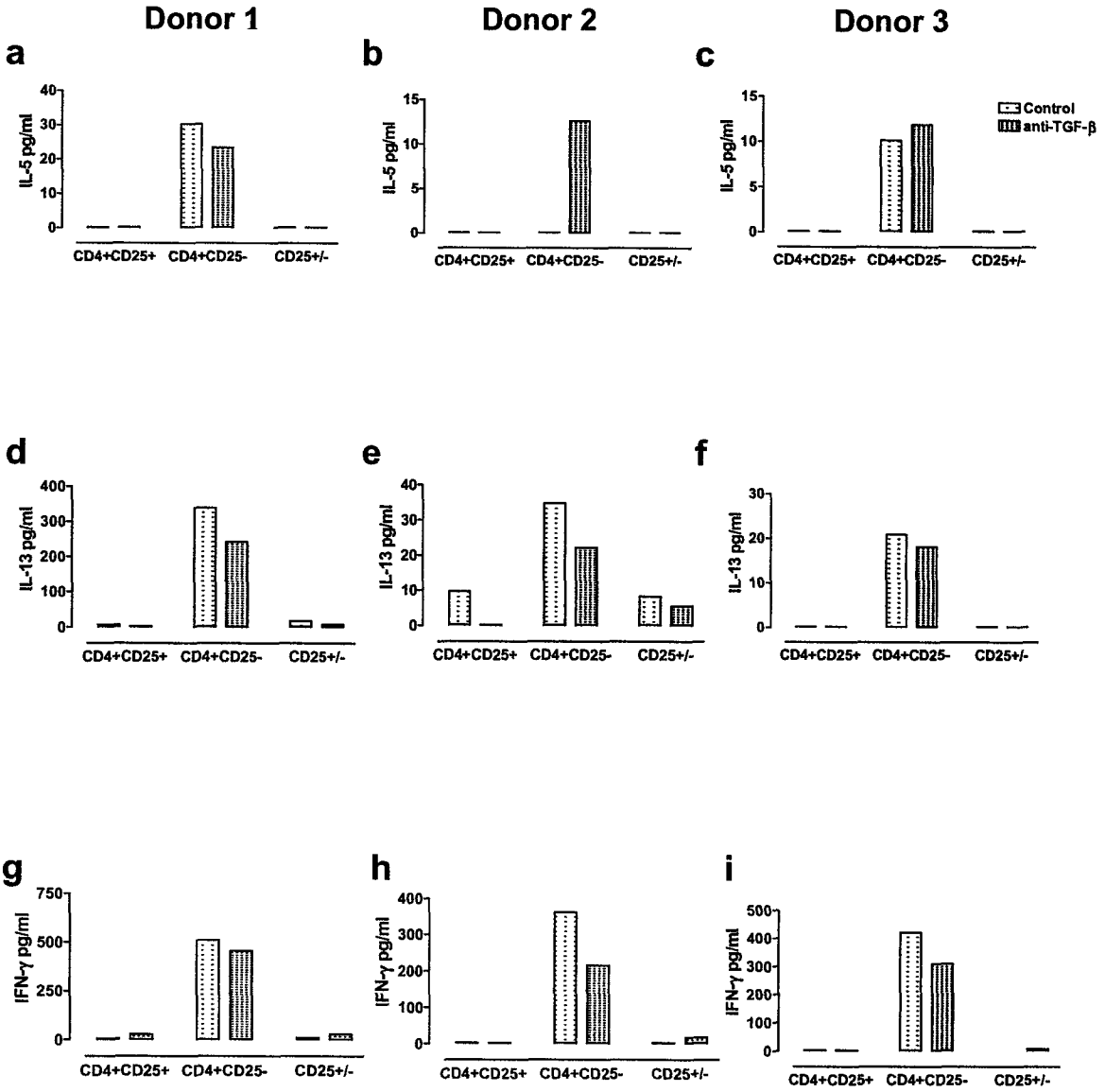


Figure 3.18. Effect of blocking TGF–β on allergen specific cytokine production by CD4+CD25+ and CD4+CD25- cells and on the suppression of cytokine production by CD4+CD25+ T cells. CD4+CD25+ T cells at 2 x 10^6 c/ml or CD4+CD25- T cells at 3 x 10^6 c/ml were stimulated with grass pollen allergen extract at 100 μg/ml or media alone. CD4+CD25+ regulatory T cells, were added to CD4+CD25- T cells at a ratio of 1:2 (marked CD25+/-). All wells contained irradiated autologous PBMC at 2 x 10^6 c/ml. Mouse IgG1 control antibody (light bars) or blocking antibody to human TGF-β 1, 2 and 3 (dark bars) was added at 10 μg/ml. Concentrations of IL-5 (a-c), IL-13 (d-f) and IFN-γ (g-i) in the day 6 culture supernatants were measured by Luminex. Results from 3 non-atopic donors are shown.

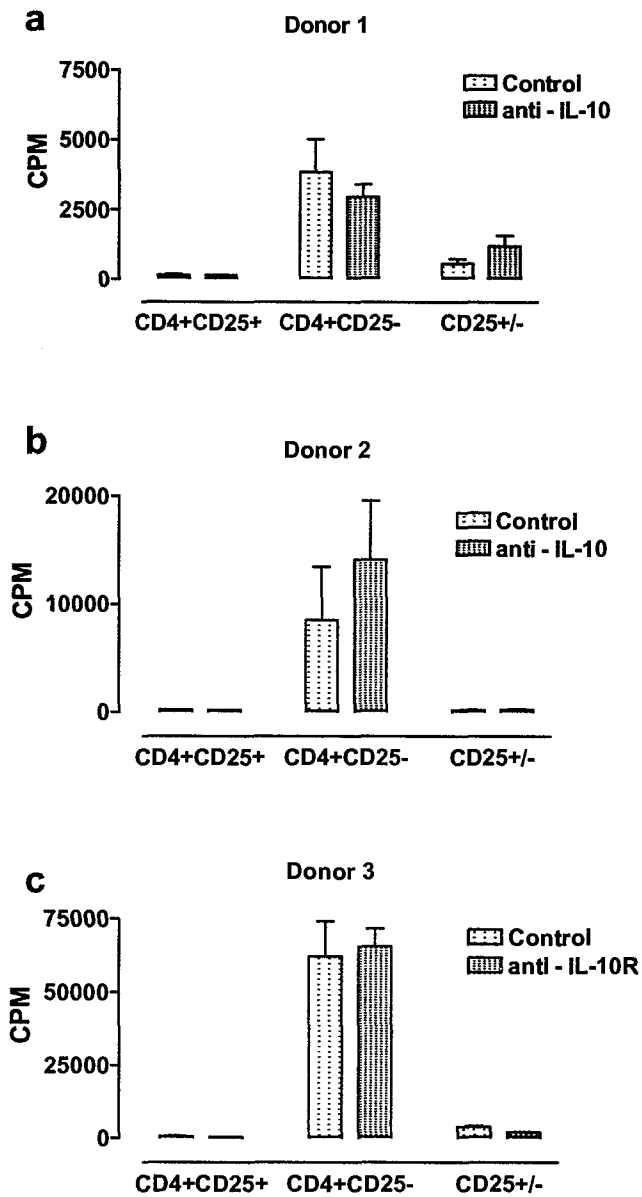


Figure 3.19. Effect of blocking IL-10 on allergen specific proliferation of CD4+CD25+ and CD4+CD25- cells and on the suppression of proliferation by CD4+CD25+ T cells. CD4+CD25+ T cells at 2 x 10^6 c/ml or CD4+CD25- T cells at 3 x 10^6 c/ml were stimulated with grass pollen allergen extract at 100 μg/ml or media alone. CD4+CD25+ regulatory T cells were added to CD4+CD25- T cells at a ratio of 1:2 (marked CD25+/-). All wells contained irradiated autologous PBMC at 2 x 10^6 c/ml. Mouse IgG1 control antibody (light bars, a-i) or blocking antibody to human IL-10 (dark bars, a + b) or IL-10 receptor (dark bars, c) was added at 10 μg/ml. Allergen specific proliferation of cells from 3 representative donors was measured by tritiated thymidine incorporation at day 7 (a-c). Mean values + SE of triplicate cultures are shown. Donors 2 and 3 were non-atopic and donor 1 was atopic.

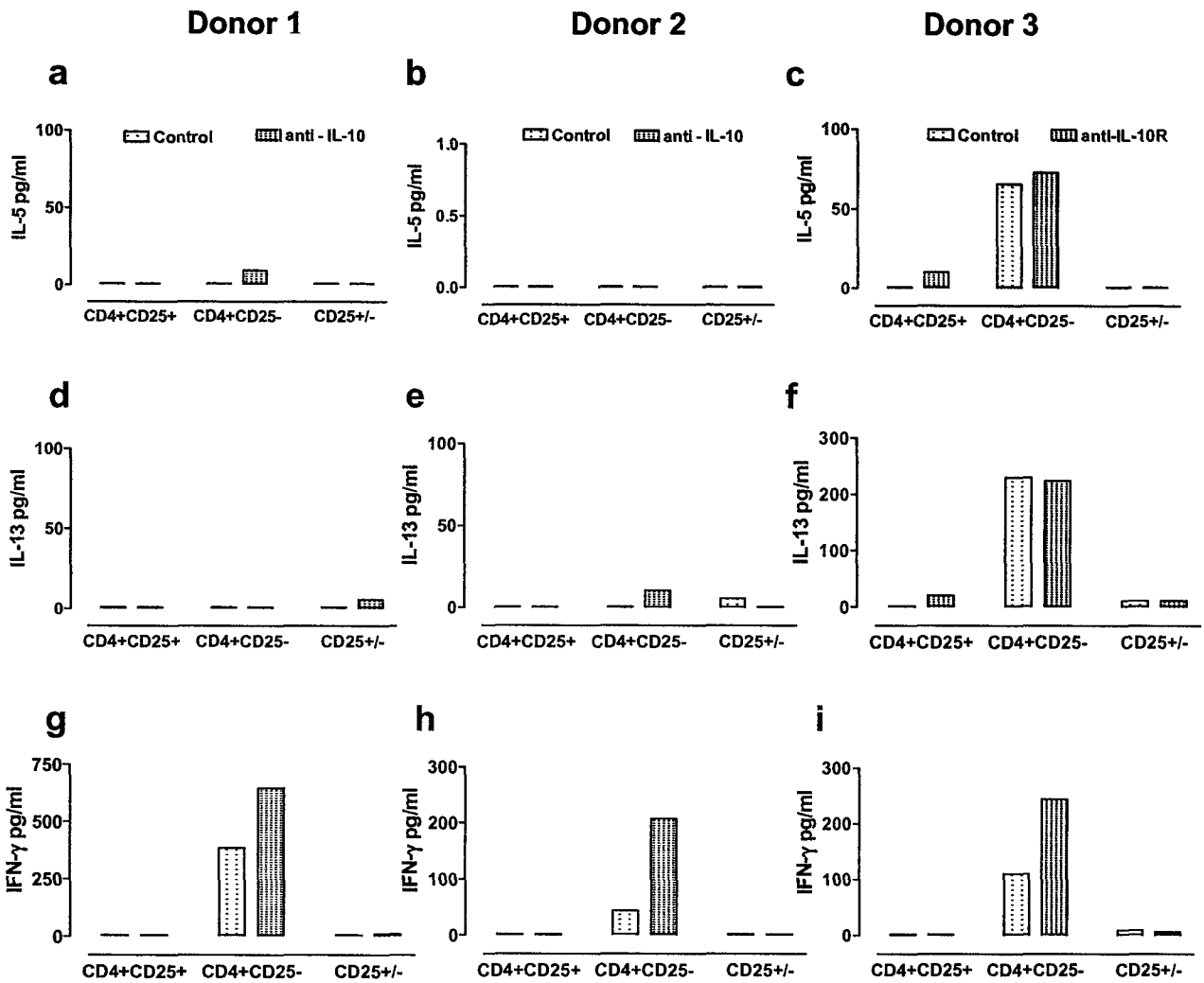


Figure 3.20. The effect of blocking IL-10 on allergen specific cytokine production by CD4+CD25+ and CD4+CD25- cells and on the suppression of cytokine production by CD4+CD25+ T cells. CD4+CD25+ T cells at 2 x 10^6 c/ml or CD4+CD25- T cells at 3 x 10^6 c/ml were stimulated with grass pollen allergen extract at 100 μg/ml or media alone. CD4+CD25+ regulatory T cells were added to CD4+CD25- T cells at a ratio of 1:2 (marked CD25+/-). All wells contained irradiated autologous PBMC at 2 x 10^6 c/ml. Mouse IgG1 control antibody (light bars, a-c) or blocking antibody to human IL-10 (dark bars, a, b, d, e, g, h) or IL-10 receptor (dark bars, c, f ,i) was added at 10 μg/ml. Concentrations of IL-5 (a-c), IL-13 (d-f) and IFN-γ (g-i) in the day 6 culture supernatants were measured by Luminex. Results from 3 donors are shown. Donors 2 and 3 were non-atopic and donor 1 was atopic.

was increased when stimulated with allergen in the presence of anti-CTLA-4 (Fig. 3.22). There was no effect on other cytokines measured in cultures of allergen stimulated CD4+CD25+ or CD4+CD25- cells. There was also no reproducible reversal of suppression of cytokine production by CD4+CD25+ cells (Fig. 3.22).

The use of a blocking antibody to GITR in this culture system partially reversed suppression of allergen driven T cell proliferation by CD4+CD25+ regulatory T cells. Addition of this blocking antibody also increased proliferation of both the CD4+CD25+ and CD4+CD25- subsets (Fig. 3.23). The effect on IL-5 production in the culture supernatant was variable. Interestingly however, there was an increase in the IL-13 concentration of all culture supernatants and a corresponding decrease in the concentration of IFN-γ in mixed cultures following stimulation in the presence of anti-GITR antibody (Fig. 3.24).

The expression of FOXP3 is associated with a regulatory T cell phenotype and over expression of this transcription factor causes both CD4+CD25+ and CD4+CD25- cells to have suppressive activity [238]. As yet there are no commercially available antibodies to block the interaction between FOXP3 and its target genes, and the majority of the studies investigating this molecule have been carried out using murine models. Therefore in the absence of such reagents, mRNA FOXP3 expression by quantitative Taqman RTPCR was studied. CD4+CD25+ cells expressed more FOXP3 mRNA than CD4+CD25- cells, indicating preferential expression of this transcription factor on the human regulatory CD4+CD25+ T cell subset (Fig. 3.25).

One way to investigate whether the suppression of T cell responses by CD4+CD25+ T cells *in vitro* is dependent on soluble mediators or cell contact dependant mechanisms is to separate the regulatory cells from the effector cells using transwell inserts (Fig. 3.26). These inserts have 0.4 μM pores that allow the passage

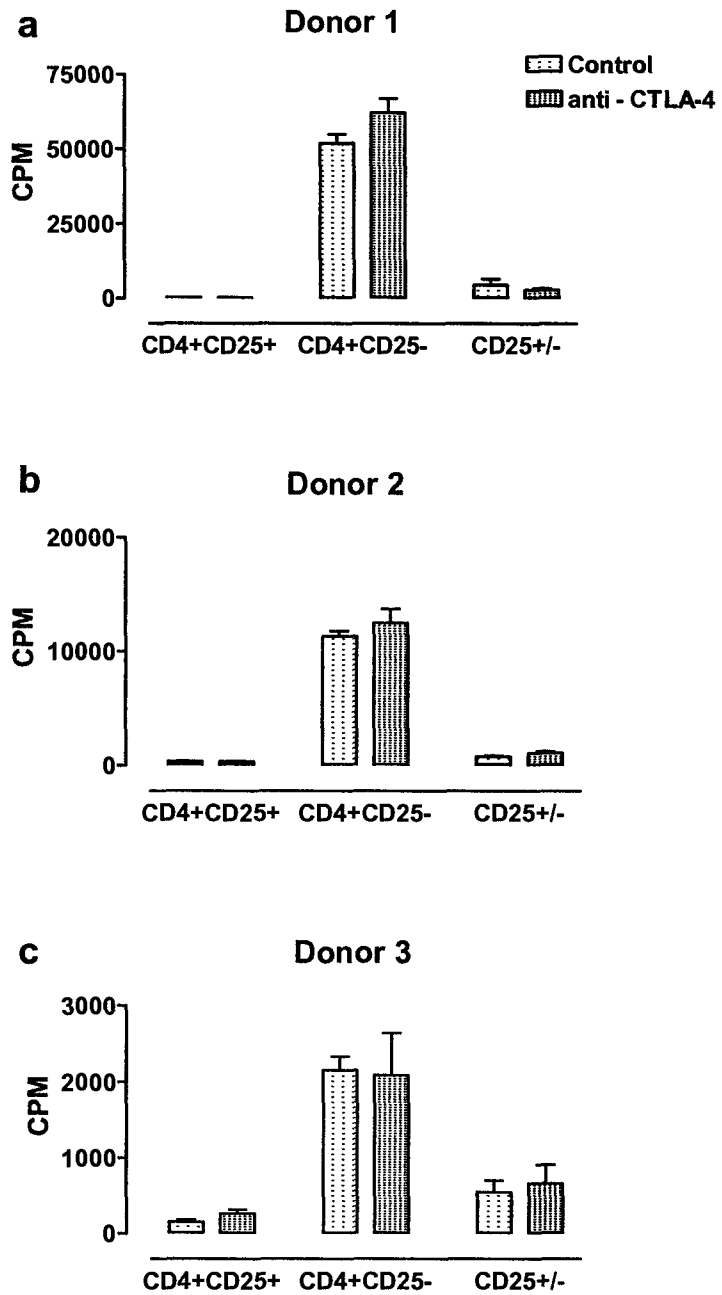


Figure 3.21. The effect of blocking CTLA-4 on allergen specific proliferation of CD4+CD25+ and CD4+CD25- cells and on the suppression of proliferation by CD4+CD25+ T cells. CD4+CD25+ T cells at 2 x 10^6 c/ml or CD4+CD25- T cells at 3 x 10^6 c/ml were stimulated with grass pollen allergen extract at 100 μg/ml or media alone. CD4+CD25+ regulatory T cells, were added to CD4+CD25- T cells at a ratio of 1:2 (marked CD25+/-). All wells contained irradiated autologous PBMC at 2 x 10^6 c/ml. Mouse IgG1 control antibody (light bars) or blocking antibody to human CTLA-4 (dark bars) was added at 10 μg/ml. Allergen specific proliferation of cells from 3 representative donors was measured by tritiated thymidine incorporation at day 7 (a-c). Mean values + SE of triplicate cultures are shown. Donors 1 and 2 were non-atopic and donor 3 was atopic.

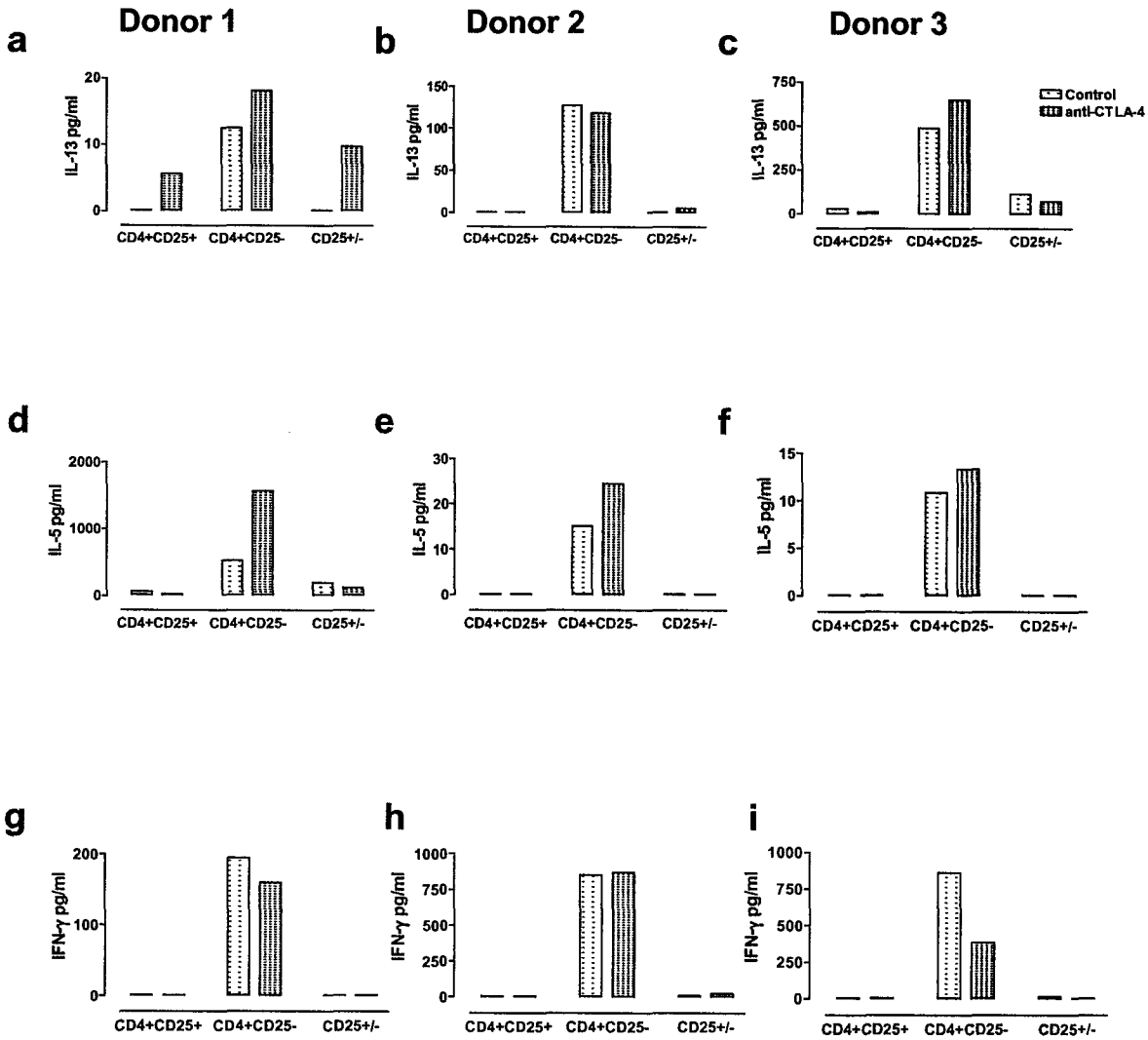


Figure 3.22. The effect of blocking CTLA-4 on allergen specific cytokine production by CD4+CD25+ and CD4+CD25- cells and on the suppression of cytokine production by CD4+CD25+ T cells. CD4+CD25+ T cells at 2 x 10^6 c/ml or CD4+CD25- T cells at 3 x 10^6 c/ml were stimulated with grass pollen allergen extract at 100 μg/ml or media alone. CD4+CD25+ regulatory T cells, were added to CD4+CD25- T cells at a ratio of 1:2 (marked CD25+/-). All wells contained irradiated autologous PBMC at 2 x 10^6 c/ml. Mouse IgG1 control antibody (light bars) or blocking antibody to human CTLA-4 (dark bars) was added at 10 μg/ml. Concentrations of IL-13 (a-c), IL-5 (d-f) and IFN-γ (g-i) in the day 6 culture supernatants were measured by Luminex. Results from 3 donors are shown. Donors 1 and 2 were non-atopic and donor 3 was atopic.

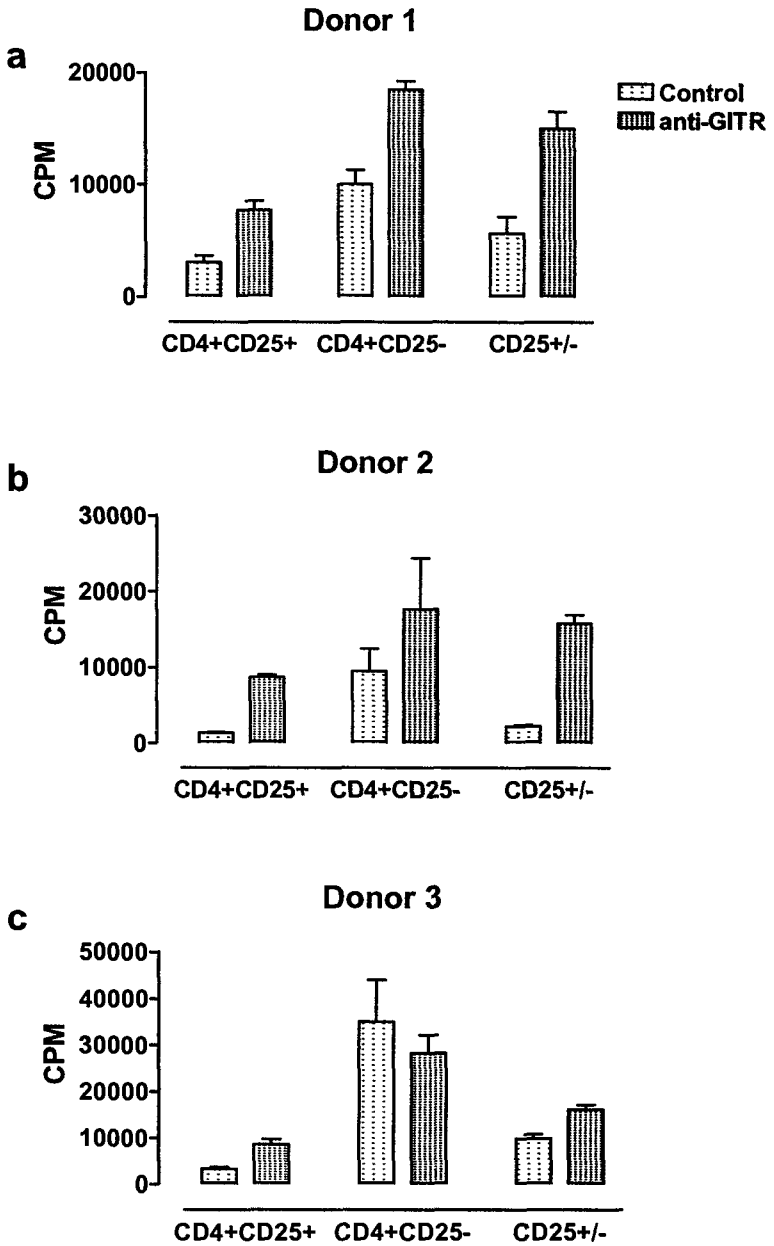


Figure 3.23. The effect of blocking GITR on allergen specific proliferation of CD4+CD25+ and CD4+CD25- cells and on the suppression of proliferation by CD4+CD25+ T cells. CD4+CD25+ T cells at 2 x 10^6 c/ml or CD4+CD25- T cells at 3 x 10^6 c/ml were stimulated with grass pollen allergen extract at 100 μg/ml or media alone. CD4+CD25+ regulatory T cells were added to CD4+CD25- T cells at a ratio of 1:2 (marked CD25+/-). All wells contained irradiated autologous PBMC at 2 x 10^6 c/ml. Mouse IgG1 control antibody (light bars) or blocking antibody to human GITR (dark bars) was added at 10 μg/ml. Allergen specific proliferation of cells from 3 representative donors was measured by tritiated thymidine incorporation at day 7 (a-c). Mean values + SE of triplicate cultures are shown. All three donors were atopic.

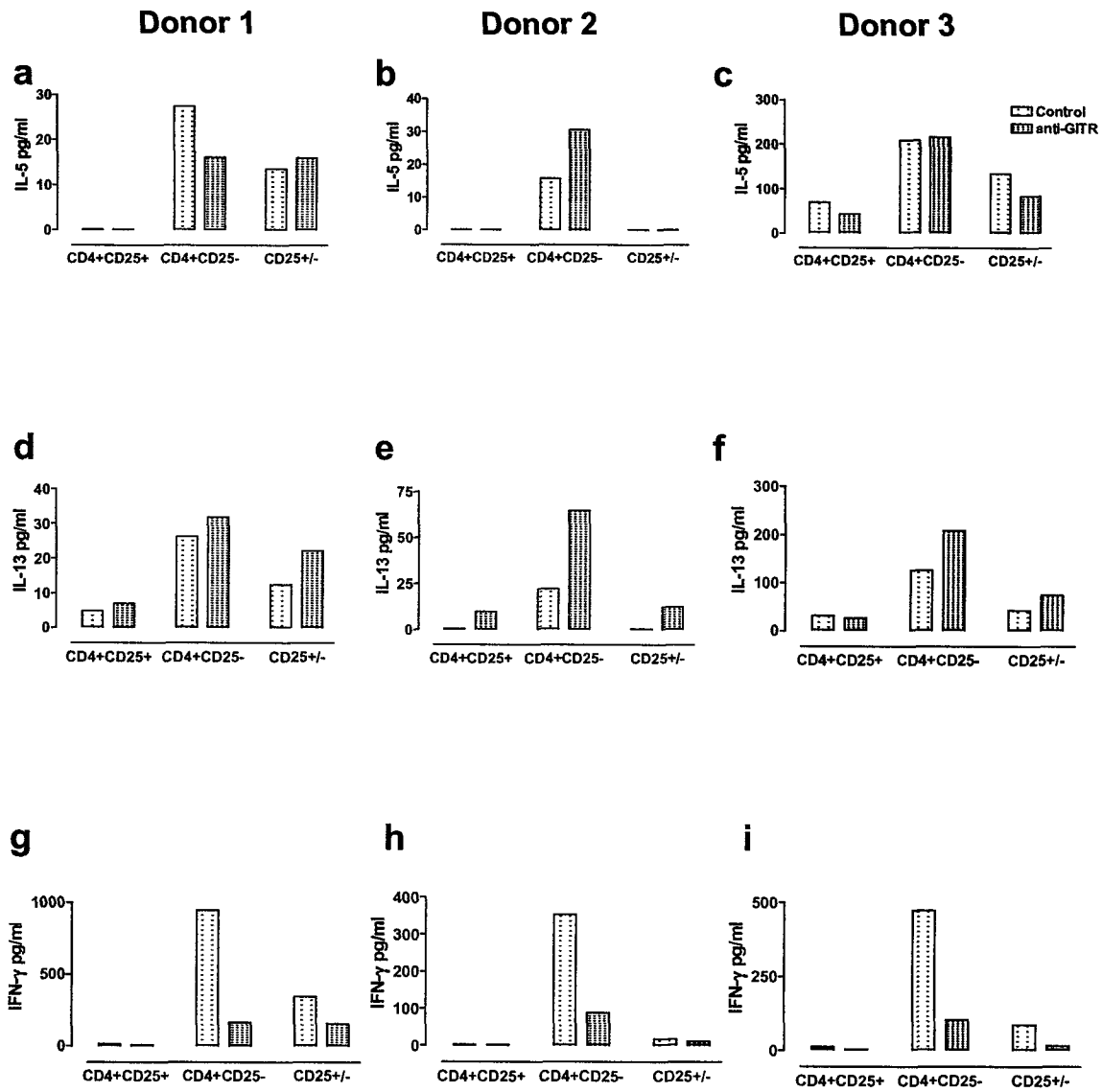


Figure 3.24. The effect of blocking GITR on allergen specific cytokine production by CD4+CD25+ and CD4+CD25- cells and on the suppression of cytokine production by CD4+CD25+ T cells. CD4+CD25+ T cells at 2 x 10^6 c/ml or CD4+CD25- T cells at 3 x 10^6 c/ml were stimulated with grass pollen allergen extract at 100 μg/ml or media alone. CD4+CD25+ regulatory T cells were added to CD4+CD25- T cells at a ratio of 1:2 (marked CD25+/-). All wells contained irradiated autologous PBMC at 2 x 10^6 c/ml. Mouse IgG1 control antibody (light bars) or blocking antibody to human GITR (dark bars) was added at 10 μg/ml. Concentrations of IL-5 (a-c), IL-13 (d-f) and IFN-γ (g-i) in the day 6 culture supernatants were measured by Luminex. Results from 3 donors are shown. All three donors were atopic.

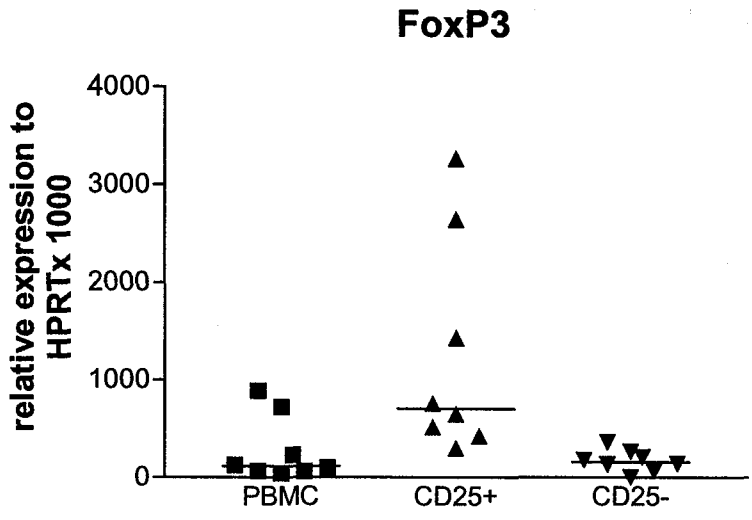


Figure 3.25. Expression of FoxP3 mRNA by PBMC, CD4+CD25+ and CD4+CD25- T cells. RNA was extracted from freshly isolated PBMC, CD4+CD25+ and CD4+CD25- T cell subsets from non-atopic donors (n = 8). RNA was then quantified and reverse transcribed. 25µl PCR reactions were carried out in triplicate with TaqMan universal PCR mastermix, FoxP3 specific primers and labelled TaqMan probe. Samples were standardised to 18S. Data is presented as expression of FoxP3 relative to expression of HPRT.

of small molecules such as cytokines but not whole cells. Using this system it was shown that the CD4+CD25+ cells can suppress allergen driven T cell responses both when they have direct contact with the CD4+CD25+ cell and when they are separated from this subset by a transwell insert (Fig. 3.27), suggesting that CD4+CD25+ T cell regulatory activity may be dependent on soluble and not cell contact dependent mechanisms.

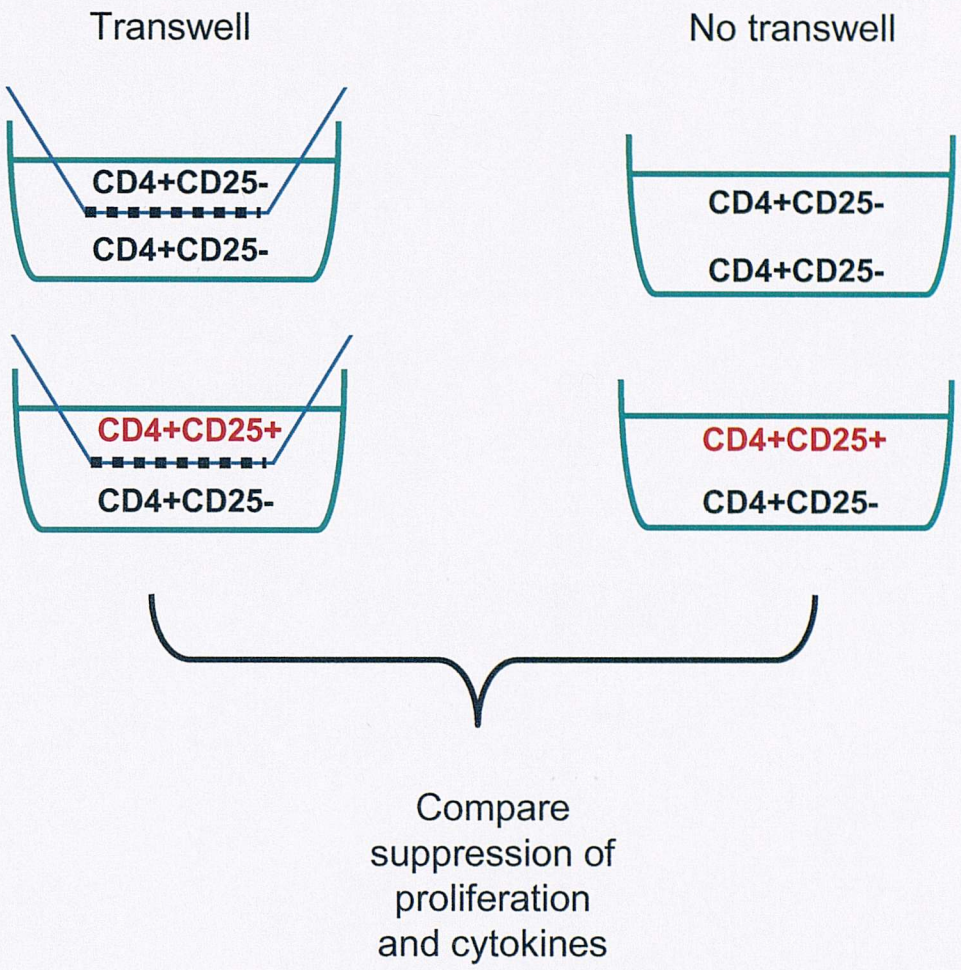


Figure 3.26. Schematic showing details of experiments investigating the ability of human CD4+CD25+ regulatory T cells to suppress allergen-driven T cell responses when separated from the CD4+CD25- responder cells by a transwell.

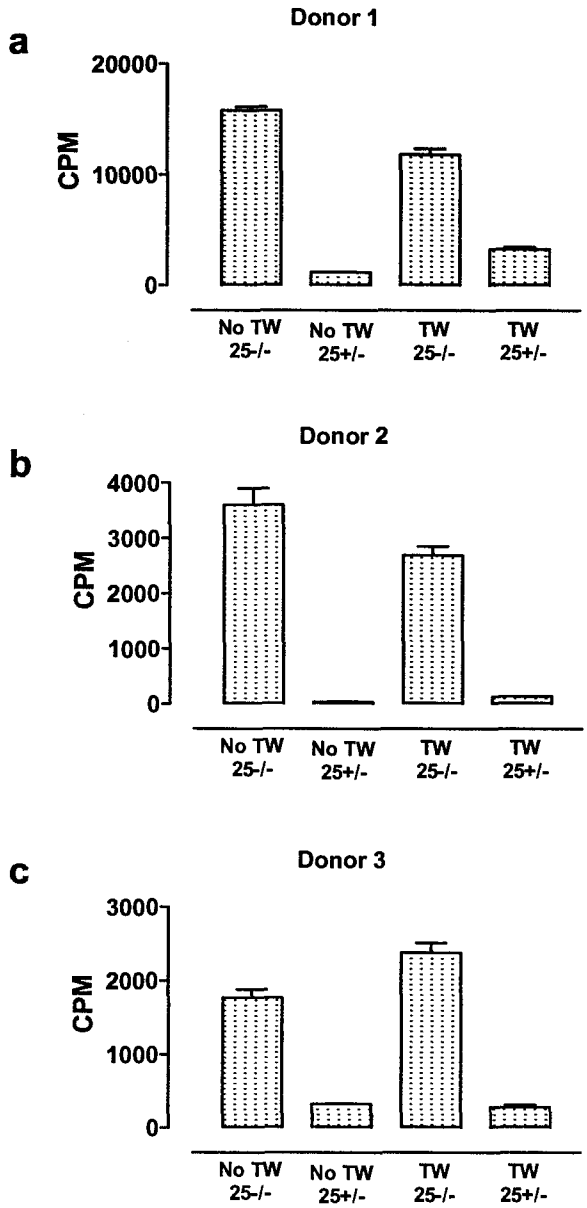


Figure 3.27. The effect of separating CD4+CD25+ regulatory T cells from CD4+CD25- responder T cells using a transwell system, on the ability of the CD4+CD25+ subset to suppress allergen driven T cell responses. CD4+CD25- T cells at 3 x 10^6 c/ml were stimulated with grass pollen allergen extract at 100 μg/ml or media alone (marked No TW 25-/-). CD4+CD25+ regulatory T cells were added to CD4+CD25- T cells at a ratio of 1:2 (marked No TW 25+/-). In some cultures, a transwell insert separated responder CD4+CD25- cells from regulatory CD4+CD25+ cells (marked TW 25+/-) or in the control, from added CD4+CD25- cells (marked TW 25-/-). All wells contained irradiated autologous PBMC at 2 x 10^6 c/ml. Allergen specific proliferation of cells from 3 representative non-atopic donors was measured by tritiated thymidine incorporation at day 7 (a-c). Mean values + SE of triplicate cultures are shown.

3.3. Discussion

3.3.1. Th2 responses to allergen by cells from non-atopic individuals

The fact that PBMCs from non-atopic individuals proliferate to *in vitro* stimulation with an allergen to which they are regularly exposed is not unexpected. What is more surprising is that there is a subset of cells within the PBMC population of non-atopic individuals, the CD4+CD25- subset, which is capable of Th2 cytokine production following allergen stimulation. Why then do non-atopic people not mount a Th2 response to allergen after stimulation *in vivo*? The data presented here supports the hypothesis that there is a population of cells within the PBMC, which suppresses the proliferation and Th2 cytokine production by CD4+CD25- T cells.

3.3.2. The suppression of Th2 responses to allergen by CD4+CD25+ regulatory T cells from non-atopic individuals

The ability of human CD4+CD25+ T cells to suppress T cell responses to polyclonal and antigenic stimulation has previously been described [228]. Although there have been several studies investigating the suppression of Th2 responses in animal models *in vivo* [58;260;312-315], the studies described here were amongst the first detailing the suppression of allergen driven T cell responses by human CD4+CD25+ T cells. During the period of the experiments described in this thesis, two other studies were published showing the suppression of house dust mite allergen [343] and grass and birch pollen allergen [344] stimulated T cell responses by human CD4+CD25+ T cells. Results described in this chapter have suggested the potential of human CD4+CD25+ T cells to suppress both the proliferation and cytokine production to the common aeroallergens cat dander and grass pollen. These data imply that naturally occurring CD4+CD25+ T cells in non-atopic individuals suppress Th2 responses to allergen,

therefore affording protection from atopic allergic reactions following allergen exposure.

3.3.3. Limitations of this in vitro assay

One criticism of the *in vitro* assay used in this study may be that the ratio of CD4+CD25+ regulatory cells to responder T cells at 1:2 is artificially high. This is certainly the case in the peripheral blood where, as is shown in Figure 3.4 and Table 3.5, the percentage of PBMC that are CD4+CD25+ is about 10-15 %. However, data from this chapter also shows that CD4+CD25+ cells have the ability to suppress allergen driven T cell responses (at least partially) at ratios of 1:4 and sometimes even 1:8 and 1:16, ratios which are perhaps more analogous to those in human peripheral blood. The ratio of 1:2 was chosen so that maximum suppression was observed and any reversal of suppression when investigating the mechanism could be clearly seen. It should also be noted that the concentration of allergen used was maximal, to give an optimal T cell response to suppress and that *in vivo,* it is unlikely that such a concentration of allergen would be encountered. The relationship between suppression by CD4+CD25+ cells and concentration of allergen should be further investigated. It is also likely that, although the percentage of CD4+CD25+ cells in peripheral blood is low, it may be higher at sites of inflammation. CD4+CD25+ T cells are highly differentiated memory cells which due to the expression of adhesion molecules such as L-selectin, CD54, LFA-1 and $\alpha_E\beta_7$ and chemokine receptors such as CCR2, CCR4, CCR5 and CCR8, would be expected to have the ability to migrate to areas of allergen challenge such as the lung and skin[324 203;325;326]. It may well be that at the site of allergen challenge in a non-atopic individual, the ratio of regulatory cells to effector cells could be as high as 1:4 or even 1:2. Further studies should investigate

the role of regulatory T cells at sites of allergic inflammation although, due to the small numbers of T cells recovered from sites such as lung and skin, these will be difficult to execute. Results from studies of CD4+CD25+ T cells in cancer and autoimmune disease however, do illustrate that these cells can be recovered from tissues [296;303].

When analysing results from *in vitro* regulatory T cell assays it is also important to take into account the fact that in such an enclosed system, growth factors such as IL-2 and IL-4 may be limiting. It may be that in the co-culture experiments, the CD4+CD25+ T cells, which don't make such cytokines, act as a sink and use up all available growth factors. A recent study has also shown that the combined activity of CTLA-4 and TGF-β1 expressed by activated CD4+CD25+ T cells is able to inhibit the expression of the α-chain of the IL-2R on target T cells, making these cells unresponsive to the growth activity of IL-2 [324]. Data from our lab however, shows that addition of exogenous IL-2 does not reverse suppression in the allergen driven system, and that there is measurable IL-2 in cultures where CD4+CD25+ T cells suppress CD4+CD25- T cell proliferation to polyclonal stimulation (Smith TRF, Ahern D and Robinson DS, personal communication), indicating that lack of proliferation is not just due to lack of growth factors. Another recent paper comparing the ability of CD4+CD25+ thymocytes to suppress Th1 and Th2 clones derived from CD4+CD25- T cells indicates that Th2 cells have a lower susceptibility than Th1 cells to the suppressive activity of CD4+CD25+ T cells. The authors suggest that this may be because Th2 cells are able to produce and to respond to growth factors distinct from IL-2, such as IL-4 and IL-9 [345]. It would therefore be interesting to block/add exogenously cytokines such as IL-4 and IL-9 in this allergen driven system.

The allergen used in the majority of these experiments was allergen extract and not purified protein. Whilst the relative concentration of major allergens and biological activity of such extracts is standardised, they may contain proteins other than grass pollen or cat dander, which could induce cells to proliferate or produce cytokines. It was therefore considered important to show that CD4+CD25+ T cells could suppress T cell responses to purified recombinant Phl PV, the major allergenic protein in grass pollen. Obviously it would have been preferable to use purified recombinant Phl PV throughout the study, however this was not possible due to cost constraints. Work from our laboratory has shown that naturally occurring human CD4+CD25+ cells are able to suppress proliferation and Th2 cytokine production from both an allergen specific and allergen-peptide specific T cell clone [346]. This data supports the fact that these cells are suppressing an allergen-specific response, as well as illustrating that they are able to inhibit responses by activated T memory cells.

