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Lung mucosal Th2 responses are regulated by CD4+CD25+ suppressor T cells

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ABSTRACT

In recent years, a subset of CD4+CD25+ T cells has been described that regulates cell mediated immune responses. Although CD4+CD25+ T cells prevent the development of autoimmunity their role in limiting allergic inflammation and their mode of action is not clearly understood. To investigate the role of regulatory T cells in airway inflammation, we have developed a model using the DO11.10 mouse which expresses a OVA specific T cell receptor.

FACS analysis revealed that 6.8% of CD4+ T lymphocytes in the lymph nodes of DO11.10 mice were CD4+CD25+. CD4+CD25+ T cells were purified by magnetic bead separaration to a purity of 70-75 %. These CD4+CD25+ T cells mediated regulatory activity since they were effective at inhibiting T cell responses to anti-CD3. Although DO11.10 CD4+CD25+ T cells were anergic it was found that they could be expanded and polarised *in vitro* in the presence of $OVA_{323-339}$ peptide and exogenous IL-2 and IL-4. Expanded CD4+CD25+ T cells expressed an OVA specific T-cell receptor (97-98% KJ1-26+) and were effective at inhibiting T cell proliferative responses to either $OVA_{323-339}$ or anti-CD3.

Total CD4+, CD4+CD25+ and CD4+CD25- T cells were analysed for the differential expression of adhesion molecules. FACS analysis revealed that CD44 was a predominant adhesion molecule expressed by all 3 groups. Total CD4+ and CD4+CD25- expressed β7 chain, CD62L and CD31 but the level of expression was lower than CD44. It was also found that PLN cells expressed CD103, but its expression was lost following the expansion of these cells in culture.

The effect of CD4+CD25+ T cells on Th2 polarization and allergic inflammation *in vivo* was examined. Th2 cells were either generated from unfractionated CD4+ T cells or CD4+ T cells depleted of CD4+CD25+ cells prior to polarization. Depletion of CD4+CD25+ T cells markedly influenced anti-CD3 stimulated cytokine production by Th2 polarised cells. The level of IL-4, IL-5 and IL-13 produced by Th2 cells generated from CD4+CD25- T cells was markedly lower than that by unfractionated CD4+ T cells. This reduced cytokine production was evident irrespective of the concentration of anti-CD3 used. Interestingly, cultured CD4+ CD25+ regulatory T cells inhibited IL-4 but not IL-5 production by Th2 cells.

The effect of depleting CD4+CD25+ T cells on airway inflammation was assessed *in vivo*. A marked pulmonary eosinophilia could be induced following transfer of DO11.10 Th2 cells if recipients were exposed to OVA aerosols. Recipient mice were exposed to aerosolised OVA for 7 consecutive days and then sacrificed on the last day. The number of CD4+KJ1-26+ T cells in the BALF and associated eosinophilia increased from no detectable inflammation until 5 days of aerosol challenge. Surprisingly, BALB/c mice that had received Th2 cells generated from CD4+CD25- cells developed a significantly higher level of pulmonary eosinophilia and less CD4+KJ1-26+T cells in the BALF than recipients of unfractionated CD4+ T cells. BALB/c mice which had received total CD4+ Th2 cells (CD4+CD25+ T cells not depleted) produced a relatively weak eosinophilia.

Collectively, these studies demonstrate that CD4+CD25+ T cells influence both Th2 polarization and the development of pulmonary inflammation.

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Abbreviations

Ab	antibody
AEC	airway epithelial cell
Ag	antigen
AIAR	aspirin-induced asthma and rhinitis
AM	alveolar macrophage
APC	antigen presenting cell
ARDS	adult respiratory distress syndrome
ASA	aspirin
BAL	bronchoalveolar lavage
BALF	bronchoalveolar lavage fluid
BSA	bovine serum albumin
BTL	B leukotreine receptor
cAMP	cyclic AMP
CCR	CC chemokine receptor
cDNA	complementary DNA
CMI	cell-mediated immunity
cpm	counts per minute
CTLA	cytotoxic T lymphocyte antigen
CXCR	CXC chemokine receptor
COX	cyclooxygenase
CysLT1, CysLT2	cysteinyl leukotreine receptors
DC	dendritic cell
DTH	delayed- type hypersensitivity
ECP	eosinophil cationic protein

EDTA	ethylene diamine tetaacetic acid
ELISA	enzyme linked immunosorbant assay
EP1 (2,3,4)	prostaglandin E receptor 1 (2,3,4)
EPO	eosinophil peroxidase
FACS	fluorescence activated cell scanner
FCS	foetal calf serum
FcγR	functional Fcγ receptor
FcεRI	high affinity IgE Fc receptor
FcεRII	low affinity IgE Fc receptor
FITC	fluorescence isothiocyanate
GM-CSF	granulocyte macrophage- colony stimulating factor
HBSS	Hank's balanced salt solution
HEPES	N-2-Hydroxyethyl piperazine-N'-2ethanesulfonic acid
Ia	MHC class II histocompatibility molecule of mice
ICAM	intracellular adhesion molecule
IgE	immunoglobulin E
IgG	immunoglobulin G
IL	interleukin
IFN-γ	interferon-γ
IM	interstitial macrophage
INDO	indomethacin
ip	intraperitoneal

IP receptor	prostacyclin receptor
Iv	intravenous
LFA	lymphocyte function associated antigen
LMC	lung mononuclear cell
LO	lipoxygenase
LPS	lipopolysaccharide
LTB4	leukotriene B4
MBP	major basic protein
M-CSF	macrophage- colony stimulating factor
2- ME	2 mercapto-ethanol
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
mRNA	messenger RNA
NEC	nasal epithelial cell
NK	natural killer cell
NO	nitric oxide
NSAID	non-steroid anti-inflammatory drugs
OD	optical density
OVA	chicken egg ovalbumin
PAF	platelet activating factor
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PE	phycoerythrin
PG (D2, E2, F2α, I2, J2)	prostaglandin
PGHS	prostaglandin H synthase
PMN	polymorphonuclear leukocyte
RT	reverse transcription
TAE	Tris-acetate electrophoresis buffer
TCR	T cell receptor
Tg	transgenic
TGF	transforming growth factor
TNF	transforming necrosis factor
TXA2 (B2)	thromboxane A2 (B2)
VLA-4	very late antigen-4

Chapter 1

Introduction

1.Asthma

Asthma is a common clinical disorder characterised by paroxysmal constriction of the airways, which is thought to be driven by chronic inflammation of the airways and is linked to atopy. It has been proposed that a type 2 response by CD4+ T cells in the airways drives this inflammatory response. Th2 cells are characterized by their secretion of IL-4, IL-5, IL-10 and IL-13, which are known to facilitate IgE production and eosinophilic inflammation that contribute to the pathogenesis of allergic disease. Much of our understanding of the mechanisms of asthma have been obtained using the model of allergen inhalation. The response to allergen inhalation is considered in two phases 1) early asthmatic response known as immediate-type hypersensitivity characterized by rapid onset of mucosal oedema, increase in airway smooth muscle tone and airway narrowing associated with mast cell degranulation (Pauwels, R., 1989), 2) late asthmatic response begins 3-6 hours after allergen challenge characterized by airway narrowing with the migration of leukocytes from the blood into the airways (De Monchy, J.G *et al.*, 1985.; Bousquet, J. *et al.*, 1990a).

1.1 CD4+ Th cell responses :

CD4+ effector T cells interact with other cells by producing soluble protein mediators (cytokines). The pattern of cytokines varies and determines the functional properties of that T cell. Once established, the cytokine profile is not stable. CD4+ effector T cells can be classified as either Th1 or Th2 cells. Th1 cells are CD4 + αβ T cells which produce IFNγ, TNF-β and IL-2 (Ahmed, R. and Gray, D., 1996). These cells

are responsible for the development of cell-mediated immunity and elimination of intracellular pathogens such as bacteria and virus. Th2 cells are CD4+ αβ TCR T cells that produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. Th2 cells are responsible for humoral immunity and are important in allergies and the elimination of parasites.

1.2.1 <u>Th1 CD4+ T cell responses:</u> Th1 and Th2 cells differentiate from precursor T cells termed as Th0 cells. Antigen presentation in the presence of the cytokine IL-12 drives Th1 development from Th0 cells. IL-12 is a 70 KDa hetero-dimer secreted by APC, neutrophils and keratinocytes (Macatonia, S.E. *et al.*, 1995). IL-4 inhibits its secretion and down-regulates the expression of the β2 subunit of the IL-12 receptor. Th1 cells inhibit cytokine production by Th2 cells (IL-4 and IL-5) directly or indirectly via the production of IFNγ, which also inhibits the expression of IL-4 receptor. IFNγ has several functions which include, promoting phagocytosis and upregulating microbial killing by macrophages. In mice, IFNγ promotes immunoglobulin isotype switching to IgG_{2a}, known to be important for the opsonization of bacteria (Heinzel, F.P. et al., 1995). In addition, IFNγ promotes the expression of FcγRI receptors which are important in phagocytosis. It also upregulates the production of NO, hydrogen peroxide and superoxide in cells actively participating in phagocytosis (Boehm, U. *et al.*, 1997). To facilitate migration of monocytes/macrophages and T cells to the site of infection, IFNγ, in association with TNF- β and LT-α, induces endothelial cells to express adhesion molecules and chemokines that specifically attract mononuclear cells (IP-10, MIG, RANTES and MCP-1) (Boehm, U *et al*, 1997; Cuff, C.A. *et al*, 1998). The development of Th1 cells from the precursor Th0 cells is a sequential step involving the interaction of TCR with

the antigen presented in the context of MHC class II by antigen presenting cells (APC). Typically, these are dendritic cells in a primary response and macrophages or B cells in the secondary response. In addition to antigen recognition, there is a need for co-stimulatory molecule interactions to elicit IL-2 production. Such interactions include B7-CD28 and CD40-CD40L. CD40-L induced on T cells interacts with CD40 constitutively expressed on the APC. The interaction of CD40-CD40L stimulates the expression of CD86 (B7.2) and later CD80 (B7.1) by APCs. The expressed B7 acts as a ligand for CD28 on T-cells. B7-CD28 ligation triggers the amplification of IL-2 secretion and induces the anti- apoptotic molecule Bcl-XL (Boise, L.H. *et al.*, 1995; Lenschow, D.J. *et al.*, 1996).

1.2.2 <u>Th2 CD4+ T cell responses:</u> The differentiation of Th2 cells from a Th0 precursor requires the presence of IL-4. IL-4 is considered dominant over IL-12 since it upregulates the expression of IL-4 receptor and inhibits the secretion of IL-12. Moreover, it downregulates the expression of the β2 subunit of the IL-12 receptor.

IL-4 is a 20kDa monomer secreted by Th2 cells, mast cells, basophils and eosinophils. It has been reported that Th2 cells can induce Th1 cells to switch to Th2 by producing IL-4 (Nakamura, T. *et al.*, 1997b). In contrast, IL-12 cannot block IL-4 production and cannot induce a Th2 cell to switch to Th1. This may be due to the fact that Th2 cells are constantly making IL-4 which downregulates the IL-12 Rβ2 subunit (O'Garra, A., 1998). The development of Th2 cells play a major role in the protection against large extracellular parasites such as helminths, via the production of IL-4 and IL-5. IL-4 acts by promoting the production of IgG_1 and IgE in mice (IgE and IgG_4 in humans) important in priming both mast cells and eosinophils (Boehm, U. *et al.*, 1997). It also promotes the upregulation of IgE receptors on mast cells, eosinophils and

macrophages and induces membrane expression of macrophages MHC class II molecules and the IL-4 receptor.

1.3 T cells in asthma

It has been demonstrated that there is an increase in CD4+ T-cells following allergen challenge of the airways (Metzger, W.J. *et al.*, 1987.; Frew, A.J *et al.*, 1990) in addition to the infiltration of eosinophils suggesting that CD4+ T-cells might be involved in antigen-induced eosinophil recruitment of airway LPR. T-cell derived cytokine IL-5 has been shown to be an important mediator that increases the migration of eosinophils into the tissue (Yamaguchi, Y. *et al.*, 1988a.; Walsh, G.M. *et al.*, 1990) and prolongs the survival of eosinophils (Yamaguchi, Y. *et al.*, 1988b). Later studies show (Nakajima, H. *et al.*, 1992) that CD4+ not CD8+ T-cells mediate antigen-induced eosinophil recruitment in the airways and that IL-5 mediates this eosinophil recruitment. Anti-murine IL-5 mAb decreased OVA-induced eosinophil infiltration in the trachea (Nakajima, H. *et al.*, 1992). Th2 type cytokine IL-4 has been detected at the mRNA and protein level in BAL and bronchial biopsies from patients with atopic asthma and during late asthmatic responses (Humbert, M. *et al.*, 1996). IL-4 induces bronchial mucus production and upregulates the expression of vascular cell adhesion molecule 1(VCAM-1) on vascular endothelium (Ying, S. *et al.*, 1997b). Taken together with the role of IL-4, especially in promoting IgE, it seems likely that the Th2 cell may be involved in the development of asthma. Conversely, Th1 cells may protect against asthma, for example, in infancy airborne endotoxin exposure may confer protection against asthma (Gereda, J.E. *et al.*, 2000.; Martinez, F.D. and Holt, P.G., 1999.; von Mutius, E. *et al.*, 2000) by promoting enhanced Th1 response and

tolerance to allergens (Reed, C.E. and Milton, D.K., 2001), although in later life, endotoxin has adverse effects on patients with asthma. Endotoxin binds to receptors on macrophages and other cells that generate IL-12 which inhibits IgE response and modulate T cells towards the Th1 phenotype.

CD4+ TH2 cells and the cytokines (IL-4, IL-5, IL-6, IL-9, and IL-13) contribute to the pathogenesis of allergic asthma directly or indirectly (Anderson, G.P., 2001). Interleukin -5 is crucial for the differentiation, migration, and activation of eosinophils (Kung, T.T. *et al.*, 1995a). IL-13 plays a key role in regulating AHR, mucus hypersecretion, eotaxin production, and eosinophilia in the allergic lung (Mattes, J. *et al.* 2001). IFN-γ is an important pro-inflammatory cytokine involved in classical delayed type hypersensitivity but also opposes the actions of IL-4 (Snapper, C.M. *et al.*, 1988.; Lopez, A.F. *et al.*, 1988.; Thompson-Snipes, L. *et al.*,1991.; Punnonen, J. *et al.*, 1994.; Maggi, E. *et al.*,1992). IL-13 shares many functions with IL-4 including upregulation of eosinophil adhesion molecules and mucus induction (Zhu, Z. *et al.*,1999) and may have a more important role than IL-4 in established disease (Dabbagh, K. *et al.*, 1999). IL-4 and IL-13 also promote the production of IgE antibody while IL-13 increases mucus hypersecretion by epithelial cells (Pene, J. *et al.*, 1988). It has been found that the majority of allergen-specific T-cell clones derived from the peripheral blood of atopic individuals produce increased amounts of IL-4 and IL-5 but lower levels of IFN-γ (Jung, T. *et al.*, 1995). Furthermore, immunohistochemistry and in situ hybridization studies have shown IL-4/IL-5 cytokine protein or mRNA in biopsies from the airways of asthmatics

(Kay, A.B. *et al.*, 1991.; Ying, S. *et al.*, 1995) and these Th2 cytokines were also increased in BAL after allergen challenge (Robinson, D.S. *et al.*, 1992). Because of its role in synthesizing IL-4 and IL-5, the Th2 cell is a potential therapeutic target in the development of new treatments for asthma (Broide, D.H., 2001).

Possible approaches include the inhibition of transcription factors such as nuclear factor kappa B and GATA-3 (Finotto, S. & Glimcher, L., 2004) that control cellular Th2 cytokine production of IL-4 and IL-5. There is already some evidence that experimental therapies that turn the immune responses away from Th2 immunity reduce asthmatic airway inflammation (Janssen, E.M. *et al.* 2000b).

The importance of T cells in the regulation of allergy and asthma has been recognised and the investigators started to address the possible role of T cells in asthma during the early 1980s. They focused on a possible defect in T cell suppression which might allow the allergic inflammatory process to proceed. It has been observed following allergen inhalation, subjects who showed single early responses had more CD8+ cells in their airways than those who showed early and late phase responses (Gonzalez, M.C. *et al.*, 1987). After thorough investigation, it became clear that T-cell infiltration and activation were features of all grades of asthma and that activation was associated with symptomatic asthma (Robinson, D.S. *et al.*, 1993a). Activated T cells were identified in the peripheral blood during exacerbations of asthma (Corrigan, C.J. *et al.*, 1988), but under stable conditions the T cell changes were largely confined to the airways. It has been observed that treatment with inhaled corticosteroids reduced both the numbers and activation status of the airway T cells (Wilson, J.W. *et al.*, 1994). Rapid expansion of understanding of cytokines and chemokines led to an appreciation of the possible function of T cells; in the context of allergy and asthma, the focus was on IL-4 because of its role in IgE switching.