Another limitation of this study was that suppression was only studied at days 6-7. This time point was chosen as it was the peak of the allergen specific proliferation and cytokine production and therefore any suppression by CD4+CD25+ T cells could be clearly detected. It would have been interesting to study the suppression at a number of different time points throughout the course of the T cell response to allergen. This was not possible however as the number of cells from each donor required for such an assay is greater than was normally isolated. To overcome this problem, proliferation could be measured by incorporation of 5-carboxyfluorescien diacetate succinimidyl ester (CFSE), instead of by tritiated thymidine incorporation. CFSE is a dye which incorporates into the cell membrane and which can be detected by flow cytometry. Intensity of the staining decreases with each cell division as the dye is split equally among the progeny. If a population of

cells were stained with CFSE and then stimulated with allergen, aliquots could be taken from the culture at different time points and levels of CFSE staining and therefore proliferation, measured by flow cytometry. Fewer cells are needed for CFSE compared to thymidine and so more time points could be studied. CFSE staining could also be used in conjunction with intracellular cytokine staining to examine the production of cytokines by proliferating cells, and cells whose proliferation had been suppressed by CD4+CD25+ cells.

Another consideration of this *in vitro* assay is that CD4+CD25+ cells were isolated by positive immunomagnetic selection using an anti-CD25 antibody attached to microbeads. Although this method of isolation has been used by others [203;224-226], it is possible that this could activate cells or affect their suppressive function. However, the manufacturer claims that this is not the case (http://www.miltenyibiotec.com/), and when the CD4+CD25+ cells are cultured with media *in vitro* there is no spontaneous cytokine production or proliferation suggesting no activation. Also, immunomagnetic selection did not affect cell viability, as shown by assessment of trypan blue exclusion. Furthermore, CD4+CD25+ cells isolated using fluorescence activated cell sorting (FACS) show the same phenotypic and functional properties as those isolated using immunomagnetic separation [347] (and personal communication D. Ahern). Using this method, it is possible to isolate cells based on their intensity of CD25 expression and/or other cell surface markers. Using FACs, it has been shown that CD4+CD25high cells are more effective at suppressing T cell responses than CD4+CD25low cells [347]. However, the disadvantages of this isolation technique are that the flow cytometer is not an enclosed unit which can lead to problems of contamination and also that the cell yield is not as high as that using immunomagnetic separation.

3.3.4. The mechanism of suppression of allergen driven T cell responses by human CD4+CD25+ regulatory T cells in vitro

Results from this chapter do not support a role for the immunoregulatory cytokines TGF-β and IL-10 as part of the mechanism by which CD4+CD25+ regulatory cells suppress T cell responses to allergens. This is in agreement with data from a large number of *in vitro* studies investigating the suppression by CD4+CD25+ cells of polyclonal and antigenic stimulated T cell responses [208;214;223-227;255;256]. It cannot be disregarded however that *in vivo*, a role for these cytokines has been implicated [53;255;287;337;338]. It may be possible that the blocking antibodies to TGF-β1, 2 and 3, IL-10 and IL-10R used in this study may not completely inhibit all the interactions between cytokine and receptor. The concentrations used were those suggested by the manufacturer and also those used in previous studies [208;214;223-227;255;256]. However it is of note that although levels of TGF-β were not measured, no IL-10 was detected in the culture supernatant of CD4+CD25+ cells stimulated with allergen in the majority of donors. This may suggest that IL-10 is not involved in suppression. However, only the day 6 culture supernatants were tested and IL-10 could be involved earlier in the response or alternatively the cells in the culture system could have consumed it. Another possibility is that cytokines may be bound to the cell surface of the regulatory cell in a latent form, and then delivered by cell-cell contact to the responder cell, a process that would not be affected by blocking antibodies. This mechanism has been proposed by Strober and colleagues who have reported that blocking the latent form of TGF-β, latency-associated peptide (LAP), reverses suppression [246]. However, recent results show that addition of LAP does not reverse suppression of allergen driven T cell responses by CD4+CD25+ regulatory T cells (Nguyen, X.D. and Robinson, D.S. JACI, in press), suggesting that in this

allergen driven system, cell surface latent TGF-β is not involved in the mechanism of suppression.

Conflicting results from *in vivo* and *in vitro* studies have also been demonstrated when investigating the role of CTLA-4 in the suppression of T cell responses by CD4+CD25+ T cells. Blockade of CTLA-4 in several murine models resulted in reversal of suppression,[53;54;287;339] whereas suppression was unaffected by blocking antibodies added to *in vitro* cultures [208;222;223]; data further supported by findings in this chapter.

GITR has been reported to play a role in suppression by murine CD4+CD25+ T cells [233;235]. Although GITR is present on human CD4+CD25+ T cells, no such role for this receptor in the mechanism of suppression has so far been reported [329]. Results from this chapter show that, although blocking antibody to GITR partially reversed suppression of allergen driven T cell responses, there was no reversal of suppression of cytokines. Addition of anti-GITR antibody also increased allergen specific proliferation of both the CD4+CD25+ and CD4+CD25- subset suggesting non-specific activation of cells. The effect of cytokine production was also dramatic with the increase in IL-13 and decrease of IFN-γ in the supernatants suggesting promotion of a Th2 response. The role of GITR in Th2 responses to allergen should be further investigated. It should be noted that all three donors whose T cells were used in the anti-GITR experiments were atopic due to a lack in availability of non-atopic donors, and therefore suppression was not maximal. Ideally, this experiment should be repeated with non-atopic donors.

The expression of the transcription factor FOXP3, which is a key factor in the generation and function of regulatory T cells [236], supports the notion that the cells used in this assay were of a regulatory phenotype. Unfortunately there are no tools

available to manipulate FOXP3 in human *in vitro* systems, limiting the investigation of the role of this transcription factor in the suppression of allergen driven T cell responses by CD4+CD25+ cells. Further experiments could be carried out however to examine the relationship between concentration of FOXP3 and degree of suppression by CD4+CD25+ cells.

Although some *in vivo* models investigating suppression by regulatory cells have shown that cytokines are important [198;239;332], *in vitro* studies [208;214;223-227;255;256] have shown that CD4+CD25+ regulatory T cells exert their suppression through cell contact dependent mechanisms. However, results described in this chapter contradict these latter findings as transwell assays showed that suppression of allergen specific T cell responses still occurred when a semi-permeable membrane separated the CD4+CD25+ and CD4+CD25- T cell subsets. The reason for the difference seen in this allergen driven system compared to other *in vitro* systems is unclear although the nature or strength of the stimulus could be a factor, as could differences in the culture system such as cell density and type of antigen presenting cell used. Either this result is erroneous or it may reflect the fact that this system mimics more effectively what happens *in vivo* than other *in vitro* systems, due to use of an allergen rather than polyclonal stimulus.

Other *in vitro* studies have shown that CD4+CD25+ regulatory T cells mediate infectious tolerance by inducing an anergic/suppressive phenotype in the responder CD4+CD25- cells, therefore amplifying the suppression [220;221]. Strikingly in these systems, although the CD4+CD25+ regulatory cell requires cell contact to suppress the CD4+CD25- responder cell, this cell can then suppress other T cells in a soluble manner through IL-10 or TGF-β. This finding could explain the conflicting data on cell contact dependent/independent mechanisms of suppression as CD4+CD25+ cells

could induce CD4+CD25- T cells to become suppressive by the time of the readout at day 7. When isolating CD4+CD25+ cells for the assays described in this chapter, the purity of the CD25+ separation was on average 80% and it could be that contaminating CD4+CD25- cells in the CD4+CD25+ transwell were suppressed by the CD4+CD25+ cells and, by the process of infectious tolerance, a soluble mediator suppressed the proliferation of the CD4+CD25- subset on the other side of the membrane. It would therefore be interesting to use blocking antibodies to IL-10 and TGF-β in conjunction with transwell assays. It is also possible that there are different populations of regulatory cell within both the CD4+CD25+ and CD4+CD25- subsets that suppress by different mechanisms, or that the nature or strength of the stimulus, the inflammatory site or cytokine milieu in which the regulatory cell is residing, may effect the mechanism of suppression.

3.3.5. Conclusion

There have been few studies reporting the suppression of Th2 responses by human CD4+CD25+ regulatory T cells. Data from this chapter has shown that CD4+CD25- T cells from non-atopic donors proliferate and produce Th2 cytokines when stimulated with allergen *in vitro* and that the CD4+CD25+ subset from these individuals is able to suppress this response. This assay was next used to further investigate the suppression of allergen driven T cell responses by CD4+CD25+ regulatory T cells from both non-atopic and atopic donors.

Chapter 4 – A comparison of the ability of CD4+CD25+ T cells isolated from non-atopic and atopic donors to suppress allergen driven T cell responses *in vitro*.

4.1. Introduction

Results presented in Chapter 3 describe the ability of CD4+CD25+ T cells to suppress allergen-specific T cell responses *in vitro*. This chapter describes an investigation of the relationship between atopic disease and suppression of T cell responses to allergen by CD4+CD25+ regulatory T cells. CD4+CD25+ T cells were isolated from atopic donors and using the assay described in Chapter 3, their ability to suppress CD4+CD25- T cell proliferation and cytokine production in response to stimulation with allergen extract *in vitro* was assessed. By isolating cells from grass allergic donors during the grass pollen season, the effect of exposure of atopic donors to allergen on the suppression of allergen-specific T cell responses by CD4+CD25+ T cells was also determined.

4.1.1. Th2 responses to allergen in atopic individuals

Increased expression of Th2 cytokines are reported to be found at sites of allergic inflammation such as the skin [106;109;110], nose [348] and lung [107;349;350], suggesting a role for these cytokines in allergic disease. PBMCs from atopic individuals also produce higher concentrations of the Th2 cytokines IL-5 and IL-13 relative to the Th1 cytokine IFN-γ than those from non-atopic donors [107;112], and IL-5 secretion by allergen stimulated CD4+ cells is associated with the severity of atopic disease [113;351].

The presence of Th2 cells at sites of allergen challenge in atopic donors implicates, but does not prove, their importance in the pathogenesis of atopic disease. Murine studies however have provided substantial evidence for this role. In mouse models of allergic asthma, airway inflammation is dependant on CD4+ cells, and more specifically, is seen when TCR transgenic Th2, but not Th1 cells are adoptively transferred [114-117]. The individual roles of Th2 cytokines have been further dissected by the use of cytokine deficient mice. Using such animal models, IL-4 was shown to be crucial for the initial priming of Th2 effector cells [116;118-121], whereas IL-5 and IL-13 are more directly involved in characteristic atopic pathology such as eosinophilia and mucus hypersecretion [121-138]. The use of a monoclonal anti-CD4 antibody, significantly improved peak expiratory flow in severe corticosteroid dependant asthmatics [352], and the roles of IL-5 and IL-13 have been further investigated by the use of blocking antibodies as a potential therapy for human allergic disease [353-357]. A summary table highlighting the importance of CD4+ Th2 cells in generating the inflammation that characterises atopic allergic disease is shown in Table 1.3 in Chapter 1.

4.1.2. The suppression of allergen driven Th2 responses by Th1 cells

It was originally thought that Th1 cells, which cross-regulate Th2 cells [67;358], suppressed allergen driven Th2 responses in non-atopic individuals, and that failure to induce Th1 responses to common pathogens in early life was associated with increased prevalence of allergy, termed "the hygiene hypothesis" [359-361].

However, although Th1 cells suppress Th2 cell cytokine production *in vitro*, the adoptive transfer of Th1 cells into rodent models of Th2 cell mediated allergic disease gives variable results [122;362], and in some models it actually enhances inflammation of the airways [363-365]. Therapeutic administration of the Th1 cytokines IFN-γ or IL-12 in

humans also has no effect on symptoms of human atopic disease [366-368]. Furthermore, populations with high rates of helminth infection, which induce Th2 responses, appear to be protected from the development of allergic disease [369;370].

Finally, there has been an increase in prevalence of Th1 driven diseases [371;372], which has mirrored that of Th2 type diseases, providing further evidence that a lack of Th1 responses alone is not solely responsible for the modern day increase in dysregulation of immune responses to allergens.

4.1.3. The association between suppression of Th2 responses by regulatory T cells and atopic disease

Regulatory T cells suppress T cell responses to polyclonal and antigenic stimuli *in vitro* (discussed further in Chapter 3) and Th1 responses in animal models *in vivo*. There are fewer studies reporting the suppression of Th2 responses by regulatory T cells, although such cells have been shown to suppress Th2 responses in animal models *in vivo* (discussed further in Section 3.1.3) Results from Chapter 3 and from two reports published since the start of the experimental work for this thesis [343;344], indicate that human CD4+CD25+ cells can suppress proliferation and Th2 cytokine production by CD4+CD25- T cells stimulated by common aeroallergens such as grass pollen, cat dander and house dust mite.

There were no published reports investigating the association between the suppression of Th2 responses by regulatory T cells and human atopic disease. However, IL-10, which is produced by and which is involved in generation and suppression by some subsets of regulatory T cells [329], is found at sites of allergen challenge [373], although some studies report decreased levels of this cytokine in atopic, compared with non-atopic individuals [374]. Furthermore, concentration of IL-10 and

expression of CD25 in nasal and skin biopsies, and cultures of allergen stimulated PBMCs from atopic donors who have previously received allergen immunotherapy treatment, are elevated compared to atopic control donors who have not received treatment and whose symptoms of atopic disease are therefore worse [176;178;375;376]. These studies (discussed further in Chapter 5) therefore suggest that regulatory T cells may play a role in the suppression of Th2 responses in atopic donors who have received allergen immunotherapy treatment, but do not address whether atopic individuals have a deficiency in the regulatory T cell-mediated suppression of Th2 responses.

4.1.4. Hypothesis

The hypothesis tested in this Chapter was that **the *in vitro* suppression of Th2-type T cell responses to aeroallergens by CD4+CD25+ regulatory T cells from atopic donors is not as effective as that by these cells from non-atopic donors.** The effect of allergen exposure on suppression of *in vitro* allergen driven T cell responses by CD4+CD25+ regulatory T cells was also examined.

4.2. Results

4.2.1. Isolation of CD4+CD25+ T cells from atopic and non-atopic individuals

To investigate whether the regulation of allergen-specific T cell responses is less effective by CD4+CD25+ T cells from atopic than from non-atopic individuals, CD4+CD25+ and CD4+CD25- T cells were isolated from such donors and their ability to suppress allergen driven T cell responses determined using the *in vitro* culture system described in Chapter 3. Non-atopic donors, as described in Chapter 3, had a negative skin prick test and no specific IgE to the allergen used to stimulate the cells *in vitro* (either cat dander or grass pollen allergen extract). These donors also had no history of allergic disease; the clinical details are shown in Table 3.3. Atopic donors were characterised by a positive skin prick test to the allergen used in the *in vitro* culture system and/or the presence of specific IgE to that allergen measured by RAST test, in addition to a history of allergic disease. The clinical details of the atopic donors are shown in Table 4.1. The blood was taken when the pollen count was low so that the grass pollen allergic donors were asymptomatic at the time of the experiments.

There was no significant difference in the purity of the isolated CD4+CD25+ or CD4+CD25- T subsets between the non-atopic and atopic donors. There was also no difference between the numbers of CD4+CD25+ and CD4+CD25- T cells recovered from 150 ml blood (Tables 4.2 and 3.4).

4.2.2. T cell responses by cells from non-atopic and atopic donors following stimulation with allergen extract in vitro

PBMCs from asymptomatic atopic donors were stimulated *in vitro* with allergen extract to which they had specific IgE (either cat dander or grass pollen) or

Donor	Gender	Age	IgE to cat dander IU/ml	IgE to grass pollen IU/ml	Total IgE IU/ml	SPT results (mm)	Allergic disease	Allergen extract used in culture
1	M	38	101	N/a	28	N/a	Y	Cat dander
2	M	35	0.5	N/a	N/a	N/a	Y	Cat dander
3	F	33	N/a	1.4	28	N/a	Y	Grass pollen
4	F	42	N/a	3.2	83	N/a	Y	Grass pollen
5	M	25	18	N/a	161	N/a	Y	Cat dander
6	M	40	7.5	N/a	147	N/a	Y	Cat dander
7	M	36	101	4.8	251	N/a	Y	Grass pollen
8	M	30	101	N/a	2893	N/a	Y	Cat dander
9	F	23	10.4	N/a	401	N/a	Y	Cat dander
10	F	20	9.3	N/a	367	N/a	Y	Cat dander
11	M	39	0.9	N/a	106	N/a	Y	Cat dander
12	M	42	0.7	N/a	90	N/a	Y	Cat dander
13	F	35	N/a	N/a	N/a	8	Y	Grass pollen
14	M	29	N/a	N/a	N/a	7	Y	Cat dander
Summary	9 = M 5 = F	35 (20 – 42)	9.85 (0.5 – 101)	3.2 (1.4 – 4.8)	106 (28 - 2893)	N/a	14 = Y 0 = N	10 = Cat 4 = Grass

Table 4.1. Clinical details of atopic donors.
Gender; F = female, M = male. IgE levels measured by RAST test in IU/ml. Skin prick tests (SPT) to allergen used in culture, diameter measured in mm. Allergic disease; Y = history of atopic disease, N = no history of atopic disease. Summary is median and range where appropriate or summary of values. N/a is unavailable data.

Donor	Amount of blood taken (ml)	Total no. of PBMC ($x10^6$)	Total no. of CD4+ cells ($x10^6$)	Total no. of CD4+ CD25+ cells ($x10^6$)	Total no. of CD4+ CD25- cells ($x10^6$)	% CD4+ CD25+ recovered from the CD4+ subset	Purity of CD4+ cells	Purity of CD4+ CD25+ cells	Purity of CD4+ CD25- cells
1	100	206.8	69.9	2.7	26.4	3.9	N/a	N/a	N/a
2	150	336	103.5	4.4	123.9	4.3	95.9	78.9	69.1
3	150	386	164.8	5.25	203.45	3.2	94.5	65.4	80.2
4	150	237	138	5.5	118.9	3.9	92.8	54.4	73.5
5	150	279	100.8	6.95	96	6.9	96.4	85.2	75.4
6	120	200	49	2.85	28.73	5.8	98.6	73.5	80.0
7	120	349.7	55.5	2.9	60.5	5.2	98.4	63.3	65.9
8	150	290.7	93.6	5.4	47.4	5.8	96.2	78.9	69.1
9	150	377.3	99	3.7	81	3.7	82.5	77.7	70.5
10	150	200	104	3.5	64.9	3.4	95.9	83.3	78.4
11	150	353.1	125.3	4.1	119.5	3.3	99.1	87.3	78.8
12	150	238	52	2.2	134.5	4.2	N/a	N/a	N/a
13	150	387	70	3.3	60.2	4.7	95.3	65.7	86.9
14	150	273.9	60	1.7	51	2.8	93.5	77.8	89.9
Summary	150 (100 – 150)	284.9 (200 – 387)	96.3 (49 – 164.8)	3.6 (1.7 – 6.95)	72.95 (26.4 – 203.45)	4.05 (2.8 – 6.9)	95.9 (82.5 – 99.1)	77.75 (54.4 – 87.3)	76.9 (65.9 – 89.9)

Table 4.2. Recovery and purity of cells isolated from atopic donors.
Total number of cells isolated was calculated by cell count. Purity of cells was calculated using flow cytometry after staining with CD4 and CD25 antibodies and isotype-matched controls, Fig. 3.4. Summary is median and range. N/a is unavailable data.

media alone. There was no significant difference in allergen-specific PBMC proliferation (proliferation to allergen, minus proliferation to medium alone), by PBMCs from these donors when compared to those from non-atopic donors. (Fig. 4.1a). There was also no difference in the concentration of cytokines in the day 6 supernatant (Fig. 4.1 b-e), although there was a surprising trend for a decrease in IL-13 and a corresponding increase in IFN-γ and IL-10 in asymptomatic donors. CD4+CD25+ T cells from both atopic and non-atopic donors were hyporesponsive to allergen stimulation and there was no difference in cytokine production by this population of cells between the two groups (Fig. 4.1 a-e). There was however, a trend towards an increase in IL-10 production by CD4+CD25+ from atopic donors (Fig. 4.1. e). CD4+CD25- cells from atopic donors proliferated significantly less than those from non-atopic donors (Fig. 4.1a) and produced significantly more IL-10 (Fig. 4.1 e). There was no significant difference in the concentration of IL-5, IL-13 or IFN-γ by allergen stimulated CD4+CD25- T cells between groups.

4.2.3. The in vitro suppression of allergen driven T cell responses by CD4+CD25+ T cells from atopic and non-atopic donors

In the previous chapter it was shown that CD4+CD25+ regulatory T cells from non-atopic donors could suppress allergen driven T cell responses *in vitro*. Using the same *in vitro* culture system, CD4+CD25- T cells from atopic donors were stimulated with an allergen extract to which they had specific IgE (either cat dander or grass pollen) in the presence of CD4+CD25+ T cells (or CD4+CD25- T cells as a cell number control). CD4+CD25+ T cells from both groups of donors were able to suppress allergen-specific proliferation of the CD4+CD25- subset (Fig. 4.2a). To compare the suppressive ability of the CD4+CD25+ T cells between groups, the

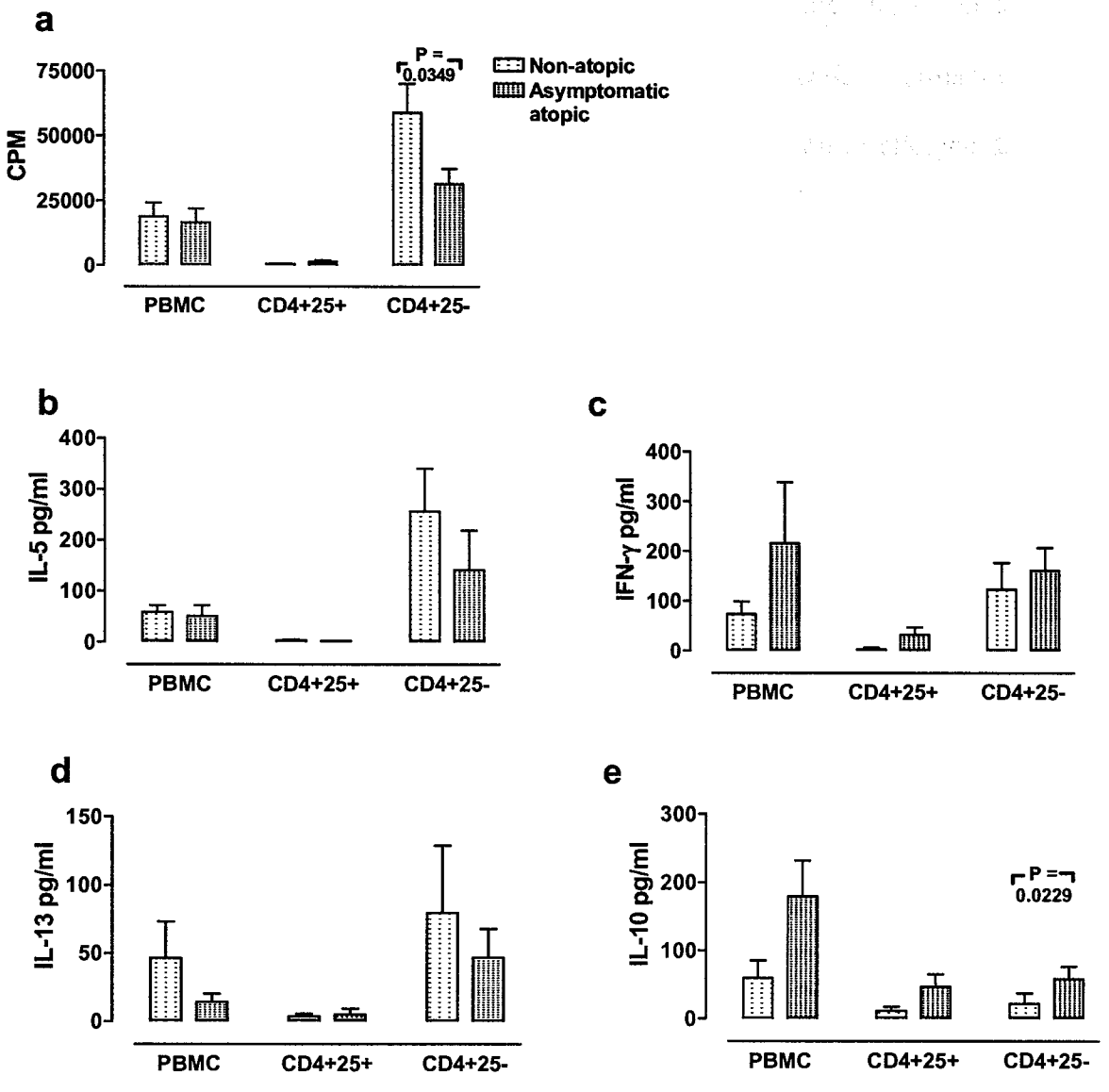


Figure 4.1. Proliferation and cytokine production by human T cell subsets from non-atopic and asymptomatic atopic donors, stimulated *in vitro* with allergen. Allergen specific proliferation was measured by thymidine incorporation on day 7 (a) and IL-5 (b), IFN-γ (c), IL-13 (d) and IL-10 (e) production measured by Luminex of day 6 supernatants, of PBMC, CD4+CD25+ and CD4+CD25- cells from non-atopic donors (n = 9) and asymptomatic atopic donors (n = 14). Cells were stimulated for 7 days *in vitro* at 2 x 10^6 c/ml with allergen extract (either cat dander or grass pollen) at 100 μg/ml or media alone. All wells contained autologous irradiated PBMC at 2 x 10^6 c/ml. Median value + 95% CI is shown.

percent suppression of proliferation was determined, as described in section 3.2.4. Briefly, the CD25+/- allergen-specific CPM was subtracted from the CD25-/- allergen-specific CPM and expressed as a percentage of the allergen-specific proliferation of cells in the CD25-/- culture. By using this calculation, suppression of allergen driven proliferation by CD4+CD25+ regulatory T cells from atopic donors was shown to be less effective than that by cells from non-atopic donors (Fig. 4.2 b).

CD4+CD25+ T cells from atopic donors, like those from non-atopic donors, were able to suppress IL-5 and IFN-γ but not IL-13 and IL-10 production by allergen stimulated CD4+CD25- T cells (Fig. 4.3 a-d). There was no difference between the two groups in the percent suppression of IL-5, IL-13 or IL-10 (Fig. 4.4 a, c, d), but CD4+CD25+ T cells from non-atopic donors were able to suppress IFN-γ production more effectively than those from atopic donors (Fig. 4.4b).

4.2.4. Responses to grass pollen allergen extract stimulation by T cells from grass pollen allergic donors during the UK grass pollen season.

To investigate the effect of exposure to allergen on suppression of allergen driven T cell responses by CD4+CD25+ T cells from atopic donors, blood was taken from grass allergic donors between May and August, during the grass pollen season when they were suffering with symptoms of hayfever (Fig. 4.5). The atopy of these donors was characterised by positive skin prick test and/or specific IgE to grass pollen. There was no significant difference in the concentration of allergen-specific IgE between these donors and the asymptomatic atopic donors (Tables 4.3 and 4.1 respectively).

The purity of the CD4+CD25+ and CD4+CD25- subsets isolated from donors with hayfever was not significantly different to those from non-atopic and

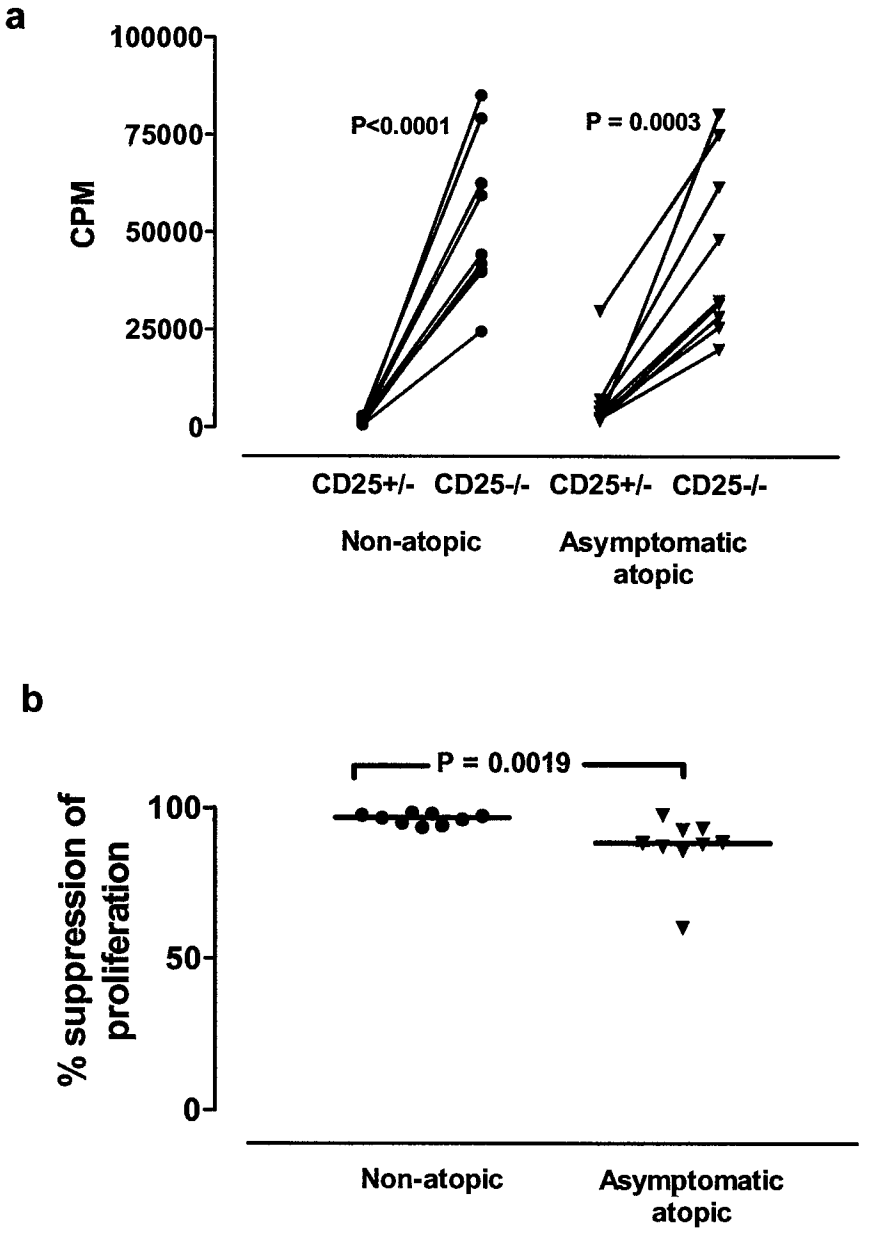


Figure 4.2. Suppression of allergen induced T cell proliferation by CD4+CD25+ T cells from non-atopic and asymptomatic atopic donors. CD4+CD25- T cells (marked 25-/-) or CD4+CD25- T cells in the presence of CD4+CD25+ T cells at a ratio of one 25+ to two 25- cells (marked 25+/-), from non-atopic donors (n = 9) and asymptomatic atopic donors (n = 9) were cultured at 3 x 10^6 c/ml in the presence of 100 μg/ml allergen extract (cat or grass), or media alone. Allergen specific proliferation was measured by tritiated thymidine incorporation on day 7. All wells contained irradiated PBMC at 2 x 10^6 c/ml, (a). Individual % suppression values for suppression of allergen specific CD4+CD25- proliferation by CD4+CD25+ cells are shown (b), a bar represents the median value.

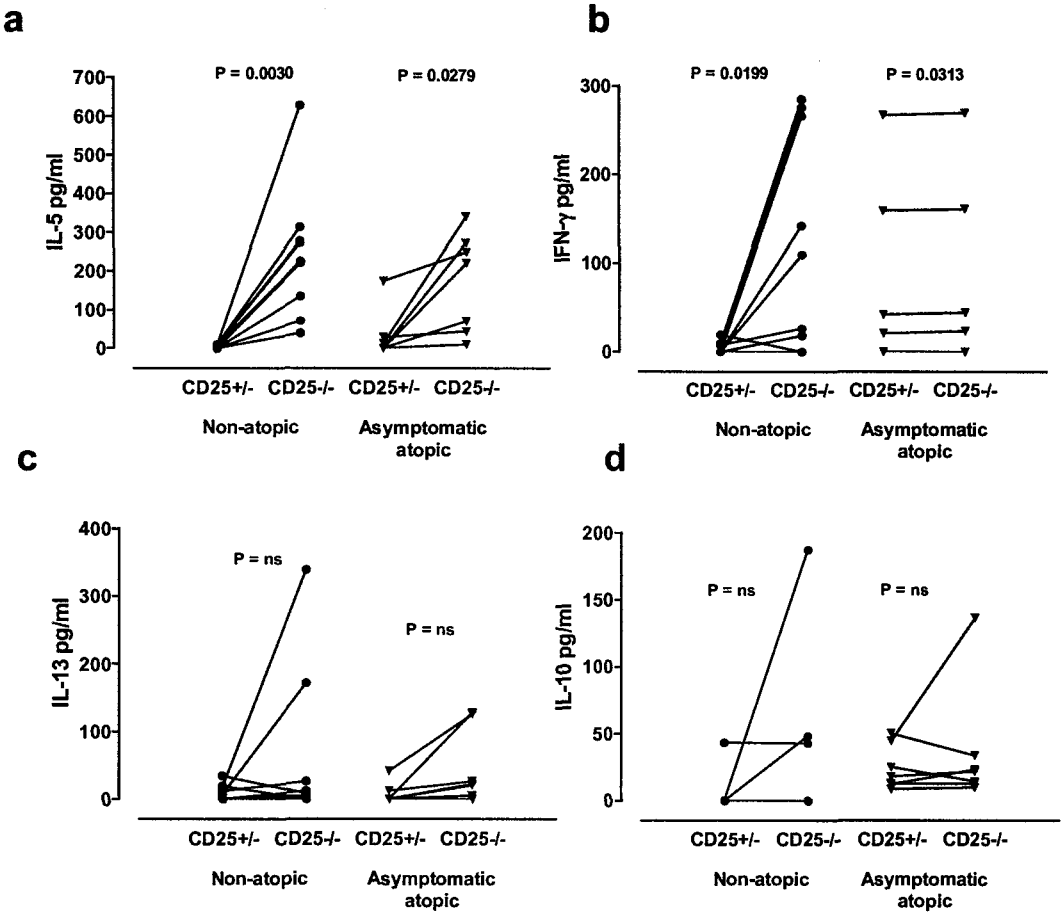


Figure 4.3. Suppression of allergen induced T cell cytokine production by CD4+CD25+ T cells from non-atopic and asymptomatic atopic donors. CD4+CD25- T cells (marked 25-/-) or CD4+CD25- T cells in the presence of CD4+CD25+ T cells at a ratio of one 25+ to two 25- cells (marked 25+/-), from non-atopic donors (n = 9) and asymptomatic atopic donors (n = 7) were cultured at 3 x 10^6 c/ml in the presence of 100 μg/ml allergen extract (cat or grass), or media alone. Allergen specific IL-5 (a), IFN-γ (b), IL-13 (c) and IL-10 (d) production was measured by Luminex of day 6 culture supernatants. All wells contained irradiated PBMC at 2 x 10^6 c/ml.

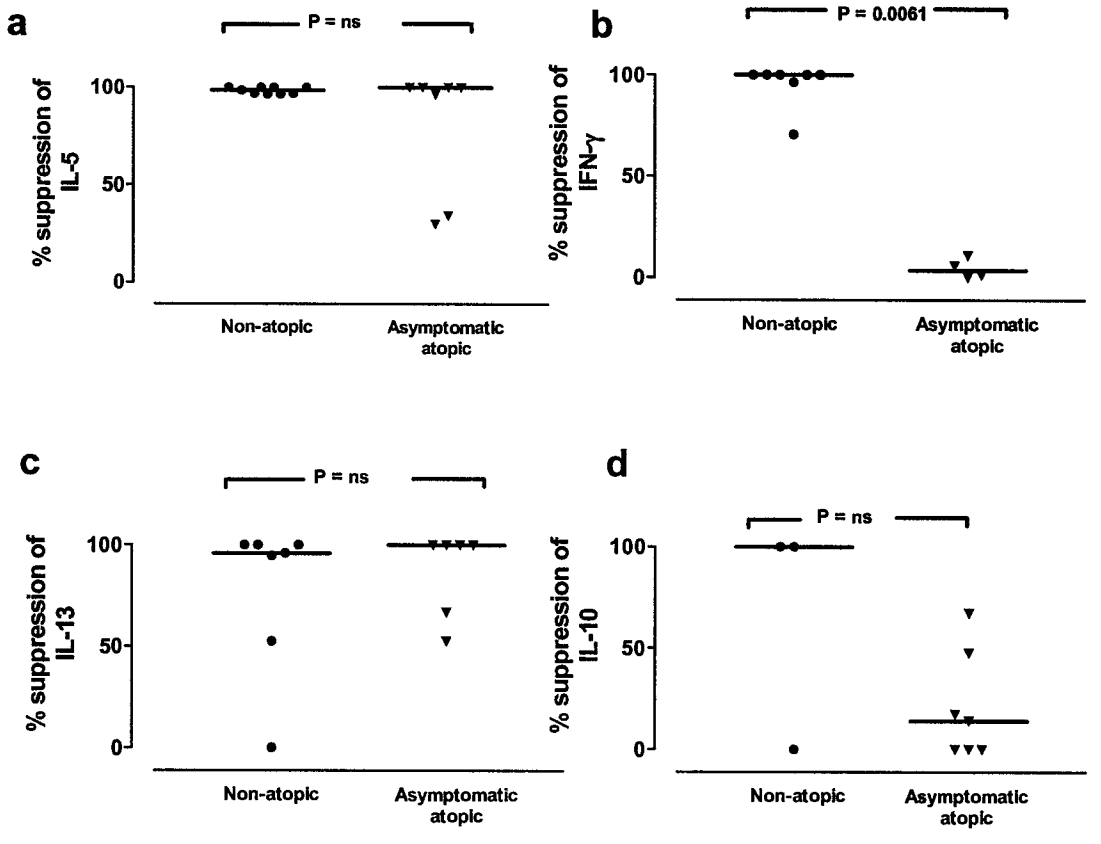


Figure 4.4. Suppression of allergen induced T cell cytokine production by CD4+CD25+ T cells from non-atopic and asymptomatic atopic donors. CD4+CD25- T cells (marked 25-/-) or CD4+CD25- T cells in the presence of CD4+CD25+ T cells at a ratio of one 25+ to two 25- cells (marked 25+/-), from non-atopic donors (n = 9) and asymptomatic atopic donors (n = 7) were cultured at 3 x 10^6 c/ml in the presence of 100 μg/ml allergen extract (cat or grass), or media alone. Allergen specific cytokine production was measured by Luminex of day 6 culture supernatants. All wells contained irradiated PBMC at 2 x 10^6 c/ml. Individual % suppression values for suppression of allergen specific CD4+CD25- IL-5 (a), IFN-γ (b), IL-13 (c) and IL-10 (d) production by CD4+CD25+ cells are shown, a bar represents the median value.

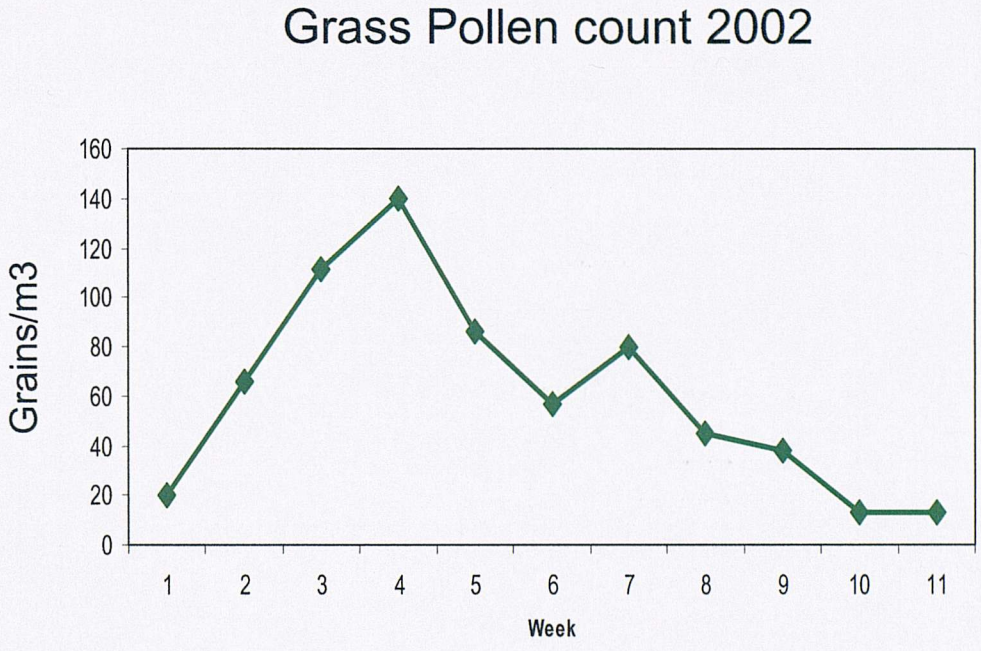


Figure 4.5. The UK grass pollen count 2002. The level of grass pollen grains in the atmosphere was measured in grains/m^3 during the weeks of summer 2002 starting from the 3rd week of May. Data kindly provided by Professor Stephen Durham.

Donor	Gender	Age	IgE to grass pollen IU/ml	Total IgE IU/ml	SPT results	Allergic disease
1	F	28	5.980	389.0	N/a	Y
2	M	24	101	319.0	N/a	Y
3	F	20	101	465.0	N/a	Y
4	M	22	101	1053.0	N/a	Y
5	F	25	0.780	208.0	N/a	Y
6	F	22	12.300	26.0	N/a	Y
7	M	43	38.9	175.0	N/a	Y
8	M	58	8.500	22.0	N/a	Y
9	F	34	N/a	N/a	N/a	Y
10	M	30	101	2893.0	N/a	Y
11	M	39	43.8	379.0	N/a	Y
Summary	6 = M 5 = F	28 (20 – 58)	41.35 (0.78 – 101)	349 (22 – 2893)	N/a	11 = Y 0 = N

Table 4.3. Clinical details of donors with hayfever.
Gender; F = female, M = male. IgE levels measured by RAST test in IU/ml. Skin prick tests (SPT) to grass; diameter of wheal measured in mm. Allergic disease; Y = history of atopic disease, N = no history of atopic disease. Summary is median and range or summary of values. N/a is unavailable data.

asymptomatic atopic donors. There was also no difference between the numbers of CD4+CD25+ and CD4+CD25- T cells recovered from 150 ml blood between groups (Tables 4.4, 3.3 and 4.2).

Grass pollen allergen extract stimulated PBMCs from donors with hayfever proliferated to the same extent as allergen stimulated PBMCs from asymptomatic and non-atopic donors (Fig. 4.6a). They produced significantly more IL-13 than those from the other two groups and there was a trend for higher IL-5 production (Fig. 4.6 d and b respectively). There was no significant difference in the production of IFN-γ by allergen stimulated PBMCs between the three groups (Fig. 4.6c) and IL-10 production was reduced compared to the asymptomatic atopic group, but comparable to that produced by cells from non-atopic donors. (Fig. 4.6e).

There was no significant difference in the allergen-specific proliferation of CD4+CD25+ T cells between donors with hayfever, asymptomatic atopic and non-atopic donors (Fig. 4.6a). The proliferation of CD4+CD25- T cells from donors with hayfever was comparable to that by CD4+CD25- T from non-atopic donors, but significantly less to that by the same subset of cells from asymptomatic atopic donors (Fig. 4.6a). There was a significant increase in the concentration of the Th2 cytokines IL-5 and IL-13 in the culture supernatant of allergen stimulated CD4+CD25+ and CD4+CD25- T cells from donors with hayfever (Fig. 4.6b and d) and a decrease in IL-10 concentration compared to that produced by cells from asymptomatic atopic donors (Fig. 4.6e). The concentration of IFN-γ produced by both allergen stimulated CD4+CD25+ and CD4+CD25- T cell subsets was not significantly different between groups (Fig. 4.6c).

Donor	Amount of blood taken (ml)	Total no. of PBMC ($x10^6$)	Total no. of CD4+ cells ($x10^6$)	Total no. of CD4+ CD25+ cells ($x10^6$)	Total no. of CD4+ CD25- cells ($x10^6$)	% CD4+ CD25+ recovered from the CD4+ subset	Purity of CD4+ cells	Purity of CD4+CD25+ cells	Purity of CD4+CD25- cells
1	150	704	225	3.5	153.9	1.6	95.9	82.4	74.9
2	150	287.1	113.4	2.05	92.5	1.8	97.6	72.6	78.5
3	150	267.3	67.2	1.9	78	2.8	96.6	72.2	76.9
4	150	572	141.6	6.1	100	4.3	87.5	81.2	67.3
5	150	296	93.6	4.15	93.6	4.4	89.4	69.4	82.1
6	150	313.5	121.6	4.65	131.5	3.8	97.8	89.1	93.4
7	150	297	110.6	2.25	66	2.0	94.3	85.3	69.5
8	150	341	85.4	3.6	130	4.2	97.8	74.2	72.7
9	150	517.5	198	7.5	148.5	3.8	96.5	82.2	91.9
10	150	591.25	186.75	4.8	103.5	2.6	85.9	88.6	77.7
11	150	253	142.4	2	132.6	1.4	98.2	67.6	87.8
Summary	150 (150 – 150)	313.5 (253 – 704)	121.6 (67.2 – 225)	3.6 (1.9 – 7.5)	103.5 (66 – 153.9)	2.8 (1.4 – 4.4)	96.5 (85.9 – 98.2)	81.2 (67.6 – 89.1)	77.7 (67.3 – 93.4)

Table 4.4. Recovery and purity of cells isolated from the peripheral blood of donors with hayfever.
Total number of cells isolated was calculated by cell count. Purity of cells was calculated using flow cytometry after staining with fluorescent CD4 and CD25 antibodies and isotype-matched controls, see Fig. 3.4. Summary is median and range.