Some unexpected insights have been provided following the attempts to demonstrate the presence of cytokine protein in human samples, the overwhelming majority of cell staining for IL-4 protein turn out to be mast cells rather than T cells (Bradding, P., *et al.*, 1992). Thus while T-cell –derived IL-4 may be essential for switching B cells to make IgE (at the time of T-B cell interactions), it may be the mast-cell- derived IL-4 that drive the differentiation of Th2 cells from precursor T-cells by providing Th2 favouring environment. Mast cells also contain significant amounts of IL-5 and IL-6 protein and it has been shown that these proteins are synthesised by the mast cell and released upon activation (Bradding, P. *et al.*, 1994). T cells do not seem to store detectable amounts of cytokine protein but manufacture cytokines *de novo* and release them rapidly. Although the genes for IL-4 and IL-5 are located very close together on chromosome 5q (Frew, A.J. and Dasmahapatra, J., 1995), recent evidence indicates that their expression is not coordinately regulated (Jung, T. *et al.*,1995).

Both allergic and non-allergic asthma is characterised by the expression of mRNA for IL-5 (Humbert, M. *et al.*, 1996), and it has been observed that there is a close correlation between T-cell activation and serum IL-5 concentrations in various forms of asthma (Corrigan, C.J. *et al.*, 1993). Lack of a really good animal or *in vitro* model of asthma has hampered the experimental studies of the role of IL-4 and IL-5 in allergic disease. However, some animal models have shown that T-cells are the main source of IL-5, but not IL-4, in the lungs of antigen- challenged mice (Garlisi, C.G. *et al.*, 1996). In addition, it has been consistently shown that monoclonal antibodies directed against IL-5 can prevent airways hyperresponsiveness in several species

including mice, guinea pigs and monkeys (Kung, T.T. *et al.*,1995a.; Mauser, P.J. *et al.*, 1993.; Mauser, P.J. *et al.*, 1995).

In bronchial biopsies, increased expression of IL-5 mRNA is one of the hallmarks of asthma (Hamid, Q. *et al.*,1991a), but IL-4 mRNA is less diagnostic. A series of studies addressing cytokine expression by BAL T- cells has shown the expression of IL-13, TNF-α and IFN-γ in unstimulated cells baseline BAL samples (Bodey, K.J. *et al.*, 1999). A minority of samples showed small amounts of IL-4 and IL-5. An increase in the expression of IL-3, IL-4 and IL-5 was observed when these BAL T-cells are stimulated with the polyclonal mitogen PHA. Twenty-four hours after local endobronchial allergen challenge, mRNA for IL-13 and GM-CSF is readily detectable in unstimulated samples, but the level of expression of IFN-γ mRNA is reduced compared to baseline samples (Bodey, K.J. *et al.*, 1999). When T cells become activated in culture CD25 expression is induced, and it was therefore assumed that the increased proportion of T cells expressing CD25 indicated active T cell involvement in asthma pathogenesis (Robinson, D.S. *et al.*, 1993b.; Corrigan, C.J. *et al.*,1988). But it became clear later that T cells can also express CD25 if they are partially activated and then become anergic and unresponsive to further stimulation (Schall, T.J. *et al.*, 1992). The expression of CD25 was compared with the production of IL-2 and IFN-γ in BAL samples, and it has been found that virtually none of the CD25+ cells were producing cytokines (Bakakos, P. *et al.*, 2002). This suggests that the CD25+ T cell population in asthmatic airways is anergic or regulatory and not participating actively in the inflammatory process.

1.4 The role of IL-10 in asthma

It has been reported that IL-10 stimulates regulatory cells 1 to produce high levels of IL-10 (Groux, H. *et al.*, 1997). It inhibits the production of pro-inflammatory cytokines including, under certain circumstances, its own production, by monocytes and enhances the production of IL-1RA (de Waal Malefyt., R. *et al.*, 1991.). This cytokine inhibits the expression of HLA-DR (Moore, K.W. *et al.*, 1993) and a number of costimulatory molecules including CD54, CD80 and CD86 on the APCs, IL-10 also exerts anti-allergic effects by inhibiting the expression of CD40 on eosinophils (Ohkawara, Y. *et al.*, 1996.), which accelerates eosinophil apoptosis, conferring an additional mechanism to prevent eosinophilic inflammation.

Low IL-10 production may contribute to the development of the characteristic inflammatory response in asthma, since, lower levels of IL-10 are formed in the BAL of patients with asthma compared to normal healthy individuals (Borish, L. *et al.*, 1996). Decreased expression of IL-10 transcripts was also detected in purified T cells from children with allergic or non-allergic asthma, as compared to those in healthy controls (Koning, H. *et al.*, 1997.).

1.5 Eosinophils, mast cells and basophils in asthma

Eosinophils are important mediators of allergic inflammation in the lung (Matsumoto, K and Saito, H., 2001). They contribute to the late asthmatic response, airway hyperresponsiveness and may also contribute to airway remodelling. Although, Th2 lymphocytes, monocytes, macrophages and epithelial cells contribute to airway inflammation, it has been very well documented that eosinophils acts as a major

effector of airway damage and dysfunction (Corrigan, C.J. and Kay, A.B., 1992). There is a strong correlation between the severity of disease and the number and the state of maturation and activation of eosinophils detected in the bronchial mucosa and in BAL of asthmatics (a).Bousquet, J. *et al.*, 1990). There is also a correlation between the resolution of eosinophilia and remission of asthma symptoms (Corrigan, C.J. and Kay, A.B., 1992.; Vignola, A.M. *et al.*, 1998).

In early stages of acute allergic inflammation, neutrophils may migrate into asthmatic airways but they cannot synthesize cysteinyl leukotrienes due to the lack of LTC4 synthase. The Cys-Lts are among the most potent of all known bronchoconstrictors and are 1000- fold potent than that of histamine (Leff, A.R., 2001).

Eosinophils release cysteinyl- leukotrienes causing bronchospasm and directly damage the airways via release of highly cationic proteins from their granules (Frigas, E. *et al.*, 1980). Over the long term, they may contribute to airway obstruction via fibrinogenic growth factors such as TGF-α and TGF-β. Eosinophils produce leukotriene C_4 (Jorg, A. *et al.*, 1982.; Weller, P.F. *et al.*, 1983.; Henderson, W.R. *et al.*, 1984) and PAF that increases vascular permeability and contracts smooth muscle (Lee, T. *et al.*,1984.; Pinchard, R.N., 1983). Leukotriene synthase contained by eosinophils serves as the predominant leukotriene delivery instrument in human asthma. cPLA2 (cytosolic phospholipase A2) is a cytosolic enzyme critical for the conversion of membrane phospholipid into arachidonic acid and Lyso-platelet activating factor (lyso-PAF) which is eventually converted into PAF (Zhu, X. *et al.*, 1999). The migration of eosinophils into the airways is closely associated with the priming of cPLA2.

Eosinophils also release a number of other soluble inflammatory mediators including vasoactive intestinal peptide (Aliakbari, J. *et al.*, 1987), substance P (Weinstock, J.V. *et al.*, 1988) and the chemokine IL-8 (Weller, P.F. *et al.*, 1996). There are four principal cationic proteins in the eosinophil granule namely MBP (major basic protein), EPO (eosinophil peroxidase), EDN (eosinophil-derived neurotoxin) and ECP (eosinophil cationic protein) (Wasmoen, T.L. *et al.*, 1988.; Barker, R.L. *et al.*, 1988). MBP is exceedingly highly basic due to the fact that it contains 17 arginines. MBP also contains 9 cysteines (Wasmoen, T.L. *et al.*, 1988.; Barker, R.L. *et al.*, 1988) and at least 4 reactive sulfhydryl groups : these groups may explain its marked tendency to aggregate with itself and with other proteins at alkaline pH. Both EDN and ECP possess ribonuclease activity (Slifman, N.R. *et al.*, 1986.; Gullberg, U. *et al.*, 1986) and demonstrate marked sequence similarity to ribonucleases from various species (Gleich, G.J. *et al.*, 1986). EPO has been partially sequenced and its light chain has been distinguished from MBP (Weller, P.F. *et al.*, 1988). *In vitro*, EPO in conjunction with H2O2 and halide kills a variety of microorganisms, and the preferred halide *in vitro* is iodide. Recent studies indicate that the effectiveness of EPO is dependant on the concentration of bromide that is present in physiological fluids (Weiss, S.J. *et al.*, 1986). Injection of eosinophil cytoplasmic granule, containing MBP, into the airway in animal models *in vivo* induces bronchial hyperreactivity, possibly by effects on respiratory epithelium (Gleich, G.J. *et al.*, 1988). EPO (Henderson, W.R. *et al.*, 1980) and MBP (O'Donnell, M.C. *et al.*,1983.; Zheutlin, L.M. *et al.*, 1984) may also have indirect pro-inflammatory effects through activation of mast cells to release histamine.

Eosinophil differentiation, activation and survival occur within the bone marrow under the influence of GM-CSF, IL-3 and IL-5, with IL-5 having the most cell-specific effects (Sampson, A.P., 2001; Campbell, H.D. *et al.*, 1987). IL-5 is essential to the haematopoeisis and survival of human eosinophils *in vivo* (Zhu, X. *et al.*, 1999.; Strek, M.E. and Leff, A.R., 1990). IL-5 also upregulates β2-integrin in human eosinophils. Hence IL-5 was considered to be a good potential target for treating asthma. It has been well established that IL-5 mediates antigen-induced eosinophilia in guinea pigs (Van Oosterhout, A.J. *et al.*, 1993) and mice.

When exposed to IL-5 by inhalation patients with asthma developed airway eosinophilia and hyperreactivity which suggests an important role both for IL-5 and for eosinophils in human asthma (Shi, H.Z. *et al.*, 1998b).

In contrast, a recent clinical trial using a humanized anti-IL-5 monoclonal antibody failed to reduce airway hyperresponsiveness or the late asthmatic response after allergen inhalation despite a very substantial reduction in blood and sputum eosinophilia (Leckie, M.J. *et al.*, 2000; Matsumoto, K. and Saito, H., 2001) suggesting that eosinophils may not be essential to the LAR. Eosinophils migrate from the peripheral circulation and are present in increased numbers in the conducting airways of chronic asthmatics. They are not present in significant numbers in the conducting airways of normal individuals (Leff, A.R. *et al.*, 1991).

Eosinophils are attracted in response to a variety of chemoattractants including lipid mediators, complement components, chemokines and cytokines. Eosinophils express receptors for complement components C3a and C5a, for chemokines including IL-8, RANTES (CCR1) and eotaxin (CCR-3), for cytokines including IL-1, IL-2, IL-3, IL-4, IL-5, IL-9, IL-16, GM-CSF, IFN-γ and TNF-α and for immunoglobulins (Ig) A,

IgG and IgE (high and low-affinity) (Weller, P.F.,1997). Eosinophils are thus well equipped to respond to a range of inflammatory stimuli.

Using recombinant murine IFN-γ and anti -IFN-γ monoclonal antibodies, it has been found that IFN-γ inhibits antigen-induced eosinophil recruitment into the tissue (Iwamoto, I. *et al.*, 1993). The intraperitoneal administration of rIFN-γ prevented antigen-induced eosinophil infiltration in the trachea of sensitized mice. The administration of rIFN-γ also decreased antigen induced CD4+ T cell but not CD8 + T-cell infiltration in the trachea. IFN-γ regulates antigen induced eosinophil recruitment into the tissue by inhibiting CD4+ T cell infiltration (Iwamoto, I. *et al.*, 1993). It is unlikely that IFN-γ directly acts on eosinophils thereby inhibiting the antigen-induced IL-5-dependant eosinophil infiltration, since it has been demonstrated that IFN-γ is an activator for eosinophils to prolong the survival and enhance cytotoxicity (Valerius, T. *et al.*, 1990). The effective inhibition of antigen-induced eosinophil recruitment into the tissues with r IFN-γ suggests that r IFN-γ would be useful for the treatment of atopic diseases such as asthma and atopic dermatitis.

Eotaxin is a CC family chemokine (Luster, A.D., 1998), a potent eosinophil attractant and can induce eosinophil superoxide and leukotriene production as well as granule release (Jose, P.J. *et al.*,1994.; Garcia- Zepeda, A. E. *et al.*, 1996.; Hisada, T. *et al.*, 1999b). Eotaxin has been found in asthmatic airways and can be upregulated by the cytokine IL-13. Later studies suggest that eotaxin-3 rather than eotaxin-1 or eotaxin-2 may contribute to the ongoing eosinophil recruitment to asthmatic airways in the later stages following allergen challenge (Berkman, N. *et al.*, 2001). The specific eosinophil recruiting chemokine eotaxin is important in the early recruitment of

eosinophils after allergen challenge (Rothenberg, M.E. *et al.*, 1997), but, a recent study with neutralizing monoclonal abs demonstrated that chemokines such as RANTES, monocyte chemotactic protein-5, and macrophage inflammatory protein-1α are also important in eosinophil tissue recruitment (Gonzalo, J.A. *et al.*,1998).

The selective recruitment of eosinophils into the tissues relies on local expression of endothelial adhesion molecules including ICAM-1 and VCAM-1. ICAM-1 binds CD11/CD18 molecules on a range of leucocyte types, while VCAM-1 is induced by IL-4 (Schleimer, R.P. *et al.*, 1992) and binds to VLA-4, which is found on eosinophils but not on neutrophils.

Mast cells and basophils also contribute to the pathogenesis of allergic asthma (Walls, A.F. *et al.*, 2001). The activation of mast cells and basophils results in the release of a range of potent mediators of inflammation and bronchoconstriction. Mast cells and basophils can be stimulated to degranulate by allergen cross-linking specific IgE bound to high-affinity IgE receptors on the cell membrane. In the airways, mast cells are abundant in the mucosal tissues, may also be present in the submucosa, particularly in the vicinity of mucus glands; and small numbers are free in the lumen, where they are well placed to respond to inhaled allergens.

There is also some evidence indicate that basophils are recruited to the bronchial mucosa and express IL-4 mRNA and protein after allergen provocation in sensitive patients with atopic asthma (Nouri-Aria, K.T. *et al.*, 2001). The IL-4 released from basophils might contribute to local IgE synthesis, and the basophils may promote tissue eosinophilia or other aspects of allergic inflammation during late responses and ongoing asthma.

Several studies have demonstrated infiltration by basophils of human allergen-induced late responses occuring in the skin, nose and bronchi (Prat, J. *et al.*, 1993.; Gibson, P.G. *et al.*, 1992.; Iliopoulos, O. *et al.*, 1992.; Guo, C.B. *et al.*, 1994.; Maruyama, N. *et al.*, 1994).

1.6 Mouse models of asthma

The increased tendency of bronchial smooth muscle to contract in response to an irritant is known as airway hyperreactivity and is a characteristic of asthma (McFadden, E.R. and Gilbert, I.A., 1992). Since an extensive study of the pathogenesis of asthma in human has been found to be impossible, it necessitated the development of appropriate mouse models. Mouse models resemble human disease in many aspects, therefore, studying asthma in the mouse may give an overall picture to understand what is happening in human. Studies in the mouse, suggest that IL-5 and IL-4 may control eosinophilia and IgE respectively (Coyle, A.J. *et al.*, 1995c.; Foster, P.S. *et al.*, 1996) whereas IL-13 and IL-9 may control mucus production and airway hyperreactivity (Cohn, L. *et al.*, 1999a.; Townsend, M.J. *et al.*, 2000a). Depletion of CD4 cells from mice can prevent AHR and eosinophilia (Gavett, S.H. *et al.*, 1994), while Th2 clones or primary CD4+ T cells secreting IL-4, IL-5 and IL-13 can induce asthmatic symptoms if adoptively transferred into unimmunized recipients (Cohn, L. *et al.*, 1999a). OVA inhalation by mice that had received the Th2 cells developed a pronounced airway eosinophilia associated with the largest increase in the number of eosinophils in the BAL (Lee, S.C., et al. 1999). An increase in the production of IL-4 and IL-5 in these mice has also been reported.

It has been demonstrated using a mouse model that much of the asthmatic Th2 response is dependant on OX40/OX40L interactions including production of high levels of IL-5, recruitment of large numbers of eosinophils and induction of IgE

(Jember, A. G. *et al.*, 2001). Notably, goblet cell hyperplasia, mucus production and AHR are all suppressed in mice not expressing OX40, demonstrating that this molecule plays a major role in the development of allergic airway inflammation.

The role of IL-5 in the selective accumulation of eosinophils and the development of pulmonary dysfunction was analysed in IL-5 knockout mice using an aeroallergen-induced lung damage model (Foster, P.S. *et al.*, 1996). Sensitized IL-5 knockout mice on a C57BL/6 background failed to develop any airway or blood eosinophilia in response to OVA aerosolization, and minimal morphological changes to pulmonary structure were observed (Foster, P.S. *et al.*, 1996). OVA -specific IgE was detected at similar levels in sera from IL-5+ and IL-5- mice after aeroallergen challenge indicating that IgE and eosinophilia are independently regulated.

Airway hyperreactivity to beta -methacholine was also abolished in aeroallergen-challenged IL-5- deficient mice. These observations establish that IL-5 plays an important role in generating blood and airway eosinophilia, the subsequent development of airway hyperreactivity and lung damage which occurs in response to aeroallergens. The role of eosinophil degranulation and peroxidase-mediated oxidation of airway proteins have been studied in knockout mice deficient for eosinophil peroxidase; the lack of EPO had no effect on the development of OVA-induced pathology in the mouse (Denzler, K.L., 2001). OVA- induced airway hyperresponsiveness was also displayed by EPO- deficient animals after provocation with methacholine. This data demonstrates that EPO activity is not important to the development of allergic pulmonary pathology in the mouse.

It has been observed in a mouse model of asthma, that allergen-induced bronchial hyperreactivity and eosinophilic inflammation can occur in the absence of IgE (Mehlhop, P.D. *et al.*, 1997).

When wild type and IgE-deficient mice were sensitized intranasally with *Aspergillus fumigatus*, both groups of animals developed bronchoalveolar lavage eosinophilia and pulmonary parenchymal eosinophilia accompanied by increased serum levels of total and Ag-specific IgE in the wild-type animals only. But AHR was significantly elevated both in wild type mice and IgE-deficient mice (Mehlhop, P.D. *et al.*, 1997.). Surprisingly, unsensitized IgE-deficient mice had increased bronchial responsiveness compared with unsensitized wild type controls. These suggest that AHR and airway inflammation can fully develop via IgE- independent mechanisms.

Greater AHR and more eosinophil infiltration in the respiratory epithelium after OVA sensitization and challenge have been reported in mice bred for high IgE production than control animals (Eum, S. Y. *et al.*, 1995). Attenuation of both eosinophilic airway inflammation and AHR in mice have been shown with the inhibition of IgE by anti-IgE (Coyle, A.J. *et al.*, 1996c). Although IgE plays an important role in the development of AHR and airway pathology, it is likely that other mechanisms take part in asthma pathogenesis.

It has been reported that the respiratory mucosa of asthmatic patients contains activated allergen-specific T- cells (Azzawi, M. *et al.*, 1990.; Gerblich, A. A. *et al.*, 1984) and a mouse model of AHR induced by repeated applications of picryl chloride has the features of a delayed type-hypersensitivity reaction and is T-cell dependant (Garssen, J. *et al.*, 1993). IL-13 has also been identified as a potent regulator of bronchoconstriction in mouse models of allergic asthma and experimental AHR (Wills-Karp, M. *et al.*, 1998.; Grunig, G. *et al.*, 1998). Although this cytokine is primarily secreted by Th2 cells, mast cells, macrophages, eosinophils and NK cells also express this cytokine (McKenzie, A.N. *et al.*, 1993.; Burd, P.R. *et al.*, 1995.; Hancock, A. *et al.*, 1998). IL-13 shares about 30% identity (at the protein level) with

IL-4 and in part, has similar biological functions through utilization of the IL-4 Rα chain (Zurawski, G. and de Vries, J.E., 1994). In mouse models of experimental asthma, blockade of IL-13 signaling by a soluble IL-13 R α 2-IgGFc function protein which specifically binds and neutralizes IL-13 has identified this cytokine as a principal regulator of AHR and mucus production in the allergic lung (Wills-Karp, M. *et al.*, 1998.; Grunig, G. *et al.*, 1998).

IL-13 was found to be sufficient to induce pathological features of asthma i.e AHR, IgE production, eosinophilia and mucus production, when instilled into the airways of naive mice or overexpressed in the lung (Li, L. *et al.*, 1999.; Grunig, G. *et al.*, 1998). IL-13 also induces eotaxin production in the lung (Li, L. *et al.*, 1999). Activation of STAT6 occurs when IL-13 signals by binding to its primary receptor chain (IL-13 Rα1) and recruiting the IL-4 Rα chain into the receptor complex (Zurawski, S.M. *et al.*, 1993.; Lin, J.X. *et al.*, 1995). In naive IL-4 Rα- deficient mice, IL-13 failed to induce asthma symptoms, which supports the concept that this receptor and subsequent signaling through STAT-6 are critical for the induction of allergic disease of the lung (Akimoto, T. *et al.*, 1998.; Kuperman, D. et al., 1998.; Tomkinson, A. *et al.*, 1999.; Foster, P.S., 1999). In contrast, IL-13 induced airways hyperreactivity independantly of the IL-4Rα chain in the allergic lung (Mattes, J. *et al.*, 2001) but IL-13 +/+ T cells did not induce disease in STAT 6-deficient mice. These results indicate that IL-13 employs a novel component of the IL-13 receptor signaling system that involves STAT 6, independantly of the IL-4- Rα chain to modulate pathogenesis. It has been shown that IL-13 signaling is dependant on T-cell activation in the lung and is critically linked to down stream effector pathways regulated by eotaxin and STAT6 (Mattes, J. *et al.*, 2001).

Recent studies in animals indicate that IL-13 can induce pathological changes reminiscent of asthma, including infiltration of eosinophils and mononuclear cells, epithelial damage, hyperplasia of goblet cells and subepithelial fibrosis (Zhu, Z. *et al.*, 1999.; Grunig, G. *et al.*, 1998.; Wills-Karp, M .*et al.*, 1998). In the airway epithelial cell line BEAS-2B, IL-13 upregulated eotaxin mRNA and protein synthesis which is STAT6 -dependant mechanism (Matsukura, S. *et al.*, 2001). As well as its role in the recruitment of eosinophils, IL-13 probably plays an important role as a mucus-stimulating cytokine (Zhu, Z. *et al.*, 1999.; Cohn, L. *et al.*, 1999a); it also induced the expression of vascular cell adhesion molecule-1 (VCAM-1) which is involved in eosinophil recruitment (Bochner, B.S. *et al.*, 1995.; Terada, N. *et al.*, 1998). Eotaxin is a C-C chemokine that binds with high affinity and specificity to the chemokine receptor CCR3 (Jose, P.J. *et al.*, 1994.; Rothenberg, M.E. *et al.*, 1995a.; Daugherty, B.L. *et al.*, 1996.; Stellato, C. *et al.*, 1997), which is expressed on important cells in allergic disease such as eosinophils, basophils, a subset of Th2 cells and mast cells (Daugherty, B.L. *et al.*, 1996.; Sallusto, F. *et al.*, 1997.; Yamada, H. *et al.*, 1997.; Ochi, H. *et al.*,1999). Eotaxin is highly expressed in the epithelium of asthmatics (Lamkhioued, B. *et al.*, 1997.; Mattoli, S. *et al.*, 1997.; Ying, S. *et al.*, 1997c), it may play a role in the recruitment of eosinophils into the airways. IL-13 induces the expression of eotaxin in the airway epithelium of mice more effectively than IL-4 (Li, L. *et al.*, 1999).

1.7 Novel approachs at the molecular level for treatment of asthma

Intramuscular injection of rats with a plasmid DNA encoding a house dust mite allergen results in its long -term expression and the induction of

specific immune responses. (Hsu, C. *et al.*, 1996). This prevented the induction of immunoglobulin E synthesis , histamine release in bronchoalveolar larvage fluid and airway hyperresponsiveness in rats challenged with aerosolized allergen. This suppression is persistent and can be transferred into naive rats by CD8+ T cells from allergen gene-immunized rats. It suggests that allergen- gene immunization is effective in modulating allergic responses and may provide a novel therapeutic approach for allergic diseases. Recent experimental evidence indicate that functionally distinct subsets of CD8+ T cells may play an important regulatory role in IgE production and suppress allergen-induced airway hyperresponsiveness (AHR) (Sedgwick, J.D. and Holt, P.G., 1985b.; Kemeny, D.M. and Diaz-Sanchez, D., 1991.; McMenamin, C. and Holt, P.G., 1993.; Renz, H. *et al.*, 1994).

1.8 Observations in patients with asthma and potential treatment

Several human studies have demonstrated a correlation between the levels of eosinophils in the peripheral blood and bronchial hyperreactivity (Durham, S.R. and Kay, A.B., 1985.; Iijima, M. *et al.*, 1985.; Taylor, K.J. and Luksza, A.R., 1987). Selective increase of eosinophils in the BAL fluid during the late-phase reaction (de Monchy, J.G. *et al.*, 1985) and the elevated ECP/albumin ratio in the BAL suggest that eosinophils degranulate during the peak of the late reaction. The presence of eosinophils in BAL has also been reported for patients with intrinsic asthma for whom allergic triggers of asthma have not been identified (Bentley, A.M. *et al.*, 1992). Neutrophils were not increased in BAL fluid in these patients. The human airway produces an array of cytokines which is diverse and occurs in response to

many stimuli. These stimuli can be cytokines such as IL-1β and TGF-β which cause the release of IL-6, IL-11 and LIF from the smooth muscle cell (Elias, J.A. *et al.*, 1997). IL-1β and TNF-α promote the release of GM-CSF (Sukkar, M.B. *et al.*, 2000) whereas TNF-α induces lL-8 (Pang, L. and Knox, A.J., 2000) and IL-6 (McKay, S. *et al.*, 2000) release.

The remodelling process is a key feature of persistent asthma which involves the deposition of extracellular matrix proteins within the airways. AHR and airway obstruction in asthmatics are associated with increases in total and antigen-specific IgE (Burrows, B. *et al.*, 1989.; Sears, M.R. *et al.*, 1991) and the genetic analysis of families indicates that AHR and IgE levels are linked (Postma, D.S. *et al.*, 1995). The development of therapeutics such as agents which block the interaction of IgE with its high affinity receptor, Fcε RI has been driven by the concept that allergen-specific IgE initiates allergic airway symptoms (Saban, R. *et al.*, 1994. ; Shields, R.L. *et al.*, 1995). IgE contributes to the pathogenesis of airways allergy in a number of ways, it is well established that IgE can initiate immediate hypersensivity reactions by triggering mast cell degranulation via FcεRI (Galli, S.J., 1993). In the airways, mast cell-derived mediators released after allergen challenge lead to immediate bronchial smooth muscle constriction, bronchial oedema and mucus hypersecretion (Kaliner, M., 1989.; Norel, X. *et al.*, 1991).

IgE-induced activation of mast cells leads to the synthesis of cytokines (Plaut, M. *et al.*, 1989. ; Burd, P.R. *et al.*, 1989). Cellular immune responses to allergen may be modulated by IgE by facilitating antigen uptake, processing and presentation by B

cells via CD23 thereby amplifying and regulating the immune response to allergens (Sutton, B.J. and Gould, H.J., 1993.; Kehry, M.R. and Yamashita, L.C., 1989).

IgE is a therapeutic target to develop new treatments for allergy and asthma as anti-IgE has been shown to attenuate both early and late-phase responses in mildly asthmatic patients after the inhalation of allergens (Boulet, L.P. *et al.*, 1997.; Fahy, J.V. *et al.*,1997), and to reduce asthma exacerbations in patients with moderate to severe allergic asthma who are taking corticosteroids (Milgrom, H. *et al.*, 1999).

A recombinant humanized anti-IgE mAb , omalizumab has been developed and used in experimental and clinical studies in asthma. It acts by binding to free IgE and blocks its interaction with mast cells and basophils (Busse, W. *et al.*, 2001).

1.9 Characteristics of regulatory T cells.

A distinct population of CD4+ T cells that constitutively express the IL-2 receptor (IL-2R) α chain (CD25) has recently been identified in mice (Sakaguchi, S., 2000. ; Asseman, C. *et al.*, 1999). CD4+CD25+ T cells are regulatory cells which constitute about 4-10 % of lymphocytes in mice and in humans. These cells play an essential role in the induction and maintenance of peripheral self-tolerance. Murine CD4+CD25+ T cells appear to mediate the suppression of effector T cell function both *in vitro* and *in vivo* via several mechanisms requiring either cell-cell contact or the production of immunosuppressive cytokines such as IL-10 and TGF-β (Sakaguchi, S., 2000. ; Asseman, C. *et al.*, 1999; Shevach, E.M., 2000). The costimulatory molecules CD28 and CTLA-4 interact with CD80 and CD86 present on APC.

CD28 is present on naïve T-cells and is important for the development of primary responses (Shahinian, A. *et al.*, 1993.; Allison, J.P., 1994.; June, C.H. *et al.*, 1994.). In contrast, CTLA-4 is induced only after T-cell activation and its ligation results in the inhibition of T-cell activation. IL-2 gene expression and cell cycle progression are inhibited as a result of concomitant cross- linking of CTLA-4 with TCR- signaling (Walunas, T.L. *et al.*, 1996.; Krummel, M.F. and Allison, J.P., 1996.), CTLA-4 contributes to maintaining immunological self-tolerance (Takahashi, T. *et al.*, 2000.); *in vivo* blockade of CTLA-4 for limited period in normal mice led to the spontaneous development of chronic organ-specific autoimmune diseases. CD25+CD4+ T cells suppressed antigen-specific and polyclonal activation and proliferation of other T cells, including CTLA-4 -deficient T-cells, when stimulated via T cell receptor *in vitro*; blockade of CTLA-4 abrogated the suppression (Takahashi, T. *et al.*, 2000.). There is another member of B7 family (B7-H1) whose ligation co-stimulated T-cell responses to polyclonal stimuli and allogeneic antigens and preferentially stimulated the production of IL-10 (Dong, H. *et al.*, 1999) although it should be noted that small amounts of IL-2 were required for the effect of B7-H1 costimulation (Dong, H. *et al.*, 1999).

CD28 –deficient CD25+CD4+T cells can also suppress normal T-cells suggesting that CD28 is not crucial for the activation of regulatory T-cells (Takahashi, T. *et al.*, 2000). The unique function of CTLA-4 could be exploited in controlling T-cell-mediated immunoregulation and thereby induction of immunological tolerance or to control autoimmunity.

1.10 Role of regulatory T cells in preventing autoimmunity

CD4+CD25+ regulatory T cells are thymus- dependent, are capable of suppressing auto-reactive T-cells (Itoh, M. *et al.*, 1999) and prevent the induction of organ – specific autoimmune diseases (Sakaguchi, S. *et al.*, 1995.; Asano, M. *et al.*, 1996.; Suri-payer, E. *et al.*, 1998.; Sakaguchi, S., 2000.). These regulatory cells constitute about 5-10% of peripheral CD4+ T cells in non-immunized naïve mice (Sakaguchi, S. *et al.*, 1995; Asano, M. *et al.*, 1996.; Suri-payer, E. *et al.*, 1998) and inhibited the proliferation of CD4+CD25- cells *in vitro* (Thornton, A.M. and Shevach, E.M., 1998.; Takahashi, T. *et al.*,1998). There is accumulating evidence demonstrating the existence of human CD4+CD25+ regulatory cells. These cells express high levels of the IL-2 receptor (Baecher-Allan, C. *et al.*, 2001) and following TCR cross-linking, CD4+CD25high cells did not proliferate but instead totally inhibited proliferation and cytokine secretion by activated CD4+CD25- responder T cells in a contact - dependant manner. The inhibition is dependant on contact between responding CD4+CD25- T cells and the CD4+CD25+T cells and not mediated by cytokines (eg IL-4, IL-10 and TGF-β) (Thornton, A.M. and Shevach, E.M., 1998.; Takahashi, T. *et al.*,1998). For the regulatory function of CD4+CD25+ T cells, the expression of cytotoxic T-lymphocyte associated antigen 4 (CTLA-4) is required (Read, S. *et al.*,2000.; Takahashi, T. et al., 2000).