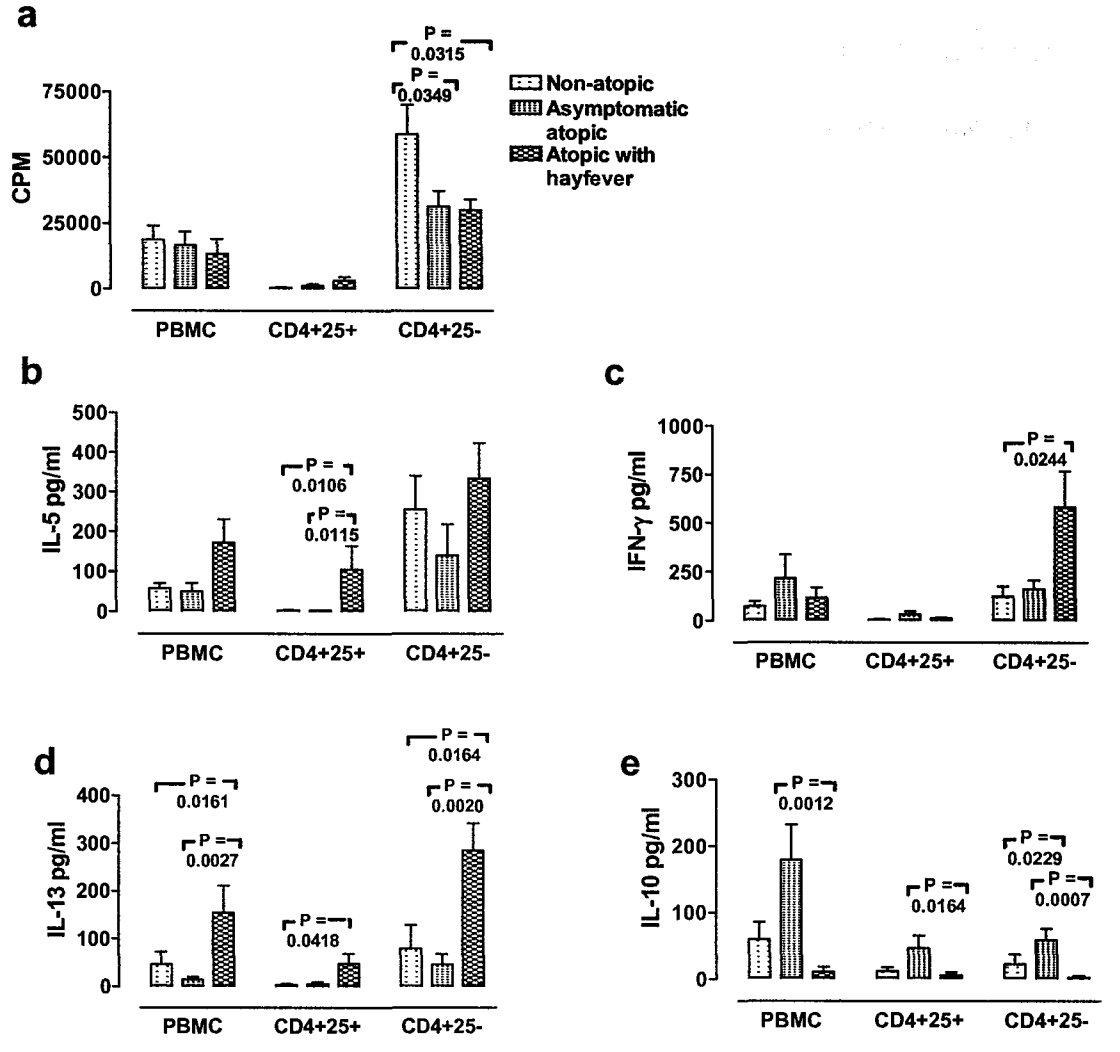


Figure 4.6. Proliferation and cytokine production by human T cell subsets from non-atopic, and asymptomatic and symptomatic atopic donors, stimulated *in vitro* with allergen. Allergen specific proliferation measured by thymidine incorporation on day 7 (a) and IL-5 (b), IFN-γ (c), IL-13 (d) and IL-10 (e) production measured by Luminex of day 6 supernatants, of PBMC, CD4+CD25+ and CD4+CD25- cells from non-atopic donors (n = 9) and asymptomatic atopic donors (n = 14) and grass allergic donors with hayfever (n = 9). Cells were stimulated for 7 days *in vitro* at 2 x 10^6 c/ml with allergen extract (either cat dander or grass pollen) at 100 μg/ml or media alone. All wells contained autologous irradiated PBMC at 2 x 10^6 c/ml. Median value + 95% CI is shown.

4.2.5. The effect of exposure to allergen on suppression of in vitro allergen driven T cell responses by CD4+CD25+ T cells from atopic and non-atopic donors

CD4+CD25+ T cells isolated from grass allergic donors with symptoms of hayfever, during the grass pollen season, were able to suppress grass pollen specific proliferation of CD4+CD25- T cells (Fig. 4.7a). The magnitude of this suppression, as expressed by percent suppression however was significantly less than when compared to suppression of allergen driven CD4+CD25- T cell proliferation by CD4+CD25+ T cells from both non-atopic and asympyomatic atopic donors (Fig. 4.7b).

In contrast to CD4+CD25+ T cells from non-atopic and asymptomatic atopic donors, those isolated from donors with hayfever were unable to suppress allergen-specific IL-5 production by CD4+CD25- cells (Fig. 4.8a and Fig. 4.9a), although in contrast to asymptomatic donors, they could suppress IFN-γ production (Fig. 4.8b and Fig. 4.9b). As was seen for non-atopic and asymptomatic atopic donors, the presence of CD4+CD25+ T cells from symptomatic atopics had no effect on the concentration of IL-13 or IL-10 in the culture supernatant, by (Fig. 4.8 c and d and Fig. 4.9 c and d).

There was no correlation between the suppression of allergen-specific CD4+CD25- T cell proliferation or cytokine production by CD4+CD25+ T cells and the concentration of allergen-specific IgE or the diameter of the skin prick test wheal (data not shown).

4.2.6. The cell surface phenotype of CD4+CD25+ and CD4+CD25- T cells from non-atopic donors, asymptomatic atopic donors and atopic donors with hayfever

It is possible that during exposure to allergen, CD4+CD25+ and/or CD4+CD25- T cells from symptomatic hayfever donors may be more activated than those from non-atopic and asymptomatic atopic donors. If this is the case, the

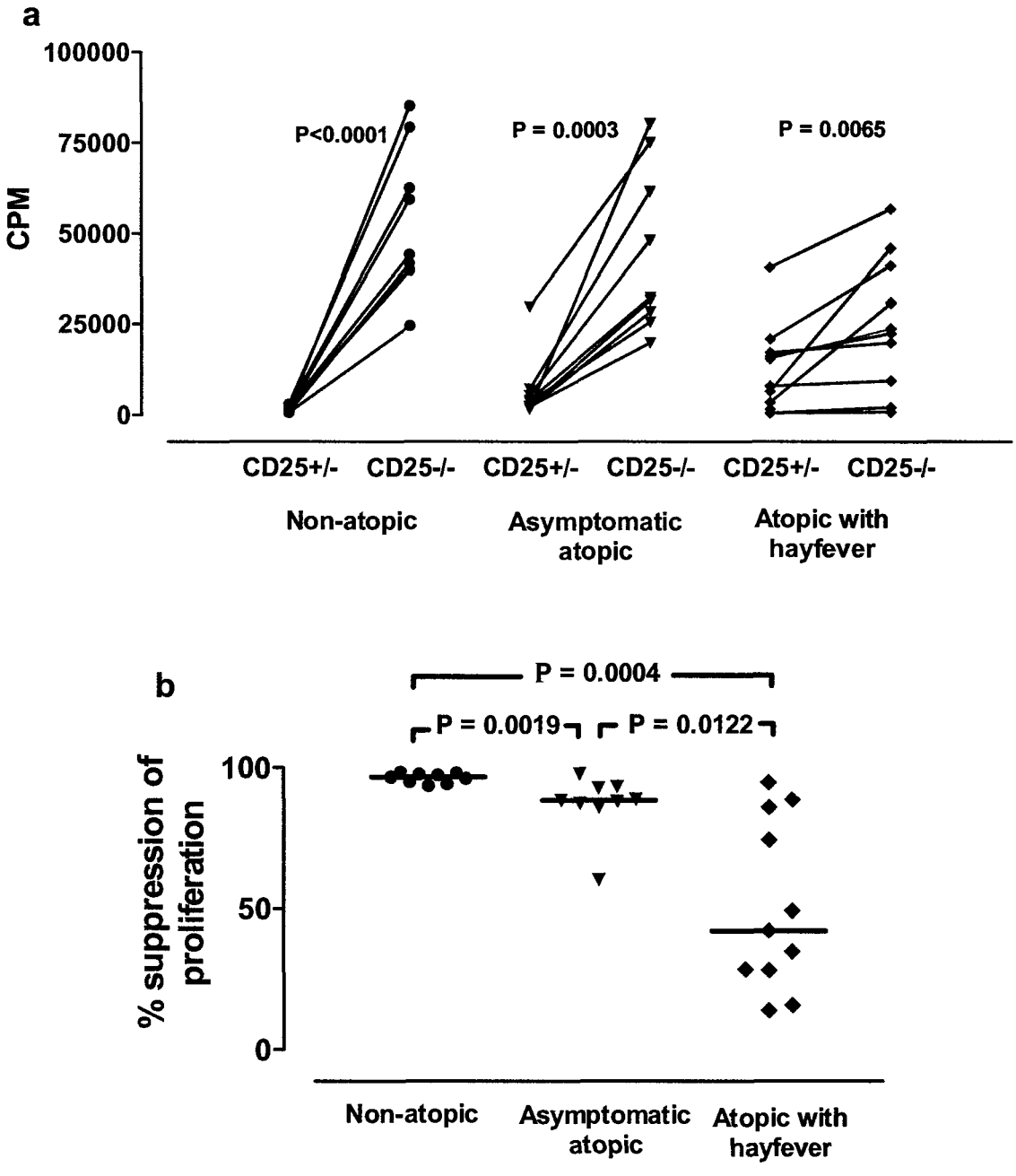


Figure 4.7. Suppression of allergen induced T cell proliferation by CD4+CD25+ T cells from non-atopic, and asymptomatic and symptomatic atopic donors. CD4+CD25- T cells (marked 25-/-) or CD4+CD25- T cells in the presence of CD4+CD25+ T cells at a ratio of one 25+ to two 25- cells (marked 25+/-), from non-atopic donors (n = 9), asymptomatic atopic donors (n = 9) and atopic donors with hayfever (n = 11) were cultured at 3 x 10^6 c/ml in the presence of 100 μg/ml allergen extract (cat or grass), or media alone. Allergen specific proliferation was measured by thymidine incorporation at day 7. All wells contained irradiated PBMC at 2 x 10^6 c/ml (a). Individual % suppression values for suppression of allergen specific CD4+CD25- proliferation by CD4+CD25+ cells are shown (b), a bar represents the median value.

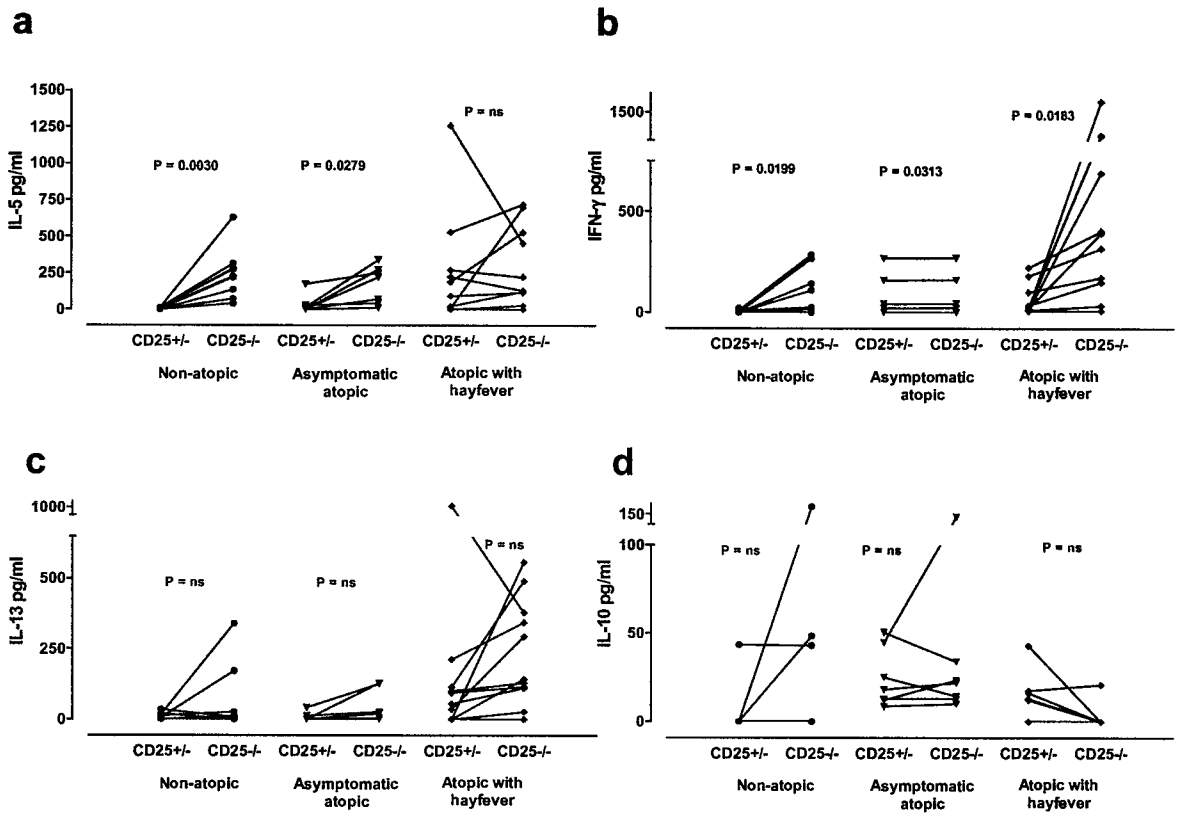


Figure 4.8. Suppression of allergen induced T cell cytokine production by CD4+CD25+ T cells from non-atopic, and asymptomatic and asymptomatic atopic donors. CD4+CD25- T cells (marked 25-/-) or CD4+CD25- T cells in the presence of CD4+CD25+ T cells at a ratio of one 25+ to two 25- cells (marked 25+/-) from non-atopic donors (n = 9), asymptomatic atopic donors (n = 7) and atopic donors with hayfever (n = 11) were cultured at 3 x 10^6 c/ml in the presence of 100 μg/ml allergen extract (cat or grass), or media alone. Allergen specific IL-5 (a), IFN-γ (b), IL-13 (c) and IL-10 (d) production was measured by Luminex of day 6 culture supernatants. All wells contained irradiated PBMC at 2 x 10^6 c/ml.

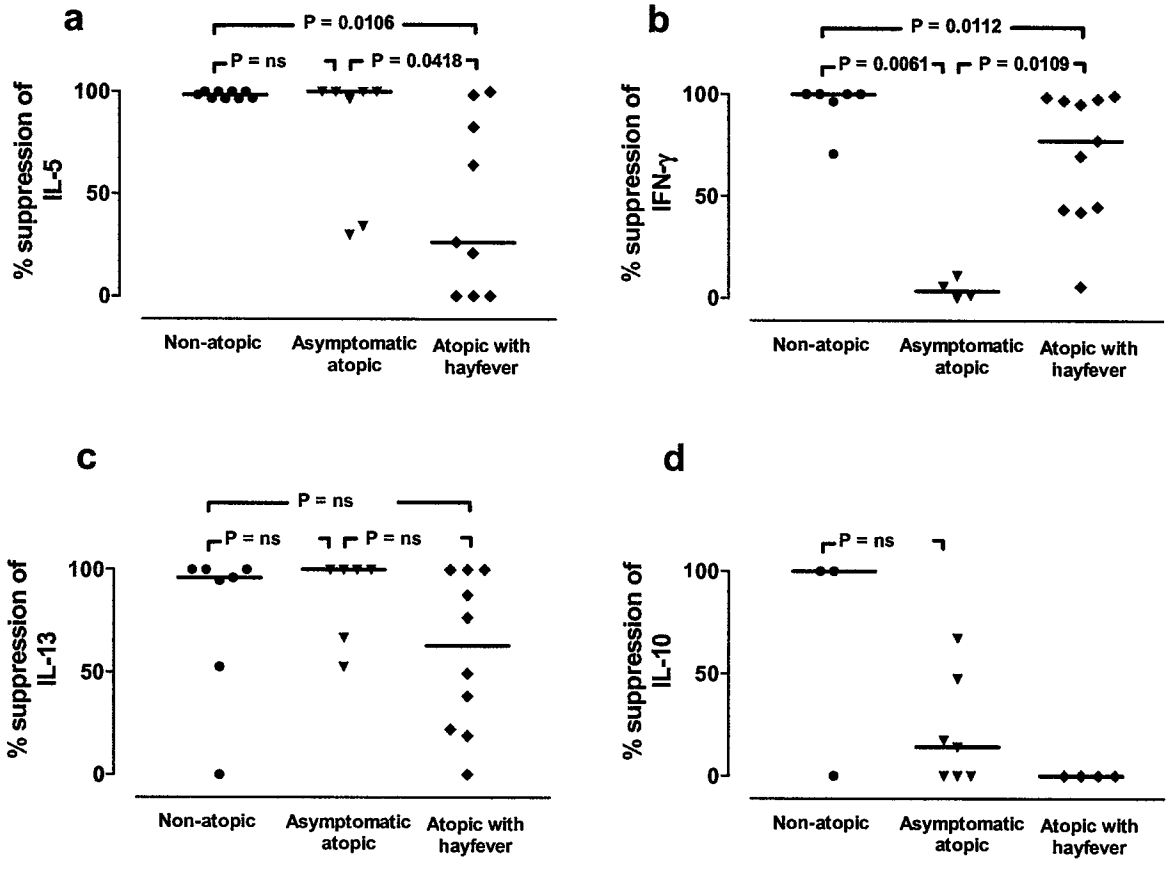


Figure 4.9. Suppression of allergen induced T cell cytokine production by CD4+CD25+ T cells from non-atopic, and asymptomatic and symptomatic atopic donors. CD4+CD25- T cells (marked 25-/-) or CD4+CD25- T cells in the presence of CD4+CD25+ T cells at a ratio of one 25+ to two 25- cells (marked 25+/-), from non-atopic donors (n = 9), asymptomatic atopic donors (n = 7) and atopic donors with hayfever (n = 11) were cultured at 3 x 10^6 c/ml in the presence of 100 μg/ml allergen extract (cat or grass), or media alone. Allergen specific IL-5 (a), IFN-γ (b), IL-13 (c) and IL-10 (d) production was measured by Luminex of day 6 culture supernatants. All wells contained irradiated PBMC at 2 x 10^6 c/ml. Individual % suppression values for suppression of allergen specific CD4+CD25- cytokine production by CD4+CD25+ cells are shown, a bar represents the median value.

CD4+CD25+ population may not contain as many regulatory cells, and highly activated CD4+CD25- cells may be harder to suppress. To investigate whether the CD4+CD25+ and CD4+CD25- T cells isolated from grass pollen allergic donors suffering from hayfever were a phenotypically different population than those isolated from non-atopic and asymptomatic atopic donors, the expression of the markers of activation CD25, CD69 and CTLA-4 were analysed by flow cytometry (Table 4.5). There was no difference in the percentage of PBMCs or CD4+ T cells that were CD25+ between the three groups, and no difference in the mean fluorescence intensity of CD25 was observed. The percentage of CD4+CD25+ and CD4+CD25- cells that expressed CD69 was also comparable between groups. Finally, intracellular staining revealed that although a higher percentage of CD4+CD25+ cells expressed CTLA-4 than did CD4+CD25- cells, there was no difference in CTLA-4 expression by either CD4+CD25- or CD4+CD25+ cells between the three groups (Table 4.5).

4.2.7. Paired data investigating the effect of seasonal exposure to grass pollen on suppression of grass pollen allergen stimulated T cell responses by CD4+CD25+ T cells from grass allergic donors

To further investigate the effect of allergen exposure on suppression of grass pollen specific T cell responses by CD4+CD25+ T cells, peripheral blood was taken from grass pollen allergic donors out of the grass pollen season (between October and March) and then from the same donors during the grass pollen season (between May and August).

When stimulated *in vitro* with grass pollen allergen extract, there was no difference between the proliferation of PBMCs, CD4+CD25+ or CD4+CD25- T cells isolated from grass pollen allergic donors during and out of the grass pollen season

	Non-atopic (n=6)	Asymptomatic atopic (n=6)	Hayfever (n=6)
MFI CD25	37.5 (11.3 - 106)	14.5 (13.4 - 18.6)	19.0 (7.9 - 25.0)
CD4+CD25+ (%)	9.8 (4.2 - 19.8)	4.7 (2.1 - 8.9)	11.3 (5.5 - 24.1)
CD4+CD25+ CD69+ (%)	2.4 (0.5 - 3.3)	5.6 (0.1 - 6.8)	2.3 (0.9 - 3.7)
CD4+CD25- CD69+ (%)	1.5 (0.1 - 1.76)	0.5 (0.0 - 1.48)	0.8 (0.0 - 5.0)
CD4+CD25+ CTLA-4+ (%)	13.4 (7.7 - 63.3)	22 (21.0 - 37.3)	21.2 (17.8- 46.3)

Table 4.5. The phenotype of CD4+CD25+ and CD4+CD25- T cells from non-atopic, and asymptomatic and symptomatic atopic donors.
Data are median (range). PBMCs were isolated from non-atopic donors (n = 6), asymptomatic atopic donors (n = 5) and atopic donors with hayfever (n = 5). Expression of cell surface CD4, CD25 and CD69 and intracellular expression of CTLA-4 were analysed by flow cytometry. Data are mean fluorescence intensity (MFI) of CD25 staining, percentage of CD4+CD25+ T cells within the PBMC and percentage positive cells relative to the isotype control antibody for expression of CTLA-4 by CD4+CD25+ and CD69 by CD4+CD25+ and CD4+CD25- T cells.

(Fig. 4.10a). Suppression of grass pollen specific T cell responses was analysed using the co-culture system previously described. CD4+CD25+ cells from grass pollen allergic donors could suppress grass pollen stimulated CD4+CD25- T cell suppression both during and out of the grass pollen season (Fig. 4.10b). Interestingly however, percent suppression of proliferation by CD4+CD25+ cells was higher by cells isolated out of the grass pollen season than those from the same donors during the pollen season (Fig. 4.11a). There was no effect on seasonal exposure to grass pollen on suppression of grass pollen stimulated T cell proliferation by CD4+CD25+ T cells from non-atopic donors (Fig. 4.11b).

When the cell culture supernatants of PBMC, CD4+CD25+ or CD4+CD25- T cells isolated from grass allergic donors and stimulated *in vitro* with grass pollen allergen extract were analysed using Luminex, no difference in the concentration of IL-5 was detected between cells isolated in and out of the pollen season (Fig. 4.12a). The addition of CD4+CD25+ T cells isolated both in and out of the grass pollen season did not reduce allergen-specific IL-5 production by CD4+CD25- T cells significantly (Fig. 4.12b). When comparing percent suppression of IL-5 in these cultures however, CD4+CD25+ cells isolated out of the grass pollen season were more effective at suppression of this cytokine than those isolated from the same donors during the grass pollen season (4.13a). There was no effect on seasonal exposure to grass pollen on suppression of grass pollen stimulated CD4+CD25- T cell IL-5 production by CD4+CD25+ T cells from non-atopic donors (Fig. 4.13b).

To investigate whether the effect of allergen exposure on the ability of CD4+CD25+ T cells from grass pollen allergic donors to suppress grass pollen induced T cell responses was due to differences in the CD4+CD25+ or CD4+CD25- T cell subset, the assay was carried out using the same donors both during and out of the

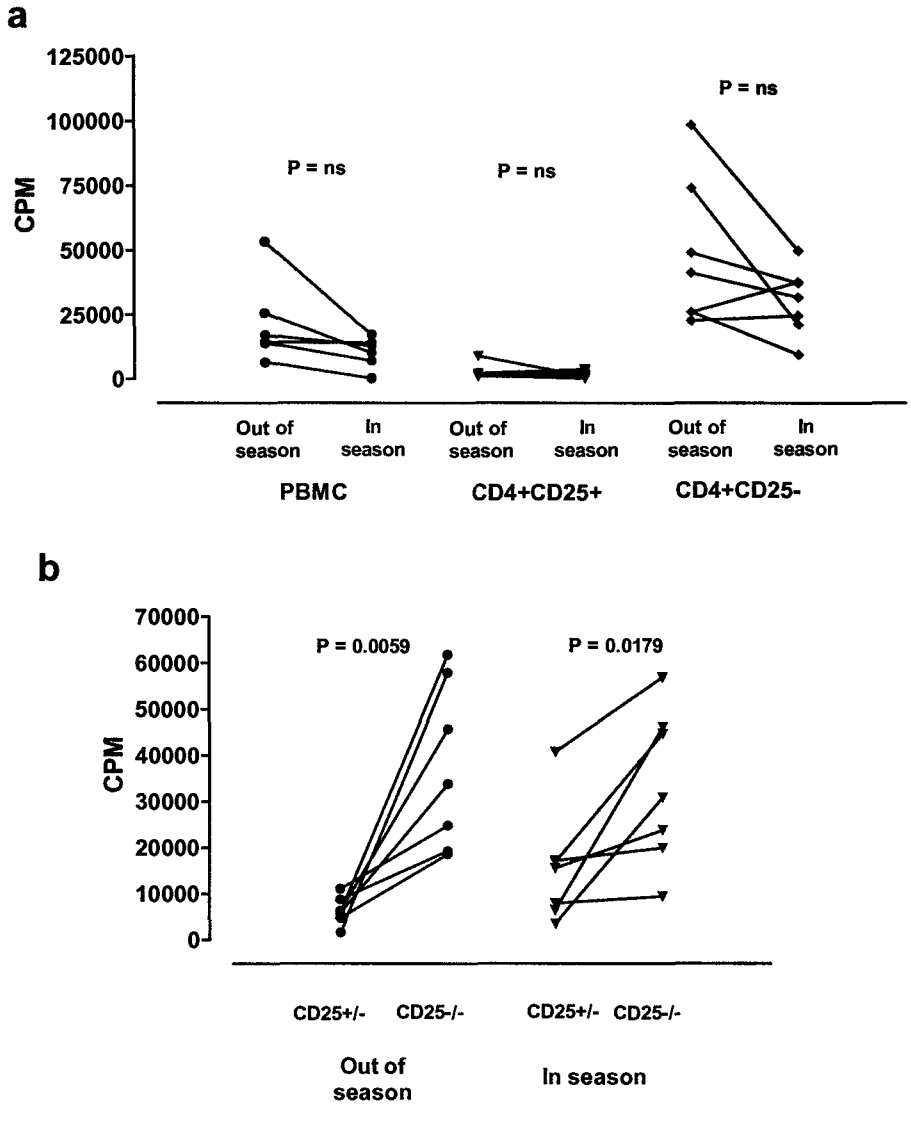


Figure 4.10. Proliferation by human T cell subsets from grass allergic donors in and out of the grass pollen season. Allergen specific proliferation measured by thymidine incorporation on day 7 of PBMC, CD4+CD25+ and CD4+CD25- cells at 2 x 10^6 c/ml (a). CD4+CD25- T cells (marked 25-/-) or CD4+CD25- T cells in the presence of CD4+CD25+ T cells at a ratio of one 25+ to two 25- cells (marked 25+/-), were cultured at 3 x 10^6 c/ml (b). Cells were isolated from grass allergic donors (n = 7) in and out of the grass pollen season and stimulated with grass pollen allergen extract at 100 μg/ml or media alone. All wells contained autologous irradiated PBMC at 2 x 10^6 c/ml.

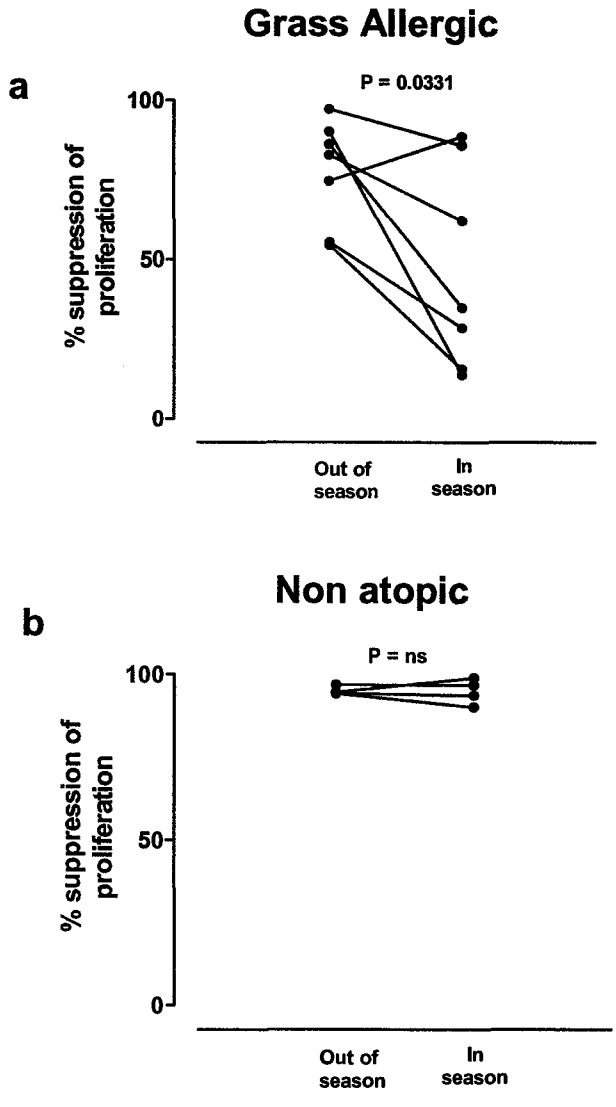


Figure 4.11. Suppression of grass pollen induced T cell proliferation by CD4+CD25+ T cells from grass pollen allergic and non atopic donors in and out of the grass pollen season. CD4+CD25- T cells or CD4+CD25- T cells in the presence of CD4+CD25+ T cells at a ratio of one 25+ to two 25- cells were cultured at 3 x 10^6 c/ml. Cells were isolated from grass allergic (n = 7) (a) and non-atopic (n = 4) (b) donors in and out of the grass pollen season Cells were stimulated with grass pollen allergen extract at 100 μg/ml or media alone and proliferation measured by incorporation of tritiated thymidine on day 7. All wells contained autologous irradiated PBMC at 2 x 10^6 c/ml. Individual % suppression values for suppression of allergen specific CD4+CD25- proliferation by CD4+CD25+ cells is shown.

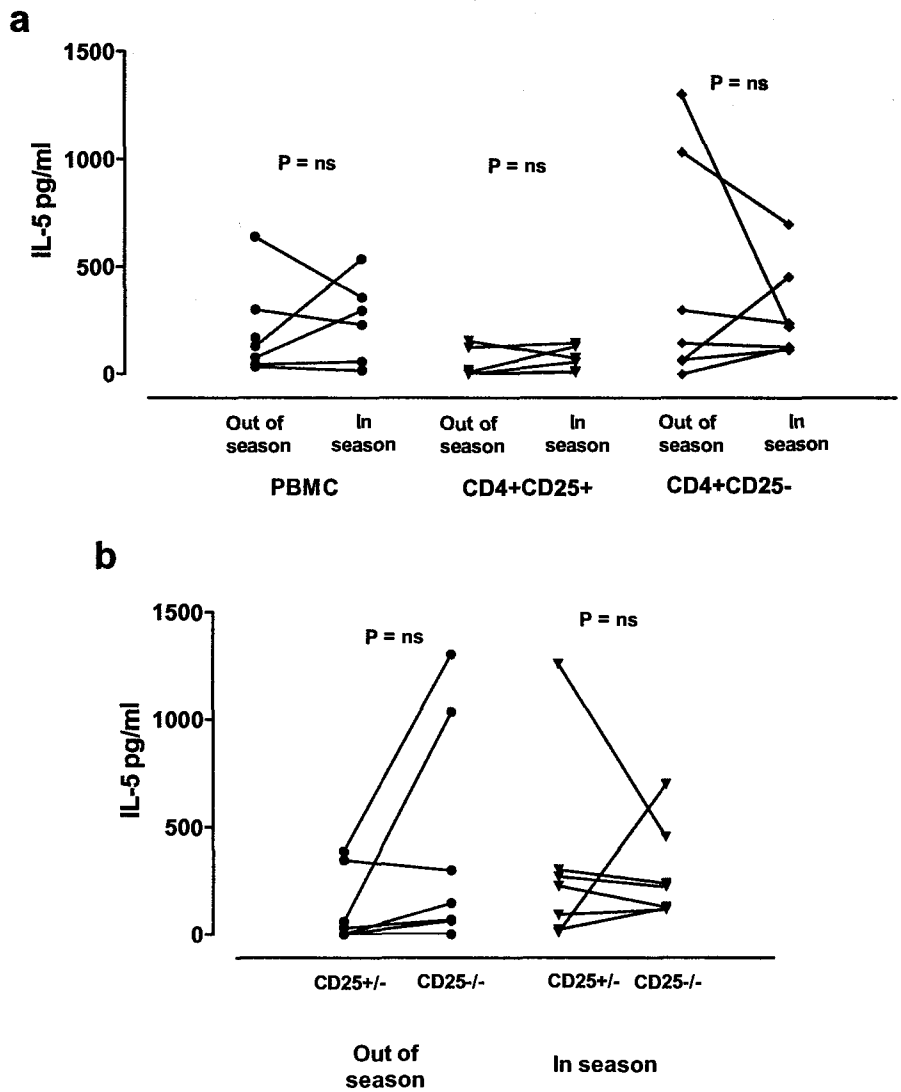


Figure 4.12. IL-5 production by human T cell subsets from grass allergic donors in and out of the grass pollen season. IL-5 production measured by Luminex of the day 6 culture supernatants from PBMC, CD4+CD25+ and CD4+CD25- cells at 2 x 10^6 c/ml (a). CD4+CD25- T cells (marked 25-/-) or CD4+CD25- T cells in the presence of CD4+CD25+ T cells at a ratio of one 25+ to two 25- cells (marked 25+/-), cultured at 3 x 10^6 c/ml (b). Cells were isolated from grass allergic donors (n = 7) in and out of the grass pollen season Cells were stimulated with grass pollen allergen extract at 100 μg/ml or media alone. All wells contained autologous irradiated PBMC at 2 x 10^6 c/ml.

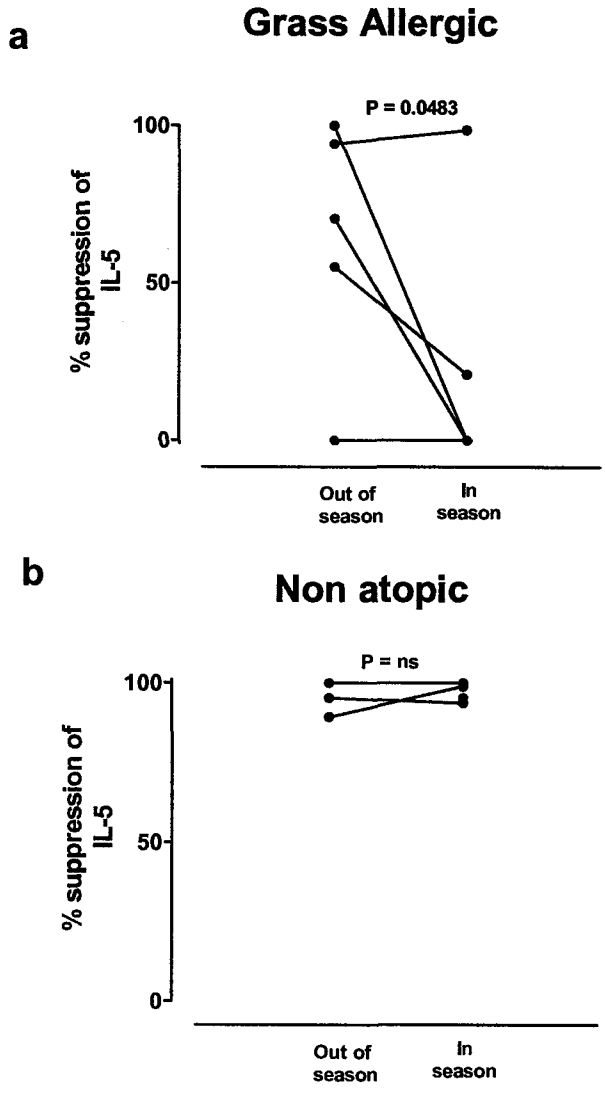


Figure 4.13. Suppression of grass pollen induced T cell IL-5 production by CD4+CD25+ T cells from grass pollen allergic and non atopic donors in and out of the grass pollen season. CD4+CD25- T cells or CD4+CD25- T cells in the presence of CD4+CD25+ T cells at a ratio of one 25+ to two 25- cells were cultured at 3 x 10^6 c/ml. Cells were isolated from grass allergic (n = 7) (a) and non-atopic (n = 4) (b) donors in and out of the grass pollen season and stimulated with grass pollen allergen extract at 100 μg/ml or media alone and IL-5 production was measured by Luminex of the day 6 culture supernants. All wells contained autologous irradiated PBMC at 2 x 10^6 c/ml. Individual % suppression values for suppression of allergen specific CD4+CD25- proliferation by CD4+CD25+ cells is shown.

grass pollen season. When the assay was performed out of the pollen season, aliquots of CD4+CD25- T cells were stored at -70°C. When the subsequent "in season" experiments were carried out, both suppression of in season and out of season CD4+CD25- T cells by in season CD4+CD25+ T cells could be examined.

The level of proliferation by CD4+CD25- cells isolated in season, out of season and by those previously stored out of season was comparable in two out of the three donors tested, indicating that freeze/thawing of CD4+CD25- T cells did not effect their proliferation to allergen *in vitro*. As previously described, CD4+CD25+ cells from all three donors were more effective at suppressing proliferation when isolated out of the pollen season. When analysing the suppression of CD4+CD25- cells not exposed to allergen, CD4+CD25+ cells from Donor A were more effective at suppressing grass pollen induced proliferation when isolated out of the grass pollen season than during it (Fig. 4.14a). There was no difference in the suppression of CD4+CD25- T cells isolated in or out of the grass pollen season, by CD4+CD25+ cells from the other two donors studied (Fig. 4.14 b and c).

4.2.8. A comparison of the suppression of T cell responses to grass pollen allergen, cat dander allergen and PPD extract stimulation by CD4+CD25+ T cells from grass allergic donors.

To investigate whether the deficiency in suppression of T cell responses by CD4+CD25+ T cells from atopic donors was limited to T cell responses to which the individual produced specific IgE, CD4+CD25+ T cells were isolated from grass allergic donors (who were not allergic to cats), and their ability to suppress T cell responses to grass pollen and cat dander allergen extract as well as to *M. tuberculosis* derived purified protein derivative (PPD), were determined. There was no significant

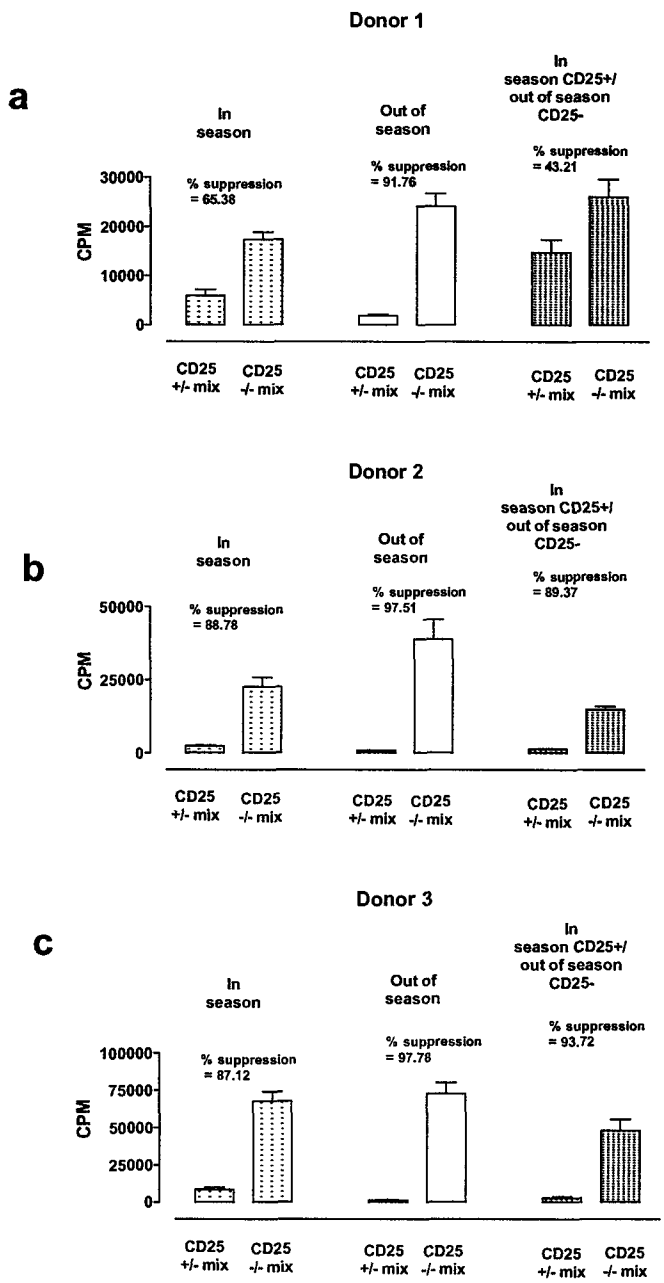


Figure 4.14. Suppression of grass pollen induced T cell proliferation by CD4+CD25+ T cells from grass pollen allergic donors in and out of the grass pollen season. CD4+CD25- T cells or CD4+CD25- T cells in the presence of CD4+CD25+ T cells at a ratio of one 25+ to two 25- cells were cultured at 3 x 10^6 c/ml. Cells were isolated from grass allergic donors in (light grey bars) and out (white bars) of the grass pollen season. CD4+CD25- T cells which had been frozen down during the grass pollen season were also used as responder cells and mixed with fresh out of season CD4+CD25+ cells (dark grey bars). Cells were stimulated with grass pollen allergen extract at 100 μg/ml or media alone and proliferation was measured by thymidine incorporation at day 7. All wells contained autologous irradiated PBMC at 2 x 10^6 c/ml. Triplicate values +SE from three individuals are shown.

difference in the proliferation of PBMC, CD4+CD25+ or CD4+CD25- T cells isolated from grass pollen allergic donors following *in vitro* stimulation with cat dander compared to grass pollen allergen extract (Fig. 4.15). CD4+CD25+ T cells from grass pollen allergic donors were able to suppress the proliferation of CD4+CD25- T cells stimulated with both grass pollen and cat dander allergen extract (Fig. 4.16a). However, percent suppression of proliferation to cat dander extract by CD4+CD25+ T cells was higher than suppression of proliferation to grass pollen allergen (Fig. 4.16b).

The suppression of PPD-stimulated T cells was next examined. There was no significant difference in the level of proliferation of PBMC, CD4+CD25+ or CD4+CD25- T cells from grass allergic donors when stimulated with grass pollen allergen extract or PPD *in vitro* (Fig. 4.17). As expected PBMC, CD4+CD25+ and CD4+CD25- T cells stimulated with grass pollen allergen extract produced higher concentrations of the Th2 cytokines IL-5 and IL-13 (Fig. 4.18 a and c) and lower concentration of IFN-γ (Fig. 4.18b) than when stimulated with PPD. These differences were not significant as supernatants from only three donors were analysed. There was also a slight increase in IL-10 production by all subsets when stimulated with PPD compared with grass pollen allergen extract (Fig. 4.18d).

CD4+CD25+ T cells from grass allergic donors were unable to suppress the proliferation of either the grass pollen allergen extract stimulated, or the PPD stimulated CD4+CD25- subsets (Fig. 4.19a) and there was no difference in the percent suppression of proliferation by CD4+CD25+ T cells of CD4+CD25- T cells activated by the different stimuli (Fig. 4.19b).

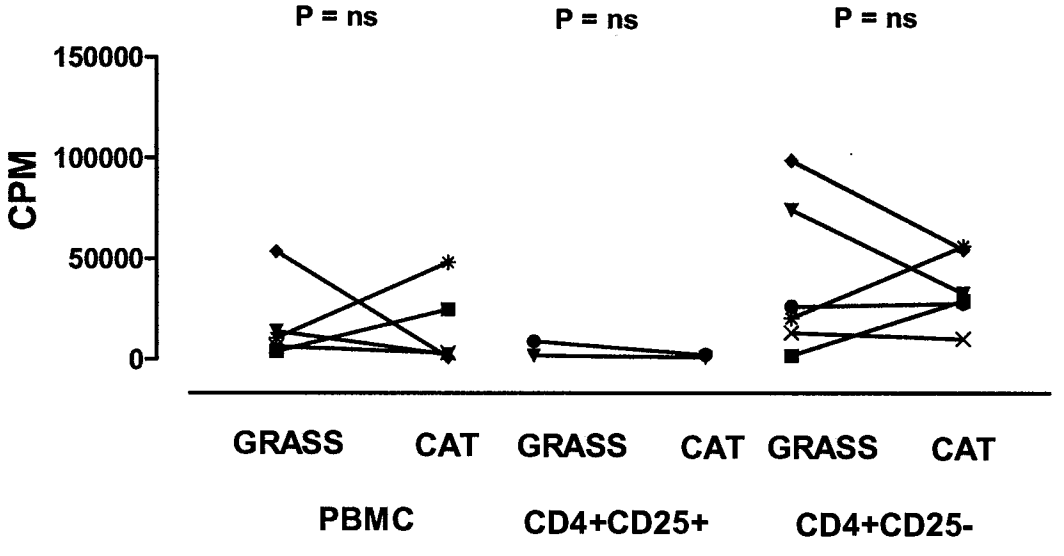


Figure 4.15. Proliferation by human T cell subsets from grass allergic donors to grass pollen and cat dander allergen extract. Allergen specific proliferation measured by thymidine incorporation on day 7 by PBMC, CD4+CD25+ and CD4+CD25- cells at 2 x 10^6 c/ml. Cells were isolated from grass (but not cat) allergic donors (n = 6) and stimulated with grass pollen or cat dander extract at 100 μg/ml, or media alone. All wells contained autologous irradiated PBMC at 2 x 10^6 c/ml.