CD4+CD25+ cells play a major role in mediating immunological self tolerance via protecting the body from being attacked by T-cells reactive against self-antigens (Sakuguchi, S., 2000). It has been proposed that two distinct populations of CD4+T

cells exist, one capable of mediating autoimmune disease and a second inhibiting such responses. Typically, the latter are dominant and maintain self-tolerance.

There have been different cell surface markers employed to identify regulatory cells, CD25 (IL-2 receptor alpha chain generally expressed on activated T-cells), CD45RB/RC (a protein tyrosine phosphatase expressed in almost all haematopoetic cells), CD5 (expressed at high levels on mature T-cells and a possible ligand for CD72) and RT6.1 (expressed on the majority of mature T-cells in rats, and having ADP- ribosylation activity (Sakaguchi, S., 2000). T-cell deficient mice or rats which were given CD4+ splenic cells depleted of CD4+, RT6.1+, CD5high or CD45 RB/RC low cells spontaneously developed various organ specific autoimmune diseases such as insulin-dependant diabetes mellitus, thyroiditis, gastritis and systemic wasting disease in a few months (Sakaguchi, S., 2000). Although, the surface markers of the regulatory cells are not directly associated with the regulatory function itself, it may indicate that the cells are in an "activated", "primed" or memory state. These cells suppress the activation and expansion of potentially pathogenic self-reactive T-cells in the normal immune system, thereby contributing to the maintainance of self –tolerance. In the thymus and the periphery, regulatory cells constitute about 4-10% of mature CD4+ T-cells. The removal of these cells leads to spontaneous activation and expansion of self-reactive T-cells and consequently to the development of various autoimmune diseases (Itoh, M. *et al.*, 1999). Functionally mature regulatory cells are produced as CD4+ CD8- cells in the thymus and are capable of preventing autoimmunity (Itoh, M. *et al.*, 1999.; Seddon, B. and Mason, D., 2000). The normal thymus seems to be continuously producing not only

pathogenic self-reactive T-cells but also functionally mature regulatory CD4+T-cells controlling them. There are some published evidence that these regulatory cells can be expanded *in vitro* in exogenous IL-2 (Chen, Y. *et al.*, 1994.; Groux, H. *et al.*, 1997). It has also been observed that the mice deficient in TGF-β or TGF-β- receptor developed various autoimmune diseases (Gorelik, L. and Flavell, R.A., 2000).

Regulatory CD4+ T- cells unlike other T-cells, constitutively express CTLA-4, a costimulatory molecule expressed on T-cell activation, and the T-cells stimulated via CTLA-4 predominantly secrete TGF-β (Chen, W. *et al.*, 1998a.; Salomon, B. *et al.*, 2000). Collectively, these observations suggest that regulatory CD4+ T-cells activated through CTLA-4 might suppress other T-cells by secreting TGF-β.

Regulatory T-cells also exert a suppressive effect on other T-cells via a cognate cellular interaction with APCs (Itoh, M. *et al.*, 1999). Anergy and clonal deletion are the main mechanisms conferring immunological tolerance in order to avoid severe autoreactivity. Interestingly, thymectomy of neonatal and adult mice results in the development of organ specific autoimmunity. Thymectomised animals reconstituted with CD25+ CD4+ regulatory T cells do not develop autoimmunity. This regulatory cell lineage is generated in the thymus and the expression of CD25 antigens distinguishes regulatory cells from other T cells. It has been very well documented that effector and suppressor T cell populations are CD4+CD8- (Smith, H. *et al.*, 1991; Smith, H. *et al.*, 1992). Although reconstitution experiments implied strongly that the development of autoimmunity was prevented by suppressor T-cells, the implication has been strengthened by the demonstration that the removal of suppressor T cells from an immune system of a normal animal resulted in disease, and that reconstitution

of the recipient with suppressor T cells triggered the reestablishment of self tolerance and prevented autoimmunity (Sakaguchi, S. *et al.*, 1985). Similarly, a role for regulatory T cells in controlling antibody production has been hypothesized i.e that antibody- mediated autoimmune diseases might develop because of the failure of regulatory T cells to control autoantibody production (Penhale, W.J. *et al.*, 1973.; Penhale, W.J. *et al.*, 1975.; Penhale, W.J. *et al.*, 1976.).

1.11 Role of Tr cells in transplantation tolerance

In organ transplantation in animals, tolerance can be acquired by transferring lymphocytes from long-term survivors to naïve recipients (Zhai, Y. and Kupiec-Weglinski, J.W., 1999).

Tolerance established either by a course of cyclosporin A treatment or a blocking anti- CD4 antibody can be transferred to naïve animals by CD4+ T cells from tolerant donors (Hall, B.M. *et al.*,1990; Qin, S. *et al.*, 1993). Encouragingly, transfer of CD4+ T cells from anti-CD4 antibody- treated tolerant mice triggered the naïve CD4+ T cells in the recipients to gradually become tolerant with the allograft sustaining the tolerant state (Qin, S. *et al.*, 1993). It is unclear whether the CD4+ regulatory T cells responsible for transplantation tolerance are similar to T cells mediating self-tolerance.

It has been demonstrated that retroviral mediated gene transfer of viral IL-10 prolongs allograft survival by decreasing donor- specific cytotoxic T lymphocyte precursor and IL-2-secreting helper T lymphocyte precursor frequency within graft-infiltrating cells

(Qin, L. *et al.* 2001). It also reports that local immunosuppression induced by viral IL-10 gene transfer is CD4+T cell and IL-4 and murine IL-10 dependent, and prevent direct alloantigen presentation through an alteration of donor type APC function.

1.12 Role of Tr cells in allergy and asthma

In a mouse model of asthma, depletion of CD4+CD25+ T cells resulted in a decreased antigen-induced eosinophil recruitment into the airways (Suto, A. *et al.*, 2001). Endogenous IL-10 suppresses allergen-induced airway inflammation and non-specific airway responsiveness demonstrated in IL-10 knock out mice and wild type counterparts (Tournoy, K.G. *et al.*, 2000).

TGF-β has been implicated in immunosuppression (Nakao, A. *et al.*, 2000.), since the blockade of TGF-β/Smad signalling in mature T-cells by expression of Smad 7 enhanced airway inflammation and airway reactivity (Nakao, A. *et al.*,2000). These observations indicate that regulation of T-cells by TGF-β was crucial for the negative regulation of inflammatory immune response. TGF-β/Smad signalling in mature T-cells has also been implicated as a regulatory component of allergic asthma (Nakao, A. *et al.*, 2000). In the immune system, TGF-β regulates growth, differentiation and the function of macrophages, T-cells, B-cells and natural killer cells (Wahl, S.M., 1992.; Miyazono, K. *et al.*, 1994.; Letterio, J.J. and Roberts, A.B., 1998). Furthermore, it has also been noticed that TGF-β null mice developed extensive inflammation in various organs and died shortly after birth, suggesting that TGF-β played a crucial role in the suppression of the immune system. (Shull, M.M. *et al.*,1992.; Kulkarni, A.B. *et al.*, 1993.). The mechanism of action of TGF-β *in vivo* is

not clear, TGF-β acts on multiple targets affecting the immune system (Wahl, S.M., 1992). An extensive study into the pathological role of TGF-β/Smad signaling in the asthmatic airways needs to be established since TGF-β/Smad signaling has been implicated in the suppression of airway inflammation in mice (Nakao, A. *et al.*, 2000).

In another mouse model, treatment with a killed Mycobacterium vaccae- suspension, generated allergen-specific CD4+CD45RB (Lo) regulatory T cells, which confer protection against airway inflammation (Zuany-Amorim, C. *et al.*, 2002). This mechanism appeared to be mediated by IL-10 and TGF-β, as antibodies against IL-10 and TGF-β abrogated the inhibitory effect of CD4+CD45RB (Lo) T cells.

1.13 Role of Tr cells in limiting colitis

Stimulation of CD4+T cells in the presence of IL-10 induces the differentiation of a unique subset of T-cells with immunoregulatory properties (Groux, H. *et al.*, 1997) defined as regulatory cells secreted high levels of IL-10, low levels of IL-2 and no IL-4. These antigen specific T-cell clones suppressed the proliferation of CD4+T cells in response to antigen, and prevented colitis induced in SCID mice by pathogenic CD4+CD45 RB high splenic T cells (Groux, H. *et al.*, 1997).

In the intestine, the regulatory cells which prevent the development of pathogenic responses to both self and intestinal antigens, have the same phenotype (CD25+CD45 RB low CD4+) as those that control autoimmunity (Read, S. *et al.*, 2000). Their immunosuppressive function *in vivo* depends on signaling via the negative regulator of T-cell activation cytotoxic T-lymphocyte –associated antigen-4 as well as the secretion of immune- suppressive cytokine transforming growth factor-β (Read, S. *et*

al., 2000). Regulatory cells constitutively express CTLA-4 , suggesting the expression of CTLA-4 by these cells is involved in their immunosuppressive function (Read, S. *et al.*, 2000).

1.14 The effects of prostaglandin on the immune system

Prostaglandins are inflammatory mediators secreted by mononuclear cells such as macrophages and dendritic cells. There are several different types of prostaglandins (PGI_2, PGE_2, $PGF_{2\alpha}$, TXB_2, PGD_2, etc) each of which has a different function. PGE_2 increases cyclic AMP in T cells, and inhibits the production of IL-2 and IFN-γ by Th1 cells but not IL-4 by Th2 cells (Betz, M. and Fox, B.S., 1991). We have preliminary *in vitro* data indicating that PGI_2 can augment the production of IL-10 by CD4+CD25+ T cells. The critical enzymes involved in the production of prostaglandins from arachidonic acid are cyclooxygenases (COX). Two isoforms of the cyclooxygenase enzyme exist specifically, COX-1 and COX-2 which catalyse the rate limiting step in the formation of prostaglandins. The expression of COX- 1 is constitutive but COX-2 expression is induced following the onset of inflammatory responses. The effect of administration of COX-2 inhibitors on Th2 cells has been recently addressed in mice.

It is well known that some asthmatics are sensitive to aspirin which inhibits both COX-1 and COX-2. This observation has encouraged previous students in our research group to conduct a study in a DO11.10 mouse model, in which they

observed that mice treated with a COX-2 inhibitor still expressed PGE_2, $PGF_{2\alpha}$, PGD_2 and TxA_2 in BALF but there was no PGI_2. We have also found that CD4+CD25+ cells express receptors for PGI_2. We have therefore hypothesised that the prostaglandin PGI_2 may help to ease the symptoms in aspirin sensitive asthmatics via augmenting the production of IL-10 by CD4+CD25+ T cells.

A variety of agents have been shown to induce COX-2 expression, including bacterial lipopolysaccharides, growth factors, cytokines and phorbol esters (O' Sullivan, M.G. *et al.*, 1992b.; Jones, D.A. *et al.*, 1993b.; Kujubu, D.A. *et al.*, 1993). The liberation of arachidonic acid from membrane bound phospholipids is the first step in the production of prostaglandins. Phospholipase enzymes, primarily phospholipase A2, are involved in the liberation of phospholipids. Phospholipase A2 is expressed in different isoforms, some are expressed constitutively and others are induced in response to inflammatory stimuli (Cirino, G., 1998). It has been proposed that phospholipase and not cyclooxygenase is the rate limiting step in prostaglandins production (Cirino, G., 1998). The ability of cells and tissues expressing COX-2 *in vitro* (Saunders, M.A. *et al.*, 1999) and *in vivo* (Hamilton, L.C. *et al.*, 1999a) to release prostanoids is greatly enhanced when phospholipase is activated. The profile of products made by cells expressing COX-1 or COX-2 is determined by the presence of different downstream synthases. However, when cells express large amounts of COX-2 , the PGH2 formed results in the formation of large amounts of PGE2 (Bishop- Bailey, D. *et al.*, 1997a) possibly by non-enzymatic conversion. This is probably due to the apparent lack of regulation of synthase enzymes at the site of inflammation.

COX-2 is undetectable in most tissues in the absence of stimulation, but is induced as an intermediate early gene in a limited range of cells, notably in monocytes, macrophages, neutrophils and endothelial cells (Fu, J.Y. *et al.*, 1990.; Hla, T. and Neilson, K., 1992.; Niiro, H. *et al.*, 1997). Expression of the COX-2 isoform is inhibited by glucocorticoids and by the anti-inflammatory cytokines interleukin 10 and IL-4 (Masferrer, J.L. *et al.*,1990.; Mertz, P.M. *et al.*, 1994.; Dworski, R. and Sheller, J.R., 1997). Administration of conventional non- steroidal anti-inflammatory drugs inhibits the synthesis of prostaglandins thought to be responsible for both the therapeutic and adverse effects (Vane, J.R., 1971.; Vane, J.R. and Botting, R.M., 1995). These compounds do not discriminate between the two isozymes; however, the therapeutic effects of this agents are related to the inhibition of COX-2 at the sites of inflammation, whereas the adverse effects in gastrointestinal tract and platelets are attributable to the inhibition of COX-1 (Masferrer, J.L. *et al.*, 1996.).

Two factors which regulate the biological effects of COX-2 are: 1) distribution of synthase enzymes and the oxidative state of the cells, 2) Distribution of prostanoid receptors on local target tissue (Mitchell, J.A. and Warner, T.D., 1999). There are several different prostanoid receptor types. The main receptor for PGI2 is the IP receptor which is linked to activation of adenylate cyclase. Thus the action of PGI2 upon IP receptors elevates cyclic- AMP, leading to an inhibition of active processes (Mitchell, J.A. and Warner, T.D., 1999.). For example, the activation of IP receptors in vascular smooth muscle promotes vasodilation while its activation in platelets leads to a reduction in aggregation and adhesion (Armstrong, R.A., 1996). *In vivo* increases in the expression of COX-2 have been associated with inflammation (Vane, J.R. *et al.*,

1994), rheumatoid arthritis (Kang, R.Y. *et al.*, 1996) and ischaemia (Planas, A.M. *et al.*, 1995). In the spinal cord, COX-2 expression is also elevated following peripheral inflammation (Beiche, F. *et al.*, 1996). TNF-α and IL-10 production was modified with PGI2 or PGE2 in mice via IP, EP2 or EP4 receptors. The production of TNF-α was also down regulated resulting in an anti-inflammatory effect (Ueno, A. and Ohishi, S., 2001). COX-2 is downregulated by glucocorticoids (Masferrer, J.L. et al., 1994) and also by related agents such as 17 β-estradiol (Morisset, S. *et al.*, 1998). COX-2 metabolites are intimately involved in the induction of pain and inflammation. The precise role of prostanoids in pain and fever is not known, but delineation of such mechanisms will facilitate the development of anti-pyretic analgesics with fewer side effects.

1.14.1 Production of prostaglandins during inflammation at the mucosal site:

The expression of COX-2 is induced by cytokines in a number of airway cells including epithelium (Mitchell, J.A. *et al.*, 1994) and underlying smooth muscle (Belvisi, M.G. *et al.*, 1998). Narrowing airways in some patients is contributed to by the excessive proliferation of airway cells , which is characteristic of asthma and other related diseases. Proliferation of human airway smooth muscle cells is inhibited by COX-2 induction suggesting a protective role of this enzyme in diseases such as asthma (Belvisi, M.G. *et al.*, 1998). In mice in which the DP receptor has been disrupted, the allergen induced airway eosinophilic inflammation, Th2 type cytokine production, and bronchial hyperresponsiveness to cholinergic stimuli were reduced, suggesting that PGD2 is an important mediator of allergic asthma (Tanaka, H. and Nagai, H., 2001). Inflammatory resolution in carageen –induced pleurisy in rats at 48

hours coincided with an increase in the production of PGD2 and 15 deoxy delta prostaglandin J2 (Ujihara, M. et al.,1988d.; Fournier, T. et al., 1997.; Mizuno, H. *et al.*, 1997), in contrast, PGE2 production was decreased. The COX-2 selective inhibitor NS-398 and non-specific COX-1/COX-2 inhibitor indomethacin inhibited inflammation at 2 hours but exacerbated inflammation at 48 hours (Gilroy, D.W. *et al.*, 1999). This was due to the reduced prostaglandin D2 and 15 deoxy delta PGJ2 concentration and was reversed by replacement of these prostaglandins, implying that they play an important role as anti- inflammatory agents.