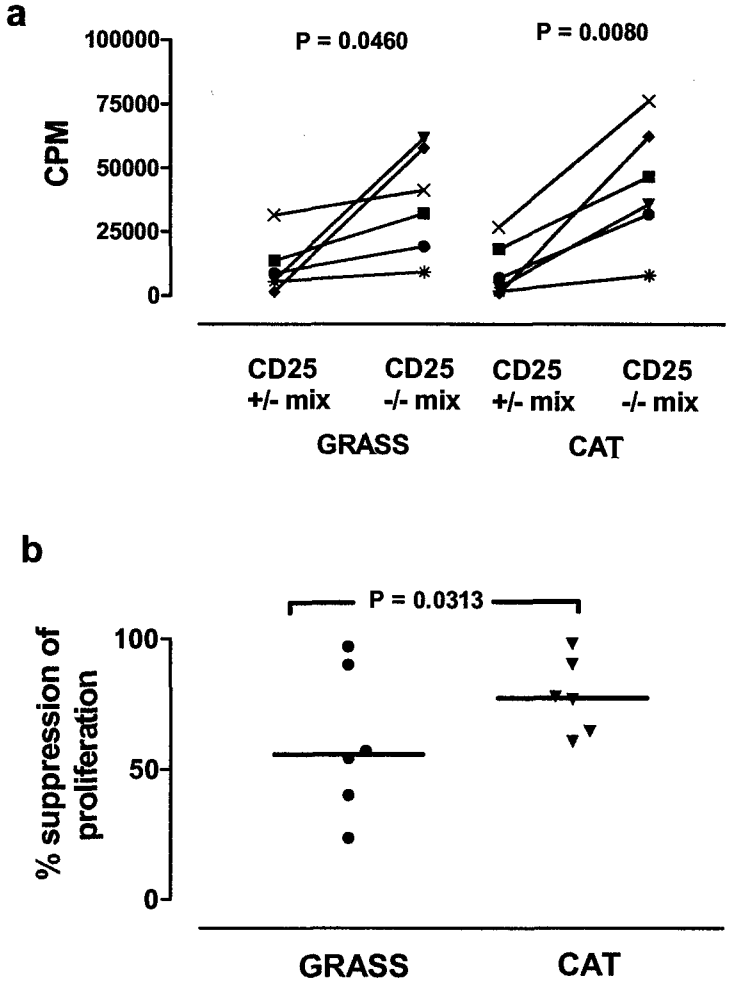


Figure 4.16. Suppression of grass pollen and cat dander allergen extract induced T cell proliferation by CD4+CD25+ T cells from grass pollen allergic donors. CD4+CD25- T cells or CD4+CD25- T cells in the presence of CD4+CD25+ T cells at a ratio of one 25+ to two 25- cells were cultured at 3 x 10^6 c/ml. Cells were isolated from grass (but not cat) allergic donors (n = 6) and stimulated with grass pollen or cat dander allergen extract at 100 μg/ml, or media alone. Proliferation was measured by incorporation of tritiated thymidine on day 7. All wells contained autologous irradiated PBMC at 2 x 10^6 c/ml (a). Individual % suppression values for suppression of allergen specific CD4+CD25- proliferation by CD4+CD25+ cells is shown (b).

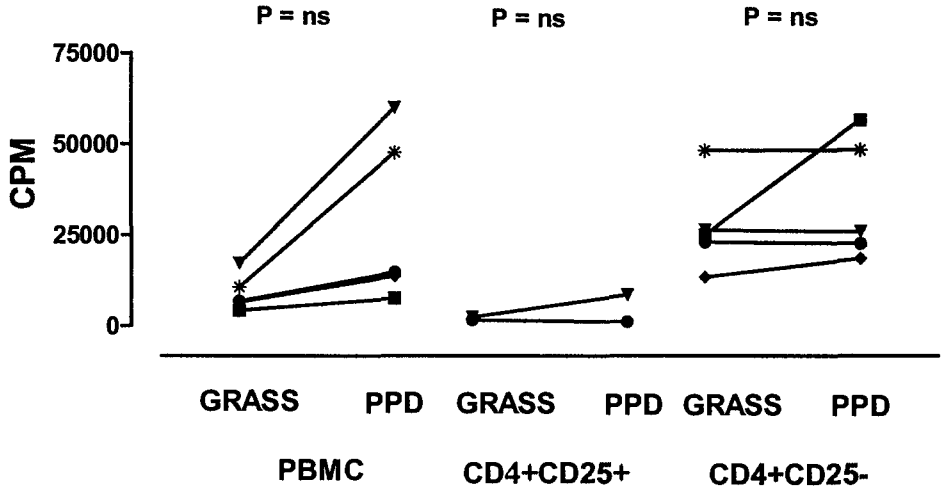


Figure 4.17. Proliferation by human T cell subsets from grass allergic donors to grass pollen allergen extract and purified PPD. Allergen/antigen specific proliferation measured by thymidine incorporation on day 7 of PBMC, CD4+CD25+ and CD4+CD25- cells at 2 x 10^6 c/ml. Cells were isolated from grass allergic donors (n = 5) and stimulated with grass pollen allergen extract at 100 μg/ml, PPD at 25 μg/ml or media alone. All wells contained autologous irradiated PBMC at 2 x 10^6 c/ml.

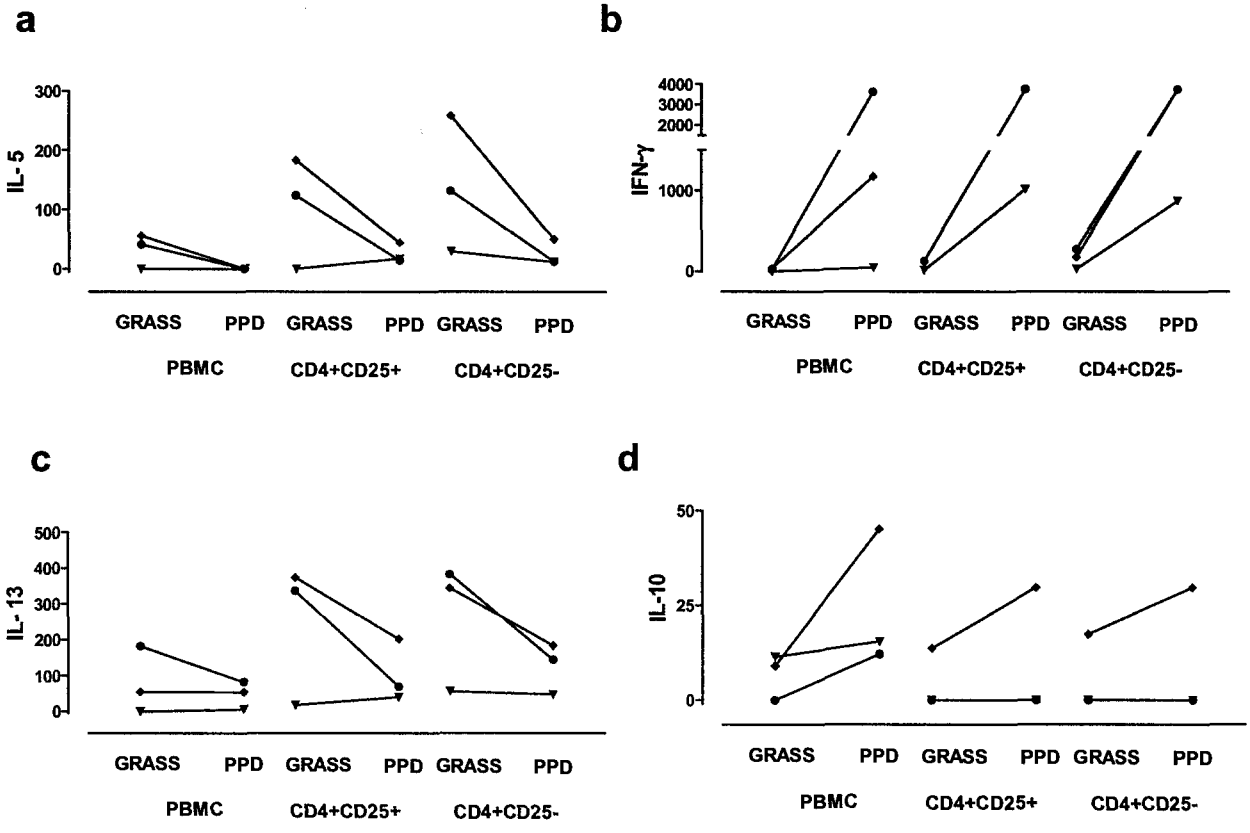


Figure 4.18. Cytokine production by human T cell subsets from grass allergic donors, stimulated with grass pollen allergen extract and purified PPD *in vitro*. Allergen/antigen specific IL-5 (a) IFN-γ (b) IL-13 (c) and IL-10 (d) measured by Luminex of the day 6 culture supernatant of PBMC, CD4+CD25+ and CD4+CD25- cells at 2 x 10^6 c/ml. Cells were isolated from grass allergic donors (n = 3) and stimulated with grass pollen allergen extract at 100 μg/ml, PPD at 25 μg/ml or media alone. All wells contained autologous irradiated PBMC at 2 x 10^6 c/ml.

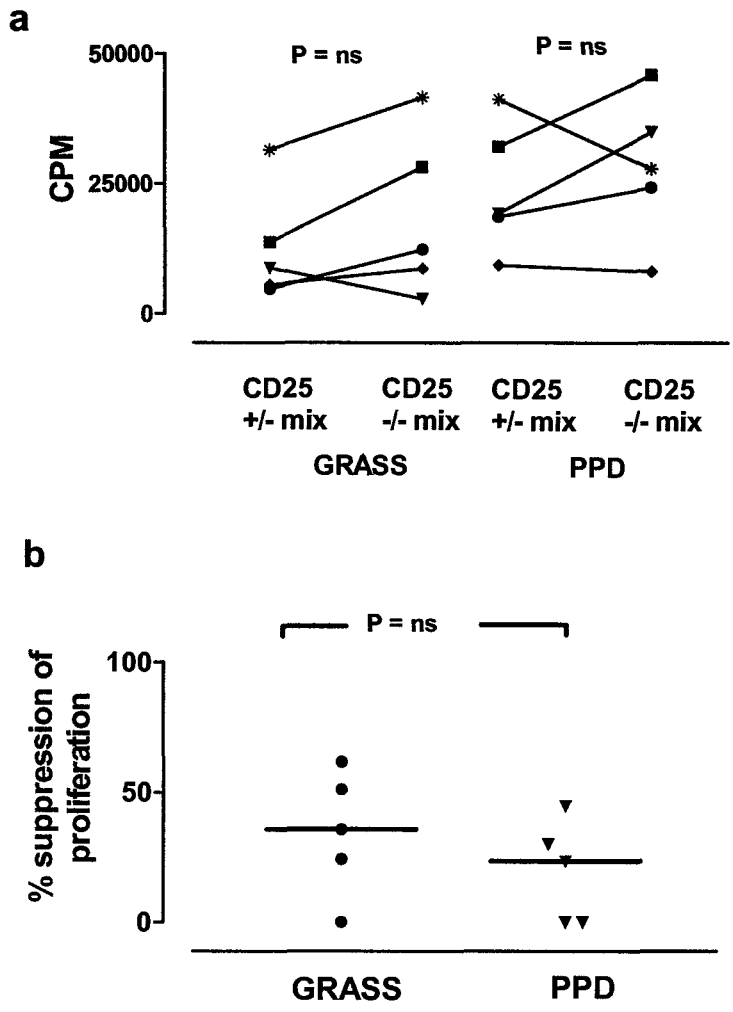


Figure 4.19. Suppression of grass pollen and PPD induced T cell proliferation by CD4+CD25+ T cells from grass pollen allergic donors. CD4+CD25- T cells or CD4+CD25- T cells in the presence of CD4+CD25+ T cells at a ratio of one 25+ to two 25- cells were cultured at 3 x 10^6 c/ml. Cells were isolated from grass allergic donors (n = 5) and stimulated with grass pollen allergen extract at 100 μg/ml, PPD at 25 μg/ml or media alone. Proliferation was measured by incorporation of tritiated thymidine on day 7. All wells contained autologous irradiated PBMC at 2 x 10^6 c/ml (a). Individual % suppression values for suppression of allergen specific CD4+CD25- proliferation by CD4+CD25+ cells is shown (b).

4.3. Discussion

4.3.1. The response of PBMC, and CD4+CD25+ and CD4+CD25- T cells from atopic donors to stimulation in vitro with allergen extract

When comparing the allergen induced proliferation and cytokine production by PBMC from non-atopic and atopic donors, the level of cellular proliferation was not different between groups. However, the secretion of Th2 cytokines such as IL-5 and IL-13 by cells from atopic donors exposed to allergen was higher. These findings are consistent with those from others [112;113] indicating that T cells from atopic donors will elicit a Th2-type response upon allergen stimulation.

The fact that CD4+CD25+ T cells from asymptomatic atopic donors were hyporesponsive to stimulation with allergen extract *in vitro* suggests that any activated Th2 cells (which may also be CD25+), isolated together with CD25+ regulatory T cells, were suppressed. When isolated from atopic donors exposed to allergen, CD4+CD25+ T cells produced higher concentrations of the Th2 cytokines IL-5 and IL-13 than did those from unexposed atopic and non-atopic donors. This raises the possibility that, in atopic donors after allergen exposure, the balance between effector Th2 and regulatory T cells within the CD25+ population may be shifted in favour of the effector cell. However, the allergen-specific proliferation of the CD4+CD25+ T cell subset was not higher in atopic donors exposed to allergen, indicating that the CD4+CD25+ T cell population isolated was not merely a population of activated Th2 cells.

Allergen-specific proliferation of CD4+CD25- T cells from both atopic and non-atopic donors was higher than that by PBMC, indicating the presence of regulatory cells within the PBMC capable of suppressing allergen-specific proliferation. The

finding that CD4+CD25- T cells from non-atopic donors proliferated to a greater extent than those from atopic donors was unexpected. It is possible however that the CD4+CD25- T cell subset from atopic donors contained cells that had previously been stimulated with allergen, and were therefore anergic upon restimulation *in vitro*. Another possibility is that the CD4+CD25- T cells from atopic donors actually proliferated more strongly than those from atopic donors and that this high level of proliferation "exhausted" them, or that the cells died due to an overcrowding effect of the enclosed *in vitro* culture system.

The interesting observation that CD4+CD25- T cells from non-atopic donors produced high concentrations of Th2 cytokines after allergen stimulation *in vitro* has already been discussed (Chapter 3). Results from Chapter 4 show that the concentrations of IL-5 and IL-13 were not significantly different from allergen stimulated CD4+CD25- T cells from atopic donors, suggesting that the Th2-associated IgE response to allergen in atopic individuals is not due to enhanced production of Th2 cytokines, but actually a lack of suppression of this response. However, after exposure to allergen, CD4+CD25- T cells from atopic donors did produce more IL-13 than unexposed atopic and non-atopic donors indicating that allergen exposure does cause expansion of activated Th2 cells in these individuals.

Another interesting observation was that PBMCs from atopic donors exposed to allergen produced lower concentrations of IL-10 when stimulated with allergen *in vitro* than those from unexposed atopic donors. This is particularly important, as IL-10 is a suppressor of T cell proliferative and cytokine responses [377], it inhibits IgE-induced activation of mast cells [378], and has been shown to both be secreted by and cause the generation of certain subsets of regulatory T cells [329]. A recent publication also shows that CD4+ T cells engineered to produce IL-10 prevent allergen induced airway

hyperreactivity and inflammation in mice [379]. The fact that there is very little IL-10 in the *in vitro* allergen stimulated cultures of cells taken from the hayfever donors is possibly part of the reason why the IL-5, IL-13 and IFN-γ levels are so high.

4.3.2. The suppression of allergen driven T cell responses by CD4+CD25+ T cells from atopic donors

The observation that suppression of allergen driven T cell proliferation by CD4+CD25+ T cells from asymptomatic atopic donors is less than that by CD4+CD25+ T cells from non-atopic individuals suggests three possibilities: 1) there is a deficiency in regulatory activity of CD4+CD25+ T cells from atopic individuals, 2) isolation of CD25+ T cells from atopic donors includes activated effector cells thereby diluting regulatory CD25+ T cells, and 3) effector T cells from atopic donors are highly activated and are therefore harder to suppress; ie the defect lies within the CD4+CD25- T cell population.

There was no phenotypic evidence for there being more activated cells and less regulatory T cells amongst the peripheral blood CD4+CD25+ T cells isolated from asymptomatic atopic donors compared to non-atopic controls. The expression of CD69 (a marker of activation [380]), and CTLA-4 (which has been shown to be constitutively expressed on regulatory T cells [54]) on the CD4+CD25+ T cell subset, did not differ between atopic and non-atopic donors, nor were there any differences in the percentage of CD4+ T cells that were CD25+, or in the intensity of CD25+ staining. However without a specific marker for regulatory cells, the possibility that there were more effector cells in the CD4+CD25+ T cell population isolated from asymptomatic atopic donors cannot be completely ruled out. Similarly, we did not see evidence of activated CD25- blood T cells in atopic individuals, since these cells did not express CD69 and

their IL-5 production to allergen stimulation *in vitro* was not different to those from non-atopic donors. They also proliferated less to allergen stimulation than those from non-atopic donors, although as discussed earlier, this may reflect the fact that some of these cells had been previously activated and were therefore anergic after secondary stimulation *in vitro*.

During the course of the experiments for this thesis, data from a similar study investigating the ability of CD4+CD25+ from atopic and non-atopic donors to suppress allergen driven T cell responses *in vitro* was published [344]. The main finding from that study was that CD4+CD25+ T cells from non-atopic and the majority of atopic donors could suppress allergen driven T cell responses. This observation confirms data presented in this chapter showing that CD4+CD25+ T cells from both non-atopic and asymptomatic atopic donors suppress T cell proliferation and cytokine production to allergen *in vitro*. The Bellinghausen study however, reported that there was no difference in suppression of allergen driven T cell responses by CD4+CD25+ T cells from atopic and non-atopic individuals, which contradicts the results presented in this Chapter. However there are differences between the two studies and it could be that similar results have been interpreted in different ways.

In the Bellinghausen experiments, the CD4+CD25+ T cells were stimulated in the presence of autologous, mature, monocyte-derived, allergen-pulsed dendritic cells before being re-stimulated in the presence of the CD4+CD25- responder cells. For the experiments described in this thesis, there was no pre-activation of the CD4+CD25+ T cells and irradiated autologous PBMC were used as antigen presenting cells. These differences in culture system could cause different results.

There are no clinical details of the atopic blood donors from the Bellinghausen study. They are only described as being "allergic to grass or birch pollen (mainly with

rhinitis)", and perhaps were not as highly allergic as those recruited for this thesis, who were mainly from an allergy clinic at the Royal Brompton Hospital. There were some atopic patients in the Bellinghausen study whose CD4+CD25+ T cells suppressed the IFN-γ production but not Th2 cytokine production or proliferation of CD4+CD25- T cells. It could be that these donors are more atopic than the others in the study, and are more similar to those studied in this thesis. There are also no details of what time of year the experiments were carried out and, as has been described in this chapter, the effect of exposure to allergen critically affects the suppression of allergen driven T cell responses by CD4+CD25+ T cells.

To investigate the nature of the deficiency in suppression by CD4+CD25+ T cells from atopic donors, it would be interesting to add the supernatant of allergen stimulated CD4+CD25+ T cells from non-atopic donors to allergen stimulated CD4+CD25- T cells from atopic donors. This would indicate whether any suppressive factor produced by CD4+CD25+ T cells from non-atopics was soluble. If a soluble mediator produced by CD4+CD25+ T cells from non-atopic donors could suppress CD4+CD25- T cells from atopic donors, it would also show that the defect was within the CD4+CD25+ and not the CD4+CD25- subset. It would obviously be preferable to set up a system in which CD4+CD25+ T cells from non-atopic donors suppressed CD4+CD25- T cells from atopics and vice versa, but this would cause an MLR reaction due to incompatibity of donor MHCs.

To further analyse the deficiency in suppression, quantitative Taqman PCR should be performed to see if there is a relative difference in expression of FOXP3 mRNA by CD4+CD25+ T cells from atopic and non-atopic donors. Expression of GITR on CD4+CD25+ T cells should also be compared between the two groups.

The experiments described in this thesis only use cells isolated from peripheral blood. CD4+CD25+ T cells have been isolated successfully from tissues such as the lung [296] and synovial fluid [303], and it may be possible to isolate them from sites of allergic inflammation. To define the relevance of this *in vitro* system, it will be important to investigate whether CD4+CD25+ T cells isolated from sites of allergic inflammation are phenotypically and functionally similar to those isolated from human peripheral blood.

The defect in suppression of allergen driven T cell responses by CD4+CD25+ T cells from atopic donors may be due to the processing and presentation of allergen by the APC. Inhaled allergen has been shown in mice to induce a population of APC in the lung, which secrete IL-10 and induce the development of regulatory T cells [330]. It is conceivable that, in non-atopic people these "regulatory APCs" normally present allergen peptides to T cells and induce the T cell to develop into a regulatory T cell and that in atopic people, this process is deficient. However, it is unlikely that the APC is responsible for causing the difference in suppression in this assay, as irradiated PBMC were used, which are unable to proliferate or produce high concentrations of cytokines and are therefore unlikely to influence T cell development and/or regulation.

It would also be of interest to establish whether drugs used for the prevention of symptoms of allergic disease function by generating CD4+CD25+ T cells or enhancing their suppressive activity. It is of note that the *in vitro* grass pollen allergen induced IL-5 production of PBMCs from grass allergic donors with rhinitis, is decreased when fluticasone is added to the cultures [348]. Recent data from our laboratory suggests that pre-incubation of CD4+CD25+ T cells from atopic donors with fluticasone increases their suppressive ability (Nguyen XD, and Robinson DS, JACI in press). Furthermore dexamethasone, which is another drug used to treat allergic disease, has been shown in

conjunction with Vitamin D3 to generate a population of regulatory cells which produces IL-10 and which can prevent central nervous system inflammation [171].

4.3.3. The effect of allergen exposure on the suppression of allergen driven T cell responses by CD4+CD25+ T cells

Results from this chapter show that after natural exposure to allergen, CD4+CD25+ T cells isolated from donors with hayfever were less efficient at suppressing both the allergen-specific CD4+CD25- T cell proliferation and IL-5 production than those from atopic donors who were not exposed to allergen. This result was confirmed by comparing the suppressive ability of the CD4+CD25+ T cell population isolated from a group of grass allergic donors in and then out of the grass pollen season.

The fact that allergen stimulated CD4+CD25+ and CD4+CD25- T cells from donors with active hayfever produced higher concentrations of Th2 cytokines than donors not exposed to allergen, suggests that the reason for the deficiency in suppression may have been due to the presence of activated Th2 cells. However, there was no other phenotypic evidence for the presence of activated CD4+CD25+ or CD4+CD25- T cells within the subsets isolated. Furthermore, paired data from the same grass allergic donors studied during and out of the grass pollen season showed that there was no difference in the allergen-specific proliferation or cytokine production of either the CD4+CD25+ or CD4+CD25- T cell subsets after natural allergen exposure. Nonetheless, it is possible that during active allergen exposure there is relative expansion of allergen-reactive T cells, and that signals from the innate immune system (possibly from mast cells), such as IL-6 [381], overcome regulation by CD4+CD25+ T cells. It will therefore be important to investigate the effect of exposure

to other allergens such as cat dander on the *in vitro* CD4+CD25+ T cell-mediated suppression of T cell responses to allergens such as cat dander and grass pollen. Also, the association between severity of allergic symptoms at the time of the experiments and the magnitude of suppression by CD4+CD25+ T cells should be investigated.

Out of season suppression of allergen-stimulated CD4+CD25- T cells from hayfever patients was similar to that seen in the asymptomatic atopic donors, but still significantly less than that seen for non-atopic donors. This may result from a defect in allergen-specific CD4+CD25+ T cells in atopic donors or from expansion of allergen-reactive CD25- T cells making these relatively resistant to suppression. CD4+CD25+ T cells are suggested to arise in the thymus [382], but may also develop in the periphery [383]. Whether CD4+CD25+ T cells that regulate allergic responses are selected by allergenic peptides during the neonatal period, represent cross-reactive T cells directed against self-antigens, or arise in the periphery requires investigation if these processes are to be manipulated for therapy.

Using a murine model of asthma, it has been shown that on exposure to respiratory allergen, pulmonary DCs present in the bronchial lymph nodes can induce regulatory T cells which block the development of asthma [58]. These cells develop through an intermediate stage that they share with developing Th2 cells, during which they produce IL-4 and IL-10. It is therefore possible that under normal circumstances, exposure to respiratory allergen results in the development of regulatory T cells and subsequent tolerance, whereas Th2 cells may develop in atopic individuals as an aberration of regulatory T cell development [384].

4.3.4. The suppression by CD4+CD25+ T cells from atopic donors of T cell responses to antigens which drive a Th1 response and allergens to which they do not make specific IgE

If a general deficiency in the suppressive capability of CD4+CD25+ T cells is causing a lack of regulation of allergen driven T cell responses, the ability of this population of cells to suppress T cell responses to other stimuli may also be hindered. However, there was no difference in the ability of CD4+CD25+ T cells from non-atopic compared with atopic donors to suppress anti-CD3 and anti-CD28 stimulated T cell proliferation [346], suggesting that this is not the case.

Data from this chapter has shown that CD4+CD25+ T cells from donors with specific IgE to grass pollen but not cat dander suppress T cell proliferation to cat dander allergen extract more effectively than they do to grass pollen allergen extract, suggesting that the defect in the suppression by CD4+CD25+ T cells is allergen-specific. However, when the suppression of cat dander stimulated T cell proliferation was compared to that by cells from non-atopic donors it was still significantly less. Unfortunately, these experiments were carried out during the grass pollen season so the donor was exposed to grass pollen but not cat dander. Further experiments need to be carried out when the donors are being exposed to either both or neither allergen, so that any effect of allergen exposure on the suppression of allergen driven T cell responses can be ruled out.

CD4+CD25+ regulatory T cells have previously been reported to suppress T cell proliferation and cytokine production to PPD [228]. It was therefore of interest to determine whether the lack of regulation of T cell responses by CD4+CD25+ T cells from atopic donors was limited to Th2 driven responses. Preliminary data in this chapter investigating the ability of CD4+CD25+ T cells from grass allergic donors to

suppress Th1 responses to PPD has shown that these donors were unable to suppress T cell responses to either grass pollen allergen extract or PPD, suggesting that any defect is not allergen specific. These experiments were also carried out during the grass pollen season however, when the donor was being exposed to grass pollen but not PPD, so they require repeating out of the grass pollen season. The suppression by CD4+CD25+ T cells from non-atopic donors of T cell responses to PPD will also need to be determined to investigate whether CD4+CD25+ T cells from atopic donors suppress these responses to the same magnitude as non-atopic donors.

4.3.5. Conclusion

There have been few reports comparing the suppressive activity of CD4+CD25+ regulatory T cells from atopic and non-atopic individuals. Data from this Chapter is the first to show that CD4+CD25+ regulatory T cells from atopic donors are not as effective at suppressing allergen driven T cell responses *in vitro* as those from non-atopic donors. Furthermore, exposure of atopic donors to allergen further reduced the ability of CD4+CD25+ T cells to suppress allergen-driven T cell responses.

Chapter 5 – The role of regulatory T cells in allergen immunotherapy treatment.

5.1. Introduction

Results presented in Chapter 4 illustrate for the first time the potential importance of CD4+CD25+ T cells in the suppression of human allergic disease. These data suggest that induction of allergen-specific regulatory T cells is a potential therapeutic approach to the treatment of allergy.

This chapter aimed to investigate whether CD4+CD25+ regulatory T cells isolated from the peripheral blood of grass allergic donors who have received allergen-specific immunotherapy treatment are more effective at suppressing grass pollen stimulated T cell responses *in vitro*, than those from donors with hayfever. A separate population of regulatory T cells, which produce IL-10 and reside in the CD4+CD25- population, will also be introduced. The relationship between the regulatory activity of this subset with expression of allergic disease by donors with hayfever and those who have received grass pollen specific allergen immunotherapy treatment, will be discussed.

5.1.1. Protection afforded by allergen immunotherapy treatment and natural high dose allergen exposure

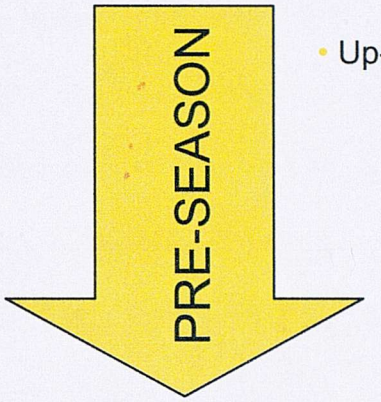
Although the symptoms of allergic disease can be treated with various pharmacological agents, specific allergen immunotherapy is the only disease modifying approach. This treatment, which was pioneered in 1911[385], involves administering increasing concentrations of allergen extract subcutaneously over a long period of time [386]. A typical protocol for this treatment is shown in Figure 5.1. It is an

extremely effective therapy for patients suffering from allergy to insect venom [387], seasonal allergic rhinitis [173;388;389] and to a lesser extent, perennial rhinitis [386;390]. Protection from allergic disease is long lasting, especially when treatment is continued for several years [173]. The use of allergen specific immunotherapy to treat allergic asthma remains controversial. Unfortunately, due to IgE cross-linking caused by the high dose of allergen administered, immunotherapy treatment itself can induce anaphlylaxis [391]. The treatment can also induce new IgE responses to proteins contained in the extract [392;393].

Interestingly, the effects of specific allergen immunotherapy treatment are mirrored by natural high dose antigen exposure, as in the case of bee-keepers who have previously been stung by high numbers of bees [177]. These individuals do not mount allergic reactions when stung by bees. Moreover, when PBMCs are isolated and stimulated *in vitro* with the major bee venom allergen phospholipase A_2 (PLA_2), they do not proliferate and they produce low levels of Th2 cytokines together with high levels of IL-10.

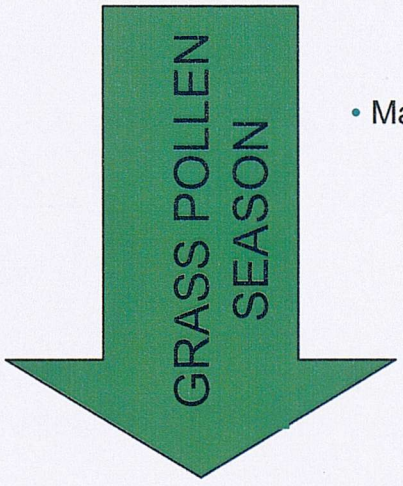
5.1.2. The mechanism of allergen immunotherapy treatment

The mechanism by which allergen specific immunotherapy exerts its beneficial effects is extremely complex. The influence of immunotherapy on T cells is thought to be central to its inhibition of the late phase response. Successful allergen immunotherapy treatment is associated with a decreased *ex vivo* allergen specific T cell proliferative response [174;387;394-396] accompanied by decreased production of the Th2



- Up-dosing
  - A total of 15 injections were given to reach the maintenance dose of 100,000 SQ Units within 7-8 weeks

Injection no:	Dosage (SQ-U)
1	20
2	40
3	80
4	200
5	400
6	800
7	2,000
8	4,000
9	8,000
10	10,000
11	20,000
12	40,000
13	60,000
14	80,000
15	100,000



- Maintenance
  - Injections of 100,000 SQ Units were given every 6 ± 2 weeks

Figure 5.1. Protocol of the immunotherapy treatment received by grass allergic blood donors involved in this study

cytokines IL-4, IL-5 and IL-13 relative to the production of IFN-γ and increased concentration of IL-10. The expression of IL-4 and IL-5 mRNA by T cells is also decreased (see Table 5.1.).

The recruitment of inflammatory cells such as mast cells, basophils and eosinophils to sites of allergic disease is decreased after treatment [178;397;398]. This is thought to be due in part to the reduced levels of the inflammatory cytokines IL-4, IL-5 and IL-13, together with increased levels of both allergen specific and total IgG4 [174]. IgG4 competes with IgE for allergen, reducing IgE receptor cross-linking and subsequent release of inflammatory mediators from mast cells and basophils [392;399;400].

CD4+CD25+ T cells with regulatory-like features can be generated by oral or intranasal administration of whole protein or peptides in mice [255;330;401]. Several groups have suggested that regulatory T cells are involved in the mechanism through which specific allergen immunotherapy protects from allergic disease. For example, elevated numbers of CD25+ cells are detected in cutaneous sites of allergen challenge from individuals who have received allergen immunotherapy treatment compared to untreated control subjects [178].

During the course of the studies described in this thesis, two papers were published that provided evidence of activation or recruitment of CD25+ T cells by allergen immunotherapy [343;402]. A higher number of CD4+CD25+ T cells were present in Phl PV stimulated cultures of PBMCs from donors who had received allergen immunotherapy treatment than from untreated control subjects, and a higher proportion of these CD4+CD25+ cells from immunotherapy treated donors were IL-10+ by intracellular cytokine staining [402]. From this study however, it was not clear whether the cells expressing the CD25 marker were truly regulatory T cells, or T cells activated

Potential Mechanism	Effect	Evidence for mechanism
Blocking IgG competing with IgE	Smaller immediate skin reactions due to: less degranulation of mast cells and basophils. Less IgE mediated allergen activation of T cells, therefore reduction of the late phase reaction	392;400 403
Reduction in T cell derived cytokines	Decreased mediator release from mast cells and basophils Decreased inflammation in the late phase response, reduction of eosinophils	398 394;395;404-407 397
Induction of Th1 cells	Suppression of Th2 cell proliferation and cytokine production	394;395;405
Induction of regulatory T cells/anergy/tolerance	Decreased T cell proliferation and cytokine production	176;395;396;406;408-411

Table 5.1. Potential mechanisms of allergen immunotherapy treatment.

during the course of the *in vitro* allergen driven culture. A further study investigated the effect of allergen immunotherapy treatment on the ability of regulatory T cells to suppress allergen specific T cell responses [343]. The results showed that after immunotherapy treatment for allergy to house dust mite, CD4+CD25+ T cells were more efficient at suppressing the proliferation of T cells stimulated with this allergen *in vitro*. This increase in suppression was dependent on IL-10 and TGF-β.

5.1.3. The effect of allergen exposure on allergic sensitisation

It has also been proposed that avoidance can be used to prevent sensitisation of individuals with negative skin test results or even from birth (ie, primary avoidance) [412-415]. The fact that exposure to allergen is necessary for sensitisation, is illustrated by the fact that children raised in a community without dust mite allergen do not become allergic to dust mites. Similarly, children raised with minimal or no exposure to cockroach allergens (eg, suburban areas of New Zealand, Delaware, or Sweden) do not become allergic to cockroaches [416]. It is not always possible to avoid exposure to allergen however. It is now clear that the major cat allergen, Fel d 1, which is carried on dander particles, spreads throughout the community. Significant quantities of Fel d 1 can be found in floor dust from schools, public buildings, and homes without a cat[416-418]. Airborne Fel d 1 can also be detected in the air of homes without a cat[419;420]. Thus not having a cat in the home is not an effective measure to prevent sensitisation to cats and indeed might have the opposite effect. A recent study showed that families who chose not to own a cat because of a family history of allergy had a higher prevalence of cat sensitisation than comparable families who had a cat [421].

Studies have also shown that although increasing exposure to allergen is associated with increased prevalence of IgE sensitisation, exposure to the highest

concentration of cat dander is associated with decreased IgE sensitisation and a higher prevalence of IgG antibody to Fel d 1[422]. This modified T-helper-2 cell response should be regarded as a form of tolerance and may be the correct objective of immunotherapy.

5.1.4. IL-10 producing regulatory T cells and allergic disease

Repeated injection of mice with antigen [267] or peptide [268] results in the generation of IL-10 producing cells. These cells fail to proliferate in response to antigen, show markedly reduced secretion of IL-2, IL-4 and IFN-gamma but produce high levels of IL-10 [269]. A similar population is generated from human naïve CD4+ T cells after repeated stimulation with allogeneic immature dendritic cells [270].

T cells that produce IL-10 suppress Th2 responses in animal models of allergic disease. Antigen specific Tr1 cells, which are induced *in vitro* by chronic activation in the presence of IL-10, inhibit Th2 cell activation in a murine model of inhaled allergen challenge [260]. In addition, mature pulmonary dendritic cells in the bronchial lymph nodes of mice induce the development of IL-10 producing regulatory T cells when exposed to respiratory allergen and these cells, termed Tr cells, block the development of asthma in a murine model [58]. Furthermore, CD4+ T-helper cells secreting IL-10 engineered by gene therapy, prevent allergen induced airway hyperreactivity and inflammation in mice by a process that is dependent on IL-10 [379].

IL-10 producing T cells are also implicated in the mechanism of allergen immunotherapy treatment of human allergic disease [177;343;375;376;402]. Moreover neutralisation of endogenous IL-10 in the PLA_2 stimulated PBMC cultures from naturally tolerant bee-keepers fully reconstituted the proliferative T cell response and cytokine production [343].

5.1.5. Blood donors recruited for this study

Three groups of blood donors were recruited to take part in this study; non-atopic donors with no history of allergy, negative skin prick test to grass pollen and/or low total and grass-pollen specific serum IgE; grass allergic donors with positive grass pollen skin prick test and/or total and specific IgE and with history and/or current symptoms of hayfever, and grass allergic donors who had received grass pollen specific allergen immunotherapy treatment and who no longer suffered from hayfever. Unless stated, all blood was taken at the height of the UK pollen season in 2003.

The donors recruited for the allergen immunotherapy treated group had received allergen immunotherapy treatment at either the Royal Brompton or St. Mary's Hospital, London. All donors were at the maintenance stage of treatment, see Figure 5.1, and therefore had been receiving treatment for at least 18 months. If donors were attending hospital for a maintenance injection, blood was taken before they received the allergen injection.

5.1.6. Hypothesis

Although it is probable that regulatory T cells are involved in the mechanism of allergen immunotherapy treatment, it is not yet clear which of the many subtypes of T cells with suppressive activity that have been described, are involved. Studies that have reported an increase in regulatory T cells after immunotherapy treatment have so far referred only to the phenotype of the cells, ie expression of CD25 or production of IL-10. To date, there has been only one functional study investigating whether allergen immunotherapy treatment increases the ability of regulatory T cells to suppress Th2 responses. Further work also needs to be carried out to determine which subtype of regulatory T cell is involved.

Therefore, this chapter addresses the hypothesis: **Regulatory T cells are involved in the protection elicited by conventional allergen immunotherapy treatment.**

5.2 Results

5.2.1. Isolation of CD4+CD25+ and CD4+CD25- T cells from the peripheral blood of grass allergic donors treated with allergen immunotherapy and untreated donors with hayfever.

PBMCs from donors with hayfever and donors who had received grass pollen allergen immunotherapy treatment were isolated and further separated into CD4+CD25+ and CD4+CD25- subsets. The clinical details of these two groups of donors are shown in Tables 5.2 and 5.3, respectively. There were no significant differences between groups in the serum concentration of grass pollen specific IgE or skin prick test wheal diameter. The recovery of CD4+CD25+ cells and the purity of both CD4+CD25+ and CD4+CD25- subsets (determined by flow cytometry), did not differ between untreated donors with hayfever and those who had received allergen immunotherapy treatment, (Tables 5.4 and 5.5)

5.2.2. CD4+CD25- T cells from immunotherapy treated donors produce more IL-10 and proliferate less than those from untreated donors when stimulated in vitro with grass pollen allergen extract.

PBMCs from donors with hayfever and donors who had received grass pollen allergen immunotherapy treatment were stimulated *in vitro* with grass pollen allergen extract or medium alone. There was no significant difference in allergen-specific PBMC proliferation (proliferation to allergen, minus proliferation to medium alone), between the two groups, (Fig.5.2a). There was however a trend for less proliferation by cells from immunotherapy donors

Donor	Gender	Age	IgE to grass pollen IU/ml	Total IgE IU/ml	Grass pollen SPT wheal diameter (mm)	Allergic disease
1	M	29	N/a	N/a	8	Y
2	M	39	N/a	N/a	10	Y
3	F	43	12.9	N/a	20	Y
4	F	33	94	296	N/a	Y
5	M	42	N/a	13	13	Y
6	M	25	N/a	67	9	Y
7	M	26	N/a	N/a	12	Y
8	M	24	2.64	1007	5	Y
9	M	30	N/a	N/a	14	Y
10	F	N/a	33	N/a	8	Y
11	F	38	20	45	7	Y
Summary	7 = M 4 = F	31.5 (24 - 43)	20 (2.6 – 94)	67 (13 – 1007)	9.5 (5 – 20)	11 = Y 0 = N

Table 5.2. Clinical details of donors with hayfever who had not received allergen immunotherapy treatment (CD4+CD25+ study).
Gender; F = female, M = male. IgE levels measured by RAST test in IU/ml. Grass pollen specific skin prick test (SPT); wheal diameter measured in mm. Allergic disease; Y = positive history of allergic disease, N = no history of allergic disease. N/a is unavailable data. Summary is median and range or summary of data.

Donor	Gender	Age	IgE to grass pollen IU/ml	Total IgE IU/ml	Grass pollen SPT wheal diameter (mm)	Allergic disease
1	F	N/a	18.2	N/a	N/a	Y
2	M	40	12.9	N/a	9	Y
3	M	37	N/a	N/a	7	Y
4	M	39	N/a	N/a	15	Y
5	M	46	4.53	N/a	12	Y
6	M	52	N/a	N/a	11	Y
7	M	38	24.3	N/a	17	Y
8	M	32	N/a	N/a	7	Y
9	M	34	12.2	N/a	12	Y
10	M	56	55.6	N/a	13	Y
11	M	46	N/a	N/a	12	Y
Summary	10 = M 1 = F	39.5 (32 –56)	15.5 (4.53 – 55.6)	N/a	12 (7 – 17)	11 = Y 0 = N

Table 5.3. Clinical details of grass pollen allergic patients who had received immunotherapy treatment (CD4+CD25+ study).
Gender; F = female, M = male. IgE levels measured by RAST test in IU/ml. Grass pollen specific skin prick test (SPT); wheal diameter measured mm. Allergic disease; Y = positive history of allergic disease, N = no history of allergic disease. N/a is unavailable data. Summary is median and range or summary of data.

Donor	Amount of blood taken (ml)	Total no. of PBMC ($x10^6$)	Total no. of CD4+ cells ($x10^6$)	Total no. of CD4+ CD25+ cells ($x10^6$)	Total no. of CD4+ CD25- cells ($x10^6$)	% CD4+ CD25+ recovered from the CD4+ subset	Purity of CD4+ cells	Purity of CD4+ CD25+ cells	Purity of CD4+ CD25- cells
1	200	303.5	36.5	1.75	44	4.8	95.8	77.7	80.5
2	200	384.3	55	12.15	48	22.1	98.7	79.1	77.1
3	150	268.8	62.3	1.8	61.5	2.9	89.5	65.7	77.0
4	200	318.6	76.2	1.65	45	2.2	93.5	78.2	80.1
5	200	270	40.8	1.85	30.5	4.5	N/a	N/a	N/a
6	200	334.6	61.8	2.3	52	3.7	95.6	82.4	76.3
7	150	264	102	2.8	42	2.7	91.1	73.1	79.2
8	200	375.6	133.5	4.7	76.5	3.5	94.3	85.5	80.4
9	200	240	102	3.73	95.5	3.7	97.7	76.8	83.2
10	200	205.85	68.75	1.25	32.7	1.8	95.2	79.1	68.2
11	200	294	129	3.45	67.5	2.7	N/a	N/a	N/a
Summary	200 (150 – 200)	294 (205.9 – 384.3)	68.65 (36.5 – 133.5)	2.3 (1.25 – 3.73)	48 (30.5 – 95.5)	3.5 (1.8 – 22.1)	95.2 (89.5 – 98.7)	78.2 (65.7 – 85.5)	79.2 (68.2 – 83.2)

Table 5.4. Recovery and purity of cells isolated from the peripheral blood of donors with hayfever who had not received allergen immunotherapy treatment (CD4+CD25+ study).
Total number of cells isolated was calculated using a haemocytometer. Purity of cells was calculated using flow cytometry after staining with fluorescent CD4 and CD25 antibodies and isotype-matched controls, see Fig. 3.4. N/a is unavailable data. Summary is median and range.

Donor	Amount of blood taken (ml)	Total no. of PBMC ($x10^6$)	Total no. of CD4+ cells ($x10^6$)	Total no. of CD4+ CD25+ cells ($x10^6$)	Total no. of CD4+ CD25- cells ($x10^6$)	% CD4+ CD25+ recovered from the CD4+ subset	Purity of CD4+ cells	Purity of CD4+CD25+ cells	Purity of CD4+CD25- cells
1	200	291	75.4	2.2	34.2	2.9	93.7	77.7	81.0
2	200	424.8	93.5	2.8	74	2.9	91.1	79.2	74.9
3	200	415	146	4.4	77.5	3.0	87.6	89.5	80.0
4	200	198	32.5	2.1	20	6.5	87.4	70.0	65.4
5	200	142.8	29	1.1	32	3.8	91.1	83.4	70.2
6	150	247.2	217.8	3	59	1.4	85.3	83.2	69.9
7	200	263	77	5.1	30	6.6	89.3	60.1	81.3
8	200	302	69	9.5	37	13.8	83.6	72.0	85.7
9	200	302.4	44.8	1.45	26.25	3.2	87.1	64.1	86.2
10	200	546	457.8	6.85	307.5	1.5	94.5	75.2	68.3
11	200	248.4	63.7	3.2	57	5.0	95.4	80.1	63.3
Summary	200 (150 – 200)	291 (142.8 – 424.8)	75.4 (29 – 457.8)	3.0 (1.1 – 9.5)	37 (20 – 307.5	3.2 (1.4 – 13.8)	89.3 (83.6 – 95.4)	77.7 (60.1 – 89.5)	74.9 (63.3 – 86.2)

Table 5.5. Recovery and purity of cells isolated from the peripheral blood of grass allergic donors who had received immunotherapy treatment (CD4+CD25+ study).
Total number of cells isolated was calculated by cell count using a haemocytometer. Purity of cells was calculated using flow cytometry after staining with fluorescent CD4 and CD25 antibodies and isotype-matched controls, see Fig. 3.4. . N/a is unavailable data. Summary is median and range.

(P=0.072), and a lower concentration of the Th2 cytokine IL-13 (Fig 5.2c, P=0.072) measured in the day 6 culture supernatant when compared to cells from donors with hayfever. There was no significant difference between groups in the concentration of IL- 5 (Fig. 5.2b), IL-10 (Fig. 5.2d) or IFN-γ (Fig. 5.2e).