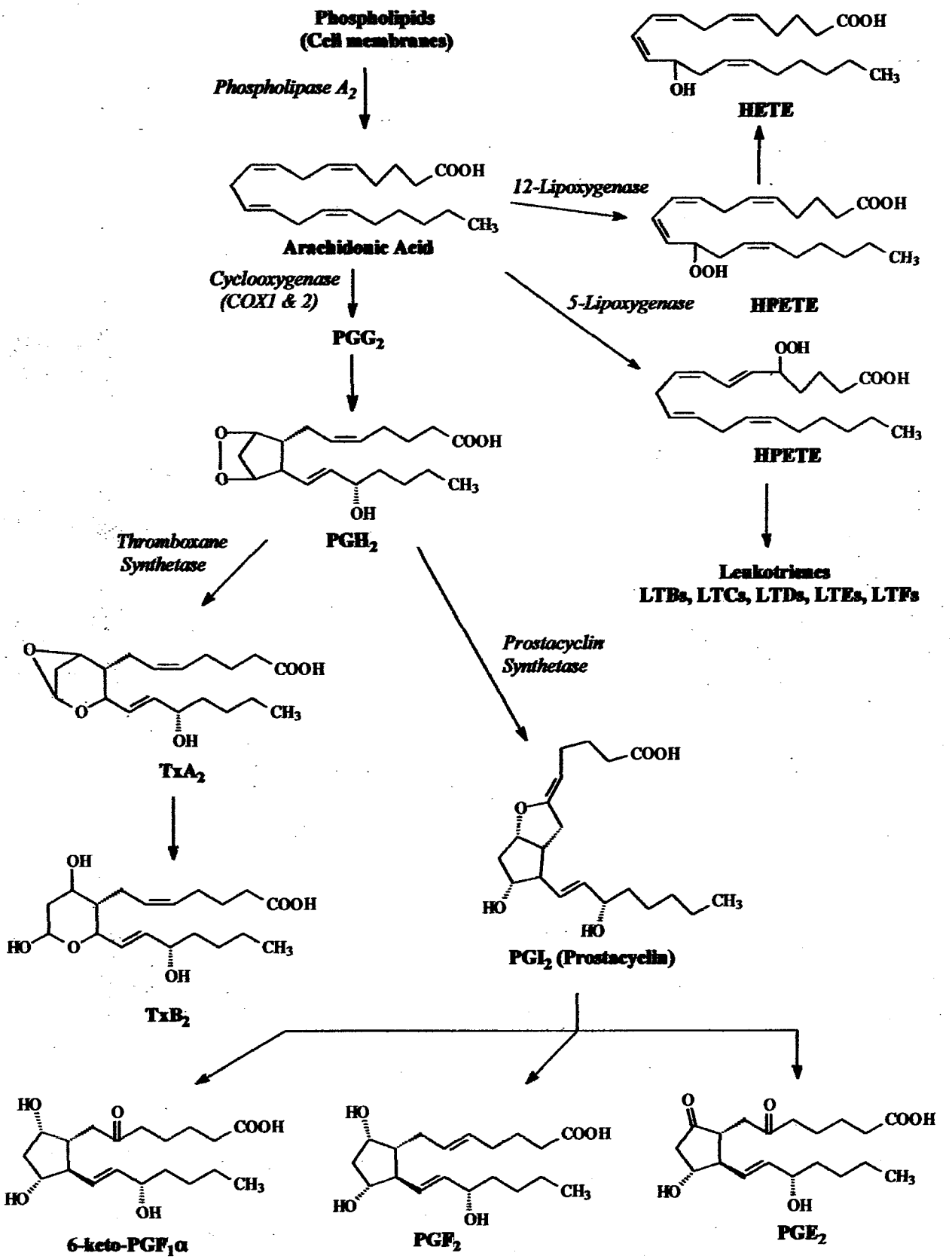
Why aspirin exacerbates symptoms in aspirin sensitive asthmatic patients is not known. There is a general level of acceptance that COX suppresses the production of leukotrienes (Kowalski, M.L., 1995). Increase in the production of leukotrienes when cyclo-oxygenase is blocked worsen the asthmatic symptoms. Aspirin sensitive asthmatics express COX-2 in their airways (Sousa, A.R. *et al.*,1997a.; Cowburn, A.S. *et al.*, 1998). There is a speculation that COX-2 in the airways leads to beneficial products that limit the production of leukotrienes. Endogenous PGI2 in gastric mucosal injury conferred protection via enhancement of calcitonin gene- related peptide (Boku, K. *et al.*, 2001) demonstrating a protective role for PGI2 at the mucosal surface.

1.14.2 Recent findings in our laboratory on evaluating the effect of COX-2 on CD4+ Th responses:

When a COX-2 inhibitor is used in the DO11.10 mouse adoptive transfer system, there was a pronounced increase in the level of pulmonary eosinophilia in mice given Th_2 cells but no effect on the inflammation evident in Th1 recipients. Analysis of BALF revealed that the COX-2 inhibitor selectively inhibited the production of PGI_2.

In contrast, the amount of PGE_2, $PGF_{2\alpha}$, PGD_2 and TxA_2 was not affected. Collectively, these observations suggest that PGI_2 may play a role in inhibiting pulmonary Th2 responses.

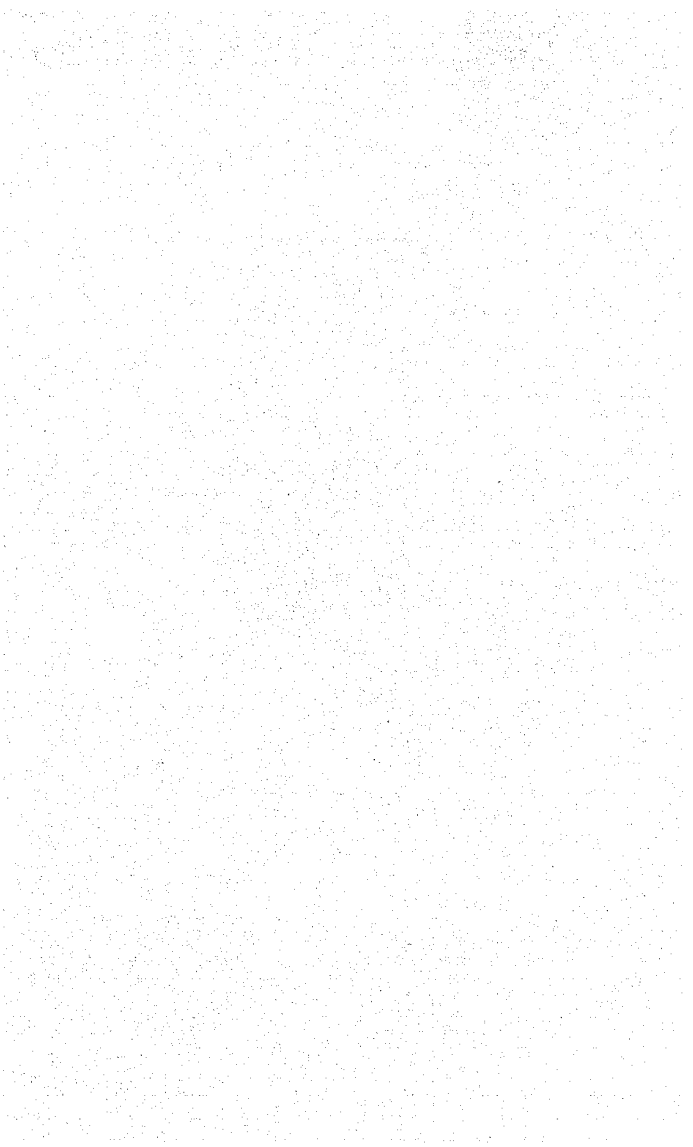
The expression of mRNA for PGI_2 receptors by purified CD4+Th2 cells has been demonstrated using the PCR technique. *In vitro*, it has been demonstrated in our laboratory that PGI_2 affects Th2 but not Th1 cells; possibly PGI_2 acts by increasing the production of IL-10 by Th2 cells. We speculate that the expression of PGI_2 receptors is induced by IL-4 and contributes to limiting Th2 responses.



The spectrum of eicosanoids produced as a consequence of arachidonic acid metabolism

Hypothesis

Lung mucosal Th2 responses are regulated by CD4+CD25+ suppressor T cells.



Aims

1. Develop and characterise a mouse model of pulmonary Th2 responses

2. Examine the effects of CD4+CD25+ regulatory T cells on:

a. Polarization of CD4+ T cells into Th2 cells
b. Pulmonary inflammation mediated by CD4+ Th2 cells

3. Examine the effect of PGI_2 on CD4+CD25+ T cells

Chapter 2

Materials and Methods I

2.0 Media

CM-RPMI (CM-complete) consisted of RPMI 1640 (Gibco BRL, Paisley, UK) supplemented with penicillin (50U/ml), Streptomycin (50μg/ml), 2mM glutamine, 2 mercapto-ethanol (5μM), 5mM HEPES and 5% foetal calf serum.

2.1 Animals

BALB/c mice were obtained from Harlan (Loughborough, UK). DO11.10 transgenic mice express a TCR recognizing OVA peptide 323-339 and backcrossed to BALB/c mice in the university of Texas Medical Branch facility were kindly provided by Dr. D. Y. Loh (Howard Hughes Medical Institute, St. Louis, MO). They were bred in a specific aseptic condition in a barrier facility at Southampton University (Southampton, UK). All mice used in these experiments were 4-8 weeks of age and were housed in our pathogen- free conditions with food and water provided.

2.2 Antigen

OVA peptide 323-339 (H-ISQAVHAAHAEINEAGR- OH) was purchased from Chiron Technologies. Peptide was dissolved in saline (pH>9.0) and purified using Sephadex G10 column (Pharmacia Biotech). PBS was used to elute the peptide. The protein was quantified by BCA protein assay (Pierce, UK). The final concentration used for proliferation assay was 1μg/ml (a dose response curve is shown in figure 2.2a), by adding 50 μl of 4μg/ml of OVA peptide into each well having a total volume of 200 μl.

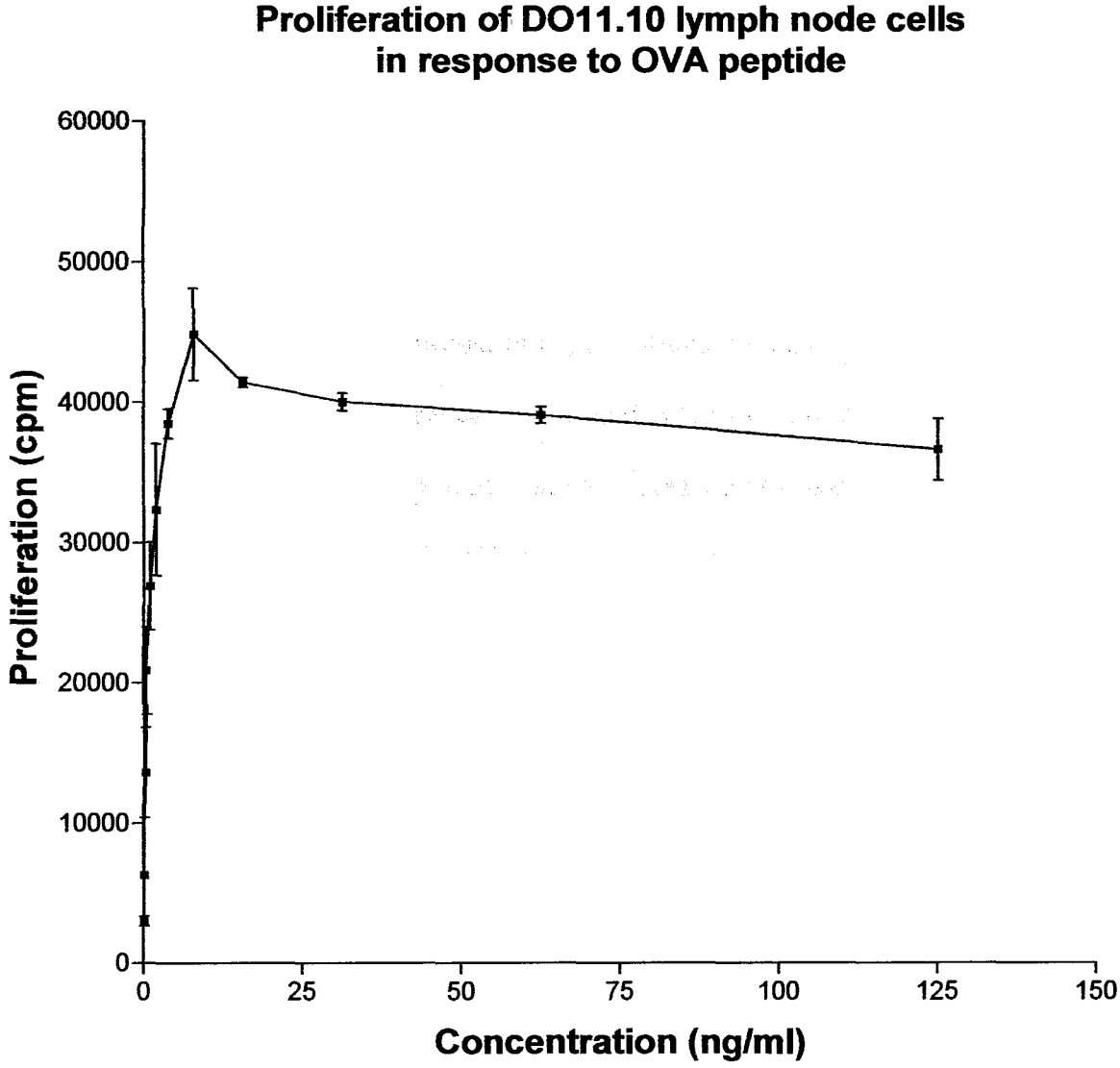


Figure 2.2a

DO11.10 lymph node cells were cultured *in vitro* with different concentrations of OVA peptide (0.06-125ng/ml), incubated at 37.8°C for 48 hours. ^{3}H-thymidine was added after 48 hours and incubated for further 24 hours. The proliferative response of DO11.10 lymph node cells to OVA peptide was measured by the level ^{3}H-thymidine incorporation and expressed as counts/min.

2.3 Preparation of CD4+ Th1 and Th2 cells from OVA specific DO11.10 mice

Peripheral and mesenteric lymph nodes were removed from OVA specific DO11.10 mice. The media containing lymph nodes were dissociated using a 5ml syringe under aseptic condition and the cells were collected through a strainer in petri dish. The cells were transferred into a sterile universal and washed in HBSS. The suspension was centrifuged at 438g, 19° C for 10 minutes and the supernatant was disposed of and resuspended in 10 ml of complete RPMI medium. Cells were counted using a neubaur chamber cell counter. For generating Th1 lines, lymph node cells were cultured at a concentration of $5x10^5$/ml with OVA peptide (1μg/ml), anti- IL-4 (5μg/ml 11B11 antibody, hybridoma supernate; American Type culture collection, Manassas, VA), mouse IL-12 (1ng/ml; R&D Systems, Abingdon, U.K.) and incubated at 37°C for 4 days.

Th_2 cell line was generated by stimulating lymph node cells ($5x10^5$/ml) with OVA peptide (1μg/ml) in the presence of anti- IFN-γ (5μg/ml; R4-6A2; American Type Culture Collection) and murine IL-4 (2ng/ml; R&D Systems). Cell cultures (Th_1 and Th_2) were removed from the incubator on the 5th day, washed in HBSS and resuspended in fresh CM-RPMI medium. Th1 cells ($3x10^5$/ml) were restimulated with mouse IL-12(1ng/ml), anti-IL-4 (5μg/ml), IL-2 (100U/ml; Cetus, Emeryville, CA) and OVA peptide (1μg/ml). Th2 cells ($3x10^5$/ml) were restimulated with OVA peptide (1μg/ml), murine IL-4 (2ng/ml), anti-IFNγ (5μg/ml) and IL-2 (100 U/ml). After 4 days of culture, CD8+ T cells and APCs were depleted by panning.

The concentration of reagents used was as published by our group (Jaffar, Z. *et al.*, 2002).

2.3.1 Removal of CD8+ T cells and APCs by panning

This was a technique used to deplete CD8+ T cells (using YTS169.4; Serotec, Oxford, U.K.) and APCs (using anti-class II Ab M5/114; American Type Culture Collection) from Th1 and Th2 cultures. Cells used to inject mice or for anti-CD3 treatment were pure CD4+Th1 and Th2 cells (10^8 cells). To polarised Th1 and Th2 cells suspended in 8 ml of CM -RPMI medium, 20μg/ml of α-CD8 and α- class II Ab (20%) were added and incubated for 30 minutes on ice. The cells were washed and resuspended in 5 ml of CM -RPMI. Both Th1 and Th2 cell suspensions were added into the petri dishes (Stratech) coated with mouse anti-rat IgG (20μg/mg; Mar 18.1) and incubated for 45 minutes.