PBMCs from both groups were then further separated into CD4+CD25- and CD4+CD25+ subsets and stimulated with grass pollen allergen extract under the same conditions, but in the presence of irradiated PBMCs to act as APCs. As described in Chapter 3, these irradiated PBMCs did not proliferate or produce cytokines when stimulated with allergen extract *in vitro*. CD4+CD25+ T cells from neither donors with hayfever nor donors who had received immunotherapy treatment proliferated in response to grass pollen allergen extract *in vitro* (Fig.5.3a). Analysis of cytokines in the day 6 supernatant indicated that there was no difference in the allergen-specific production of IL-5 (Fig. 5.3b) IL-13 (Fig. 5.3c), IL-10 (Fig.5.3d) or IFN-γ (Fig. 5.3e) by CD4+CD25+ T cells between groups.

The allergen-specific proliferation by CD4+CD25- T cells from immunotherapy donors was significantly less than by CD4+CD25- T cells from donors with hayfever (Fig. 5.3a, P = 0.0489), which may be in part due to a higher concentration of IL-10 produced by this population of cells (Fig. 5.3d, P = 0.0265). There was no difference in allergen-specific IL-5 (Fig. 5.3b), IL-13 (Fig.5.3c) or IFN-γ (Fig. 5.3e) production by CD4+CD25- T cells.

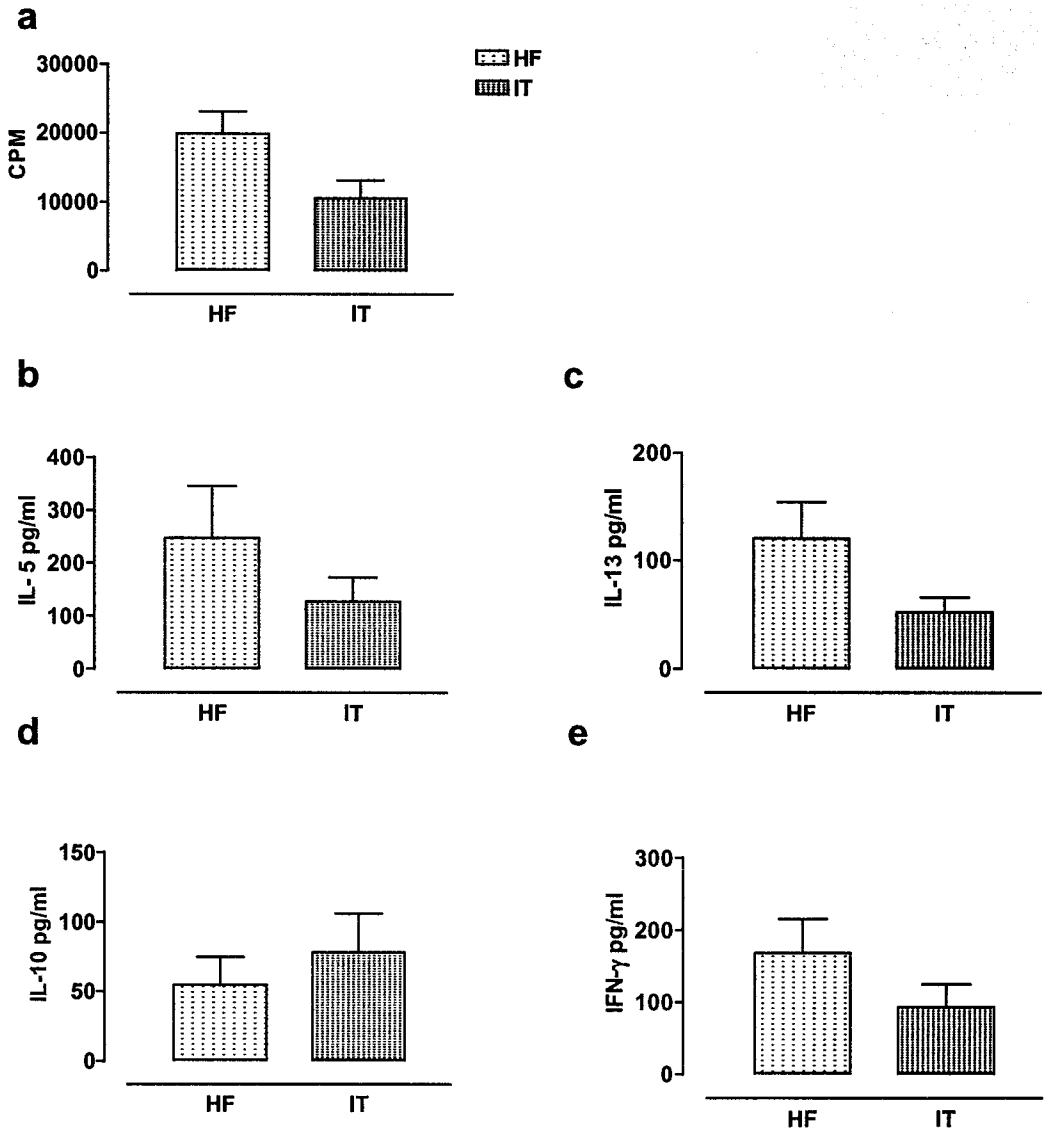


Figure 5.2. Grass pollen specific proliferation and cytokine production by PBMCs from immunotherapy treated donors and untreated donors with hayfever. PBMCs isolated from peripheral blood of allergen specific immunotherapy treated donors (IT, n = 11) and untreated donors with hayfever (HF, n = 11) were stimulated *in vitro* with grass pollen allergen extract at 100 ug/ml or media alone for 6 days at 2 x 10^6 c/ml. Allergen specific proliferation, (proliferation to allergen minus proliferation to media alone) was measured by tritiated thymidine incorporation in CPM (a), and concentration of IL-5 (b), IL-13 (c), IL-10 (d) and IFNγ (e) in the day 6 culture supernatants measured by Luminex in pg/ml. Median + 95% CI is shown.

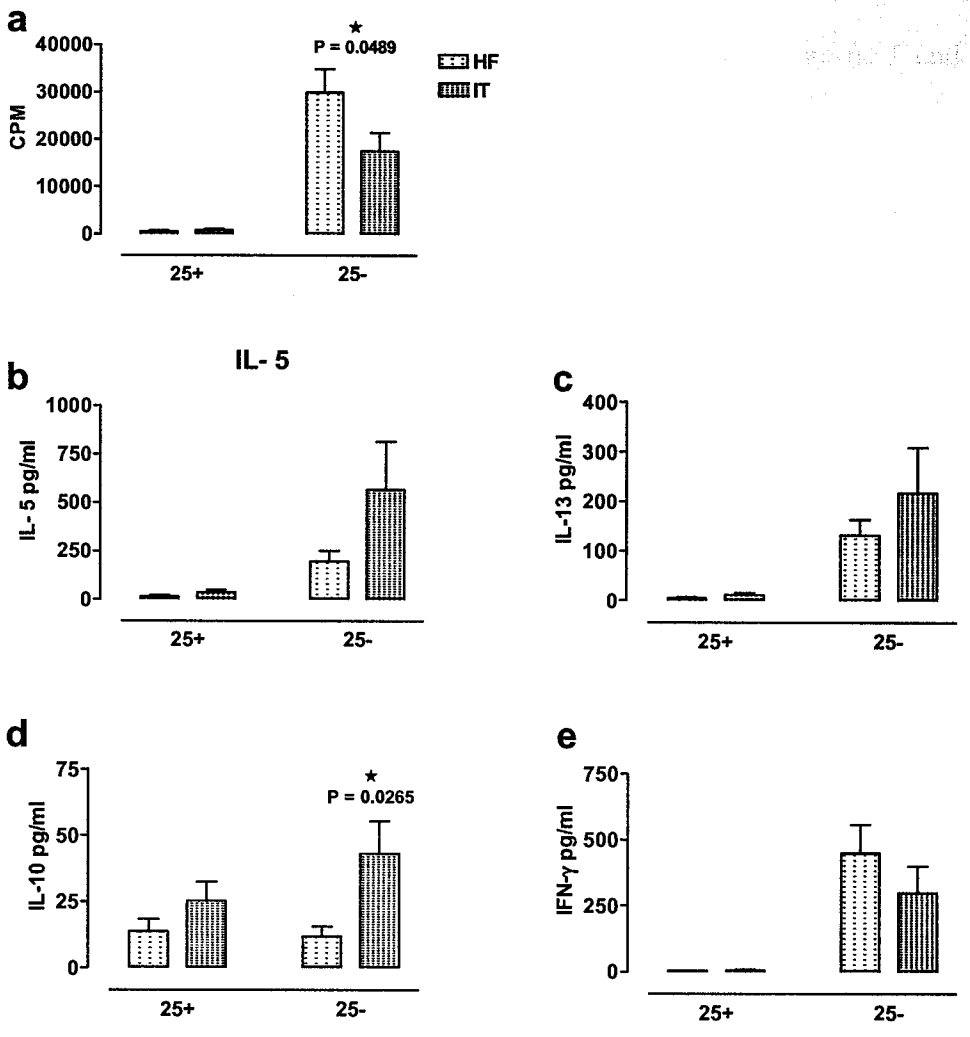


Figure 5.3. Grass pollen specific proliferation and cytokine production by CD4+CD25+ and CD4+CD25-T cells from immunotherapy treated donors and untreated donors with hayfever. CD4+CD25+ (25+) and CD4+CD25- (25-) cells isolated from peripheral blood of immunotherapy treated donors (IT, n = 11) and untreated donors with hayfever (HF, n = 11) were stimulated *in vitro* with grass pollen allergen extract at 100 ug/ml or media alone for 6 days at 2 x 10^6 c/ml in the presence of irradiated PBMCs. Allergen specific proliferation, (proliferation to allergen minus proliferation to media alone) was measured by tritiated thymidine incorporation in CPM (a). Concentration of IL-5 (b), IL-13 (c), IL-10 (d) and IFNγ (e) in the day 6 culture supernatants was measured by Luminex in pg/ml. Median + 95% CI is shown. * = P <0.05 IT vs HF.

5.2.3. Allergen immunotherapy treatment does not alter the suppression of grass pollen stimulated T cell responses by CD4+CD25+ T cells

To investigate whether CD4+CD25+ cells from immunotherapy treated, grass pollen allergic donors, could suppress grass pollen specific T cell responses more effectively than untreated donors, CD4+CD25+ cells from both groups were added to autologous CD4+CD25- T cells and stimulated with allergen extract. The allergen-specific proliferation and cytokine production of the CD25+/- culture was compared to that of CD25- cells alone cultured at the same cell density, $3x10^6$ c/ml (marked CD25-/-). Figures 5.4 a and b show proliferation data of CD25+/- and CD25-/- mixed cultures from a representative donor with hayfever and an allergen immunotherapy donor respectively, and indicate that CD4+CD25+ cells from both were able to suppress proliferation of allergen stimulated CD4+CD25- T cells. Proliferation data from all donors (Fig. 5.4c), indicates that immunotherapy treatment did not affect the proliferation of cells in the CD25+/- mix, although as expected, there was a trend for less proliferation of cells in the CD25-/- cultures from the immunotherapy donors ($P = 0.0660$).

When comparing the suppressive ability of the CD4+CD25+ cells between groups, the percent suppression of proliferation was determined, as described in section 3.2.4. There was no significant difference between the percent suppression of allergen specific CD4+CD25- T cell proliferation by CD4+CD25+ cells from hayfever donors compared to recipients of allergen immunotherapy treatment (Fig. 5.4d).

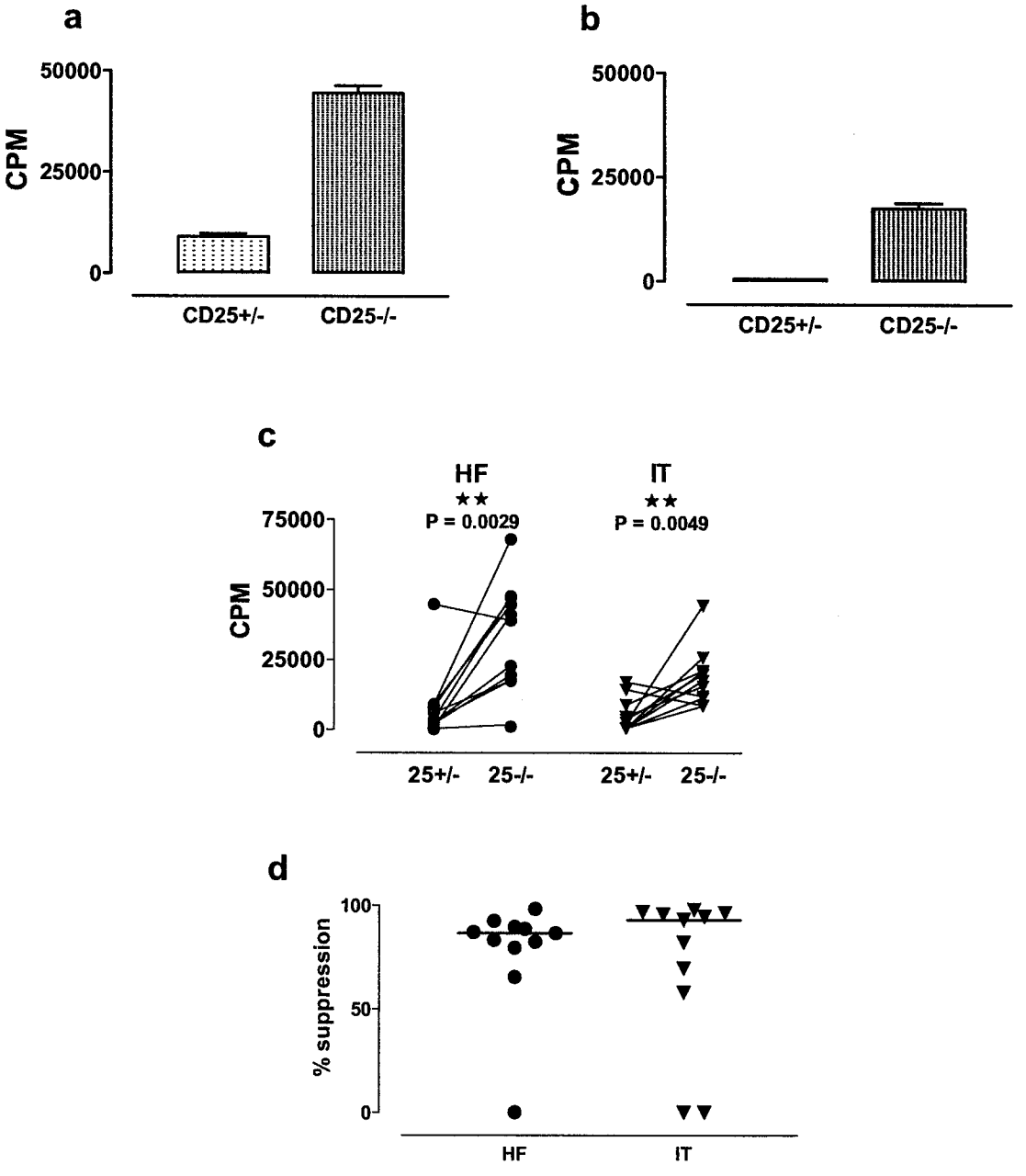


Figure 5.4. Suppression of grass pollen specific T cell proliferation by CD4+CD25+ cells from immunotherapy treated donors and untreated donors with hayfever. Proliferation of CD4+CD25- and CD4+CD25+ cells isolated from the peripheral blood of immunotherapy treated donors (IT, n = 11) and untreated donors with hayfever (HF, n = 11), in mixed cultures at a ratio of two CD25- cells to one CD25+ cell (CD25+/-) and CD4+CD25- cells alone (CD25-/-) stimulated *in vitro* with grass pollen allergen extract at 100 ug/ml or media alone for 6 days at 3 x 10^6 c/ml in the presence of irradiated PBMCS, was measured by tritiated thymidine incorporation. Representative data from a donor with HF (a) and from an IT donor (b), mean of triplicate cultures + SEM is shown. Individual allergen specific proliferation, (proliferation to allergen minus proliferation to media alone) from mixed cultures of all donors (c), % suppression of allergen specific proliferation by cells from individual donors (d), a line represents the median value.

Next, the concentration of cytokines in the supernatant of the mixed CD25+/- and CD25-/- cultures was analysed. There was no difference between cells from donors with hayfever compared to those treated with allergen immunotherapy in the concentration of IL-5 (Fig. 5.5a), IL-13 (Fig.5.5b), or IFN-γ (Fig. 5.5d) in the CD25+/- or CD25-/- cultures. There was however a trend for higher levels of IL-10 in the CD25+/- mixed culture of cells from immunotherapy treated donors compared to untreated donors with hayfever (Fig 5.5c, $P = 0.193$). Furthermore, there was significantly more IL-10 in the CD25-/- culture of cells from allergen immunotherapy donors (Fig. 5.5c). When percent suppression of the cytokines produced by the CD4+CD25- subset was calculated, there was no difference found between suppressive activity by CD4+CD25+ cells between groups (Fig. 5.6 a-d).

5.2.4. Isolation of CD4+CD25-IL-10+ cells from human peripheral blood

There was no difference in the suppressive activity of CD4+CD25+ T cells from grass allergic donors who had received allergen immunotherapy compared to grass allergic donors who had not. The decrease in proliferation and increase in IL-10 production by allergen stimulated CD4+CD25- cells isolated from immunotherapy donors however, raised the possibility that immunotherapy treatment induced or activated an IL-10-producing subset of T cells within this CD4+CD25- subset. To investigate whether such a population of cells could suppress Th2 responses, CD4+CD25- IL-10 producing cells were isolated from the peripheral blood of non-atopic donors (clinical details in Table 5.6), and their ability to suppress allergen specific Th2 responses tested.

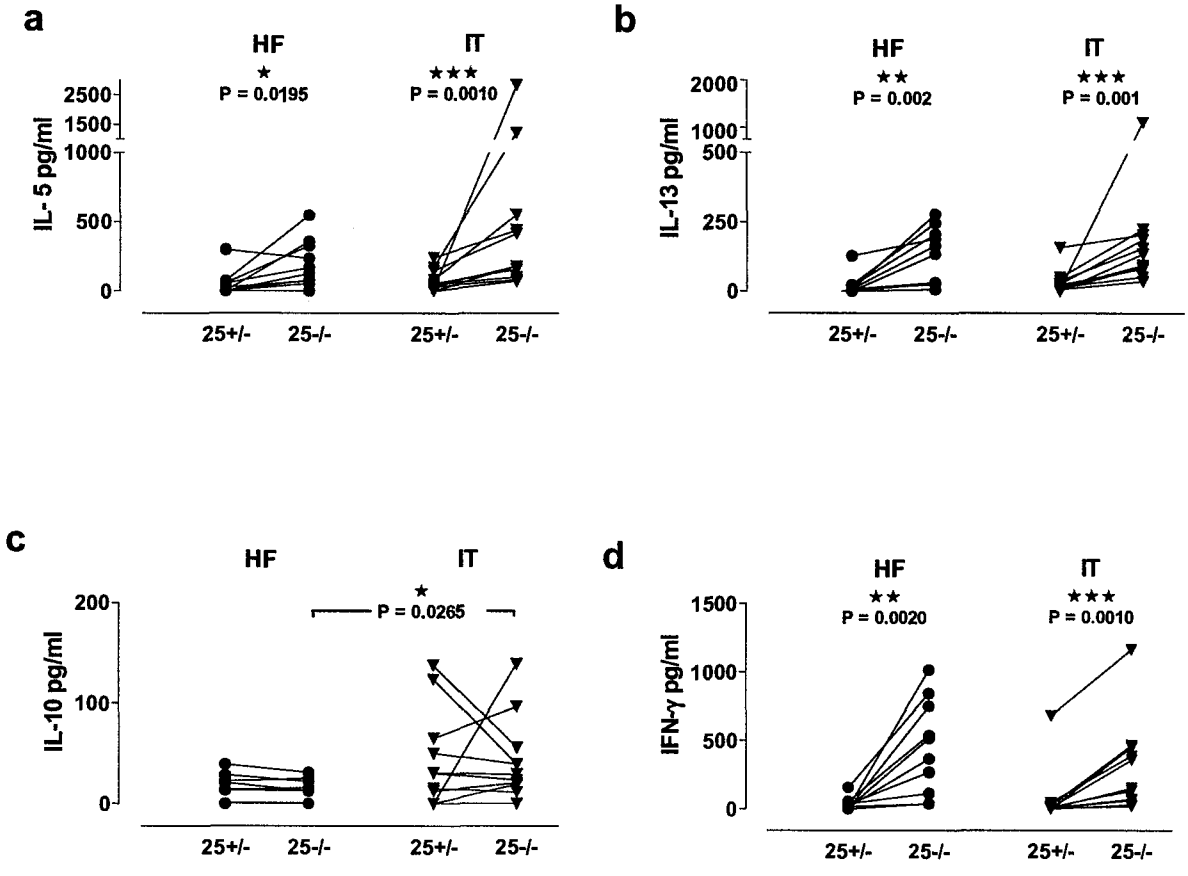


Figure 5.5. Cytokine production by grass pollen stimulated, mixed cultures of CD4+CD25- and CD4+CD25+ T cells from immunotherapy treated donors and untreated donors with hayfever. Cytokine production by CD4+CD25- and CD4+CD25+ T cells isolated from peripheral blood of immnuotherapy treated donors (IT, n = 11) and untreated donors with hayfever (HF, n = 10) in mixed cultures at a ratio of two CD25- cells to one CD25+ cell (CD25+/-) and CD4+CD25- cells alone (CD25-/-) stimulated *in vitro* with grass pollen allergen extract at 100 ug/ml for 6 days at 3 x 10^6 c/ml in the presence of irradiated PBMCs, measured by Luminex analysis. Concentration of IL-5 (a), IL-13 (b), IL-10 (c) and IFNγ (d) by individual mixed cultures from all donors.

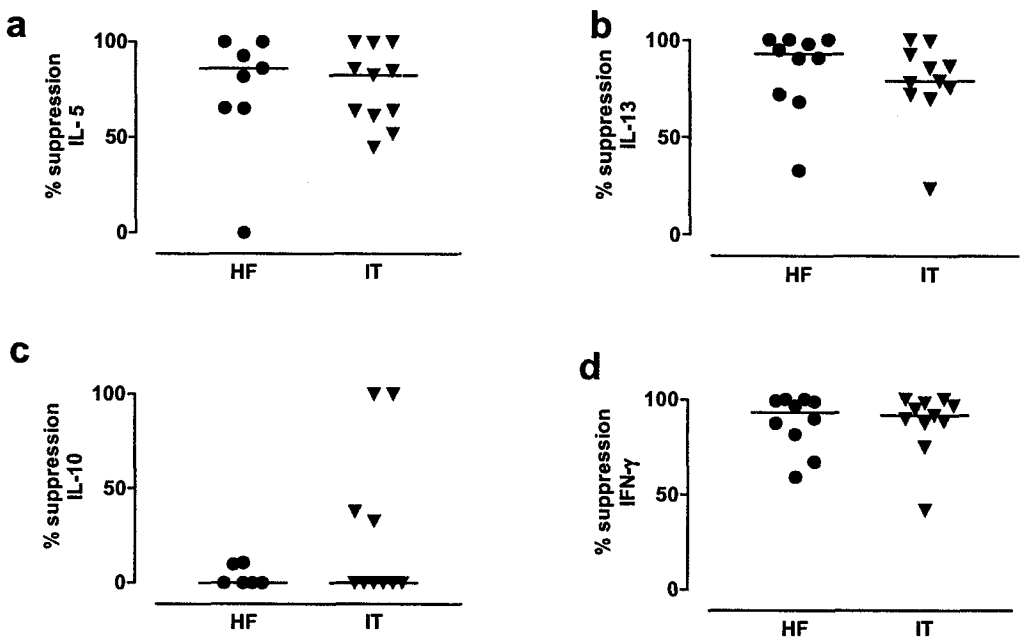


Figure 5.6. Percent suppression of grass pollen specific, *in vitro* T cell cytokine production by CD4+CD25+ cells from immunotherapy treated donors and untreated donors with hayfever. Cytokine production by CD4+CD25- and CD4+CD25+ cells isolated from the peripheral blood of immunotherapy treated donors (IT, n = 11) and untreated donors with hayfever (HF, n = 10) in mixed cultures at a ratio of two CD25- cells to one CD25+ cells and CD4+CD25- cells alone stimulated *in vitro* with grass pollen allergen extract at 100 ug/ml for 6 days at 3 x 10^6 c/ml in the presence of irradiated PBMCs, was measured by Luminex analysis. % suppression of grass pollen stimulated CD4+CD25- cell IL-5 (a), IL-13 (b), IL-10 (c) and IFNγ (d) production by CD4+CD25+ T cells is shown, a line represents the median value.

CD4+CD25- T cells from non-atopic donors were cultured overnight in media alone and the following day IL-10 secreting cells were isolated using an IL-10 secreting cell isolation kit from Miltenyi Biotech. The total number of CD4+CD25-IL10+ cells isolated ranged from 0.4 - 1.7 x 10^6 c/ml and was equivalent to 0.3 – 2.1% of the CD4+CD25- population analysed (Table 5.7).

The experiments using CD4+CD25-IL10+ cells in this thesis will describe a spontaneously occurring population, as there was no stimulation of the cells before isolation. CD4+CD25-IL10+ cells were also isolated from CD4+CD25- cells that had been stimulated *in vitro* for 12 hours with grass pollen allergen extract. Using this method there was no difference in numbers recovered, or suppressive ability to those isolated without stimulation with allergen (data not shown, n = 3).

5.2.5 Proliferation and cytokine production by CD4+CD25-IL-10+ and IL-10- cells stimulated in vitro with grass pollen allergen extract

CD4+CD25-IL10+ and CD4+CD25-IL10- cells were isolated from the peripheral blood of non-atopic donors and stimulated *in vitro* with grass pollen allergen extract, PPV or media for 6 days in the presence of autologous irradiated APCs. On day 6 proliferation of cells was measured by tritiated thymidine incorporation and culture supernatant was removed for cytokine analysis.

CD4+CD25-IL10- cells from non-atopic donors proliferated significantly more than CD4+CD25-IL10+ cells when stimulated with either grass pollen allergen extract (Fig. 5.7a) or recombinant PPV (Fig. 5.7b). Analysis of

Donor	Gender	Age	Grass pollen SPT wheal diameter (mm)	Allergic disease
1	M	42	0	N
2	M	27	0	N
3	F	23	0	N
4	F	46	0	N
5	F	49	0	N
6	F	42	0	N
Summary	2 = M 4 = F	42 (23 – 49)	0	6 = N 0 = Y

Table 5.6. Clinical details of non-atopic donors (CD4+CD25-IL10+ study). Gender; F = female, M = male. IgE levels measured by RAST test in IU/ml. Grass pollen specific skin prick test (SPT) result is the wheal diameter (mm). Allergic disease; Y = positive history of allergic disease, N = no history of allergic disease. Summary is median and range or summary of data.

Donor	Amount of blood taken (ml)	Total no. of PBMC ($x10^6$)	Total no. of CD4+ cells ($x10^6$)	Total no. of CD4+ CD25+ cells ($x10^6$)	Total no. of CD4+ CD25- cells ($x10^6$)	Total no. of CD4+ CD25- IL10+ cells ($x10^6$)	Total no. of CD4+ CD25- IL10- cells ($x10^6$)	% CD4+ CD25- IL10+cells recovered from the CD4+25- subset
1	150	340.2	107.0	1.7	55.9	0.7	42.8	1.3
2	150	180.9	73.2	2.0	73.5	0.6	51.8	0.8
3	150	290.2	203.8	2.1	117.9	0.4	24	0.3
4	150	403.3	315.5	3.1	155.7	0.4	46	0.3
5	150	598.4	201.6	6.4	92.8	1.7	35.4	1.8
6	150	379.5	117.6	2.3	46.8	1.0	28	2.1
Summary	150	359.9 (180.9 – 598.4)	159.6 (73.2 – 315.5)	2.2 (1.7 – 6.4)	83.15 (46.8 – 155.7)	0.65 (0.4 – 1.7)	39.1 (24 – 51.8)	1.05 (0.3 – 2.1)

Table 5.7. Recovery and purity of cells isolated from the peripheral blood of non-atopic donors (CD4+CD25-IL10+ study).
Total number of cells isolated was calculated by cell count using a haemocytometer. Purity of cells was calculated using flow cytometry after staining with fluorescent CD4 and CD25 antibodies and isotype-matched controls, see Fig. 3.4. Summary is median and range.

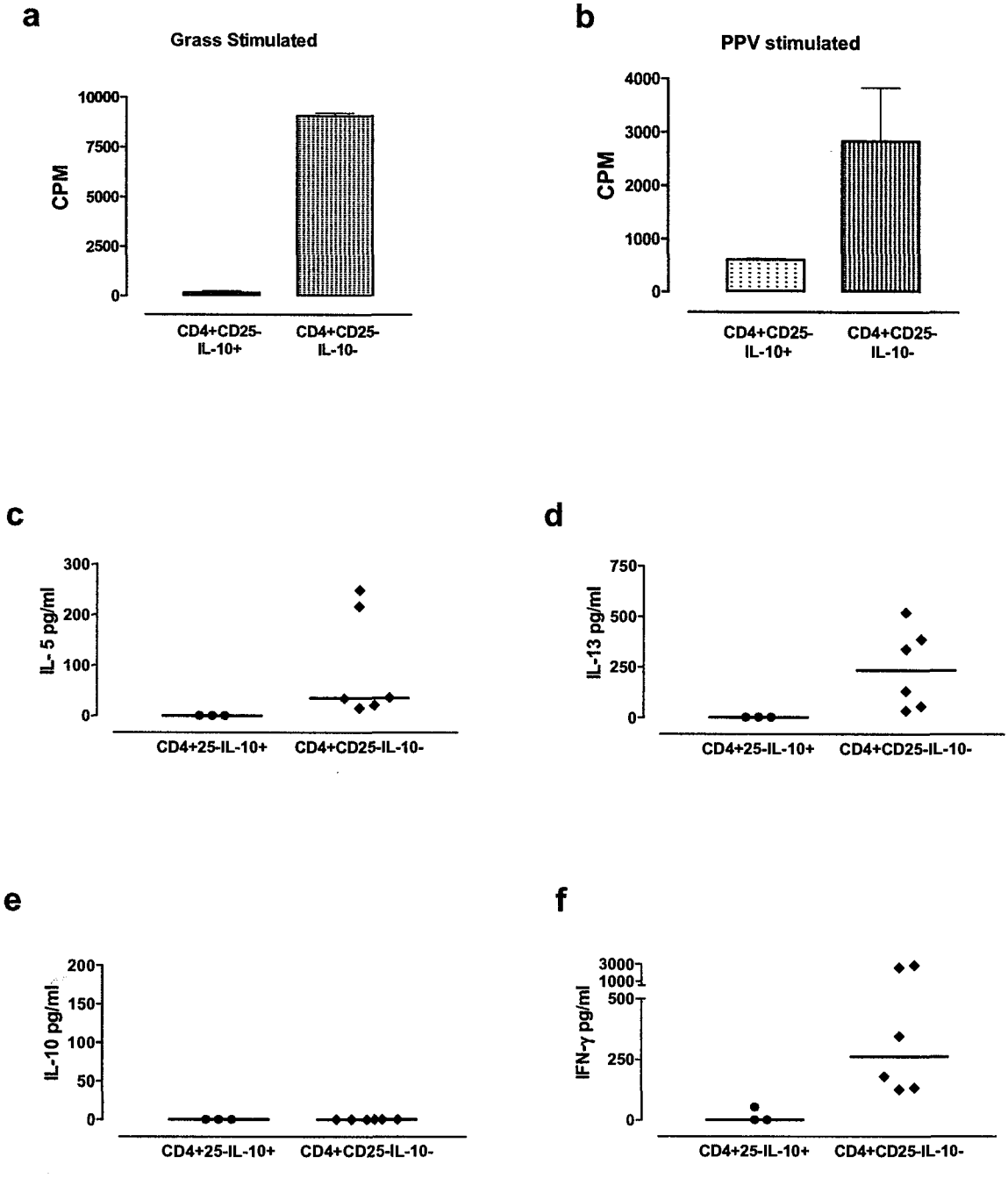


Figure 5.7. Proliferation and cytokine production by CD4+CD25-IL-10- and CD4+CD25-IL-10+ T cells from non-atopic donors in response to stimulation by grass pollen allergen extract or recombinant PPV *in vitro*. Isolated CD4+CD25-IL-10- and CD4+CD25-IL-10+ T cells from peripheral blood of non-atopic donors (n = 6) were stimulated *in vitro* with 100 ug/ml grass pollen allergen extract (a, c-e), 7.5 ug/ml purified recombinant PPV or media alone at 2 x 10^6 c/ml (b) for 6 days at 2 x 10^6 c/ml in the presence of irradiated PBMCs. Allergen specific proliferation (proliferation to allergen minus proliferation to media alone), was measured by incorporation of tritiated thymidine, representative proliferation results from one individual donor is shown (a + b), mean of triplicate cultures + SEM. IL-5 (c), IL-13 (d), IL-10 (e) and IFNγ (f) concentration was measured in day 6 culture supernatants by Luminex data from individual donors is shown, a line represents the median value.

cytokines revealed no detectable IL-5, IL-13, IFN-γ or even IL-10 present in the culture supernatant of grass pollen stimulated CD4+CD25-IL10+ cells (data not shown n = 4 A and 5 NA). CD4+CD25-IL10- cells did not produce any measurable cytokines when cultured with media alone for 6 days (data not shown). When stimulated with grass pollen extract however, they produced IL-5 (Fig. 5.7c), IL-13 (Fig. 5.7d) and IFN-γ (Fig. 5.7f). There was no detectable IL-10 in the supernatant of allergen-stimulated CD4+CD25-IL-10- cells (Fig 5.7e).

5.2.6. CD4+CD25-IL-10+ cells isolated from peripheral blood of non-atopic donors suppress proliferation of allergen stimulated CD4+CD25-IL-10- cells.

To investigate whether CD4+CD25-IL-10+ cells from non-atopic donors could suppress allergen specific T cell responses, these cells were isolated, added to autologous CD4+CD25-IL-10- cells at a ratio of 1:2 and stimulated with grass pollen allergen extract or medium alone, (for protocol, see Figure 5.8). Allergen-specific proliferation of the IL-10+/- culture was then compared to that of IL-10- cells alone (IL-10-/-) cultured under the same conditions and at the same cell density, $3x10^6$ c/ml. Figure 5.9 shows representative plots depicting that CD4+CD25-IL-10+ T cells can suppress the grass pollen allergen (a) and recombinant PPV (b) -specific proliferation by CD4+CD25-IL10- cells. Figure 5.9c shows allergen-specific proliferation of the mixed IL-10+/- and IL-10-/- cultures from all non-atopic donors. The suppressive ability of the CD4+CD25-IL-10+ cells was then calculated as percent suppression of proliferation and this was determined as described for the CD4+CD25+ experiments, see Chapter 3. CD4+CD25-IL10+ cells from all but one non-atopic donor were able to suppress

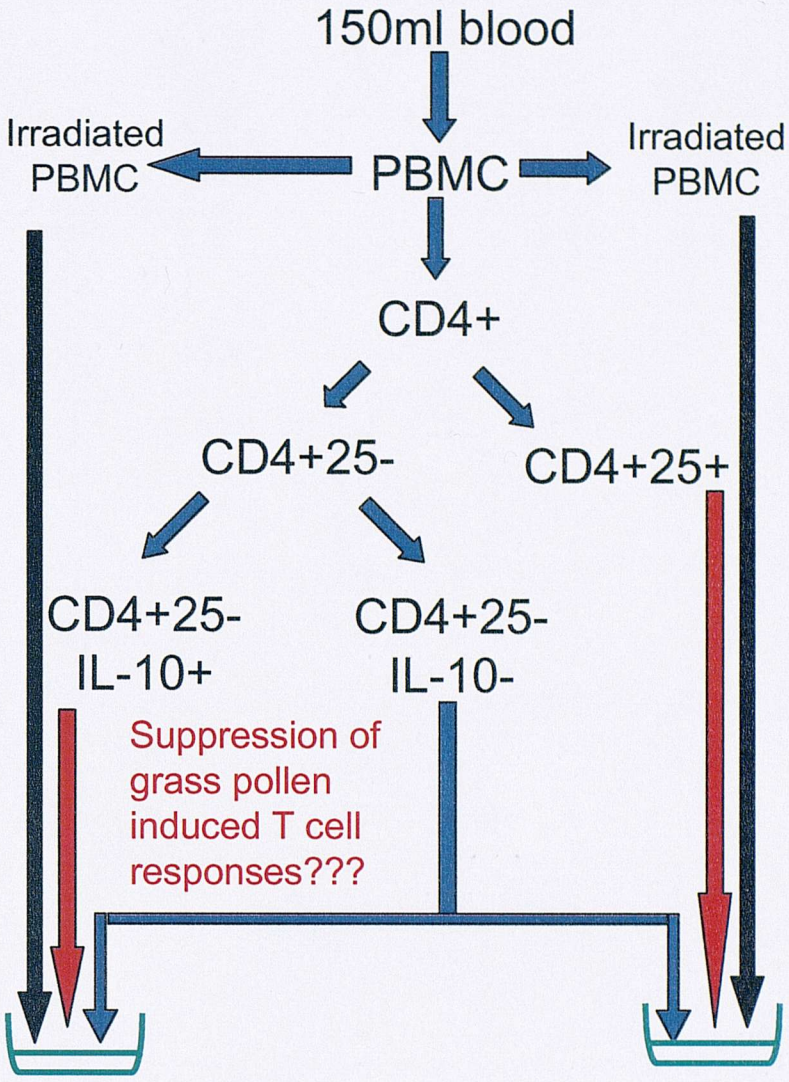


Figure 5.8. A schematic to show the assay used to investigate the ability of CD4+CD25-IL-10+ to suppress allergen driven T cell responses *in vitro*.

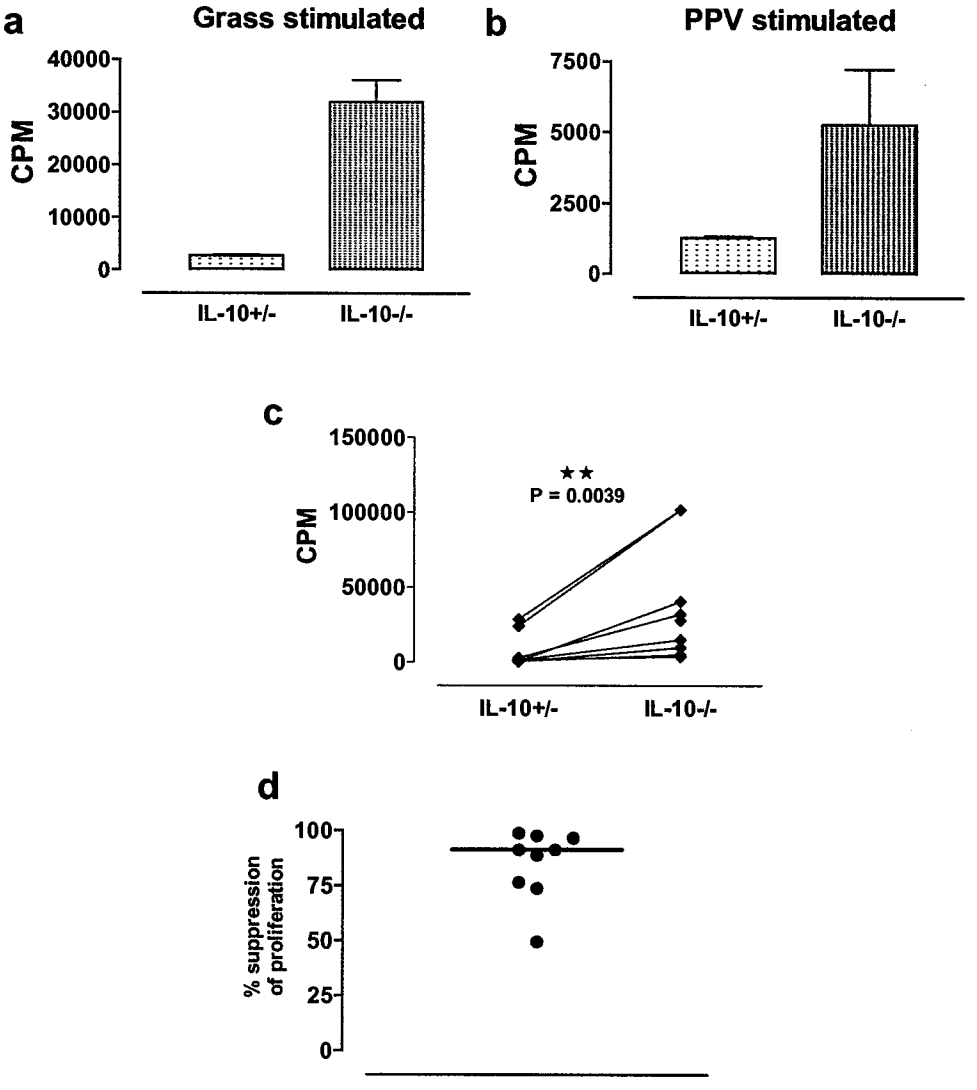


Figure 5.9. Suppression of grass pollen or recombinant PPV stimulated T cell proliferation by CD4+CD25-IL-10+ T cells from non-atopic donors. CD4+CD25-IL-10- and CD4+CD25-IL-10+ cells isolated from the peripheral blood of non-atopic donors (n = 9) in mixed cultures at a ratio of two IL-10- cells to one IL-10+ cell (IL-10+/-) and CD4+CD25-IL-10- cells alone (IL-10-/-) were stimulated *in vitro* with grass pollen allergen extract (a, c + d) or 7.5 ug/ml purified recombinant PPV (b) for 6 days at 3 x 10^6 c/ml in the presence of irradiated PBMCs. Allergen specific proliferation (prolfieration to allergen minus proliferation to media alone) was measured by incorporation of tritiated thymidine. Representative allergen specific proliferation results from one individual donor are shown (a + b), mean of triplicate cultures + SEM. Individual allergen specific proliferation results from mixed cultures of all donors (c), % suppression of CD4+CD25-IL-10- T cell allergen specific proliferation (d) by CD4+CD25-IL-10+ T cells from each individual donor, a line represents the median value.

the allergen-specific proliferation of the CD4+CD25-IL-10- subset by more than 70%, (Fig. 5.9d).

5.2.7. CD4+CD25-IL-10+ cells isolated from peripheral blood of non-atopic donors suppress in vitro cytokine production of allergen stimulated CD4+CD25-IL-10- cells.

By analysing the cytokines in the day 6 supernatants of the allergen stimulated mixed IL-10+/- and IL-10-/- cultures from non-atopic donors, it was established that there was a significantly lower concentration of the cytokines IL-5 (Fig. 5.10a) IL-13 (Fig. 5.10b) and IFN-γ (Fig. 5.10d) when CD4+CD25-IL-10+ cells were present. IL-10 was not detected in any of the IL-10-/- mixed cultures and only one of the IL-10+/- mixed cultures (Fig. 5.10c).

The degree of suppression of cytokine secretion was then calculated. CD4+CD25-IL-10+ cells from non-atopic donors suppressed allergen-driven CD4+CD25-IL-10- T cell IL-5 (Fig. 5.11a) and IL-13 (Fig. 5.11b) production by more than 80% by cells from all but one donor and IFN-γ production was suppressed by more than 65% in all cases, (Fig. 5.11c).

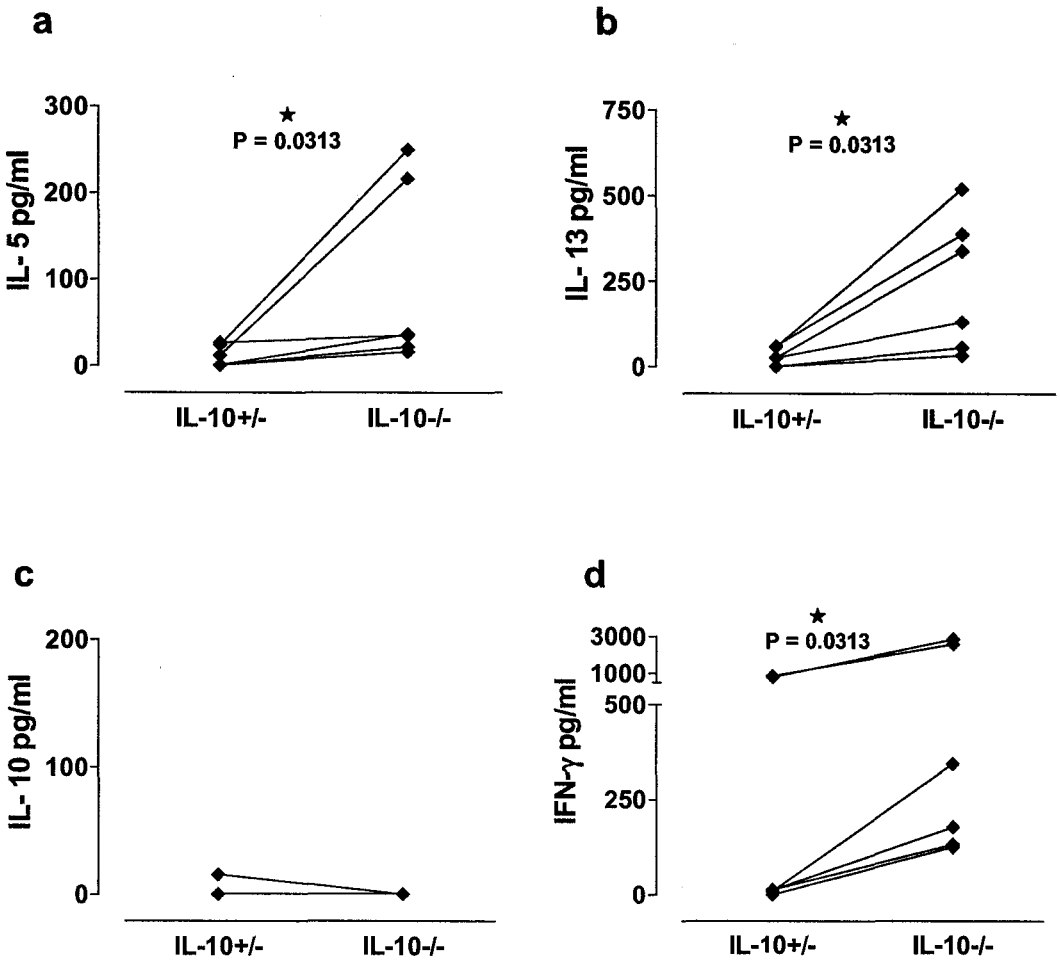


Figure 5.10. Cytokine production by grass pollen stimulated, mixed cultures of CD4+CD25-IL-10+ and IL-10- T cells from non-atopic donors. CD4+CD25-IL-10- and CD4+CD25-IL-10+ cells isolated from peripheral blood of non-atopic donors (n = 6) in mixed cultures at a ratio of two IL-10- cells to one IL-10+ cell (IL-10+/-) and CD4+CD25-IL-10- cells alone (IL-10-/-) were stimulated *in vitro* with 100 ug/ml grass pollen allergen extract for 6 days at 3 x 10^6 c/ml in the presence of irradiated PBMCs. Cytokine production was measured in day 6 culture supernatants by Luminex. IL-5 (a), IL-13 (b), IL-10 (c) and IFNγ (d) production from mixed cultures of cells from individual donors.