Non-adherant cells were collected, centrifuged and resuspended in CM-RPMI media. The pure Th1 and Th2 cells were used for injection and cytokine assessment.

The concentration of reagents used was as published by our group (Jaffar, Z. *et al.*, 2002).

2.4 Adoptive transfer of cells

CD4+ effector cells were transferred into histocompatible BALB/c mice by intravenous injection via the lateral tail vein. $10x10^6$ Th1 or Th2 cells in 200μl HBSS were adoptively transferred intravenously into each BALB/c recipient.

2.5 Animal Sensitization

We used the ovalbumin (OVA) specific – T cell receptor transgenic mouse, DO11.10, to model lung mucosal T cell responses. Mice were exposed to aerosolized 0.5% ovalbumin solution (Grade V, Sigma-Aldrich, Poole, UK) for 20 minutes a day for 7

consecutive days using a Wright's nebulizer (Buxco Europe, Petersfield, U.K). Mice were sacrificed on day 7 by using Diethyl ether inhalation anesthetized until dead. Bronchoalveolar lavage fliud was collected and the lung and peripheral lymph node cells were analysed by FACS for the expression of different receptors.

The percentage of ovalbumin used was as published by our group (Jaffar, Z. *et al.*, 2004).

2.6 Bronchoalveolar Lavage Fluid (BAL fluid)

An open tracheotomy method was used. Lungs were lavaged through a tracheal tube by cannulating the trachea of the mice and injecting with 0.5 ml PBS at 37^0 C via a cannula and retrieving the lavage.

This procedure was repeated three times for each mouse. The volume and number of cells in the BALF was assessed. The BAL was centrifuged at 503 g for 10 minutes at 4^0C. The BAL cells in each group were resuspended in 1 ml of PBS which were used for the preparation of cytospin slide staining and eosinophil peroxidase analysis. Aliquots of BALF supernatants were kept at -80^0C for biochemical analysis.

2.6.1 BAL Cells Staining and Differential Count

BAL cells were cytospin onto glass slides at 280g (Cytospin 3, Shannon). Slides were air-dried and stained using modified Wright stain and the cell differential percentages were determined by light microscopic evaluation of stained cytospin preparations and expressed as absolute cell numbers.

2.6.2 An assessment of eosinophilia (EPOassay) and cytochemical evaluation of eosinophil infiltration into BALF.

A colourimetric method was used to determine the eosinophil peroxidase (EPO) activity in BALF. Bronchoalveolar lavage fluid was centrifuged at 503g, 20^0C for 10 minutes, supernatant was poured off and the cell suspension was resuspended in 1ml of PBS. Two- fold dilutions (spanning 1/2 - 1/256) of BALF (100µl in each well) were prepared in PBS in 96 wells, flat bottomed ELISA plates (NUNC). Substrate (Trizma hydrochloride 50mM , Triton (0.1%; Sigma), hydrogen peroxide 1mM (Sigma) and OPD (orthophenylene diamine dihydrochloride , 0.1mM; Sigma) was added (100µl) into all wells which contained diluted and neat samples and incubated in the dark at room temperature for 30 minutes. The reaction was stopped by adding 50µl of 4M sulphuric acid (BDH) and the intensity of the colour was read in a plate reader at 495nm.

2.7An evaluation of IL-10 production by Th2 cells driven either in IL-10 or anti-IL-10 with or without indomethacin

DO11.10 T cells were polarised in Th2 favouring culture condition as described in 2.3 but in the presence of either IL-10 (10ng/ml), IL-10+ indomethacin (2µg/ml, Sigma), anti-IL-10 (5µg/ml) or anti-IL-10+ indomethacin. On day 8, Th2 polarised cells were washed in HBSS and resuspended in CM-RPMI. Th2 cells depleted of CD8+T and APCs as described in 2.3.1 and stimulated with immobilized anti-CD3 (2µg/ml) for 48 hours. IL-10 production in the supernatant was measured by ELISA as described in 2.9. The concentration of reagents used was as published by our group (Jaffar, Z. *et al.*, 2002.; Jaffar, Z. *et al.*, 2004).

2.8 Anti-CD3 plate setup to perform cytokine (IFN-γ, IL-4, IL-5 and IL-10) assessment on Th1 and Th2 cells.

An anti-CD3 plate was setup at a concentration of 2μg/ml in PBS, a day before the cell cultures were removed from the incubator and incubated at 37^0 C for 24 hours. Next day, cells were washed twice with PBS and added into the wells at a concentration of 0.5 X 10^6 cells/ml, 2ml /well. The anti-CD3 treatment was setup in parallel with wells containing OVA (1μg/ml) or control wells using media. The treated cells were incubated for 48 hours at 37^0 C, the supernatant was carefully removed from each well and frozen for future analysis.

2.9 An assessment of IFN-γ, IL-4, IL-5 and IL-10 by ELISA:

Capture antibody (anti-IFN-γ, anti-lL-4, anti-lL-5 or anti-IL-10) was diluted to 2.5μg/ml in carbonate buffer (1 mM, pH 9.6). Plates were coated with diluted antibody at 50μl/well, wrapped in a cling film and incubated overnight at 4^0C. Next day, plates were washed in PBS/0.1%Tween, dried and blocked with blocking buffer (1% BSA which had been heat-treated, Sigma) 200μl/well. Plates were incubated overnight at 4^0 C. Plates were washed, dried and the standards (50-0.8 ng/ml) and samples were added at 50μl/well in triplicate. Samples were added to plates either undiluted (IL-5 and IL-10) or diluted 1/10 IFN-γ and IL-4). Plates were wrapped in a cling film and incubated overnight at 4^0 C. Plates were washed, dried and 50μl of biotinylated detection antibody (2.5μg/ml) anti-IFNγ (XMG1 ab, Pharmingen),

anti-IL-4, anti-IL-5 and anti-IL-10 were added. Plates were incubated at room temperature for 45 minutes, washed and dried. Streptavidin-alkaline phosphatase (1:2000) was prepared in blocking buffer and added to all wells at 100µl/well. Plates were incubated for 30 minutes at room temperature and then plates were washed and dried. Plates were developed using an amplification system (KIT, GIBCO), amplifier substrate (GIBCO BRL, Paisley, Scotland) was added (50µl/well) and incubated for 30 minutes at room temperature. Amplifier was added (50µl/well) and the plates incubated until colour developed fully (less than 30 minutes). Stop solution (0.3 M sulphuric acid) was added at 50µl/well and plates were read at 492 nm.

2.10 An assessment of IFN-γ production by PLN cells

Peripheral lymph node cells were stimulated either with OVA peptide (1µg/ml) or OVA peptide + murine IL-4 (0.4 or 2 or 10ng/ml) or OVA peptide+ IL-4+INDO (2µg/ml) in 24 well plate for 48 hours. The level of IFN-γ in the supernatant was measured by standard ELISA as described in 2.9.

2.11 Measurement of IL-2 using the CTLL Bioassay

The IL-2 dependent cell line, CTLL was maintained in CM-RPMI with exogenous IL-2 (10U/ml) at 37^0C, 5% CO2. CTLL cells ($5x10^3$/well) were added to a 96-flat bottom plate. To assay supernates, 50µl of sample was added to wells and the well volume adjusted to 200µl. To calibrate the assay recombinant human IL-2 (CETUS) was added to wells at known concentrations. The proliferation of the cells was

determined by adding 1uCi of ^{3}H-thymidine to each well after 24 h. After a further 18h, the plate was harvested using the Dynaltech Harvester and the ^{3}H-TdR incorporation was measured by the β-counter.

Chapter 3

Materials and Methods II

3.0 Purification of CD4+CD25+ regulatory T cells

CD4+CD25+ and CD4+CD25- T cells were purified from DO11.10 mice by magnetic bead separation (MACS) using both biotinylated anti-CD25 Abs 3C7 and 7D4, labeled with streptavidin-FITC (BD PharMingen, San Diego, CA)and anti-FITC magnetic microbeads (Miltenyi Biotec, Bisley, Surrey, U.K.) : Peripheral and mesenteric lymph nodes were removed from OVA specific DO11.10 mice.

Lymph nodes were dissociated using a 5ml syringe under aseptic condition and the cells were collected through a strainer in a petri dish. The cells were washed with HBSS and centrifuged at 500g, 19^0 C for 10 minutes and the cells resuspended in 10 ml of CM-RPMI medium. Total lymphocytes were incubated with αCD8 (20μg/ml) and α-MHC class II (M5114 supernatant, 20%) for 30 minutes, washed twice in HBSS and added onto a plate coated with mouse α-rat ab (20μg/mg) and incubated for another 60 minutes at room temperature to deplete CD8 lymphocytes and APCs. Unbound cells were harvested from the plate, washed twice in HBSS and added onto another plate coated with α-mouse Ig to deplete the remaining B- cells. APCs, B-cells and CD8 depleted T- lymphocytes were then incubated with 7D4 (biotinylated anti-CD25 antibody labeled with avidin-FITC; BD PharMingen, San Diego, CA) for 10 minutes and with 3C7 (biotinylated anti-CD25 antibody labeled with avidin-FITC; BD PharMingen, San Diego, CA) for another 10 minutes. After 20 minutes incubation, cells were washed twice in multisort buffer (phosphate buffered saline supplemented with 0.5% bovine serum albumin and 2mM EDTA, pH 7.2), incubated with streptavidin FITC for 20 minutes. After washing the cells twice, goat anti-rat FITC was added and incubated for 20 minutes. MACS MultiSort anti-FITC MicroBeads (Miltenyi Biotec, Bisley, Surrey, U.K.) were then added to the thoroughly washed cells, mixed well and incubated for 15 minutes at 6^0-12^0C. Cells

were washed carefully and resuspended in 3ml of MultiSort buffer (LS^+VS^+ Magnetic Separation column used) and proceeded to magnetic separation.

A positive selection column was placed in a separater and the column was prepared for separation with 3 ml of multisort buffer. Cell suspension in 3 ml of multisort buffer was applied onto the column and the negative cells passed through the column were collected in a universal. Column was rinsed with 3x3ml of MultiSort buffer to flush out the unbound negative cells. Column was removed from separater and placed on a suitable collection tube. About 5ml of Multisort buffer was pipetted onto the column and the positive cells were flushed out using the plunger supplied with the column. A fraction of positive cells were analysed by FACS for making the purity assessment.

3.1 Characterization of CD4+CD25+ regulatory T cells

Purified CD4+CD25+ T cells ($5x10^5$/ml) were polarised and expanded *in vitro* in the presence of OVA peptide (1μg/ml) + splenic APCs ($5x10^5$ /ml) + murine IL-4 (2ng/ml; R&D Systems, Abingdon, U.K.) + IL-2 (100 U/ml; Cetus, Emeryville, CA)+ anti-IFN-γ (5μg/ml) or OVA peptide + splenic APCs + IL-2+ anti-IFN-γ. After 8 days, Tr cells were added to Th2 cells and stimulated with immobilized anti-CD3 (2μg/ml) for 48 hours. The level of cytokines secreted was measured by ELISA.

To examine whether CD4+CD25+ T cells inhibit proliferation, DO11.10 lymph node cells ($2x10^5$) were cultured in 96 wells-flat bottom plate (Falcon)
in the presence of different numbers of either freshly isolated (day 0) or expanded (day 8) CD4+CD25+ T-cells. The proliferative response to immobilized anti-CD3

(2μg/ml) coated as described in 2.10 or to OVA peptide (1μg/ml) was measured by the level of [^{3}H]-thymidine (1uCi; Amersham International) incorporation after 3 days [^{3}H]: the plate was harvested onto a glass-fibre filter (Whatman International) in a Dynatech harvester. Dry filter paper discs were transferred to scintillation vials (Packard) and 2 ml of Optiscint scintillation fluid (Wallac) was added. [^{3}H] incorporation was determined in a β-counter (2000 CA, Packard).
Method employed was as published by our group (Jaffar, Z. *et al.*, 2004).

3.2 Preparation of splenic antigen presenting cells

Spleens of BALB/c mice were dissociated using a 5ml syringe under aseptic conditions and the cells were collected through a strainer in a petri dish.
The cells were transferred into a sterile universal and washed in HBSS.
The suspension was centrifuged at 438g , 19^{0}C for 10 minutes, the supernatant was disposed of and the cell pellet resuspended in 10 mls of complete RPMI medium.
RBC's, CD4+, and CD8+ T cells were depleted from the spleen cells prior to irradiation.

3.2.1Removal of RBCs (red blood cells) from the cell suspension prior to depletion of CD4+ and CD8+ T cells.

Into a sterile universial, 5 ml of Ficol (stock) was added under aseptic condition.
Spleen cells suspension was added slowly onto the top of the Ficol (lympho-sep mouse lymphocyte), centrifuged at 800g, 19^{0}C for 20 minutes. After centrifugation, the supernatant containing RBCs depleted cells was transferred into a fresh universal.
HBSS was added to the supernatant to dilute the remaining Ficol (otherwise, it is difficult to get the pellet containing the cells) and centrifuged at 500g, 19^{0}C for

10 minutes. Supernatant was discarded and the cells were suspended in 10 ml of RPMI . Cells were counted using a neubaur chamber cell counter.

3.2.2 Depletion of CD4+ and CD8+ T cells using complement

RBCs depleted cells were treated with complement to deplete CD4+ and CD8+ T cells and irradiated (3000 rad). Irradiated splenic APCs ($5x10^5$ cells/ml) were then added to the culture.

3.3 *In vivo* activity of Regulatory T cells

DO11.10 Th2 cells prepared from either total or CD4+CD25- cells were injected intravenously into BALB/c mice (10^7/mouse). Mice (four to six per group) were then intranasally challenged by exposure to aerosolized solutions of OVA (0.5% Grade V; Sigma-Aldrich, Poole, U.K.) for 20 minute a day over 5 or 7 consecutive days using a Wright's nebulizer (Buxco Europe, Petersfield, U.K.). Control mice were exposed to OVA aerosols but did not receive DO11.10 Th2 cells. AHR was measured on day 7 in response to methacholine inhalation by whole-body plethysmography (Buxco Europe, Petersfield, U.K.). Animals were placed in chambers and exposed to nebulized PBS (baseline) followed by increasing concentrations of methacholine. Enhanced pause (Penh) was measured after each 3-min exposure. Mice were killed on day 7, and BAL fluid was collected for analysis. Eosinophil peroxide (EPO) levels in the BAL fluid was determined by colorimetric analysis as described in 2.6.2. The number of lymphocytes, macrophages and the neutrophils were also determined in BAL by light microscopic evaluation of stained cytospin preparations.

The number of CD4+, DO11.10 T cells in the lymph node and in the lung tissue were also analysed by FACS.

3.3.1 "Isolation of the Lymph Node Cells"

Peripheral lymph nodes (brachial, axillary, and inguinal LNs) were taken from mice and placed in CM-RPMI. Lymph nodes were dissociated using a 5 ml syringe and the cells were collected through a strainer in petri dish. The cells were then transferred into a universal, centrifuged at 503g, 4^0C for 10 minutes. Cells were counted and resuspended in CM-RPMI.

3.3.2 Preparation of Mouse Lung Cells

Lungs of 3-6 mice were collected and placed in a universal tube containing CM-RPMI (10-20ml). Lungs were placed in fresh media in petri dish and chopped finely for 15 minutes. Chopped lung fragments were transferred into clean universal containing fresh media. To digest the tissue, 0.1% collagenase (Sigma type IV) was added and incubated for 1 hr 10 minutes at 37 ^{0}C with gentle stirring. Lung fragments were shaken vigorously several times during to help in the digestion procedure. Sample was filtered to remove undigested fragment, the supernatant was harvested and washed with HBSS.

In parallel, light and heavy Percoll was prepared and the cells were resuspended in 14 ml of light Percoll. Into 2 universal tubes, 7ml of heavy Percoll was added (into each tube) and then 7ml of light percoll containing cells were overlaid. Tubes were topped with a final 7 ml of media. Tubes were spun at 800 g, 19^0C for 30 minutes. The middle layer of cells was carefully collected using pasteur pipette (Alpha Laboratories LTD) by avoiding top layer (epithelium and fibroblasts) and bottom pellet (RBC). Cells were made up to 20ml with PBS in two tubes, spun at 500g, 19^0C

for 10 minutes (brake 9 and acceleration 9). Cells in the two tubes were pooled, resuspended with 10 ml of RPMI and counted.