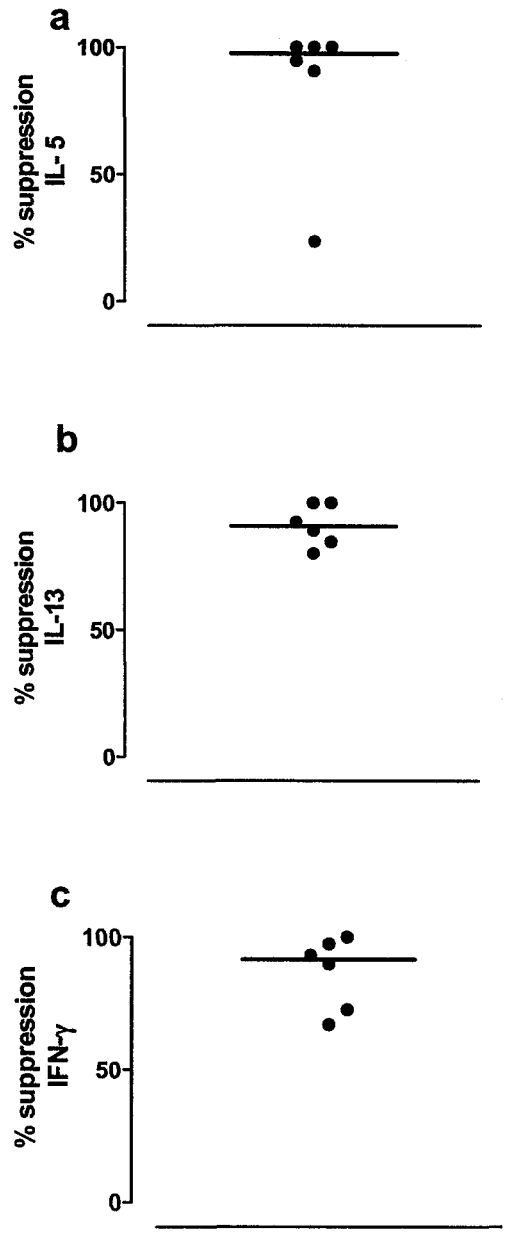


Figure 5.11. Suppression of grass pollen stimulated T cell cytokine production by CD4+CD25-IL-10+ T cells from non-atopic donors. CD4+CD25-IL-10- and CD4+CD25-IL-10+ cells isolated from the peripheral blood of non-atopic donors (n = 6) in mixed cultures at a ratio of two IL-10- cells to one IL-10+ cell (IL-10+/-) and CD4+CD25-IL-10- cells alone (IL-10-/-) were stimulated *in vitro* with 100 ug/ml grass pollen allergen extract for 6 days at 3 x 10^6 c/ml in the presence of irradiated PBMCs. Cytokine production was measured in day 6 culture supernatants by Luminex. % suppression of allergen stimulated CD4+CD25-IL10- IL-5 (a), IL-13 (b) and IFNγ (c) production by CD4+CD25-IL10+ cells, a line represents the median value.

5.2.8. CD4+CD25-IL-10+ cells from the peripheral blood of non-allergic donors are more effective at suppressing allergen specific T cell proliferation but not Th2 cytokine production than those from donors with hayfever.

To investigate whether IL-10-producing CD4+CD25- cells from non-atopic donors could suppress Th2 responses to allergen more effectively than those from donors with hayfever, blood was taken from both non-atopics and grass-allergic donors with symptoms of hayfever. The clinical details of the nonatopic donors and donors with hayfever used for this study are shown in Tables 5.6 and 5.8 respectively. There was no significant difference in numbers of cells recovered between non-atopic donors and donors with hayfever (Tables 5.7 and 5.9)

Isolated IL-10+ cells were added to autologous IL-10- cells and stimulated with grass pollen allergen extract, as described in section 5.2.6. As previously described, CD4+CD25-IL-10+ cells from non-atopic donors suppressed the allergen specific proliferation by CD4+CD25-IL-10- T cells (Fig.5.12a). The same population of cells from a donor with hayfever was not as effective at suppressing allergen-specific proliferation (Fig. 5.12b), results from representative donors are shown. When the percent suppression of proliferation was calculated, the difference between groups was striking (Fig 5.12c + d).

When cytokine levels in the supernatants of the mixed cultures were analysed by Luminex, very little IL-10 in the IL-10+/- or IL-10-/- cultures from both non-atopic donors and donors with hayfever were detected, (Fig. 5.13c). There was also no significant difference in the concentration of the Th2 cytokines IL-5 (Fig. 5.13a) and IL-13 (Fig. 5.13b) in the IL-10+/- or IL-10-/-

Donor	Gender	Age	IgE to grass pollen IU/ml	Total IgE IU/ml	Grass pollen SPT wheal diameter (mm)	Allergic disease
1	M	24	2.64	1007	5	Y
2	M	30	N/a	N/a	9	Y
3	M	29	N/a	N/a	20	Y
4	F	38	20	45	7	Y
5	F	N/a	33	N/a	8	Y
Summary	3 = M 2 = F	29.5 (24 – 38)	20 (2.64 – 33)	N/a	8 (5 – 20)	5 = Y 0 = N

Table 5.8. Clinical details of donors with hayfever (CD4+CD25-IL10+ study).
Gender; F = female, M = male. IgE levels measured by RAST test in IU/ml. Grass pollen specific skin prick test (SPT) is the wheal diameter measured in mm. Allergic disease; Y = positive history of allergic disease, N = no history of allergic disease. N/a is unavailable data. Summary is median and range or summary of data.

Donor	Amount of blood taken (ml)	Total no. of PBMC ($x10^6$)	Total no. of CD4+ cells ($x10^6$)	Total no. of CD4+ CD25+ cells ($x10^6$)	Total no. of CD4+ CD25- cells ($x10^6$)	Total no. of CD4+ CD25- IL10+ cells ($x10^6$)	Total no. of CD4+ CD25- IL10- cells ($x10^6$)	% CD4+ CD25- IL10+cells recovered from the CD4+25- subset
1	200	375.6	133.5	4.7	53.5	0.55	25	1.0
2	200	240	102	3.73	80	1.5	23.1	1.9
3	200	303.5	36.5	1.75	44	2.5	8.4	5.7
4	200	294	129	3.45	46	0.95	25	2.1
5	200	205.9	68.8	1.25	32.7	1	11.25	3.1
Summary	200	294 (205.9 – 375.6)	102 (36.5 – 133.5)	3.45 (1.25 – 4.7)	46 (32.7 – 80)	1 (0.55 – 2.5)	23.1 (11.25 – 25)	2.1 (1 – 5.7)

Table 5.9. Recovery and purity of cells isolated from the peripheral blood of donors with hayfever (CD4+CD25-IL10+ study).
Total number of cells isolated was calculated by cell count using a haemocytometer. Purity of cells was calculated using flow cytometry after staining with fluorescent CD4 and CD25 antibodies and isotype-matched controls, see Fig. 3.4. Summary is median and range.

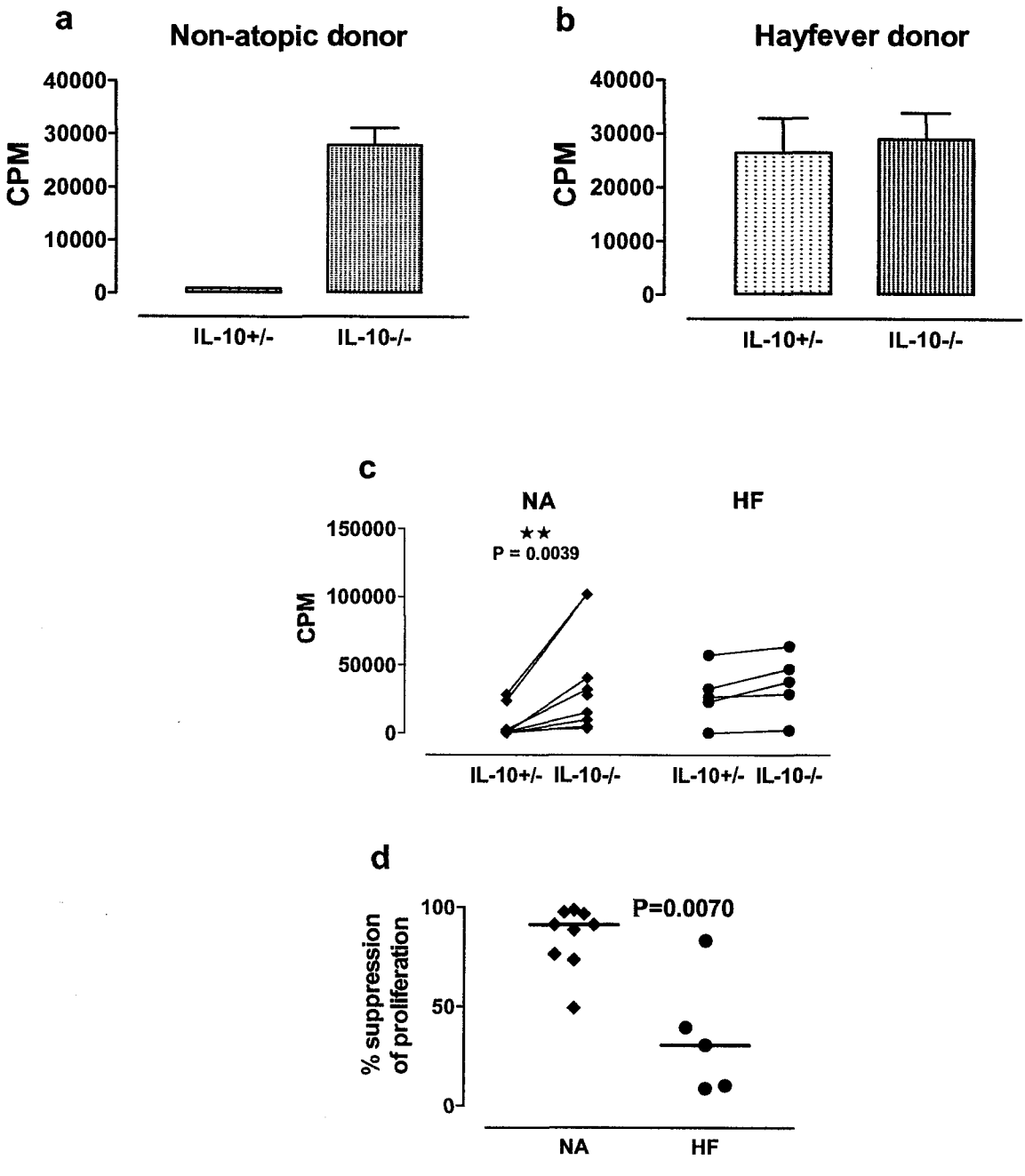


Figure 5.12. Suppression of grass pollen stimulated T cell proliferation by CD4+CD25-IL-10+ T cells from non-atopic donors and donors with hayfever. CD4+CD25-IL-10- and CD4+CD25-IL-10+ cells isolated from peripheral blood of non-atopic donors (NA, n = 9) and donors with hayfever (HF, n = 5) in mixed cultures at a ratio of two IL-10- cells to one IL-10+ cell (IL-10+/-) and CD4+CD25-IL-10- cells alone (IL-10-/-), were stimulated *in vitro* with 100 ug/ml grass pollen allergen extract for 6 days at 3 x 10^6 c/ml in the presence of irradiated PBMCs. Allergen specific proliferation (proliferation to allergen minus proliferation to media alone), was measured by incorporation of tritiated thymidine. Allergen specific proliferation of cells from an individual representative non-atopic (NA) donor (a) and a donor with hayfever (HF) (b). Allergen specific proliferation from mixed cultures (c), and % suppression of CD4+CD25-IL10- cell proliferation by CD4+CD25-IL-10+ cells from the same donors (d), a line represents the median value.

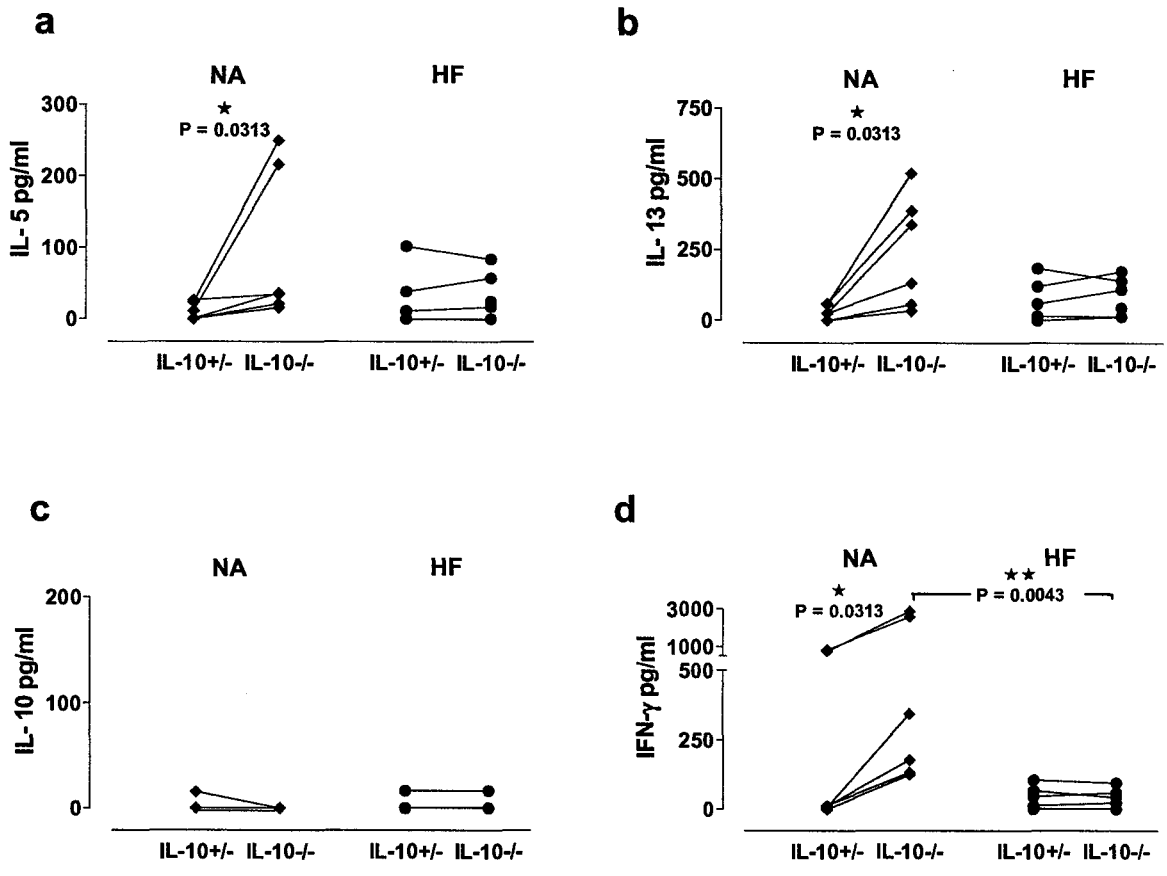


Figure 5.13. Cytokines present in the mixed IL-10+/IL-10- culture supernatants of cells from non-atopic donors and donors with hayfever. CD4+CD25-IL-10- and CD4+CD25-IL-10+ cells from the peripheral blood of non-atopic donors (NA, n = 6) and donors with hayfever (HF, n = 5) in mixed cultures at a ratio of two IL-10- cells to one IL-10+ cell (IL-10+/-) and CD4+CD25-IL-10- cells alone (IL-10-/-), were stimulated *in vitro* with 100 ug/ml grass pollen allergen extract for 6 days at 3 x 10^6 c/ml in the presence of irradiated PBMCs. Cytokines were measured from day 6 culture supernatants by Luminex. IL-5 (a), IL-13 (b), IL-10 (c) and IFNγ (d) concentrations in mixed cultures of cells.

cultures. There was however, significantly less IFN-γ present in the IL-10-/- culture of donors with hayfever compared to those from the non-atopic individuals (Fig. 5.13d). When percent suppression of allergen-specific T cell cytokine production by CD4+CD25-IL-10+ cells was calculated, no significant difference between suppression of IL-5 (Fig. 5.14a) or IL-13 (Fig. 5.14b) was found. However, the groups were small and there was a trend for greater suppression of IL-13 by cells from non-atopic donors compared to those from donors with hayfever, (P=0.0823). Suppression of IFN-γ was significantly greater by CD4+CD25-IL-10+ cells from non-allergic donors (Fig. 5.14c). However, this may reflect the fact that the IL-10-/- mix of cells from donors with hayfever contained very low levels of IFN-γ and therefore any inhibition will represent a high percent suppression value.

5.2.9. CD4+CD25-IL-10+ cells from allergen immunotherapy treated donors are more effective at suppressing allergen specific T cell proliferation but not Th2 cytokine production than untreated donors with hayfever.

A decrease in allergen-specific proliferation and an increase in IL-10 production by CD4+CD25- T cells from donors who had received allergen immunotherapy treatment, compared to untreated donors with hayfever has previously been described, see section 5.2.2. To investigate whether this difference was due to the activity of the IL-10 producing regulatory CD4+CD25- T cell subset, the ability of this population of cells from immunotherapy treated and untreated donors with hayfever, to inhibit allergen-specific T cell responses was compared. Clinical details of the donors with hayfever and those who had

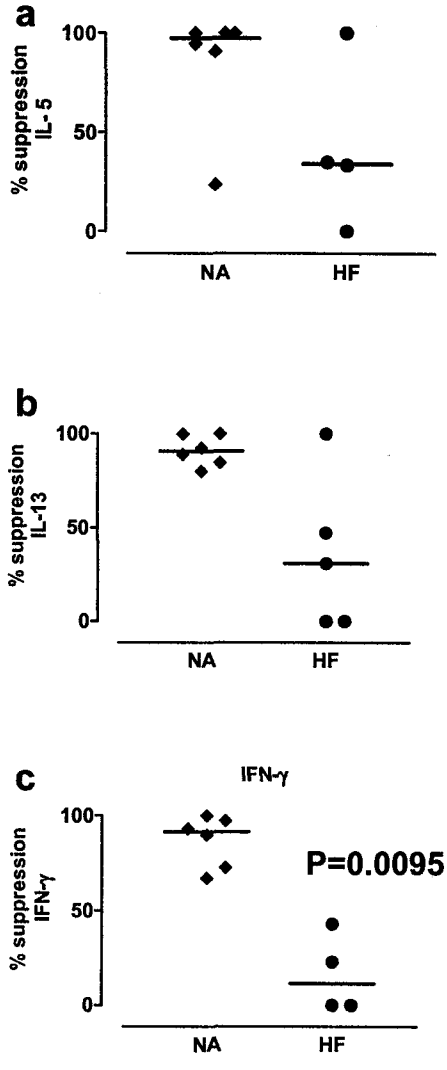


Figure 5.14. Suppression of grass pollen stimulated T cell cytokine production by CD4+CD25-IL-10+ T cells from non-atopic donors and donors with hayfever. CD4+CD25-IL-10- and CD4+CD25-IL-10+ cells isolated from the peripheral blood of non-atopic donors (NA, n = 6) and donors with hayfever (HF, n = 5) in mixed cultures at a ratio of two IL-10- cells to one IL-10+ cell (IL-10+/-) and CD4+CD25-IL-10- cells alone (IL-10-/-), were stimulated *in vitro* with 100 ug/ml grass pollen allergen extract for 6 days at 3 x 10^6 c/ml in the presence of irradiated PBMCs. Cytokines were measured from day 6 culture supernatants by Luminex. % suppression of allergen specific CD4+CD25-IL-10- IL-5 (a), IL-13 (b), and IFNγ (c) production by CD4+CD25-IL10+ cells, a line represents the median value.

received allergen immunotherapy treatment are shown in Tables 5.8 and 5.10 respectively. There was no significant difference between the two groups in the numbers of CD4+CD25-IL10+ cells recovered, (Tables 5.9 and 5.11)

As previously described, CD4+CD25-IL-10+ cells from a donor with hayfever did not significantly suppress allergen-specific proliferation by the CD4+CD25-IL-10- subset (Fig. 5.15a). The same subset of cells from a donor who had received immununotherapy however was able to suppress allergen specific T cell proliferation completely (Fig. 5.15b).

Although there was no significant difference in the proliferation of the mixed cultures between groups (Fig. 5.15c), there was a trend for less proliferation in the IL-10+/- cultures from donors who had received allergen immunotherapy treatment (P= 0.0932). When values of percent suppression of proliferation were calculated however, donors who had received immunotherapy treatment suppressed the allergen-specific proliferation of the CD4+CD25-IL-10- cells more effectively than those from donors with hayfever did (Fig. 5.15d).

Cytokines from cultures were then analysed by Luminex. There was no significant difference in the concentration of IL-5 (Fig 5.16a), IL-13 (Fig. 5.16b) or IFN-γ (Fig. 5.16d) in the supernatants from either the IL-10+/- or IL-10-/- cultures, between immunotherapy treated and untreated donors with hayfever. Moreover, there were very low (and often undetectable) levels of IL-10 in all samples (Fig. 5.16 c). When values for percent suppression of allergen-specific T cell cytokine production by CD4+CD25-IL10+ cells were calculated, no significant difference in the suppression of IL-5 (Fig. 5.17a) or IL-13 (Fig. 5.17b) between hayfever and allergen immunotherapy donors was found. The suppression of allergen-specific IFN-γ however was more effective by donors who had received allergen immunotherapy

treatment compared to those who had not, (Fig. 5.17 c). As mentioned previously however, the percent suppression of IFN-γ by CD4+CD25-IL-10+ cells from hayfever donors may be artificially low as there is very little IFN-γ present in the IL-10-/- mixed culture to suppress.

Donor	Gender	Age	IgE to grass pollen IU/ml	Total IgE IU/ml	Grass SPT wheal diameter (mm)	Allergic disease
1	M	39	12.9	N/a	9	Y
2	M	36	N/a	N/a	7	Y
3	M	45	4.53	N/a	12	Y
4	F	43	18.2	N/a	N/a	Y
5	F	45	0.35	68	10	Y
6	M	52	36.7	N/a	8	Y
7	M	38	N/a	N/a	5	Y
8	F	42	13.2	N/a	7	Y
Summary	5 = M 3 = F	42.5 (36 – 52)	13.05 (0.35 – 36.7)	N/a	8 (5 – 12)	8 = Y 0 = N

Table 5.10. Clinical details of grass allergic donors who had received immunotherapy treatment (CD4+CD25-IL10+ study).
Gender; F = female, M = male. IgE levels measured by RAST test in IU/ml. Grass pollen specific skin prick test (SPT) result is the wheal diameter (mm). Allergic disease; Y = positive history of allergic disease, N = no history of allergic disease. N/a is unavailable data. Summary is median and range or summary of data.

Donor	Amount of blood taken (ml)	Total no. of PBMC ($x10^6$)	Total no. of CD4+ cells ($x10^6$)	Total no. of CD4+ CD25+ cells ($x10^6$)	Total no. of CD4+ CD25- cells ($x10^6$)	Total no. of CD4+ CD25- IL10+ cells ($x10^6$)	Total no. of CD4+ CD25- IL10- cells ($x10^6$)	% CD4+ CD25- IL10+ cells recovered from the CD4+25- subset
1	200	424.8	93.5	2.8	74	0.95	27	1.3
2	200	415	146	4.4	77.5	0.8	58.5	1.0
3	200	142.8	29	1.1	32	2.15	9.45	6.7
4	200	126.5	75.35	2.15	36.15	2.1	16.8	5.8
5	150	397.5	55.5	3.2	38	0.6	17.8	1.6
6	200	360	59.4	2.35	38	0.45	38.8	1.2
7	200	298.8	89.1	3.75	26	0.7	24.6	2.7
8	200	243.8	117	4.15	67	0.55	31.6	0.8
Summary	200 (150 – 200)	329.4 (126.5 – 424.8)	82.23 (29 – 146)	3 (1.1 – 4.4)	38 (26 – 77.5)	0.75 (0.45 – 2.15)	25.8 (9.45 – 58.5)	1.45 (0.8 – 6.7)

Table 5.11. Recovery and purity of cells isolated from the peripheral blood of grass pollen allergic donors who had received allergen immunotherapy treatment (CD4+CD25-IL10+ study).
Total number of cells isolated was calculated by cell count using a haemocytometer. Purity of cells was calculated using flow cytometry after staining with fluorescent CD4 and CD25 antibodies and isotype-matched controls, see Fig. 3.4. Summary is median and range.

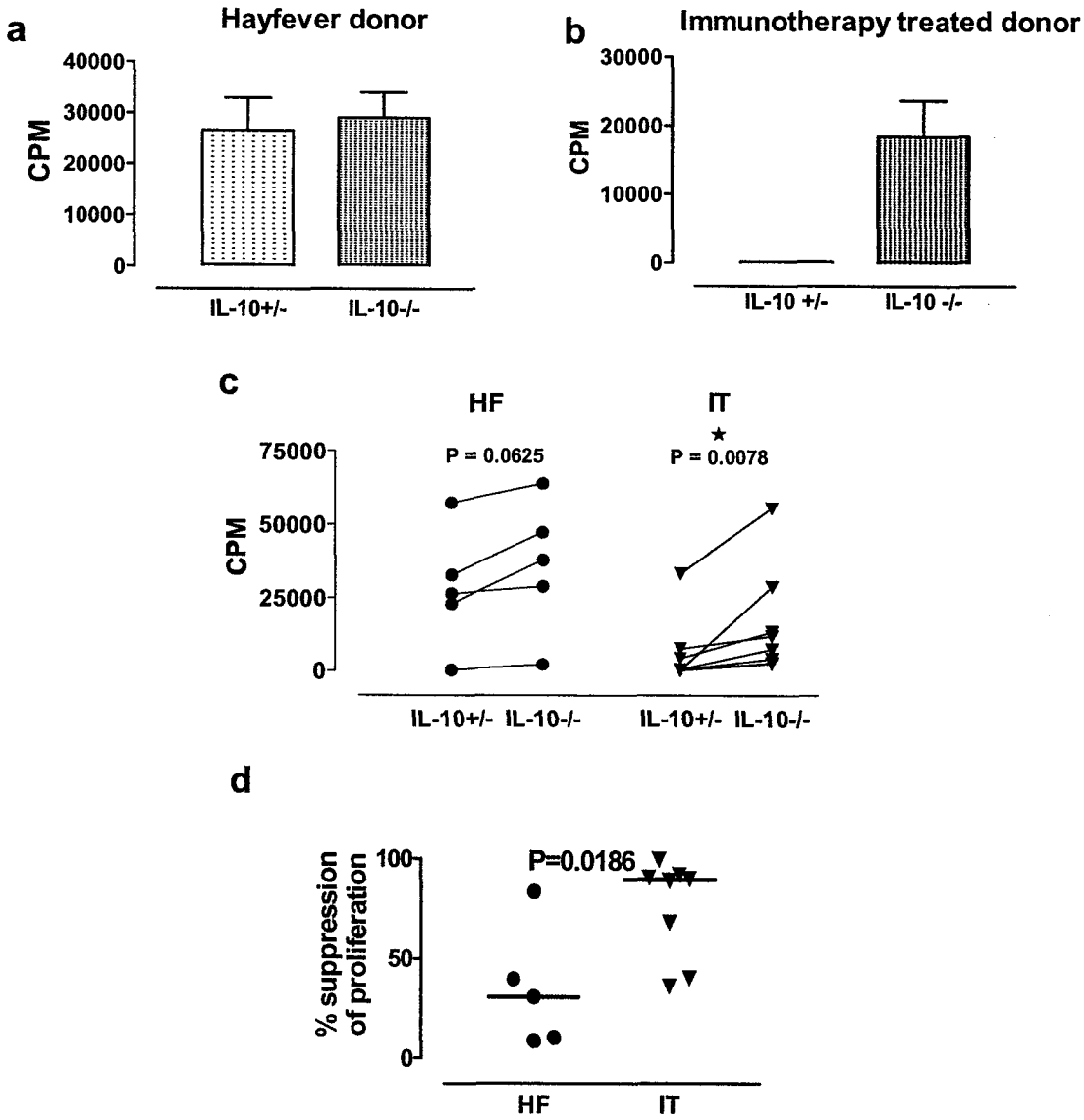


Figure 5.15. Suppression of grass pollen stimulated T cell proliferation by CD4+CD25-IL-10+ T cells from immunotherapy treated donors and untreated donors with hayfever. CD4+CD25-IL-10- and CD4+CD25-IL-10+ cells isolated from peripheral blood of immunotherapy treated donors (IT, n = 8) and donors with hayfever (HF, n = 5) in mixed cultures at a ratio of two IL-10- cells to one IL-10+ cell (IL-10+/-) and CD4+CD25-IL-10- cells alone (IL-10-/-), were stimulated *in vitro* with 100 ug/ml grass pollen allergen extract for 6 days at 3 x 10^6 c/ml in the presence of irradiated PBMCs. Allergen specific proliferation (prolfieration to allergen minus proliferation to media alone), was measured by incorporation of tritiated thymidine. Allergen specific proliferation of cells from an individual representative donor with hayfever (a), and a donor who has had allergen immunotherapy treatment (b). Allergen specific proliferation of mixed cultures from individual donors (c), and (d) % suppression of allergen specific CD4+CD25-IL-10- T cell proliferation by CD4+CD25-IL-10+ T cells from the same donors as (c), a line represents the median value.

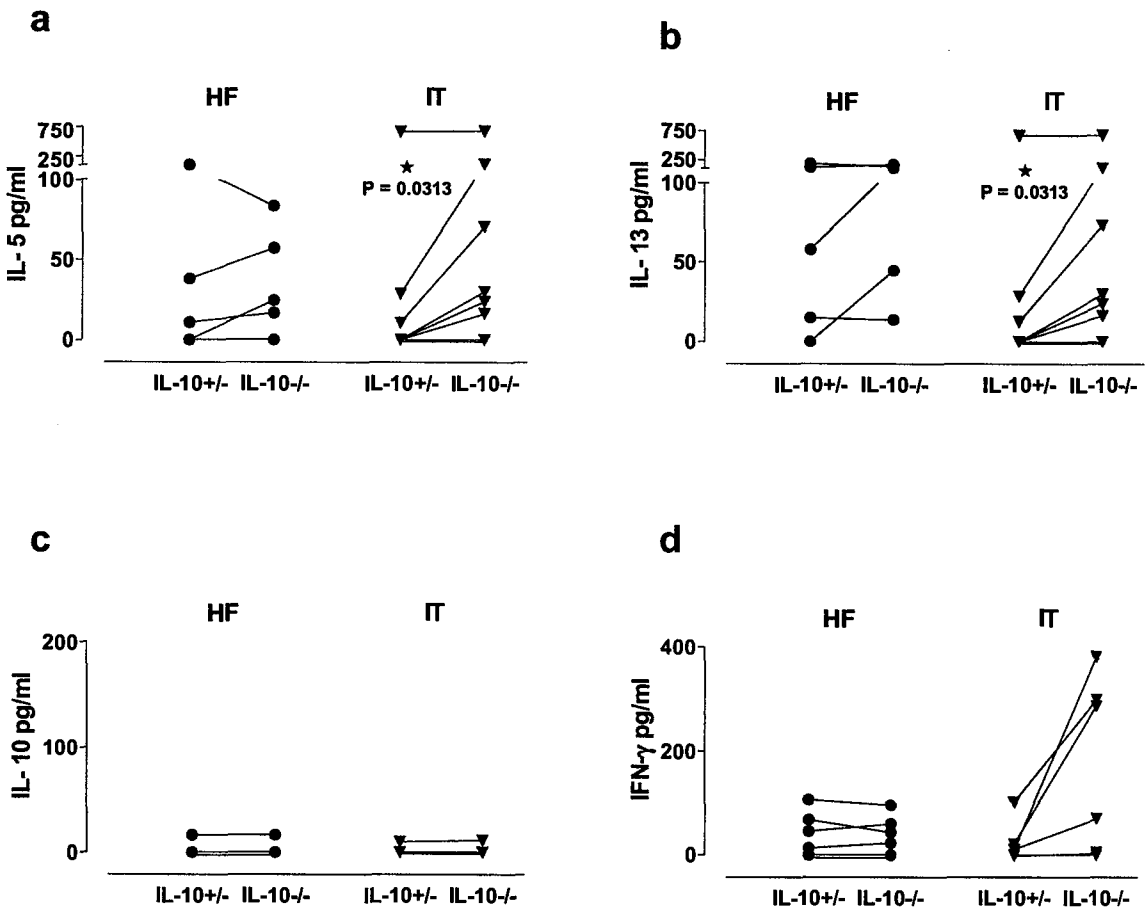


Figure 5.16. Cytokines present in the mixed IL-10+/IL-10- culture supernatants of immunotherapy treated donors and untreated donors with hayfever. CD4+CD25-IL-10- and CD4+CD25-IL-10+ cells isolated from the peripheral blood of immunotherapy treated donors (IT, n = 8) and untreated donors with hayfever (HF, n = 5) in mixed cultures at a ratio of two IL-10- cells to one IL-10+ cell (IL-10+/-) and CD4+CD25-IL-10- cells alone (IL-10-/-), were stimulated *in vitro* with 100 ug/ml grass pollen allergen extract for 6 days at 3 x 10^6 c/ml in the presence of irradiated PBMCs. Cytokines were measured from day 6 culture supernatants by Luminex. IL-5 (a), IL-13 (b), IL-10 (c) and IFNγ (d) concentrations in mixed cultures of cells from individual donors.

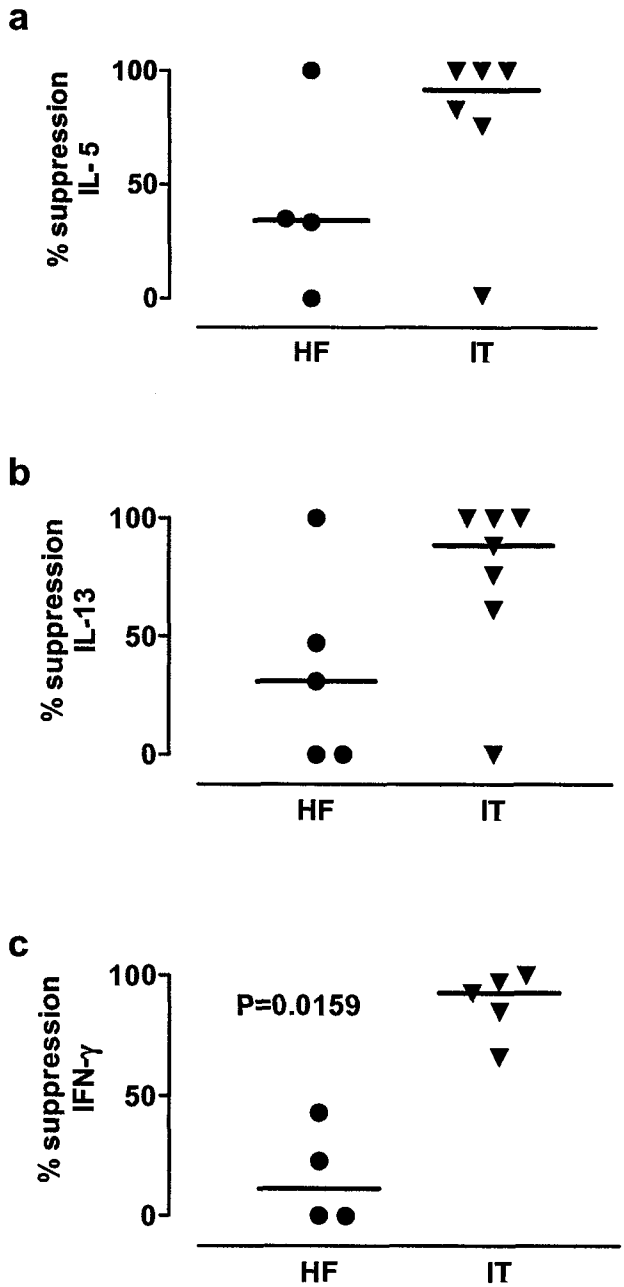


Figure 5.17. Suppression of grass pollen stimulated T cell cytokine production by CD4+CD25-IL-10+ T cells from immunotherapy treated and untreated donors with hayfever. CD4+CD25-IL-10- and CD4+CD25-IL-10+ cells isolated from peripheral blood of immunotherapy treated donors (IT, n = 8) and untreated donors with hayfever (HF, n = 5) in mixed cultures at a ratio of two IL-10- cells to one IL-10+ cell (IL-10+/-) and CD4+CD25-IL-10- cells alone (IL-10-/-), were stimulated *in vitro* with 100 ug/ml grass pollen allergen extract for 6 days at 3 x 10^6 c/ml in the presence of irradiated PBMCs. Cytokines were measured from day 6 culture supernatants by Luminex. % suppression of allergen stimulated CD4+CD25-IL-10- cell IL-5 (a), IL-13 (b), and IFNγ (c) production by CD4+CD25-IL10+ T cells, a line represents the median value.

5.3 Discussion

Although the effect of allergen immunotherapy treatment on PBMC responses to *in vitro* allergen stimulation has been previously described [174;387;394-396], to date there has been only one publication reporting the effect of this treatment on the CD4+CD25+ and CD4+CD25- T cell subsets [343]. In this chapter, the effect of grass pollen allergen immunotherapy treatment on CD4+CD25+-mediated suppression of allergen stimulated T cell responses has been described. The existence of a unique population of naturally occurring regulatory T cells in humans, which are CD4+CD25- and can be isolated due to spontaneous low-level production of IL-10, has also been demonstrated. Furthermore, these cells isolated from non-atopic donors and grass allergic donors treated with allergen immunotherapy were found to suppress Th2 responses to allergen more effectively than those from untreated donors with active allergic disease.

5.3.1. The effect of allergen immunotherapy treatment on PBMC and CD4+ T cell responses to allergen stimulation in vitro.

It has previously been reported that treatment for allergy to cat dander [176], house dust mite [177] and grass pollen [173] by allergen-specific immunotherapy is associated with decreased proliferation and increased IL-10 production by PBMCs stimulated with allergen *in vitro.* Data described in this chapter confirms this. However, this study also showed a significant increase in IL-10 production and a significant decrease in proliferation by allergen stimulated CD4+CD25- but not CD4+CD25+ cells from immunotherapy treated donors compared to control donors with active hayfever. This suggests that it may be the induction of a population of IL-10 producing cells within the CD4+CD25- subset, which suppress Th2 responses to

allergen and lead to a reduction in symptoms of allergic disease after allergen immunotherapy treatment. IL-10 producing regulatory T cells, derived under defined regimens of antigenic stimulation are involved in the control of autoimmune pathologies, such as EAE [171;268;401]. Most of the IL-10-producing cells, described to date are heterogeneous populations or clones [171;259;267;268;333;401;423-425], however they can be derived from mice lacking CD4+CD25+ regulatory T cells [171].

There was a slight decrease in the Th2 cytokine production (IL-5 and IL-13) by allergen stimulated PBMCs from immunotherapy treated donors compared to those from untreated control subjects, which was expected due to the increase in IL-10 production. Surprisingly, production of Th2 cytokines by the CD4+CD25- T cell subset from these donors was slightly increased. This may reflect the fact that CD4+ T cells isolated from untreated allergic donors with active allergic disease have been responding to grass pollen allergen *in vivo* and when they are re-stimulated with allergen *in vitro* they are "exhausted" and are unable to produce high concentrations of Th2 cytokines.

5.3.2. A comparison of the suppressive ability of CD4+CD25+ T cells from donors with active allergic disease and individuals who have received allergen immunotherapy treatment.

Jutel *et al* [343] have shown that after specific allergen immunotherapy treatment, CD4+CD25+ cells suppress allergen-specific proliferation of T cells more effectively than those isolated before treatment. This is in contrast to data in this chapter, which shows no difference in the ability of CD4+CD25+ T cells taken from donors treated with allergen immunotherapy compared to untreated controls, to suppress allergen stimulated T cell responses. There are several possible reasons for the contradictory results from these two studies: Firstly, Jutel *et al* investigated the

effect of specific immunotherapy to house dust mite, whilst the study described in this chapter focussed on grass pollen allergen immunotherapy treatment. Different allergenic antigens may cause different *in vitro* effects, especially because house dust mite allergen is ubiquitous and perennial whereas grass pollen is a seasonal allergen. Secondly, in the culture system used by Jutel and colleagues CD4+CD25+ or CD4+CD25- cells are added to allergen stimulated PBMCs to suppress or enhance proliferation and cytokine production. In contrast, the culture system used in this study investigated the direct suppression by CD4+CD25+ cells of the CD4+CD25- T cell population. Finally, the Jutel study sampled a much smaller population (5 donors) before and after house dust mite immunotherapy treatment whereas 11 treated donors and 11 untreated donors with active allergic disease were studied for this project. Furthermore, the results described in this chapter have been verified by a similar study using an identical culture system to investigate the suppression of cat allergen extract stimulated T cell responses by CD4+CD25+ T cells taken before and after peptide immunotherapy treatment for allergy to cats. This study has also shown that there is no difference between the suppressive ability of CD4+CD25+ T cells isolated before and after allergen immunotherapy treatment (Smith, TRF, *et al.* Allergy in press).

5.3.3. The existence of a novel population of regulatory T cells residing within the CD4+CD25- T cell subset.

The fact that immunotherapy treatment was found to be associated with increased IL-10 production and decreased proliferation by cells in the CD4+CD25- subset led to the hypothesis that there was a population of regulatory cells within the CD4+CD25- subset which was induced or expanded after allergen immunotherapy treatment. To demonstrate that such a population existed, CD4+CD25- T cells

spontaneously secreting IL-10 were isolated from the peripheral blood of non-atopic donors. These cells were similar to IL-10 producing regulatory T cells isolated after high dose allergen/peptide treatment in animal models as they were hyporesponsive to stimulation and could suppress Th2 responses *in vitro* [260;401]. This is the first description of such cells being isolated from human peripheral blood and clearly this novel population of regulatory T cells needs to be further characterised, in order to answer the following questions:

1) What markers do they express?

Results from this chapter have shown that this population of cells is CD3+, CD4+ and CD25-. Time permitting, further experiments would be carried out to determine whether these cells express other markers that have been described on regulatory cells such as GITR [233;235], CCR4 [326], FoxP3 [236;237;321] and CTLA-4 [52-54]. A recent paper illustrates that in contrast to CD4+CD25+ regulatory T cells, IL-10 producing regulatory T cells obtained using either *in vitro* or *in vivo* regimens of antigenic stimulation do not express FoxP3 [426].

2) What cytokines do they secrete?

When stimulated with allergen, the CD4+CD25- IL-10 producing T cells did not produce either the Th2 cytokines IL-5 and IL-13, or the Th1 cytokine IFN-γ. The fact that they produced very low levels, if any, of IL-10 was surprising considering that they were isolated on their ability to produce this cytokine. This may be a technical problem with the assay as the MACs cytokine secretion kit uses an anti-IL-10, PE-labelled antibody, which binds to the cell membrane and captures IL-10 as it is secreted by the cell. This enables IL-10 secreting cells to be captured on a magnetic

column after an incubation step with anti-PE microbeads. It may be that the anti-IL-10 capture antibody remains on the cell surface throughout the culture period and captures IL-10 secreted by the cells, therefore inhibiting its detection. Another possibility is that the anti-IL-10 capture antibody is cleaved from the cell surface and stays in the culture supernatant throughout the culture period, "mopping up" any IL-10 in the supernatant and causing it to be undetectable by the Luminex cytokine beads. Alternatively, these cells may produce IL-10 early in the culture system and it may be used up by day 6 when the supernatant is taken, (as is the case for CD4+CD25+ T cells - see Chapter 3). Ideally, a time course of cytokine production following stimulation with allergen extract should be established. The removal of supernatant at day 6 of allergen exposure is based on time course data from allergen stimulated CD4+CD25+ cells, at which time the levels of Th2 cytokines are at their highest. However, a time-course experiment with this novel cell population would be difficult due to the small numbers of cells recovered: typically 0.4 – 1.7 x 10^6 cells per donor, which is only enough for mixing experiments (Table 5.7). CFSE-labelling in combination with intracellular cytokine staining may be the only way to track the cytokine production and proliferation of this population of cells during the course of allergen stimulation *in vitro* as fewer cells are needed using this method. Recent TaqMan data from another laboratory (D Wraith, personal communication), has suggested that these cells are IL-10 mRNA positive and express IL-10 at low levels.

3) By what mechanism do these cells suppress T cell responses?

As has been discussed in detail in Chapter 3 there have been many cell contact dependent and independent mechanisms proposed by which CD4+CD25+ T cells may suppress T cell responses *in vivo* and *in vitro*. The mechanisms by which this

CD4+CD25- IL-10 producing subset can suppress T cell responses clearly need to be elucidated. IL-10 is an obvious candidate as this cytokine suppresses T cell responses *in vitro* and *in vivo* [377]. However, preliminary experiments using either blocking antibodies to IL-10, IL-10R or soluble IL-10 receptor have shown no role for IL-10 in the suppression of T cell responses *in vitro*, (Nguyen XD and Robinson DS personal communication). This is in agreement with the IL-10 independent suppression of T cells by IL-10 producing regulatory T cell populations previously described [171;426]. Although FoxP3 is associated with the suppressive activity of CD4+CD25+ cells [237;321], it has recently been shown that IL-10 producing regulatory T cells suppress CD4+CD25- T cell proliferation *in vitro* to a similar extent as CD4+CD25+ regulatory T cell despite the lack of this transcription factor [426]. This *in vitro* suppression was independent of IL-10 and was cell contact dependent [426]. The roles of CTLA-4, GITR and cell surface bound TGF-β in mediating suppression by this cell subset should therefore be investigated.