3.3.2a Preparation of heavy and light Percoll:

Osmolarity of Percoll was corrected by adding 20 ml of stock solution of Percoll (Pharmacia) into a universal tube (Sterilin) containing 2ml 10x PBS. Light Percoll (41.7%) was prepared by adding 10 ml of osmolarity corrected Percoll into a universal tube containing 14 ml of RPMI. Heavy Percoll (67.6%) was prepared by adding 10 ml of Percoll into a tube containing 4.8 ml of RPMI.

3.3.3 Flow cytometry

Lung or lymph node cells were analysed on a FACSCalibre (BD Biosciences) using CellQuest software to enumerate CD4+ T cells (GK1.5-PE; BD PharMingen), clonotypic T cells (KJ1-26-FITC), and CD25+ T cells (anti-7D4-biotin and avidin-FITC). Polarised CD4+ T cell subsets were also analysed on a FACSCalibre using CellQuest software for differential expression of adhesion molecules, CD31(MEC 13.3-FITC, rat IgG), CD62L (MR1, rat IgG), β7 chain (M393-FITC, rat IgG), and CD44 (KM81-FITC, rat IgG) . Antibodies were obtained from PharMingen (Oxford, U.K). The cell subsets were compared to their isotype control Ig.

3.4 Statistical analysis

Data are expressed as means ± SEM. Data obtained from adoptive transfer experiments were analysed using the Mann-Whitney U test, and the differences were considered statistically significant with $p \leq 0.05$. *In vitro* data were evaluated using a two-way analysis of variance, followed by Student's t test for comparison between two groups. A probability value of $p \leq 0.05$ was considered statistically significant.

Chapter 4

Characterisation and validation of a DO11.10 mouse model.

4.1 INTRODUCTION

DO11.10 mouse is a transgenic model express a TCR recognising OVA peptide 323-339 and it was characterised with particular reference to Th1 and Th2 cells. Th1 and Th2 cells differentiate from CD4+ Th lymphocytes, defined by functional attributes that reflect their different cytokine profiles (Mosmann, T.R. and Coffman, R.L., 1989). Th1 cells produce IFN-γ and provide protection against intracellular pathogens and viruses. Th2 cells produce IL-4, IL-5, and IL-13, mediate IgE production and eosinophilic inflammation, which contribute to the elimination of extracellular parasites and the pathogenesis of allergic disease such as asthma (Robinson, D.S. *et al.*, 1992.; Beasley, R. *et al.*, 1989.; Jaffar, Z. *et al.*, 1999.).

IL-2 receptor is induced on the polarised Th1 and Th2 cells, the cytokine IL-2 binds with IL-2 receptor and enhance the proliferation of T cells. But, IL-2 gene expression and cell cycle progression are inhibited as a result of concomitant cross- linking of CTLA-4 with TCR- signaling (Walunas, T.L. *et al.*, 1996.; Krummel, M.F. and Allison, J.P., 1996.).

To validate DO11.10 mouse model, airway eosinophilia was assessed by measuring eosinophil peroxidase (EPO) activity since eosinophils are important mediators of allergic inflammation in the lung (Matsumoto, K and Saito, H., 2001) responsible for the late asthmatic response, airway hyperresponsiveness and may also contribute to airway remodelling. Although Th2 lymphocytes, monocytes, macrophages and epithelial cells contribute to airway inflammation, it has been very well documented that eosinophils act as a major effector of airway damage and dysfunction (Corrigan, C.J. and Kay, A.B., 1992).

4.2 AIMS:

1). Characterise DO11.10 mice with particular reference to Th1 and Th2.

2). Validate the adoptive transfer experiments.

4.3 Results

4.3.1 Cytokine production and receptor expression by polarised Th1 and Th2 cells.

Lymph node cells from DO11.10 mice expressing OVA- specific T cell receptor were polarized *in vitro* for 8 days in IL-12+OVA peptide+anti-IL-4 or in IL-4+ OVA peptide + anti-IFN-γ. On day 8, cells were stimulated either with α-CD3 or with media. After 48 hours, the supernatant was harvested and the level of cytokines (IFN-γ, IL-4, IL-5) was measured by standard ELISA since it was important to confirm that the cells cultured *in vitro* under different conditions were true Th1 and Th2 cells.

Th1 polarised cells produced higher levels of IFN-γ and little IL-4 and IL-5 (figures 4.3.1, 4.3.2, 4.3.3; table 4.3.1 in the appendix). In contrast, Th2 polarised cells produced little IFN-γ and higher levels of IL-4 and IL-5 (figures 4.3.1, 4.3.2, 4.3.3; table 4.3.1 in the appendix). In parallel, we analysed Th1-polarised and Th2-polarised cells for the expression of OVA-specific T cell receptor (KJ1-26) or IL-2 receptor (figure 4.3.4). Th1-polarised cells were 90% positive for OVA-specific receptor +CD4 (assessed in relation to CD4+molecule) and about 74.26% positive for IL-2 receptor whereas Th2-polarised cells were 99% positive for OVA- specific receptor +CD4 and about 87.47% positive for IL-2 receptor. In figure 4.3.4, 25% of Th1-polarised cells were CD4- negative (assessed in relation to CD4 and CD4+KJ1-26+) , but Th2-polarised cells were only 5% negative for CD4. Likewise, Th1-polarised cells were 10% negative for KJ1-26 whereas Th2-polarised cells were 1% negative for KJ1-26.

The most likely explanation for this discrepancy would be that the Th2-polarised cells were depleted of CD8+T cells and antigen presenting cells (prior to FACS staining) whereas Th1-polarised cells were not depleted of CD8+T cells and antigen presenting cells. The presence of CD8+ T cells and APCs in Th1-polarised cells appear to account for the increased percentage of KJ1-26-negative and CD4-negative T-cells. These results show that the polarisation procedure succeeded in generating polarised T cell population with the appropriate cytokine profiles of Th1 and Th2 cells.

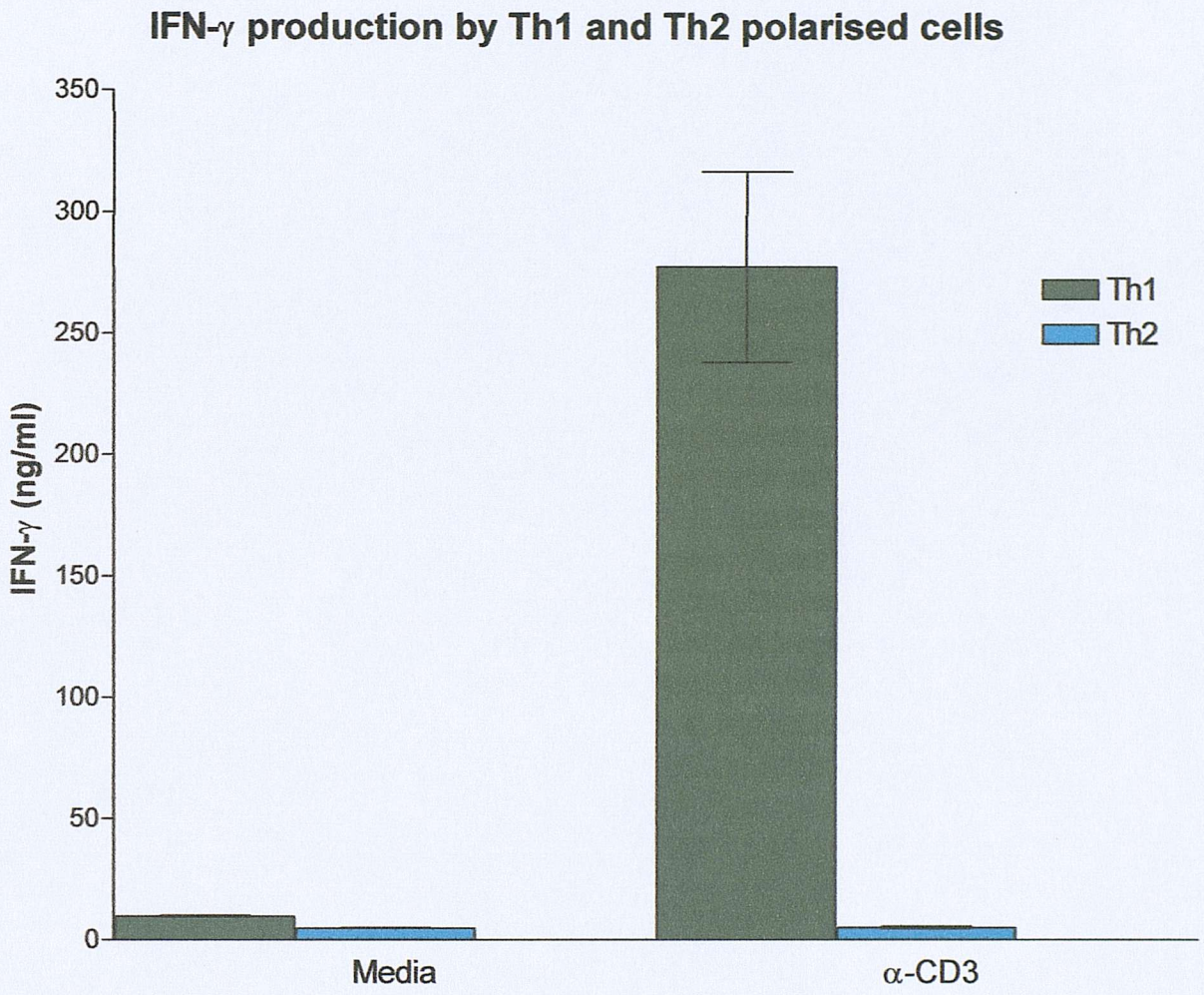


Figure 4.3.1

The level of IFN-γ production by Th1 and Th2 polarised cells was measured by stimulating polarized cells with media or anti-CD3. The quantitative assessment of IFN-γ was by ELISA. Data are shown for 1 experiment and are representative of 3 independent experiments (see appendix (4.3.1)). Data are means ± SEM (n=3). Number of mice used per experiment was 10.

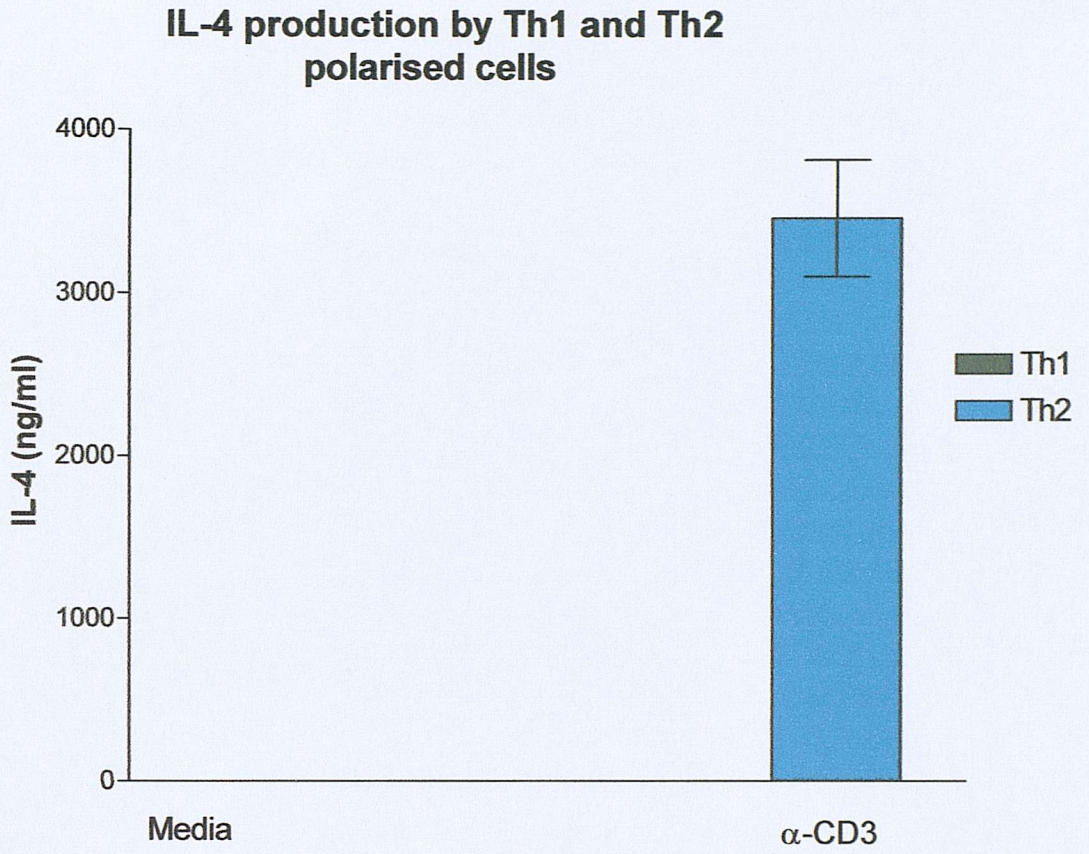


Figure 4.3.2

The level of IL-4 production by Th1 and Th2 polarised cells was measured by stimulating polarised cells with media or anti-CD3. The quantitative assessment of IL-4 was by ELISA. Data are shown for 1 experiment and are representative of 3 independent experiments (see appendix (table 4.3.1)). Data are means ± SEM (n=3). Number of mice used per experiment was 10.

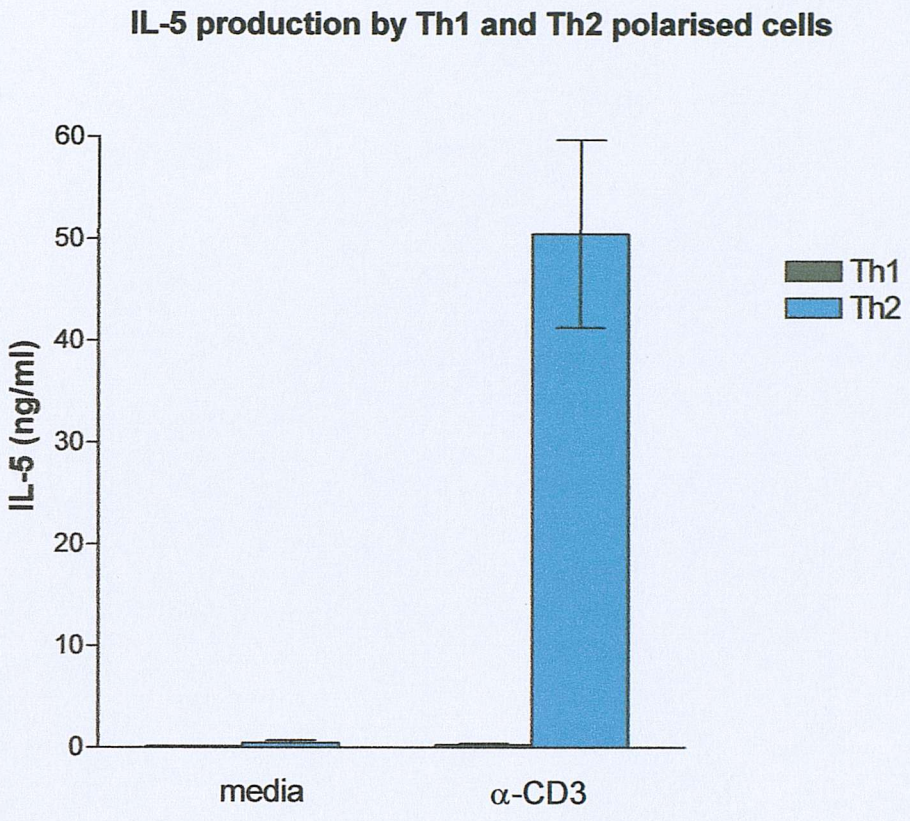


Figure 4.3.3 The level of IL-5 production by Th1 and Th2 cells was measured by stimulating polarized cells with media or α-CD3. Data are shown for 1 experiment and are representative of 3 independent experiments (see appendix (table 4.3.1)). Data are means ± SEM (n=3). Number of mice used per experiment was 10.