4) What other T cell responses can they suppress?

Results from this chapter have shown that this novel population of cells suppress T cell responses to both whole grass pollen allergen extract and recombinant Phl PV protein. IL-10 producing cells can also suppress T cell proliferation to PPD, MBP (Wraith DC personal communication) and to polyclonal stimulation with anti-CD3 coated beads [426]. It would be interesting to see whether these cells can also suppress T cell responses to other allergens, such as cat dander or house dust mite, or to non-self antigens during an MLR.

5.3.4. The effect of active allergic disease on the ability of CD4+CD25- IL10 producing cells to suppress allergen driven T cell responses.

The fact that IL-10 producing CD4+CD25- T cells from grass allergic donors with symptoms of hayfever are not as effective at suppressing allergen-induced T cell responses than those from non-atopic donors, mirrors results obtained using CD4+CD25+ T cells, (see Chapter 4). There may be several reasons for this difference in suppressive ability by CD4+CD25- IL-10 producing cells between atopic and non-atopic donors. Firstly, there may be more activated effector cells in either the responder or the regulatory population in individuals with active allergic disease. This explanation was ruled out in the CD4+CD25+ system by flow cytometry data, which indicated that neither the CD4+CD25+ nor the CD4+CD25- subset from atopic donors contained more activated (CD69) positive cells than those from non-atopic donors. The activation status of the CD4+CD25- IL-10+ cells also needs to be compared in atopic and non-atopic donors.

Experiments involving the CD4+CD25- IL-10 producing T cells described in this chapter were carried out during the height of the grass pollen season. It would be interesting to investigate whether, when isolated from atopic donors out of the grass pollen season, this population of cells is more effective at suppressing grass pollen driven T cell responses *in vitro*. If this is the case, is there still an inherent deficiency in suppression by these cells when compared to those from non-atopic donors as was noted with CD4+CD25+ regulatory cells? If the suppressive ability of these cells is impaired, it would be interesting to see whether this was only true for suppression of allergen driven responses to which the individual makes IgE. Can these cells suppress responses to other allergens to which the individual has no IgE response as effectively

as a non-atopic individual, and what about responses to other antigens such as PPD or polyclonal stimuli?

5.3.5. The association between allergen immunotherapy treatment and the suppression of allergen driven T cell responses by CD4+CD25- IL10+ cells.

Data from this chapter demonstrates that although allergen immunotherapy treatment is not associated with increased suppression by CD4+CD25+ T cells, it is associated with enhanced suppression by the CD4+CD25-IL-10+ subset. It seems that although both CD4+CD25+ and CD4+CD25-IL10+ cells from non-atopic donors can suppress Th2 responses, these populations of cells from donors with active allergic disease are unable to do so. After allergen immunotherapy treatment, the ability of the CD4+CD25-IL10+ subset to inhibit Th2 responses to allergen appears to be restored, (see Fig 5.18).

The fact that cells that produce IL-10 are associated with the protection afforded by immunotherapy treatement is not surprising, as many studies have reported that levels of this cytokine are increased after treatment [176-178]. The result illustrating that the enhancement of suppression is within the CD4+CD25- subset and not the CD4+CD25+ subset however is unexpected. This contradicts a study by Francis *et al* [402], which reported that production of IL-10 is increased only by CD4+CD25+ cells after allergen immunotherapy treatment and that no detectable IL-10 is produced by CD4+CD25- cells. The reason for this finding may be that, whereas I isolated naturally occurring IL-10 secreting cells from peripheral blood, Francis *et al* characterised the phenotype of the cells after stimulation with recombinant Phl PV. Stimulation with allergen will cause the cells to become activated and express CD25. It could be that the CD4+CD25+IL-10+ cells detected using this system were

CD4+CD25- before stimulation with Phl PV. Ideally it would have been possible to isolate IL-10 producing cells from both the CD4+CD25+ and CD4+CD25- cell subsets, however the small number of CD4+CD25+ cells recovered per donor made this experiment unpractical.

It remains to be seen whether the population of CD4+CD25-IL-10+ cells which is enhanced/expanded after immunotherapy treatment is the same as that isolated from non-atopic donors. It could be that immunotherapy treatment expands a separate lineage of IL-10 secreting cells to compensate for a deficiency in the population that is normally present in atopic donors. Alternatively, immunotherapy treatment could enhance the suppressive ability of the IL-10 producing cells, enabling them to inhibit the proliferation and cytokine production of excessively activated Th2 cells. It should also be noted that immunotherapy may well have an effect on the responder cells, making them less activated and therefore easier to suppress. Another cell type that should be considered is the APC. Immunotherapy may enhance signals from the APC to the T cell making the threshold for activation of allergen specific regulatory cells lower. Further work needs to be undertaken to investigate this possibility.

5.3.6. Conclusion

A more thorough understanding of the regulation of Th2 responses to allergen could lead to novel treatments for human allergic disease. Data from this chapter has shown that effective allergen immunotherapy treatment is not associated with an increase in the ability of CD4+CD25+ T cells to suppress Th2 responses. However, a separate novel population of IL-10 secreting T cells within the CD4+CD25- subset do suppress Th2 responses more effectively when isolated from atopic donors who have

received allergen immunotherapy treatment compared to those from untreated atopic control subjects. Manipulation of the balance between effector and regulatory T cell responses to allergen should be considered as a strategy to prevent or treat allergic disease.

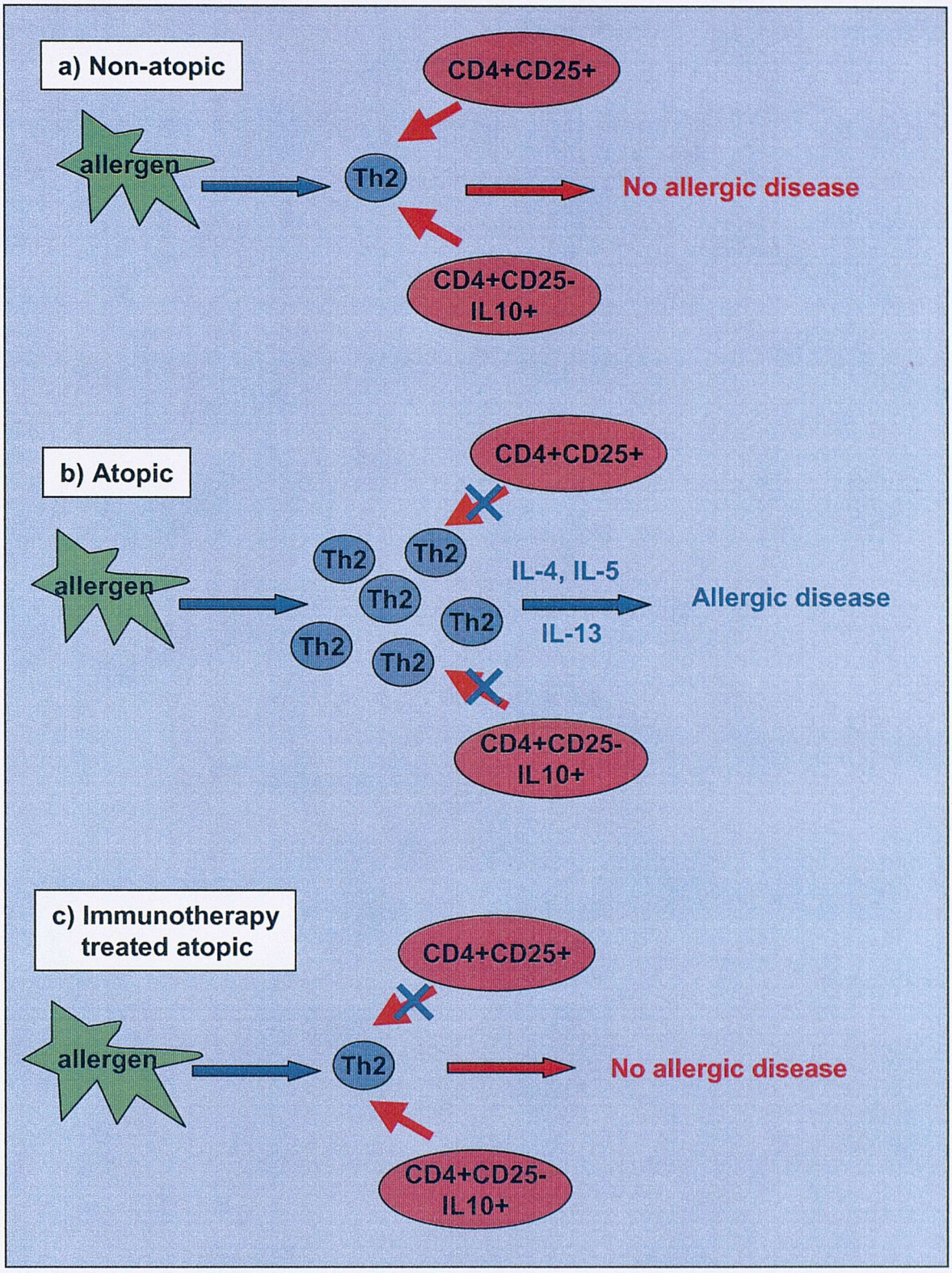


Figure 5.18. Schematic illustrating potential mechanisms of the suppression of Th2 cell responses to allergen in: a) a non atopic donor, b) an atopic donor and c) an allergen immunotherapy-treated atopic donor.

Chapter 6 General Discussion

Work from this thesis suggests a significant role for regulatory T cells in the suppression of human allergic disease. Over the past five years, interest in the field of regulatory T cells has grown considerably with many reports implicating their importance in the suppression of immune pathology in various disease models. Therapeutic manipulation of this population of cells could lead to novel treatments for many immune based disorders including atopic allergic disease. However, this is a relatively new field and further work is required to understand how regulatory T cells suppress allergen driven T cell responses, whether it would be possible to manipulate them for therapeutic benefit, and if so what effects, positive and negative any such treatment would cause. These considerations will be discussed in the following section.

6.1. The role of regulatory T cells in preventing the development of allergic sensitisation

Results from this thesis describe a role for human regulatory T cells in the suppression of Th2-type responses to common aero-allergens. Another question that needs to be addressed is whether these cells also play a role in the prevention of allergic sensitisation? The development of atopic allergic disease depends on both genetic and environmental factors, both of which may affect the development and/or function of regulatory T cells.

6.1.1. Do regulatory T cells fit in with the hygeine hypothesis?

The "hygiene hypothesis" suggests that improved hygiene in industrialised societies, together with improved public health measures and the use of vaccines and

antibiotics, have reduced the incidence of infections that stimulate the immune system and protect against the development of allergic disease[427]. It is suggested that *Mycobacterium tuberculosis*[428], hepatitis A [429] and measles infection[359] during childhood prevent the development of allergic disease in later life. Originally, the proposed mechanism for this hypothesis was that infections would cause the weakly Th2-biased [430] immune system of newborns to develop into more Th1-biased immune responses[431]. However, Th2 dominated immune responses such as helminth infections are associated with protection against the development of atopic disease[369;432] and the incidence of Th1 mediated autoimmune diseases has increased in parallel with atopic allergic disease[433 434], indicating that a lack of Th1 stimulating immune responses is not completely to blame.

The hygiene hypothesis has not been completely abandoned however, as microbial exposure has been found to have a huge impact on regulatory T cell development[435]. Germfree rodents for example, are unable to elicit tolerance to allergen[436] and are highly sensitive to arthritis induction compared with specific pathogen free (SPF) or conventional animals [437]. The hygiene hypothesis may therefore be explained by the fact that a lack of exposure to microbial antigen hampers the development of regulatory T cells and therefore regulation of both Th1 and Th2 responses.

The mechanism by which microbial exposure induces regulatory T cell development may include signalling through toll-like receptors (TLRs). Mycobacteria, which are present in soil, water and dust in a natural environment signal through pattern recognition receptors such as TLR2 and TLR4 present on host phagocytes and immune cells[438]. Activation of these receptors can aid the development of adaptive immunity, including generation of regulatory T cells.

Indeed, exposure to endotoxin has also been found to promote regulatory T cell survival and proliferation, and to enhance their suppressive functions [439].

Results from animal models support a role for regulatory T cells in the protection from atopic disease afforded by mycobacteria. For example, treatment of mice with heat-killed Mycobacterium vaccae during allergen immunisation reduced airway hyperresponsiveness, eosinophilia, IgE and production of Th2 cytokines. This down-regulation was dependent on production of IL-10 and TGF-β by regulatory T cells[331]. The relationship between mycobacterial infection and the development of allergy and asthma in humans is controversial. Although *M. tuberculosis* infection rates are significantly inversely correlated with the prevalence of atopic disease[440], whether bacillus Calmette-Gurin (BCG) vaccination is associated with protection against development of atopy is still a matter of debate[360;441;442].

If a lack of microbial infection hampers the development of regulatory T cells, why are atopic individuals not sensitised to many different allergens? It is possible that the microbial environment in which the individual is originally exposed to allergen may affect the development of allergen specific regulatory T cells. For example, if the APC presenting grass pollen allergen to a T cell has recently come in to contact with endotoxin and has therefore been stimulated through its TLR4, it may induce the development of grass-pollen specific regulatory T cells. In the same individual, if the presentation of cat allergen for the first time does not coincide with exposure to microbial infection, cat allergen-specific regulatory T cells will not develop and Th2 responses to subsequent cat allergen exposure will therefore develop unchecked.

6.1.2. Regulatory T cells and the "modified-Th2" hypothesis of allergen sensitisation

Another major environmental factor that effects the development of allergic sensitisation is exposure to allergen. It has been proposed that avoidance measures can be used to prevent sensitisation of individuals with negative skin test results or even from birth (ie, primary avoidance) [412-415]. It is not always possible to avoid exposure to allergen however. The major cat allergen, Fel d 1 for example, which is carried on dander particles, spreads throughout the community. Thus not having a cat in the home is not an effective measure to prevent sensitisation and indeed might have the opposite effect. The fact that families who chose not to own cats because of a family history of allergy had a higher prevalence of cat sensitisation than comparable families who had a cat [421], may also be related to regulatory T cell development. This observation may be explained by the fact that, although increasing exposure to allergen is associated with increased prevalence of sensitisation, exposure to high concentrations of cat dander is associated with decreased sensitisation and a higher prevalence of IgG antibody to Fel d 1[422]. A population of regulatory T cells may drive this modified T-helper-2 cell response. High dose antigen exposure has previously been shown in many models to induce regulatory T cells [260;401] or, as in the case of Chapter 5 of this thesis, to increase the suppressive ability of such a population.

6.1.3. Regulatory T cells and genetic susceptibility to atopic allergic disease

As well as the environmental factors already mentioned, genetic factors also play a role in the pathogenesis of allergic disease. For example, one study suggests that 73% of asthma susceptibility is due to genetic factors [443]. The genetic basis is likely to be highly complex however as atopic diseases are multi-factorial by nature. In fact atopy is linked to regions of several chromosomes including 5, 6, 11, 14 and

12. The chromosomal region 5q31 is particularly important as it contains genes for many cytokines and receptors including IL-12p40, IL-9 and the IL-4 cytokine cluster, containing the genes for IL-4, IL-5 and IL-13 [444]. Taking into account results from this thesis which suggest that regulatory T cells from non-atopics can suppress T cell responses to allergen more effectively than those from atopic donors, the relationship between genes associated with regulatory T cell development and/or function, with the development of atopic disease, should be investigated. Prime candidates would include IL-10, TGF-β, CTLA-4, GITR, FOXP3, IL-2 and IL-2R.

It is also possible that the susceptibility genes within the IL-4 cluster, which are mentioned above, may render the Th2 cells unable to be suppressed by regulatory T cells. The genetic defect would therefore lie within the effector T cell subset and not the regulatory population.

6.2. The generation of allergen specific (?) regulatory T cells in vivo.

6.2.1. Thymic vs peripheral development of regulatory T cells

CD4+CD25+ T cells are detectable in the periphery of normal mice from day 3 after birth. Thymectomy on day 3 substantially reduces peripheral CD4+CD25+ T cell numbers and results in autoimmune diseases similar to those induced by depletion of CD4+CD25+ T cells. The innoculation of CD4+CD25+ T cells from normal mice prevents the development of autoimmune disease in these models [206;342]. Such findings provide evidence that the thymus produces CD4+CD25+ T cells with the ability to suppress immune responses to self-antigen.

Our immune system comes into contact with many new non-pathogenic foreign antigens on a daily basis to which the production of an immune response is not necessary. Mucosal areas such as the gut and the lung are prime sites for such

interactions, but the majority of individuals do not mount unnecessary immune responses. This state of tolerance is also due in part to the induction of regulatory T cells [262]. Whether such T cells are generated in the thymus, as in the case of self-antigen specific regulatory T cells, or in the periphery is a matter of debate. As peripheral generation of regulatory T cells could lead to a novel therapeutic strategy for the treatment of both allergy and auto-immune diseases, more information on the "natural" generation of such cells *in vivo* would be advantageous.

Regulatory T cells can be induced by *in vivo* induction of oral tolerance (termed Th3 cells) [262] or they can be generated from peripheral blood T cells by *ex vivo* stimulation with antigens in the presence of particular cytokines (termed Tr1 cells) [258]. These two populations of regulatory T cells are however apparently functionally distinct from the naturally occurring CD4+CD25+ subset. Treatment of CD4+CD25- T cells with immature, cytokine (IL-10/TGF-β)-treated DCs or those expressing a Notch-ligand however induces CD4+CD25+ regulatory T cells with functional and phenotypic properties similar to naturally occurring CD4+CD25+ T cells[270;445;446]. How these artificially generated regulatory T cells relate to naturally occurring regulatory T cells in terms of phenotype and function is currently unclear.

A model recently suggested by Bluestone *et al* [447] explains the phenotypic, functional and developmental heterogeneity among regulatory T cells in terms of origin as well as specificity and effector mechanisms. They suggest that natural self-antigen specific regulatory T cells develop during thymic selection, where their regulatory fate is determined after a relatively strong TCR/MHC interaction with a self-peptide (Figure 6.1). These cells survive as a long-lived population in the periphery and recirculate through lymph nodes sustaining self-tolerance. Adaptive regulatory T cells however, develop in the periphery upon T cell activation under

particular conditions such as the presence of IL-10, immature DCs or TLR4 signalling. The origin of these adaptive regulators is uncertain; they may derive from naturally occurring thymically derived regulators, from naïve cells, activated effector memory cells or regulatory precurser cells (Figure 6.1). It is possible that there are different types of adaptive regulatory cells that have different origins.

A recent study by Huehn *et al* [448] suggests that the expression of the integrin $\alpha_E\beta_7$ can be used to differentiate between murine naturally occurring regulatory T cells, which do not express $\alpha_E\beta_7$, and adaptive regulatory T cells which do. Expression of $\alpha_E\beta_7$, correlated with a highly differentiated developmental state, according to T cell receptor excision circle (TREC) number and effector/memory markers. Furthermore L-selectin expression, which enables entry into lymph nodes, was high on CD25+ cells that did not express $\alpha_E\beta_7$ and low on those that did. In contrast, $\alpha_E\beta_7$ expressing cells bound more effectively to E- and P-selectin, suggesting that these cells extravasate into inflamed areas [449]. Finally, $\alpha_E\beta_7$ expressing CD25- regulatory T cells showed a significantly higher migratory response than CD25+ $\alpha_E\beta_7$ –negative regulatory T cells, towards the inflammatory chemokines CXCL9, CCL17 and CCL20, which bind to the chemokine receptors CXCR3, CCR4 and CCR6, respectively. Expression of $\alpha_E\beta_7$ at high levels is a hallmark of intraepithelial lymphocytes residing in the gut wall and other epithelial compartments such as skin and lung [450]. It is possible that $\alpha_E\beta_7$ expressing regulatory T cells either originate from, or migrate to, such epithelial sites in order to suppress local inflammation.

Results from Chapter 3 of this thesis illustrate that CD4+CD25+ T cells from the peripheral blood of non-atopic donors are able to suppress T cell responses to the common aero-allergens cat dander and grass pollen. Whether these cells are generated in the thymus or the periphery is as yet unknown. Their expression of CD25 and

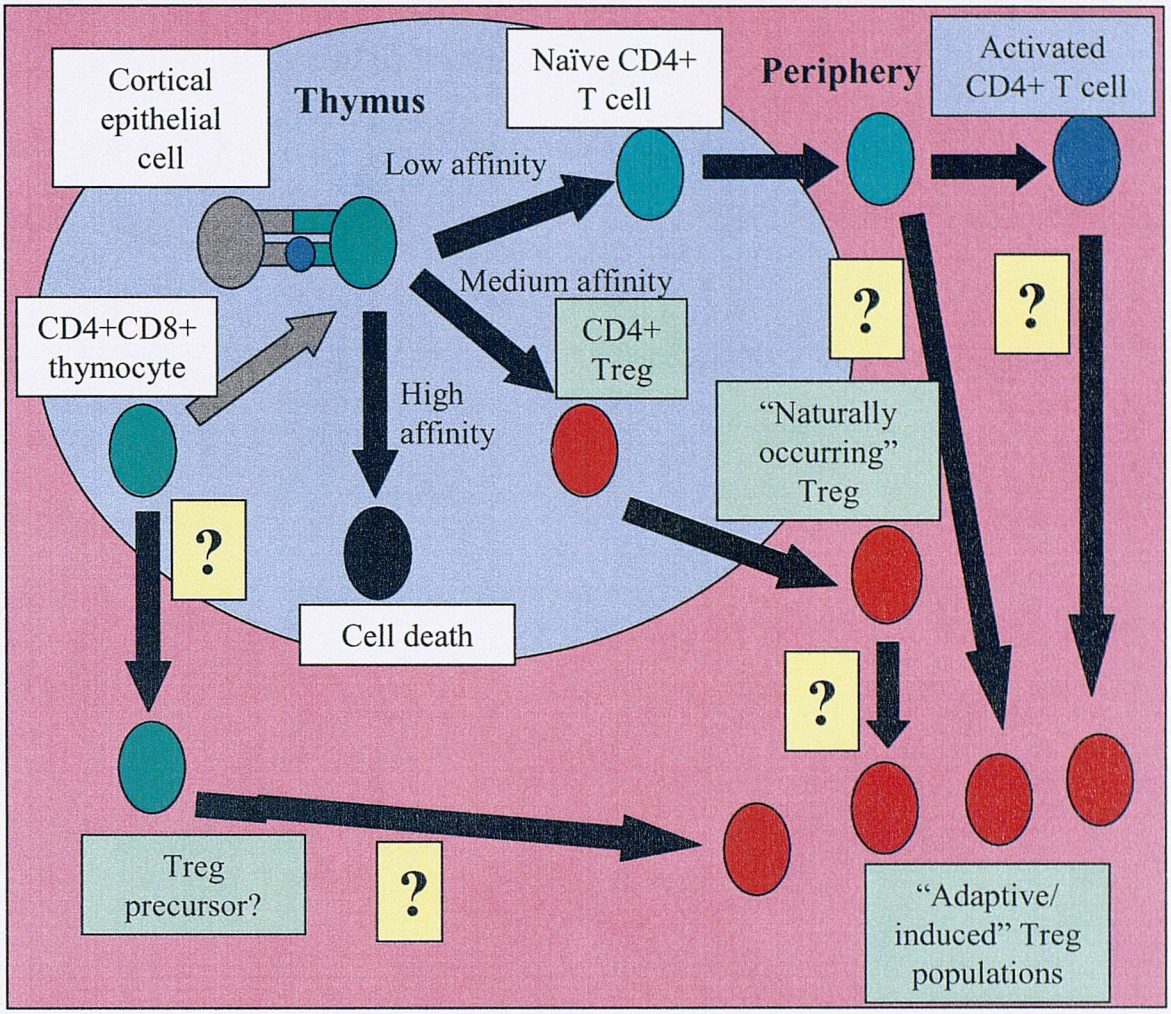


Figure 6.1. Induction of CD4+ regulatory T cell subsets in the thymus and the periphery. CD4+ "naturally occurring" regulatory T cells (Treg) are generated after a medium affinity interaction with the peptide MHC complex presented by cortical epithelial cells in the thymus. The origin of "adaptive" or "induced" CD4+ regulatory T cells is unknown; they may derive from "naturally occurring" thymically derived regulatory T cells, from naïve cells, activated effector memory cells or regulatory precurser cells.

FOXP3 however, suggests that they may be of similar lineage to those thymically derived self-antigen specific regulatory T cells. The IL-10 producing regulatory T cells described in Chapter 5 however, which were induced after allergen immunotherapy treatment of grass-allergic donors, are distinct from the naturally occurring CD4+CD25+ subset as they are CD25-FOXP3-. These may be adaptive regulatory T cells and their expression of $\alpha_E\beta_7$ should be investigated. Further work should be carried out to determine the integrin and chemokine receptor profile of both the regulatory T cell subsets described in this thesis. It would also be interesting to study the chemotactic responsiveness of these subsets and to ascertain whether they can be isolated from sites such as lung or skin.

6.2.2. Antigen specificity of regulatory T cells

It is important to determine whether both naturally occurring and adaptive regulatory T cells are specific to antigen or whether they suppress inflammatory responses in a general manner. Therapeutically, in the case of treatment for allergic disease, it would be beneficial to increase the suppressive ability of regulatory T cells specific to the allergen to which the individual makes IgE.

CD4+CD25+ T cells in normal mice are as diverse in the usage of TCR α- and β-chain genes as CD4+CD25- T cells [213;451]. Studies using transgenic mice strains in which every class II MHC molecule binds the same single peptide show that the single peptide/MHC can positively select functional CD4+CD25+ regulatory T cells with a TCR repertoire as broad as CD4+CD25- T cells. Strikingly, the regulatory CD4+CD25+ T cells generated in this system have higher avidity for this ligand compared with other T cells selected by the same ligand [452]. This high reactivity and

broad repertoire of CD25+CD4+ regulatory T cells ensures that they can dominantly control immune responses.

Alloantigen specific CD4+ T cells of both CD25+ and CD25- phenotype inhibit specific allograft rejection [453]. Similarly, TCR-transgenic mice with TCR specificity for myelin basic protein (MBP) Ac1-9 peptides on a RAG-/- background develop spontaneous EAE, and injection of normal spleen or thymocytes into such mice inhibits the induction of disease [454]. The CD4+ regulatory cells in this system are specific for MBP [455], providing further evidence that some populations of regulatory T cells are specific for self-antigen.

However, few of the models described allow the antigen specificity to be assessed and there are no reports as yet of antigen specificity of human regulatory T cells and in particular of the regulatory T cells described in this thesis which suppress T cell responses to allergen. Moreover when specificity is described, it refers to the sensitisation and not the exertion of suppression. Tolerance induced to soluble auto-antigens can "spread" to other autoantigens which are expressed by the same cell but which are devoid of any structural relationship with the initial antigen [456]. For example, tolerance induced by oral administration of MBP induces resistance to MBP-initiated EAE, but also induces tolerance to other myelin antigens such as proteolipid protein [457]. It could be that when allergen is presented to T cells, the suppression of Th2-type T cell responses depends on regulatory T cells, which are not specific for allergen itself but are scouring the area for evidence of potential autoimmune reactions. If these cells are present and activated, they will be able to suppress immune responses to other antigens or allergens non-specifically and this may be how the CD4+CD25+ regulatory T cells described in Chapter 3 suppress allergen driven T cell responses. Whether this CD4+CD25+ subset or the

CD4+CD25-IL10+ induced regulatory T cells described in Chapter 5 are allergen specific however is at present unknown and further work needs to be undertaken to clarify this.

6.3. The suppression of allergen driven T cell responses by regulatory T cells in vivo

6.3.1. Is this in vitro allergen-driven system indicative of what happens in vivo?

In vitro models of regulatory T cell suppression allow a precise dissection of cellular and molecular events that occur during the interaction between the regulatory T cell and the cell it is regulating. However, it is important to take into account the major differences that may occur between regulation *in vitro* and *in vivo*. The fact that inhibition of CD4+CD25- T cell proliferation *in vitro* does not require the presence of cytokines such as IL-10 or TGF-β, whereas the importance of such soluble mediators in animal models *in vivo* has been demonstrated extensively, is one example of such variation. *In vivo*, many other cell types will be present and interacting both through cell-cell interactions and the production of an array of soluble factors, so the fact that differences occur is not surprising.

One advantage of using an *in vitro* model is that it is possible to regulate the environment in which the suppression is taking place. It is possible to add exogenous factors and block cell surface or soluble molecules using specific antibodies. It is also possible to select and study regulatory and "regulatee" T cells, and APCs of a specific phenotype. The use of cells from genetically manipulated animals also allows the roles of particular genes to be analysed, and as has been previously mentioned, TCR-transgenic mice can give important information on the antigen-specificity of regulatory T cells.

Another reason why *in vitro* models of regulation are crucial is that it is currently not possible to analyse the suppression by human regulatory T cells *in vivo* and therefore any investigation using human cells must be carried out *in vitro*. Animal models have provided important information regarding the role of regulatory T cells in many models of disease, but the immune systems of mice and humans are markedly different. Furthermore, laboratory mice are kept in clean environments, so their T cells are mostly of a naïve phenotype. This may be immensely important when studying regulatory T cells as factors such as LPS and other microbial products are very important in driving the generation of regulatory T cells.

When studying regulatory T cells in human disease, as in this thesis, the use of an *in vitro* model allows a comparison of the suppressive ability of regulatory T cells between different patient groups to be made. Therefore, although we cannot be sure that human regulatory T cells suppress Th2 responses to allergen in this way *in vivo*, we can at least say that regulatory T cells from atopic individuals are not as effective at suppressing Th2-type T cell responses to allergen as those from non-atopic individuals.

Overall, it is important to realise that an *in vitro* model can never replicate what is happening during an immune response *in vivo*. However, information gathered from such studies can be used in conjunction with those gleaned from animal models to build up a broader picture of the role of regulatory T cells in human disease.

6.3.2. Do regulatory T cells suppress cells of the innate as well as those of the adaptive immune system

Regulatory T cells have been shown to have suppressive effects on both CD4+ and CD8+ T cells [458;459], and APCs [460]. However, there have been no reports of their suppression of innate cells such as mast cells, eosinophils or basophils, which play a major role in driving allergic disease. It would be interesting to investigate the effect of regulatory T cells on the allergen-induced mediator release by such granulocytes. If regulatory T cells could be isolated from tissue such as the lung it would also be interesting to analyse their effect on effector cells such as smooth muscle cells and fibroblasts during the course of allergic asthma.

6.3.3. Do other types of regulatory T cell play a role in the suppression of allergen driven T cell responses?

This thesis has concentrated on CD4+ regulatory T cells, but as has been outlined in Chapter 1, many other types of regulatory cell have been described. As yet there are few reports of the role of other phenotypes of regulatory T cells in the suppression of allergic disease. However, CD8+ T cells inhibit IgE responses and IL-4 production in a murine ovalbumin challenge model [149]. NKT cells and γδ T cells [384] may also have a role to play in the regulation of allergic disease

It would be interesting to see whether there are other subsets of regulatory T cells, apart from those that are CD4+, which suppress Th2 responses in the allergen-driven *in vitro* system set up in this thesis and if so, whether these cells isolated from atopic donors are as effective at suppressing such responses as those isolated from non-atopic donors.

6.4. Regulatory T cells – a novel treatment for allergic disease?

The role of regulatory T cells in the suppression of many murine models of immune mediated diseases has been well described. An absence of intrinsic regulatory T cells results in autoimmune disorders [203;334] and regulatory T cells also play a role in allograft acceptance and/or tolerance [453;461;462]. Furthermore, limited regulatory T cell depletion induces the rejection of immunogenic tumours without giving rise to life-threatening autoimmune disease [294;295]. Results from this thesis suggest that human regulatory T cells also play a significant role in the suppression of Th2 responses to allergen in non-atopic individuals and that atopic disease may occur as a result of deficient suppression of Th2-type responses to allergen by regulatory T cells. Increasing suppression of Th2 responses to allergen by regulatory T cells may therefore represent a novel therapeutic strategy for the prevention and/or treatment of atopic allergic disease.

6.4.1. The role of regulatory T cells in current therapies for the treatment of allergic disease.

Many of the current treatments for allergic disease function by suppressing immune responses in a general manner. Recent studies, including work presented in this thesis, suggest that some of these treatments, including corticosteroids and specific allergen immunotherapy may work by increasing regulatory T cell activity. Further work needs to be undertaken to investigate whether the suppressive effects of such treatments can be enhanced or more specifically targeted to the inhibition of inflammation to the allergen in question.

Corticosteroids

To suppress allergic inflammation, topical glucocorticoids given systemically are efficacious in the treatment of many allergic diseases. Glucocorticoids suppress inflammation and increase IL-10 production both *in vivo* and *in vitro* by a process that may involve regulatory T cells [171]. More research on the *in vivo* induction of regulatory T cells may lead to new methods for inducing more effective regulatory T cells during treatment of allergic disease. Data from our lab has shown that pretreatment of CD4+CD25+ regulatory T cells with fluticasone propionate increases the ability of CD4+CD25+ regulatory T cells from atopic donors to suppress T cell responses (Nguyen and Robinson JACI in press). IL-10 producing regulatory T cells, which prevent IgE and Th2 cell expansion in a mouse model of allergic disease can also be generated by activation in the presence of IL-10 [260], and a different IL-10-secreting population is induced by a combination of dexamethasone and Vitamin D3 [171]. It may be possible to induce regulatory T cells or increase their suppressive ability by *in vivo* allergen exposure in the face of tailor-made immunosuppressive agents. These induced cells would then suppress allergen driven T cell responses in place of the defective or ineffective CD4+CD25+ T cell population.

Allergen immunotherapy treatment

The role of regulatory T cells in the protection afforded by allergen immunotherapy treatment is described in detail in Chapter 5. The results showed that although allergen immunotherapy treatment was not associated with an increase in the allergen-specific suppressive capacity of CD4+CD25+ regulatory T cells, a population of CD25- T cells which were separated on the basis of IL-10 production did suppress more effectively after treatment. More work is needed to characterise

this novel regulatory T cell population. The phenotype of these cells needs to be analysed, as does their mechanism of suppression. The antigen specificity of these cells and their lineage is also unknown and although it has been shown that they suppress T cell proliferation and cytokine production, their suppressive effects on other cell types has not been determined. It would be interesting to investigate whether they migrate to target organs such as the lung, nose and skin and, if so, how they get there. The numbers of such cells at target organs and their ability to suppress allergen driven T cell responses should also be compared between atopic donors who have and have not received allergen immunotherapy treatment.

It may be that this population is similar to IL-10 producing regulatory T cells induced in animal models after nasal or oral delivery of protein or peptide [401 463]. If so, it may be possible to develop more efficient ways of inducing this regulatory T cell population during immunotherapy treatment of human allergic disease. Perhaps immunotherapy treatment could be used in conjunction with IL-10 or TGF-β treatment, or with glucocorticoids, which as mentioned above have been shown to induce regulatory T cell development. Other approaches could include combining immunotherapy treatment with adjuvants such as TLR-stimulating CPG oligonucleotides, or mycobacterial products, both of which have been shown to induce the development of regulatory T cells. Increased co-stimulation through ICOS or CTLA-4 could also increase regulatory T cell numbers, as could increasing the expression of the transcription factor FOXP3. If phenotypic analysis the CD4+CD25-IL10+ population of cells shows enhanced migratory capacity to certain chemokines, as in the case of the $\alpha_E\beta_7$ expressing CD25- T cells, perhaps topical application of chemokines to areas of inflammation during treatment could increase migration of these cells to sites of inflammation. Representative animal models of immunotherapy

treatment need to be set up in order to further investigate the role of regulatory T cells in the mechanism of allergen immunotherapy treatment. These models can then be used to assess novel approaches to treatment and the possibility of combination therapy.

This project has focussed on the suppression by CD4+ regulatory T cells. Further work should also be carried out to establish whether any other regulatory T cell populations (CD8+, γδ, NKT cells) have a role to play in the protection afforded by allergen immunotherapy treatment.

6.4.2. Utilising regulatory T cells - potential new strategies for the treatment of atopic allergic disease?

Induction of regulatory T cells ex vivo.

In the previous section, *in vivo* induction of regulatory T cells was suggested as a method that could be used in conjuction with other therapies, to increase the efficacy of such treatment. It is also possible that such cells could be generated *ex vivo* from peripheral blood, using such mechanisms. The precise phenotype of regulatory T cells could then be determined, and it may be feasible to transfer these autologous cells back to the patient. The antigen specificity of such cells would need to be determined in advance, but even if the cells were not specific to the relevant antigen, because of bystander suppression, inflammation may be inhibited anyway. This could however provoke safety concerns, as the transferred or induced regulatory T cells may also suppress T cell responses to pathogens or cancer antigens, which may cause the patient to become susceptible to such diseases. The effects of this treatment should therefore be investigated extensively in animal models of allergic disease. If

successful, this treatment may also be applicable for the suppression of other inflammatory diseases such as autoimmune disease and transplant rejection.

Increasing the suppressive capacity of regulatory T cells

Results from this thesis have shown that regulatory T cells from atopic donors have a reduced capacity to suppress allergen specific T cell responses. Future work should involve comparing the cellular and molecular phenotype of regulatory T cells from atopic and non-atopic donors to try to discover any defect in this cell type in atopic individuals. This could be accomplished by using techniques such as comparative microarray analysis or phage display technology.

If either a defect in regulatory T cells from atopic donors or the mechanism by which regulatory T cells from non-atopic donors suppress allergen driven T cell responses are conclusively elucidated, it may be possible to enhance regulatory T cell suppression. This could be achieved by inducing the expression of the molecules involved in suppression, be they cytokines or cell surface receptors. TGF-β for example has been found to increase the expression of FOXP3 [464]. Another potential mechanism may include enhancing the expression of co-stimulatory ligands such as ICOSL on APCs, or enhancing signals directly to the T cell by treatment with activating antibodies. Stimulation with CTLA-4 for example has been shown to increase the suppressive ability of regulatory T cells *in vitro* [246]. Blocking the interactions between activating co-stimulatory molecules using antibody therapy may also lead to an increased suppressive phenotype of regulatory T cells. Such reagents are available and should be tested in the *in vitro* allergen model described in this thesis to attempt to increase the ability of CD4+CD25+ regulatory T cells from atopic donors to suppress allergen driven T cell responses.

Studies which have shown increases in the suppressive ability of regulatory T cells, such as steroid treatment, (Nguyen and Robinson JACI, in press) should now focus on how this treatment has modified the cells. Microarray analysis could be carried out pre- and post-treatment of the cells to ascertain which genes are up- or down-regulated after treatment. These genes should then be analysed further as they may be the key to the elusive mechanism of suppression by regulatory T cells.

6.4.3. Doing the job of regulatory T cells for them

Regulatory T cells have been found to be highly effective at suppressing many of the pathways that lead to the development and symptoms of allergic disease. Such cells suppress IgE production, [260] and as described in this thesis, Th2-type T cell expansion and cytokine production. If regulatory T cells from atopic individuals are not as effective at suppressing such responses, then it may be possible to do their job for them by devising therapeutic procedures to suppress such responses.

Anti-IgE treatment of allergic disease

Cross-linkage of IgE molecules by prevailing allergen, results in the dimerisation of the high-affinity IgE receptors on mast cells and basophils, causing cell activation and the secretion of various inflammatory mediators and cytokines [465]. Both mouse and human regulatory T cells have been shown to suppress the production of IgE, but it is not known whether regulatory T cells act directly on B cells to suppress the secretion of IgE, or whether perhaps more likely, their suppression of Th2 cytokines indirectly inhibits IgE class switching. In either case, if CD4+CD25+ regulatory T cells from atopic donors are not as effective at suppressing allergen specific responses, this may be the reason why IgE levels in those with atopic

disease are elevated. Therefore other strategies to reduce IgE levels in atopic individuals should be developed.

Preliminary studies of IgE as a therapeutic target are promising. Administration of a humanised monoclonal antibody against the Cε3 region of IgE, which binds the α-chain of the FCεR1, results in sequestration of circulating IgE and eventual loss of cell-bound IgE in tissues [466]. Anti-IgE treatment has produced striking clinical improvement in adults and children with steroid-requiring asthma [467;468] and has recently been approved for clinical use in the USA. Advancements to this therapeutic approach involve the development of peptide vaccines, which induce a therapeutic antibody response to cell-bound IgE/Cε3 domains [469].

Suppression of Th2 responses

If CD4+CD25+ regulatory T cells from atopic individuals fail to suppress Th2 responses, it may be possible to develop therapeutics that can perform this task. Such drugs could target extracellular molecules such as cytokines or cytokine receptors or intracellular targets such as transcription factors, which are important in the development of Th2 responses.

The most direct and simple approach to reduce Th2 responses may be to remove or neutralise circulating Th2 cytokines such as IL-4, IL-5, IL-9 or IL-13. IL-5 is an example of such a cytokine that is being actively investigated as a potential therapeutic target in the treatment of Th2 mediated diseases. In support of a role for IL-5 in atopy, various experimental studies have shown a reduction in eosinophilic inflammation in IL-5-deficient animals or animals treated with anti-IL-5 monoclonal antibodies [125;470;471]. However, clinical administration of a humanised monoclonal antibody against IL-5 to treat asthma did not affect airway hyperresponsiveness [356]. A

recent study has confirmed these results and suggested that the therapy merely reduced rather than eliminated eosinophils [354].

Another major problem with targeting individual Th2 cytokines is the redundancy and overlap in function that exist between them. Single, double, triple and quadruple knockout mice for genes encoding the Th2 cytokines IL-4, IL-5, IL-9 and IL-13 showed a surprising degree of plasticity in the capacity to generate Th2 responses [472]. Therefore, a therapy based on the removal of a single cytokine may be over-simplistic. In fact, blocking only IL-4 [473] or IL-5 (as described above) has shown any positive clinical effect.

Receptors for certain Th2 cytokines including IL-13 and IL-4 are found, not only on the surface of cells, but also extracellularly in soluble form. Such receptors may act as "decoys" *in vivo* to control the magnitude of Th2 responses [474]. However, although therapeutic administration of soluble IL-4R has been shown to alter IgE production in asthmatics [473], and IL-13Rα2 ameliorates Th2 responses and IL-13-induced fibrosis [475], there are potential problems with the clinical use of recombinant monomeric soluble receptors. Such constructs often have a short *in vivo* half-life and cytokine receptors, together with the complications of redundancy, consist of two heterogeneous chains [476]. Cytokine trap technology however, which involves a complex of the extracellular domains of two different cytokine receptors fused to the constant region of human IgG1, may solve both these problems as these constructs have longer *in vivo* half lives and more potent cytokine binding affinities [477].

CpG DNA is another new therapeutic strategy for the treatment of allergic disease. The cytokine environment induced by this treatment, a combination of IL-12, IL-18, IFN-α, IL-6 and IL-10 is highly effective at reducing the levels of Th2 cytokine receptors. In mouse models of allergy, CpG DNA administration reverses

the Th2 cytokine expression, eosinophilic inflammation, epithelial mucus production and prevents the development of nasal symptoms and airway hyperresponsiveness [478;479]. Human studies with CpG and a modified CpG-allergen conjugate [480] in human allergic disease are currently in progress.

There are several key intracellular targets, which are involved in the initiation of Th2 responses. Signal transducer and activator of transcription- (STAT) 6 is one such transcription factor. In the absence of STAT-6, there is a marked block in Th2 cell development [93]. Other potential intracellular targets are suppressors of cytokine signalling (SOCS) 3, which correlates with Th2 cytokine expression [481], and SOCS 5, which is able to affect Th2 cell development by inhibiting STAT-6 activation [482]. Nuclear factors of activated T cells (NFATs) are also important in Th2 cell development. Naïve T cells deficient in both NFATc2 and NFATc3 are biased to differentiate into Th2 cells without IL-4 stimulation or the requirement for CD28 costimulation [483]. Therefore altering transcription of gene enhancer sites and elements involved in chromatin remodelling could provide an alternative to targeting proteins. The transcription of cytokines in Th2-differentiated cells for example, is partly controlled by CpG demethylation and in the absence of CpG-binding domain protein-2, Th cells have altered differentiation [484]. Strategies targeting T cell specific transciption factors may therefore be possible. The recent use of gene silencing by RNA interference (RNAi), which has shown promising results in the treatment of mouse models of autoimmune disease [485], might also offer a new approach to treat atopic disease by the selective removal of Th2 related genes.

6.4.4. Therapeutic administration of cytokines or cell surface molecules involved in the suppression by regulatory T cells.

Therapeutic manipulation of the regulatory T cell mechanism of suppression

Although there is as yet no definitive mechanism for the suppression of immune responses by regulatory T cells, several molecules have been shown to play a role in different systems and these have been discussed in detail in Chapter 3. The therapeutic manipulation of some of these molecules has been shown to suppress disease progression in various models of autoimmune disease and they therefore may be potential targets to increase regulatory T cell suppression of Th2 responses in human allergic disease.

Cross-linking of GITR by a specific mAb, together with TCR stimulation, abrogates *in vitro* CD4+CD25+ T cell mediated suppression and induces spontaneous organ-specific autoimmune disease. However, addition of GITR to previously activated CD4+CD25+ T cells has no inhibitory effect on their effector function [235]. Furthermore as shown in Chapter 3, although it partially reversed suppression of allergen driven T cell responses, anti-GITR also increased proliferation of both the CD4+CD25+ and CD4+CD25- T cell subset indicating that it may be involved in the development of suppressive functions rather than in mediating suppressive effects. In support of this notion, CD4+CD25+ T cells from GITR knockout mice show increased proliferation upon anti-CD3 activation [486]. Therefore, although stimulation of GITR may aid in the development of regulatory T cells, any such treatment may have to be used as a preventative treatment rather than to suppress ongoing inflammatory responses.

Blockade of CTLA-4 by anti-CTLA-4 antibody or fragment of antibody, elicits autoimmune diseases similar to those seen by depletion of CD4+CD25+ T cells [54;215]. CTLA-4 plays two synergistic roles in attenuating T cell responses; transduction of a negative signal to activated T cells and an activating signal to regulatory T cells [342]. The activation of this molecule could therefore be of possible therapeutic benefit for the treatment of both autoimmune and allergic disease. Studies have shown that antibody mediated depletion of CD25+ T cells in conjunction with CTLA-4 blockade facilitated the induction of tumour immunity [487]. However, as shown in Chapter 3, blocking antibodies to CTLA-4 did not reverse suppression of human allergen specific regulatory T cell activity. Furthermore, CD4+CD25+ CTLA-4 deficient mice exhibit significant suppressive activity *in vitro*, suggesting that this strategy may not be effective [54].

FOXP3 (introduced in Chapter 3) appears to be a master control gene for the development and function of natural CD4+CD25+ regulatory T cells and as such is an exciting potential therapeutic target. It is likely that in IPEX patients, mutations of the FOXP3 gene abrogate both the development and regulatory function of regulatory T cells. This is so far the clearest example of a genetic abnormality in human naturally occurring regulatory T cells that leads to autoimmune disease, IBD or allergy. It would be interesting to compare levels of FOXP3 expression on CD4+CD25+ regulatory T cells from atopic and non-atopic donors and also to investigate whether atopy is associated with mutations in FOXP3.

Therapeutic administration of cytokines produced by regulatory T cells

The cytokines IL-10 and TGF-β are important in the suppression afforded by regulatory T cells in animal models of various immune-mediated diseases. Tr1 cells

for example suppress IgE and Th2 cell responses in an immediate hypersensitivity model by a process that is dependent on IL-10, and the suppression of airway eosinophilia by killed Mycobacterium vaccae-induced allergen-specific regulatory T cells is also dependent on both IL-10 and TGF-β. The therapeutic administration of these cytokines may therefore effectively suppress allergic disease.

IL-10 displays strong suppressive effects upon the immune response [377]; inhibiting the production of pro-inflammatory cytokines and chemokines by monocytes, macrophages, neutrophils and eosinophils. *In vivo*, IL-10 displays strong suppressive and anti-inflammatory effects, as demonstrated in various experimental models for lethal endotoxemia, autoimmune thyroiditis and antigen-induced airway inflammation [488-490]. Decreased levels of IL-10 are also found in the bronchiol alveolar lavage fluid from atopic individuals compared with those from non-atopic control donors [374]. Clinical studies using IL-10 therapy on humans have not yet been carried out, but an animal model of allergic mucosal sensitisation, showed that expression of IL-10 inhibited inflammatory airway responses [491].

Although administration of TGF-β [492] in a rat model of EAE successfully prevented the development of disease and CD4+ T cells secreting TGF-β decreased allergic inflammation in a mouse model [493], there are few studies showing the effect of TGF-β administration in allergic disease. Furthermore, it is important to note that although TGF-β and IL-10 may be of importance in the down-regulation of inflammatory responses, under certain circumstances their presence is associated with pathological features of allergic disease [494-496]. For example, TGF-β is associated with basement membrane thickening and fibroblast replication and it has been suggested that this cytokine may play a role in airway remodelling in allergic asthma[496]. Cytokine treatment may therefore cause unwanted side effects. Furthermore, the lack

of studies investigating the effects of recombinant cytokines illustrates the high cost of producing these molecules, another reason why such a therapeutic strategy may not be viable. The induction of IL-10 or TGF-β producing regulatory T cells *in vivo* or *ex vivo* may be a more practical solution, a strategy that has already been discussed (Section 6.4.2.).

6.5. Conclusions

New therapeutic strategies to control Th2-mediated responses are required to address the increasing incidence of atopic disease. Data from this thesis is amongst the first to highlight the ability of human regulatory T cells to suppress allergen driven Th2-type T cell responses *in vitro*. The fact that CD4+CD25+ regulatory T cells isolated from atopic individuals were less able to suppress allergen-driven T cell responses than those isolated from non-atopic individuals illustrates the potential therapeutic importance of this population of cells. Furthermore, the discovery of a novel CD25- regulatory T cell population whose regulatory activity was increased after allergen immunotherapy treatment, may provide a clue to the mechanism by which such treatment exerts its clinical effects. Therapeutic manipulation of the balance between regulatory T cells and activated effector T cells during the allergic response may therefore provide hope for better prevention, treatment or even cure of human allergic disease.

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Declaration

The studies described in this thesis were performed in the departments of Allergy and Clinical Immunology at the National Heart and Lung Institute, and Leukocyte Biology, within Imperial College London by E. Ling, under the supervision of Dr. Douglas Robinson.

E. Ling performed blood separation, T cell isolation, cell culture, proliferation assays, cytokine analysis (by Luminex and ELISA), flow cytometry and assisted with TaqMan experiments. J. Arbery, V. Carr, K. Powrie, R. Ali, A. Menzies-Gow, Y. Ong, C.Alexander, M. Calderon, M. Tarzi and D. Robinson carried out clinical characterisation and phlebotomy at The Royal Brompton and St. Mary's Hospitals, London. K. Powrie, V. Carr and M. Calderon also executed specific allergen immunotherapy treatment at the above hospitals, under the clinical supervision of D. Robinson and S. Durham.

D. Nguyen assisted with flow cytometry in some of the larger experiments and C. Pridgeon established and optimised TaqMan RT-PCR methods under the supervision of M. Dallman.

Publications and awards arising from this thesis

Allergen immunotherapy treatment increases the ability of a population of CD4+CD25- IL-10 producing T-regs, but not CD4+CD25+ T-regs, to suppress allergen driven T cell responses. Ling *et al.* Paper in preparation.

Relation of CD4+CD25+ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease.
Ling EM, Smith T, Nguyen XD, Pridgeon C, Dallman M, Arbery J, Carr VA, Robinson DS. **Lancet. 2004 Feb 21;363(9409):608-15.**

Transforming growth factor-beta 1: its anti-inflammatory and pro-fibrotic effects. Ling, E and Robinson, DS. (Review) **Clin Exp Allergy. 2002 Feb;32(2):175-178.**

December 2002 **BSI Promega Prize**
2nd place in the National Young Immunologist of the Year Award 2002

March 2003 **Schering Plough Travel Award**
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March 2004 **Indoor Biotechnologies Travel Award**
To fund my attendance at the 2004 AAAAI conference in San Francisco

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To fund my attendance to the 2004 FOCIS conference in Montreal

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To be presented at the 2004 EAACI meeting in Amsterdam

Mechanisms of disease

Relation of CD4+CD25+ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease

Eleanor M Ling, Trevor Smith, X Dao Nguyen, Carol Pridgeon, Margaret Dallman, Justine Arbery, Victoria A Carr, Douglas S Robinson

Summary

Background Allergic diseases are frequent and rising in prevalence, and result from activation of T-helper (Th) 2 cells by allergens. CD4+CD25+ regulatory T cells suppress T-cell activation in vitro and prevent pathological findings in animal models of disease. We aimed to investigate whether the amount of inhibition of allergic responses by CD4+CD25+ T cells was related to atopy and allergic disease.

Methods Blood CD4+CD25+ and CD4+CD25– T cells were isolated from three groups of donors: non-atopic individuals; those atopic with no present symptoms; and patients with hayfever studied during and out of the grass-pollen season. We investigated the ability of CD25+ T cells from these donors to suppress allergen-stimulated T-cell proliferation and cytokine production in vitro.

Findings CD4+CD25+ T cells from non-atopic donors suppressed proliferation and interleukin 5 production by their own allergen-stimulated CD4+CD25– T cells. Inhibition of proliferation by CD4+CD25+ T cells from atopic donors was significantly reduced (p=0·0012), and was even more diminished by CD4+CD25+ T cells isolated from patients with hayfever during the pollen season (p=0·0003). In patients with hayfever, out-of-season suppression remained less than that seen by regulatory cells from non-atopic donors.

Interpretation Allergic disease can result from an inappropriate balance between allergen activation of regulatory CD4+CD25+ T cells and effector Th2 cells. This imbalance could result from a deficiency in suppression by regulatory T cells or strong activation signals could overcome such regulation. Treatment to enhance regulatory T-cell responses, in concert with reduction of Th2 cell activation, might be useful in prevention and treatment of allergic disease.

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Introduction

Atopic allergic diseases such as asthma, rhinitis, and atopic dermatitis are characterised by presence of specific IgEs to allergens such as grass pollen, cat dander, or house dustmites.[1] Activation of T helper (Th) 2 cells is thought to have a major role in allergic sensitisation, because such cells can mediate IgE synthesis via interleukin 4 and eosinophilic inflammation via interleukin 5, which, together with interleukin 13, contribute to airway hyper-responsiveness and other clinical features of allergic disease.[1,2] T-cell proliferation to allergen can be detected in primary cultures of peripheral blood mononuclear cells (PBMCs) from both atopic and non-atopic individuals. Such widespread priming is thought to be because many allergens implicated in asthma and rhinitis, such as cat dander and grass pollen, are widely distributed in the environment. Only PBMCs from atopic donors show increased production of Th2 CYTOKINES interleukin 4, interleukin 5, and interleukin 13.[3-5] We do not know why these individuals make a Th2-type T-cell response while other (non-atopic) people do not. One possibility is that regulatory mechanisms prevent IgE responses to allergens in non-atopic individuals. Potential mechanisms include suppression of Th2 responses by REGULATORY T CELLS.

Regulatory CD4+CD25+ T CELLS were characterised in mice as T cells that prevent development of organ-specific autoimmune disease in vivo and inhibit activation of CD4+CD25– T cells by polyclonal or antigenic stimulation in vitro.[6-8] A similar population of suppressor CD4+CD25+ T cells has been described in human beings from both peripheral blood and thymus.[9-11] CD25 is an imperfect marker for regulatory T cells, because it is also expressed by recently activated or memory T cells. Furthermore, other—possibly separate—regulatory T-cell populations have been described, including Th3 cells and T-regulatory-1 (Tr1) or interleukin 10-producing T cells.[12-14] The suppressive phenotype of CD4+CD25+ T cells is associated with expression of the transcription factor FOXP3, which may, thus, be used as a marker for these regulatory T cells.

Balance between activation and suppression of T-cell responses can be crucial in determining outcome of many immune-mediated diseases. Naturally occurring human regulatory T cells could have a role in prevention of transplant rejection,[9] but may also prevent control of tumours.[15] Findings of studies suggest that human regulatory T cells can also suppress responses to recall antigens, including allergens.[16-18]

We aimed to investigate the relation between activity of regulatory T cells and disease in human beings. Furthermore, we looked at the mechanism of suppression of allergen-induced T-cell activation by human CD4+CD25+ T cells. We postulated that CD4+CD25+

GLOSSARY

ATOPY
Tendency to make IgE to common environmental proteins such as house dustmite or grass-pollen allergens. Atopic individuals can develop atopic allergic diseases such as asthma, rhinitis, and atopic dermatitis.

CD4+CD25+ T CELLS
T lymphocytes are cells of the immune system that control immune responses by recognition of specific sequences from foreign (or self) molecules presented by MHCs on antigen-presenting cells such as dendritic cells or macrophages. CD4 is a marker of the helper T cell subset that interacts with B lymphocytes to induce immunoglobulin production or activate other immune cells via cytokines. CD25 is the α chain of the receptor for interleukin 2; CD4+ T cells that express this receptor have been characterised as regulatory (see below). However, CD25 is also induced on recently activated T cells.

CYTOKINES
Small proteins produced by T cells that act as signals to other cells of the immune system or structural cells. They can activate responses—eg, T helper (Th) 2 cytokines interleukin 4 and interleukin 13 activate B cells to make IgE, and interleukin 5 activates eosinophils. Another subset of CD4+ T cells, termed Th1, make interferon γ, which activates macrophages to kill intracellular pathogens and switch on IgG1 production from B cells. Other cytokines can suppress immune cell function—eg, interleukin 10 and transforming growth factor β.

REGULATORY T CELLS
T lymphocytes that suppress activity of other T cells. This process can be either beneficial (eg, preventing activation of T cells that can interact with self antigens or allergens and which could cause disease), or disadvantageous (eg, suppression of T-cell killing of cancer cells). Several different regulatory T cell types have been described, including CD4+CD25+ T cells and interleukin 10-producing T cells.

T cells generally inhibit potentially pathogenic Th2 responses to allergens, and that this process is deficient or overcome in individuals with atopic allergic diseases.

Methods

Participants

Between November, 2001, and September, 2003, we recruited non-atopic and atopic volunteers by advertisement and from staff of the National Heart and Lung Institute, London, and patients with seasonal allergic rhinitis to grass pollen (hayfever) from the allergy clinic of the Royal Brompton Hospital, London, and by advertisement. All volunteers completed a standard questionnaire about allergic symptoms and, to establish presence or absence of ATOPY, had skin-prick tests to common aeroallergens (cat, house dustmite, grass pollen, aspergillus; ALK, Horsholm, Denmark) and serum-specific IgE testing (Phadebas RAST, Pharmacia, Milton Keynes, UK). We defined atopic donors on the basis of a positive IgE or skin test and a history of allergic symptoms, whereas non-atopic donors had no history of allergic symptoms, negative skin-prick tests, and normal amounts of serum IgE. Hayfever patients had a positive skin test to grass pollen extract but not other allergens. We studied these individuals between June and August when the pollen count was high and they had symptoms of hayfever and again between December and March when they were not exposed to pollen. All studies were approved by the Royal Brompton NHS Trust local ethics committee and all volunteers gave written informed consent.

Procedures

For cell separation and culture, we obtained 150 mL blood samples with heparinised syringes. We did basic cell culture with standardised allergen extract (either cat dander or grass pollen; ALK) as described.[5] Briefly, we separated PBMCs by density centrifugation on Histopaque (Sigma Chemicals, Poole, UK). We isolated CD4+ T cells by negative immunomagnetic selection, followed by positive selection for CD25+ T cells by magnetic cell sorting (MACS; Miltenyi, Bisley, UK), as described.[9–12] We enriched CD4+ cells to a median of 96% pure (range 91–99) and CD25+ to a median of 82% (54–95). We cultured cells in 96-well flat-bottomed plates in triplicate at a density of 2×10^6 cells per mL in RPMI 1640 medium with 5% human AB serum, penicillin, streptomycin, and L-glutamine (Sigma Chemicals) for 6 days with allergen extracts added at 100 mg/L (results of pilot experiments showed that these conditions were best for proliferation and cytokine production). We added autologous irradiated mononuclear cells as antigen-presenting cells to all cultures at a density of 2×10^6 cells per mL. In all cases, we included triplicate medium control wells (no allergen) as a negative control. Cultures were of PBMCs, CD4+CD25+ cells alone, CD4+CD25– cells alone, or mixed CD4+CD25– and CD4+CD25+ T cells at a ratio of two to one, or CD4+CD25– at 3×10^6 cells per mL to control for amplified cell density of the CD25+CD25– cultures. At day 6, we obtained 100 μL of supernatant for cytokine analysis with the Luminex bead system (Luminex, Riverside, CA, USA) by labelled cytokine capture antibody pairs (Biosource International, Camarillo, CA, USA). We then added ^{3}H-thymidine (Amersham, Chalfont St Giles, UK) for the final 6 h of culture, after which cells were harvested, and we counted incorporated radioactivity as index of proliferation. We expressed data as counts per min (cpm) in allergen-stimulated wells with medium control wells subtracted (median counts for PBMCs cultured with medium alone were 802 cpm).

To compare regulation of non-allergen-driven cultures between atopic and non-atopic donors, we cultured CD4+CD25+, CD4+CD25– T cells, or mixed CD4+CD25+ and CD4+CD25– cells in a one to two ratio, for 72 h with plate-bound antiCD3 (BD Pharmingen, Oxford, UK) at 5 mg/L and soluble antiCD28 (BD Pharmingen) at 1 mg/L, as described.[11–15]

We generated a cat allergen-reactive Th2 clone from an atopic donor by limiting dilution from tertiary T-cell lines. This Th2 clone produced interleukin 5 (20 μg/L) and interleukin 13 (5 μg/L) on restimulation with cat allergen extract, whereas secretion of interleukin 10 and interferon γ were undetectable. A cat allergen (Fel d1) peptide (KALPVVLENARILKNCV)-reactive Th0 clone was generated from a non-atopic donor, which produced interferon γ (430 ng/L), interleukin 2 (200 ng/L), interleukin 5 (18 ng/L), and interleukin 13 (43 ng/L) on restimulation with peptide. In mixing experiments, clones (2×10^4 cells per well) were combined with autologous CD4+CD25+ T cells at a ratio of two CD4+CD25+ T cells to one cloned cell. We cultured cells with 10 μg/L allergen or peptide and 1×10^5 irradiated PBMCs for 72 h.

In at least three separate experiments for every antibody, we added blocking antibodies to interleukin 10 (R&D Systems, Abingdon, UK), transforming growth factor β (R&D), cytotoxic T lymphocyte antigen (CTLA) 4 (Ancell, Baycourt, MD, USA), glucocorticoid-induced tumour necrosis factor receptor (R&D), or isotype control antibodies at a concentration of 10 mg/L (as suggested by the suppliers) to cultures with allergen extract. We undertook flow cytometry of CD4+CD25+ T cells and CD4+CD25– T cells (FACScaliber; Becton Dickinson, Oxford, UK). We used labelled antibodies and isotype controls to CD4, CD25, and CD69 (BD Pharmingen), and antibodies against CTLA 4 (Ancell).

	Non-atopic (n=9)	Atopic (n=12)	Hayfever (n=11)
Age (years)	29 (24–43)	35 (20–42)	28 (20–58)
Men	6	8	6
Disease			
Rhinitis	0	3	9
Asthma and rhinitis	0	9	2
Total serum IgE (IU/L)	37 (4–111)	154 (28–2893)	354 (22–2893)
Specific IgE (IU/L)	0·34 (0·34–0·55)	4·75 (0·39–101)	56·65 (0·78–101)
Allergen extract used in culture			
Grass	5	6	11
Cat	4	6	0

Data are median (range) or number of patients.

Table 1: **Clinical characteristics of donors**

We extracted RNA from CD4+CD25+ and CD4+CD25– cell pellets with a kit (Absolutely RNA; Stratagene, La Jolla, CA, USA). We quantified RNA and then reversed transcribed it (SuperScript First Strand Synthesis system; Life Technologies) with both Oligodeoxythymidine and random hexamer primers. We did 25 μL PCR procedures in triplicate (ABI Prism 7700 sequence detection system; Applied Biosystems, Foster

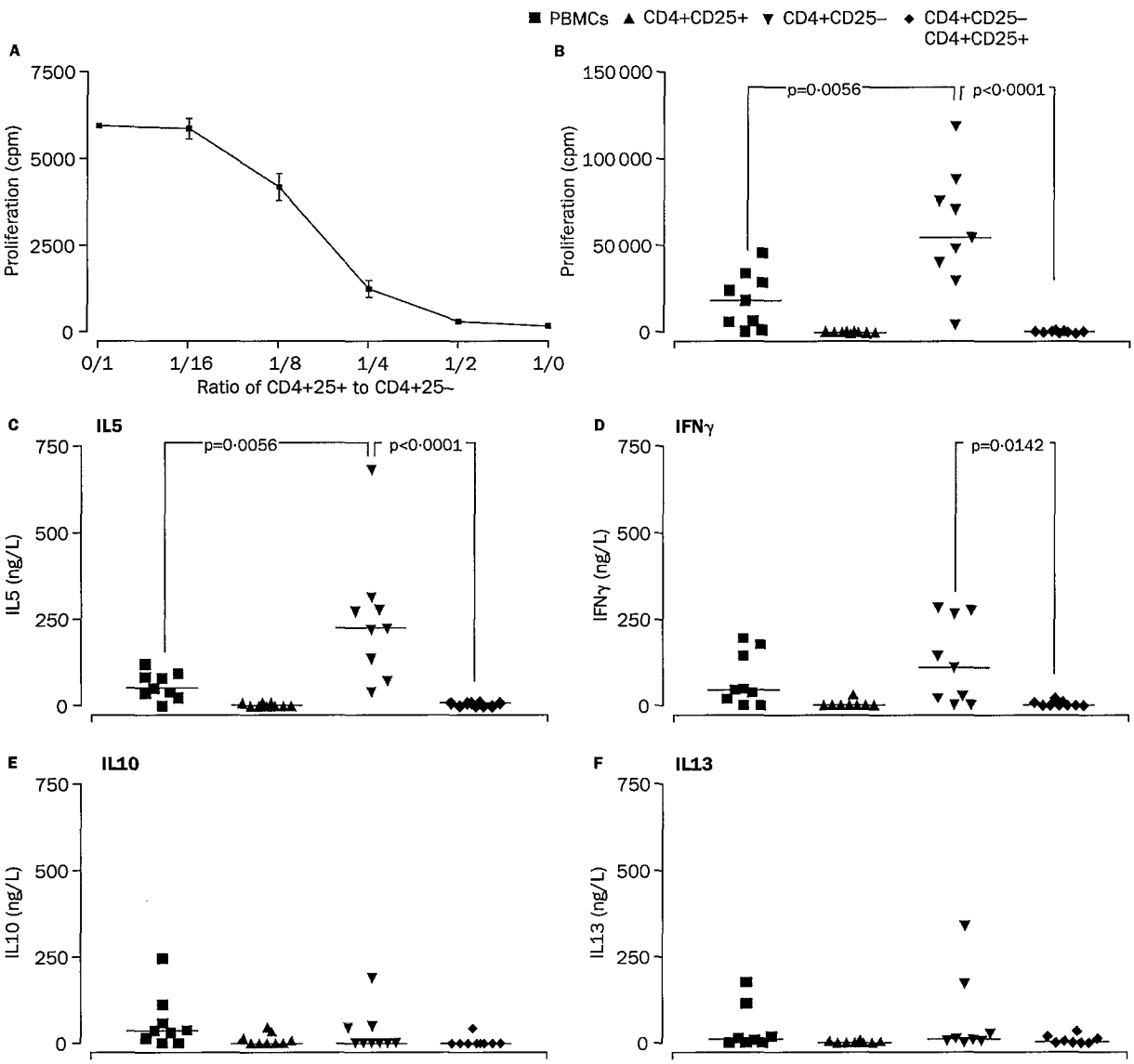


Figure 1: **Proliferation and cytokine production after allergen-stimulation of PBMCs, CD4+CD25+, CD4+CD25–, and mixed cultures of CD4+CD25+ and CD25– T cells from non-atopic donors**

IL=interleukin. IFN=interferon. Error bars=SE of triplicate cultures. (A) Dose-dependent suppression of allergen-driven proliferation of CD4+CD25– T cells by addition of differing ratios of CD4+CD25+ T cells (one experiment representative of three). (B–F) Data are individual values with median horizontal bars from nine separate non-atopic donors.

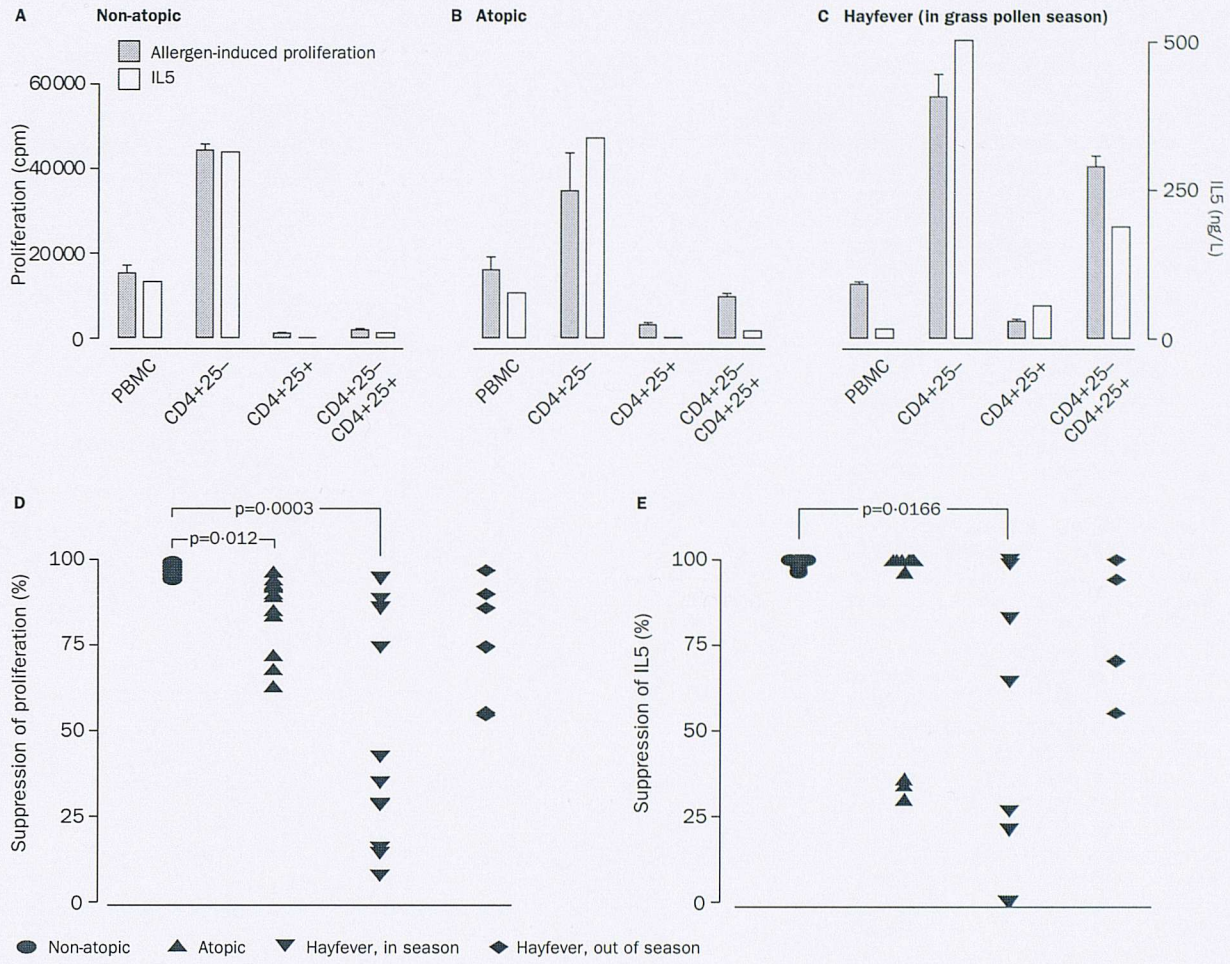


Figure 2: **Allergen-induced proliferation and production of interleukin 5 by PBMCs, CD4+CD25– T cells, CD4+CD25+, and mixed CD25+ and CD25– cells, and percentage suppression by CD4+CD25+ cells**

Error bars=SE of triplicate cultures. Allergen-induced proliferation (closed bars) and interleukin 5 (IL5) production (open bars) from (A) a non-atopic donor, (B) atopic donor, and (C) a hayfever patient studied during the grass-pollen season. Percentage suppression by CD4+CD25+ cells for proliferation (D) and interleukin 5 production (E).

City, CA, USA) with TaqMan universal PCR mastermix (Applied Biosystems), FOXP3-specific primers, and 6-carboxyfluorescein-labelled TaqMan probe (Applied BioSystems). We standardised samples to 18S (Applied Biosystems) and quantified the FOXP3 message with the comparative threshold for detection method (http://appliedbiosystems.com).

Statistical analysis

We compared proliferation data and cytokine concentrations between groups with the non-parametric Mann-Whitney *U* test, with Prism software (version 3; Graph Pad, San Diego, CA, USA). We expressed suppression by CD4+CD25+ T cells as difference in cpm (or cytokine concentration) from mixed CD4+CD25+/CD4+CD25– T cells as a percentage of cpm (or cytokine concentration) obtained with CD4+CD25– T cells alone.

Role of the funding source

The sponsor had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

We recruited nine non-atopic donors, 12 atopic allergic volunteers, and 11 allergen-exposed people with hayfever. Table 1 shows participants' characteristics.

We first compared PBMCs, CD4+CD25–, and CD4+CD25+ T cells from non-atopic donors in allergen-

	Non-atopic (n=6)	Atopic (n=6)	Hayfever (n=6)
MFI CD25	37·5 (11·3–106)	14·5 (13·4–18·6)	19·0 (7·9–25)
CD4+CD25+ (%)	9·8 (4·2–19·8)	4·7 (2·1–8·9)	11·3 (5·5–24·1)
CD25+CD69+ (%)	2·4 (0·5–3·3)	5·6 (0·1–6·8)	2·3 (0·9–3·7)
CD25–CD69+ (%)	1·5 (0·1–1·76)	0·5 (0·0–1·48)	0·8 (0·0–5·0)
CD25+CTLA4+ (%)	13·4 (7·7–63·3)	22·0 (21·0–37·3)	21·2 (17·8–46·3)

Data are median (range). Flow cytometry data are mean fluorescence intensity (MFI) of CD25 staining on CD4+ T cells, percentage of CD4+CD25+ cells in total PBMCs, and percentage positive cells relative to isotype control antibody for expression of CTLA4 by CD4+CD25+ and CD69 by CD4+CD25+ and CD4+CD25– T cells.

Table 2: **Phenotype of CD4+CD25+ and CD4+CD25– T cells from non-atopic and atopic donors**

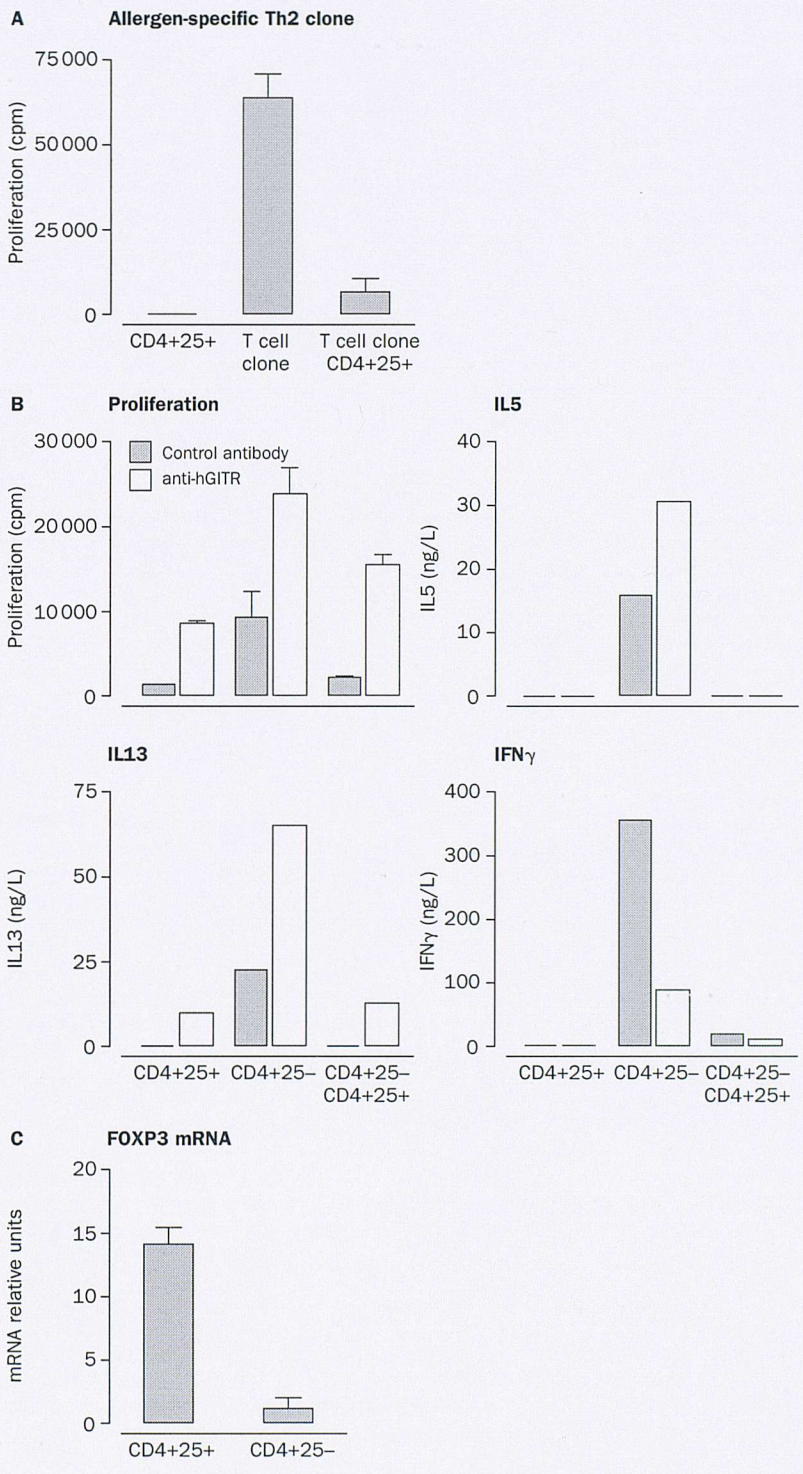


Figure 3: **Mechanism of suppression**
(A) Proliferation of Th2 cell clone by CD4+CD25+ T cells from an atopic individual. Representative data for three separate experiments. Data are mean, error bars represent SE. (B) Allergen-induced T-cell proliferation and cytokine production by CD4+CD25+ T cells with and without antibody to glucocorticoid-induced tumour necrosis factor receptor (GITR). Data from one donor, representative of three independent experiments all with cells from non-atopic donors. Note that scales for axes are different. Data are mean, error bars represent SE. (C) Expression of FOXP3 mRNA from CD4+CD25+ and CD4+CD25– T cells. Data are mean, error bars represent SE, from three separate non-atopic donors.

driven cultures. CD4+CD25+ T cells showed little or no proliferation to allergen stimulation. By contrast, culture of CD4+CD25– T cells with allergen showed substantially enhanced proliferation and production of interleukin 5 when compared with unseparated PBMCs (figure 1). Addition of CD4+CD25+ T cells suppressed allergen-driven proliferation of CD4+CD25– T cells, and this inhibition was dose-related—ie, it varied with the ratio of CD4+CD25+ T cells to CD4+CD25– T cells (figure 1). CD4+CD25+ T cells also suppressed production of interleukin 5 and interferon γ.

We then compared non-atopic individuals with asymptomatic and symptomatic atopic allergic patients. Percentage suppression of allergen-driven proliferation of CD25– T cells by CD25+ T cells was substantially reduced when cells from atopic donors were compared with those from non-atopic volunteers (figure 2). Suppression of interleukin 5 by CD4+CD25+ T cells in cultures from asymptomatic donors was variable, but did not differ greatly from that seen in non-atopic volunteers. In patients with active allergic rhinitis (hayfever), CD4+CD25+ T-cell inhibition of both grass pollen allergen-stimulated proliferation and production of interleukin 5 by CD4+CD25– T cells was substantially less than that from non-atopic individuals and asymptomatic atopic patients (figure 2). When the same individuals were studied out of season, suppression of proliferation was greater than during the season, but remained significantly less than that by cells from non-atopic donors ($p=0{\cdot}0028$) and similar to that seen in the other atopic allergic volunteers (figure 2). Inhibition of grass-pollen responses by CD4+CD25+ from non-atopic volunteers studied during the season was effective (median suppression of proliferation 96·2%, range 92–99, n=4).

Since immunomagnetic cell separation does not necessarily distinguish between CD25high regulatory T cells and CD25intermediate effector T-cell populations, we investigated whether a different cell population was isolated from atopic allergic and non-atopic donors. By flow cytometry, no difference was noted in proportion of CD4+ cells positive for CD25+ between groups nor in expression of CD69 (a marker of activated T cells) or intracellular CTLA4 (expressed at a higher level on regulatory T cells than activated T cells). CD4+CD25– T cells also

did not differ in expression of CD69 between groups (table 2). Inhibition of antiCD3-driven plus antiCD28-driven proliferation by CD4+CD25+ T cells did not differ between non-atopic and atopic donors (mean suppression 77·3% [SD 4·7] and 82·5% [3·2], respectively; n=6 for each group).

To test the ability of CD4+CD25+ T cells to suppress activated allergen-specific T cells, we derived allergen-specific T-cell clones from an atopic and non-atopic donor and noted that autologous peripheral blood CD4+CD25+ T cells suppressed proliferation of the Th2-like clone from the atopic donor (figure 3). Furthermore, CD4+CD25+ T cells from a non-atopic donor inhibited proliferation of a peptide-specific Th0-like clone from the same non-atopic donor (by a median of 50% [range 45–75], when mixed at a ratio of two CD25+ T cells to each clonal cell in three experiments with the same clone).

When looking at mechanisms of suppression, we noted that antibody to glucocorticoid-induced tumour necrosis factor receptor reversed CD4+CD25+ T-cell suppression of allergen-induced proliferation (figure 3). However, antibodies against this receptor did not reverse inhibition of allergen-induced interleukin 5, interleukin 13, or interferon γ production. Antibodies against interleukin 10, CTLA4, and transforming growth factor β had no effect on regulation by CD4+CD25+ T cells in our system (data not shown). Moreover, by quantitative RT-PCR, mRNA expression for *FOXP3* was 15-fold higher in CD4+CD25+ T cells than CD4+CD25– T cells (n=3; figure 3).

Discussion

We have shown that suppression by CD4+CD25+ T cells relates to clinical manifestation of allergy, since inhibition of allergen-driven responses by cells from atopic donors was less than those from non-atopic individuals, and least of all by those isolated from patients with active allergic disease (hayfever).

Although atopic and non-atopic donors show proliferation of PBMCs to allergen in culture, these groups differ by production of Th2 cytokines, particularly interleukins 5 and 13.[3–5] We have confirmed that human CD4+CD25+ T cells can suppress allergen-driven proliferation and cytokine production.[17,18] Our finding that CD4+CD25– T cells from non-atopic donors can produce interleukins 5 and 13 on culture with allergen, to a substantially greater extent than that for unseparated PBMCs, means that Th2 cytokine expression to allergen could be usually inhibited by regulatory T cells in non-atopic individuals. We have shown an association between clinical expression of allergic disease and ability of CD4+CD25+ T cells from different donors to suppress allergen-stimulated T cells. Our data could also be relevant to other T-cell-mediated diseases, such as diabetes and rheumatoid arthritis, and transplant rejection.

We used an in-vitro culture system with isolated CD4+CD25+ T cells similar to that reported by others.[9–11,16] Although such a system can give information on potential immunological mechanisms, it is artificial, so the behaviour of cells in the conditions and at the ratios used in vitro might not be the same in vivo. CD25 is the best available marker for a subset of regulatory T cells, but remains unsatisfactory, since CD4+CD25+ T cells that are isolated are a mixed population. Despite these limitations, in-vitro data accord with findings in animal models, and we think our results and those of other groups set important principles for understanding of immune regulation.

Our observation that suppression of allergen-driven T-cell activation by CD4+CD25+ T cells is reduced in atopic versus non-atopic individuals, and even more diminished when they are exposed to allergen, suggests three possibilities. First, there is a deficiency in regulatory activity of CD25+ T cells in atopic donors. Second, during allergen exposure, isolation of CD25+ T cells includes recently activated effector cells, so dilution of regulatory CD25+ T cells is taking place. Third, allergen exposure activates and expands effector T cells to a degree that overcomes regulation by CD4+CD25+ T cells—ie, the deficit lies with CD4+CD25– T cells. Noted differences in suppression between groups suggest that such an effect is an active process and not simply attributable to dilution of cultures with non-responsive cells.

One possible reason for the reduced ability of CD4+CD25+ T cells from patients with hayfever to suppress allergen-stimulated T-cell responses was that their CD25– T cells had been activated by mechanisms such as interleukin 6 production or Toll receptor ligation that overcome regulation, and were therefore refractory to regulation.[19] The threshold of activation versus suppression could vary with allergen exposure and intensity of antigenic stimulus.[20] Out of season, when they were not exposed to allergen in the environment, inhibition of grass-pollen-driven cultures by CD4+CD25+ T cells from patients with hayfever was still significantly less than that by CD4+CD25+ T cells from non-atopic donors. This finding suggests a possible inherent defect in suppression.

CD4+CD25+ T cells can arise in the thymus,[21] but may also develop in the periphery. Are these cells selected by allergen peptides during the neonatal period? Do they represent crossreactive T cells directed against self-antigens? Do they arise in the periphery? These three questions need to be answered if these processes are to be manipulated for treatment.

If there is a deficiency in ability of CD4+CD25+ T cells to regulate T-cell responses to allergens in atopic allergic patients, we need to establish whether this deficient response is restricted to allergens. We saw no difference between groups in terms of regulation of antiCD3-driven plus antiCD28-driven cultures. This stimulus will activate all CD4+CD25+ T cells in culture rather than those specific to allergen, which suggests that differences between groups are restricted to allergen-specific regulation. To examine regulation of other antigens or allergen to which an atopic individual does not make IgE would be of interest. It is noteworthy that findings of some epidemiological studies show a positive association between asthma and type 1 diabetes, and in non-obese diabetic mice, diabetes can partly result from a defect in immune regulation by CD4+CD25+ T cells.[22,23]

Suppression by CD4+CD25+ T cells infiltrating lung cancers and by those isolated from synovial fluid from patients with rheumatoid arthritis[15,24] shows that regulatory T cells can be found in tissues. Further studies are needed to establish whether regulatory CD4+CD25+ T cells act at sites of allergic disease, such as the airway.

Several mechanisms could account for why some children develop atopic allergic sensitisation and others do not. These include an absence of infection-related Th1 responses during the neonatal period (the hygiene hypothesis).[25] This idea has been modified to postulate that there is a deficiency in development of regulatory T cells to allergen stimuli, leading to unchecked Th2 responses.[25] Our data also suggest that a defect in regulation of allergen Th2-type responses by CD4+CD25+ T cells is implicated in atopic sensitisation.

RELEVANCE OF THIS PAPER TO PRACTICE

BACKGROUND

Whether an individual becomes sensitised (atopic) to certain proteins or not depends on a series of interactions within the immune system. One part of this system is a certain type of T cell which carries antigens CD4 and CD25, and which is thought to act as a regulator of other cells.

This paper studies three groups of people; those without atopic symptoms, those with symptoms, and those with hayfever, a particular type of atopy. Individuals were studied both in and out of the hayfever season. In normal individuals the regulatory T cells were able to efficiently suppress activation of other T cells that cause an immune response; however the cells from atopic individuals and those with hayfever, especially during the hayfever season, were not able to act as well as suppressor cells.

IMPLICATIONS

Dissecting out a specific part of the immune pathway in this way raises the possibility of designing therapies that can target this particular pathway, or perhaps bypass it.

Successful allergen injection immunotherapy is associated with enhanced numbers of CD25+ T cells that produce interleukin 10 in response to allergen exposure, so this treatment could induce or activate regulatory T cells.[18,26] However, expression of CD25 by these interleukin 10-producing T cells may represent activation of T cells during culture rather than the starting phenotype of the regulatory T cell. CD4+CD25+ cells could represent an inherent regulatory population, possibly derived from the thymus,[21] while a separate population of regulatory T cells producing interleukin 10 are expanded by immunotherapy.[14,18] We have isolated such interleukin 10-producing T cells that could also suppress allergen-stimulated T cells, suggesting that several different regulatory populations can exist. Different regulatory mechanisms probably work at varying thresholds of activation of T-cell responses or in response to signals from the innate immune system or to differing extents in different donors.

Mechanism of suppression by CD4+CD25+ T cells remains uncertain and could differ depending on intensity of stimulus and other factors. Findings of studies in mice show that inhibition of some autoimmune models, such as colitis, by CD4+CD25+ T cells is dependent on interleukin 10, transforming growth factor β, and the T-cell costimulation molecule CTLA4.[6–8] However, CD4+CD25+ T cells from mice in which genes for interleukin 4, interleukin 10, or transforming growth factor β had been knocked out could still suppress in-vitro proliferation of CD4+CD25– T cells.[6–8] In accordance with other human in-vitro systems,[9–11] we recorded that inhibition of allergen-driven proliferation and cytokine production by human CD4+CD25+ T cells could not be reversed by blocking antibodies to interleukin 10, transforming growth factor β, or CTLA4. In most cases, interleukin 10 was not detected in culture supernatants from CD4+CD25+ T cells stimulated with allergen. However, blocking antibody studies cannot completely rule out a role for suppressive cytokines in regulation by human CD4+CD25+ T cells. Crosslinking of the glucocorticoid-induced tumour necrosis factor receptor abrogates inhibition by murine CD4+CD25+ T cells in vitro and in vivo.[8] Our data extend these observations to human CD4+CD25+ T cells and show that antibodies to glucocorticoid-induced tumour necrosis factor receptor can partly overcome the suppressive phenotype of CD4+CD25+ T cells. However, it is noteworthy that inhibition of cytokine production was not affected. Amplified proliferation of CD4+CD25– T cells in the presence of antibodies to glucocorticoid-induced tumour necrosis factor receptor suggests that there is an additional regulatory population in this subset. Moreover, we show that human CD4+CD25+ T cells express FOXP3, which has an important role in regulation by murine CD4+CD25+ T cells.[27–29] Ectopic expression of FOXP3 in CD25– T cells rendered them suppressive.[27–29] How this transcription factor acts to cause a regulatory phenotype is uncertain, but it could represent a target for therapeutic manipulation of T-cell function.

Our findings suggest that CD4+CD25+ T cells can prevent pathological Th2 activation by aeroallergens in non-atopic donors and that atopic allergic disease could result from failure of such suppression. Such alterations of the balance between inhibition and activation of potentially pathogenic T cells could determine expression of other T-cell-mediated diseases.

Contributors

E M Ling did laboratory experiments on primary T-cell regulation of allergen-driven cultures and helped write the report and prepare figures. T Smith did regulation experiments on T-cell clones and with antiCD3 and antiCD28. X D Nguyen assisted with cell culture and flow cytometry. C Pridgeon did PCR analysis, supervised by M Dallman, who also helped write the report. J Arbery and V A Carr recruited and characterised patients. D S Robinson designed and supervised the study and wrote the report.

Conflict of interest statement

None declared.

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Clinical picture

Tuberculous paravertebral abscess

Valérie Martinez, Eric Rolland, François Bricaire, Eric Caumes



A 44-year-old man who came from Mali and had been living in France for 19 years presented with back pain 10 days after a physical effort. We noticed two dorsolumbar tumours on examination (figure). He reportedly had had mediastinal tuberculosis 17 years previously. He complained of recent asthaenia and weight loss. Blood tests showed an increased erythrocyte sedimentation rate and C-reactive protein. His skin tuberculin test was strongly positive. Spinal MRI showed L1-L2-L3 neural arch lysis with numerous cysts infiltrating the aponeurosis. We drained the abscesses and grew *M tuberculosis* in culture. We surgically explored the abscesses and treated the patient with triple therapy for 16 months.

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