Receptor expression by polarised Th1 and Th2 cells

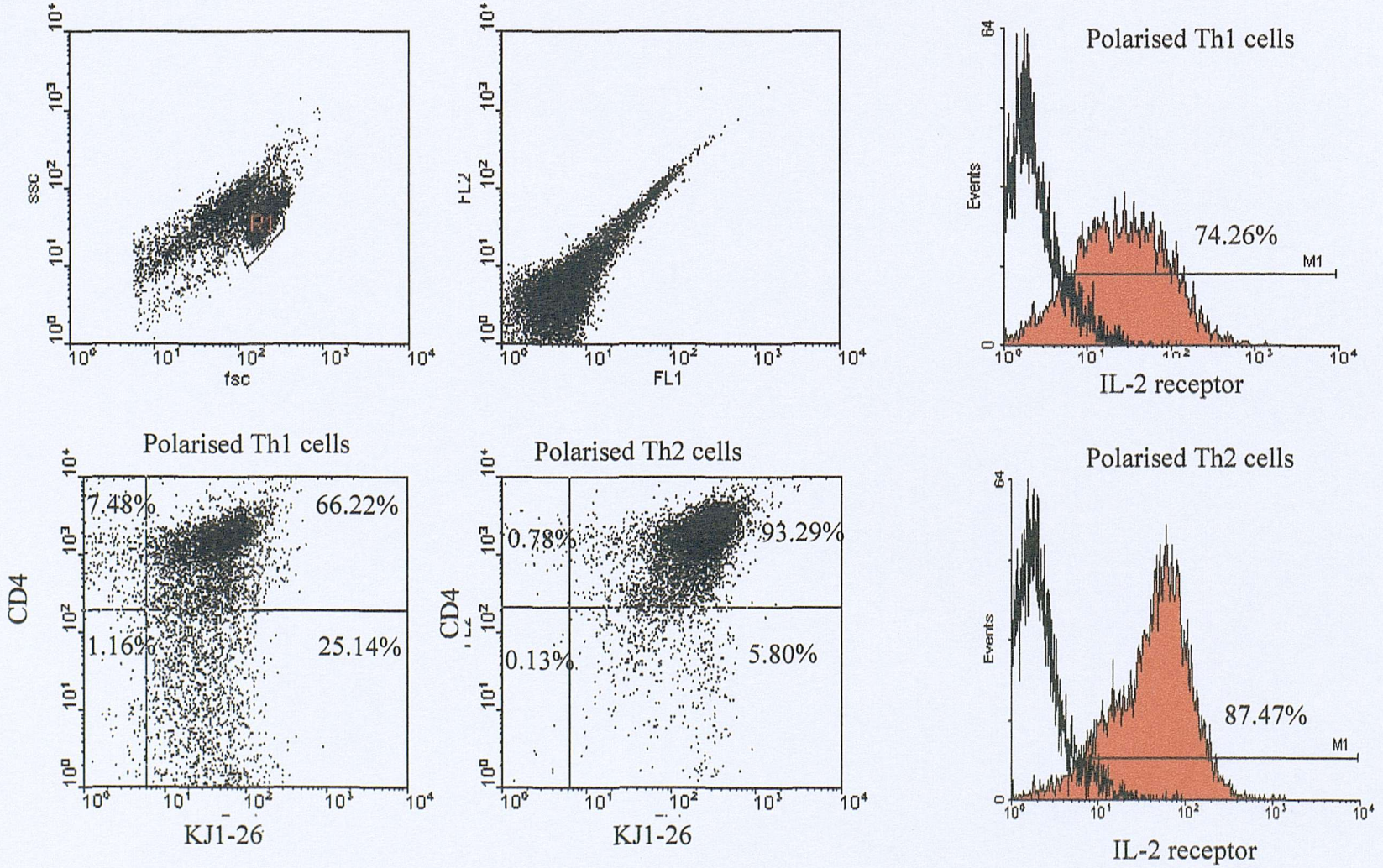


Figure 4.3.4

Polarised Th1 and Th2 cells were stained with either KJ1-26 FITC +CD4 PE or IL-2 FITC and analysed by FACS. The figure is representative of two independent experiments. Number of mice used per experiment was 10.

4.3.2 The induction of pulmonary inflammation by the adoptive transfer of Th2 cells.

OVA-specific DO11.10 polarized Th1 and Th2 cells were adoptively transferred into naive BALB/c mice. The mice were exposed to OVA-aerosol challenge for 7 consecutive days, sacrificed on the last day and then BAL was performed. Airway eosinophilia in each group was measured by EPO assay. The results indicate that the mice that received Th2 cells showed a greatly heightened eosinophilia compared to the mice that received Th1 cells (figure 4.3.5; table 4.3.5 in the appendix). Many previous studies have documented that Th2 cells mediate atopic asthma and favour the development of eosinophils in the bone marrow influenced by the cytokine IL-5.

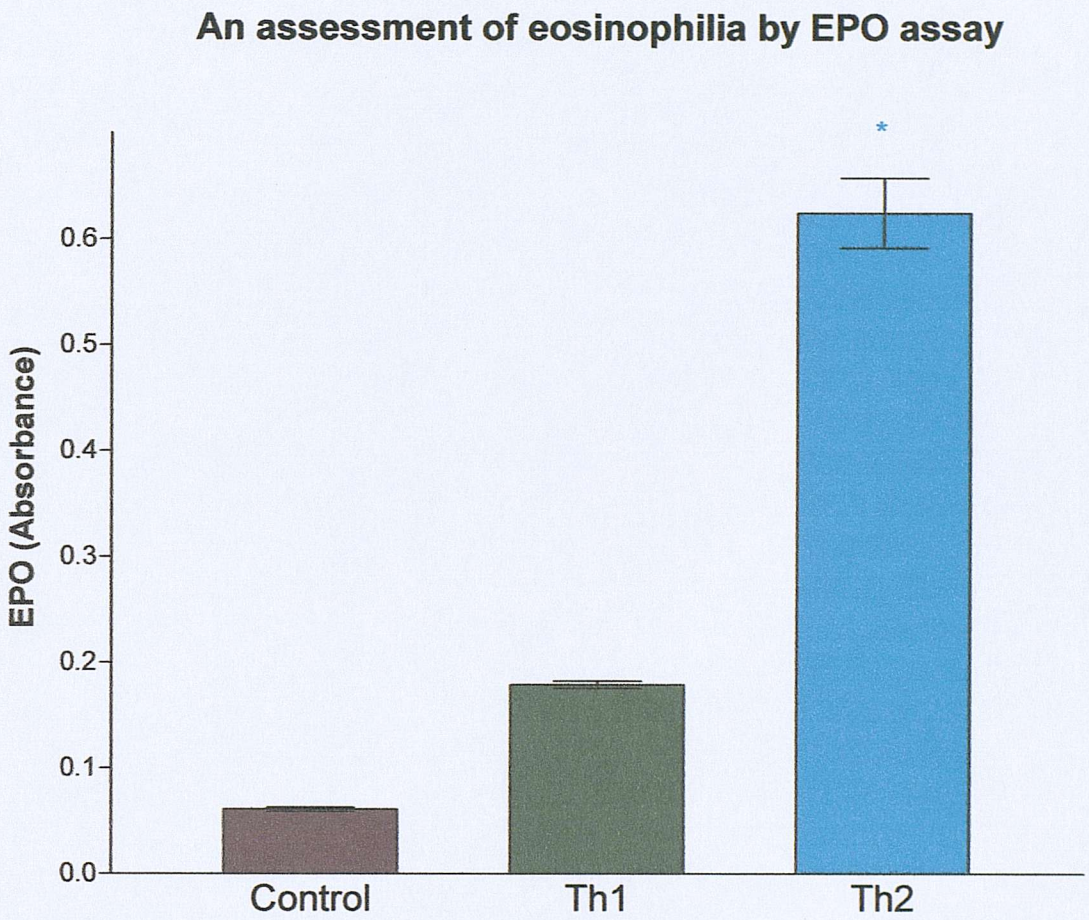


Figure 4.3.5

The level of eosinophilia in the bronchoalveolar lavage was measured by EPO assay. BALB/c mice were given either $10x10^6$ Th1 or Th2 cells. Recipients were exposed to OVA aerosol and the level of EPO in the bronchoalveolar lavage fluid determined at day 7. Data are shown for 1 experiment and are representative of 3 independent experiments (see appendix (table 4.3.5)). Data are means ± SEM (n=3). Number of mice used in each group per experiment was 5. *, $p \leq 0.05$ compared with recipients of Th1 cells.

4.4 Summary

DO11.10 mice was characterised with particular reference to Th1 and Th2 cells. This was achieved by culturing DO11.10 lymph node cells in Th1 and Th2 favouring environment and analysing the polarised populations for the production of different cytokines and receptor expression. In parallel, DO11.10 mice was validated by measuring the level of eosinophilia in the adoptive transfer model. The principal observations were:

1. Th1- polarised cells produced higher levels of IFN-γ and little IL-4 and IL-5 whereas Th2-polarised cells produced little IFN-γ and higher levels of IL-4 and IL-5.
2. In terms of receptor expression, Th1-polarised cells were 90% positive for OVA-specific receptor +CD4 and 74.26% positive for IL-2 receptor whereas Th2-polarised cells were 99% positive for OVA- specific receptor +CD4 and 87.47% positive for IL-2 receptor.

3. Validation of theDO11.10 mouse model resulted in that BALB/ c mice that received DO11.10 Th2-polarised cells produced a greatly heightened eosinophilia compared to the mice that received Th1-polarised cells.

Chapter 5

The production of the regulatory T cell cytokine IL-10 in Th1 and Th2 cell populations.

The effect of prostaglandins and the availability of exogenous IL-10 on the production of IL-10 by polarised Th2 cells.

5.1 INTRODUCTION

Next, production of the regulatory T cell cytokine IL-10 was assessed in these Th1+Th2 polarised cell populations. IL-10 is produced by many cell types, including T cells, has been shown to suppress mucosal immune responses (Asseman, C. *et al.*, 1999.; Asano, M. *et al.*, 1996).

Lower concentrations of IL-10 are found in bronchoalveolar lavage from asthmatic patients than in normal control subjects as a result of defective IL-10 secretion from alveolar macrophages (Lim, S. et al., 2004).

In this experiment, IL-10 production by polarised Th1 and Th2 cells was assessed in a number of different culture conditions.

The effect of prostaglandins and the availability of exogenous IL-10 on the production of IL-10 by polarised Th2 cells was also assessed . Prostaglandins are inflammatory mediators secreted by mononuclear cells such as macrophages and dendritic cells. There are several different types of prostaglandins (PGI_2, PGE_2, $PGF_{2\alpha}$, TXB_2, PGD_2, etc) each of which has a different function. It has been reported that treatment with PGI_2 or its stable analog, carbaprostacyclin augments the production of IL-10 by Th2 cells (Jaffar, Z. *et al.*, 2002). T cells at mucosal sites are subject to immune regulation, partly due to the actions of cyclooxygenase(COX)- derived prostanoids (Newberry, R.D. *et al.*, 1999) and cytokines such as IL-10 and TGF-β (Asseman, C. *et al.* 1999. ; Khoo, U.Y. *et al.*, 1997). Since prostaglandins have been shown to influence immune responses and, in particular, they stimulate IL-10 production from T-cells, we hypothesised that PGs might influence IL-10 production by Th2 polarised cells. It was also addressed whether IL-10 production was affected by the availability of IL-10 during the polarization process. PLN cells were polarised towards Th2 phenotype as

in the previous experiments, but with the addition of either IL-10 or anti-IL-10 during the polarization period. Indomethacin (2μg/ml) was also present in some cultures during the polarization process. The concentration of indomethacin used throughout the culture conditions was as published by our group (Jaffar, Z. *et al.*, 2002).

5.2 AIMS

1). Assess the production of regulatory T cell cytokine IL-10 in Th1 and Th2 polarised cell populations.

2). Assess the effect of prostaglandins and the availability of exogenous IL-10 on the production of IL-10 by polarised Th2 cells.

5.3 Results

5.3.1 The level of IL-10 production by polarised Th1 and Th2 cells

Polarised Th1 and Th2 cells (prepared from OVA-specific DO11.10 mice) were stimulated with α-CD3 and IL-10 was measured in supernatants after 48 hours. Th2 polarised cells produced approximately 10 times more IL-10 than Th1 polarised cells (figure 5.3.1; table 5.3.1 in the appendix). In order to assess the importance of antigen presenting cells in this experiment, we performed parallel experiments in which the Th2 polarised cells were depleted of APCs by one of 2 methods:

1) Th2 cells were incubated on a mouse anti-rat Ig-coated plate and after 45 minutes, the non adherent cells were removed and stimulated with α-CD3.

2) Th2 cells treated with monoclonal antibodies directed against CD8 and MHC class II were incubated on a mouse anti-rat Ig-coated plate and after 45 minutes, the non-adherent cells were stimulated with α-CD3.

Both treatments led to a reduced IL-10 production in response to α-CD3 (figure 5.3.1; table 5.3.1 in the appendix). These results suggest that the presence of APCs is important in enabling Th2 polarised cells to produce maximal amounts of IL-10. An alternative explanation for this reduced IL-10 production is APCs produced significant amounts IL-10.

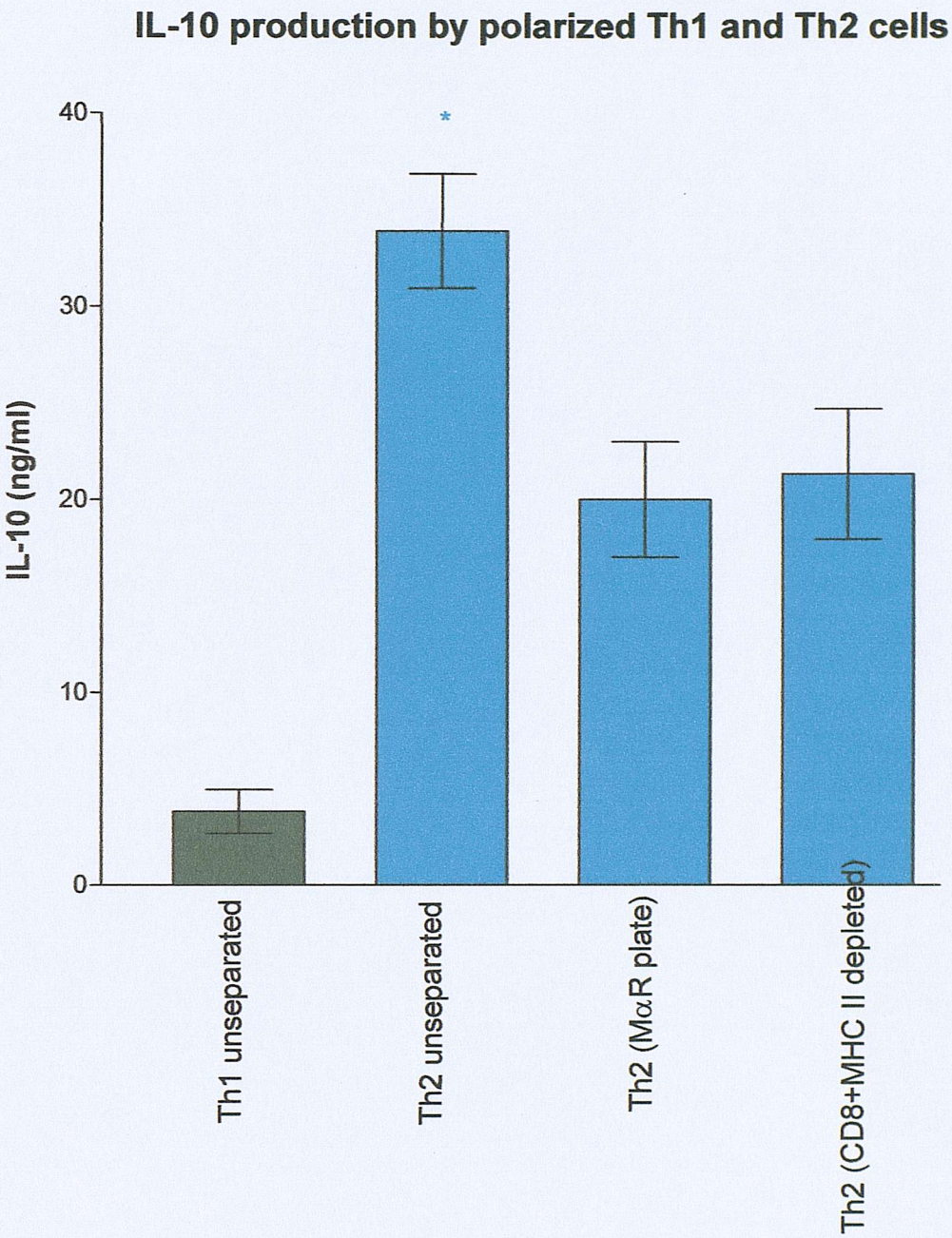


Figure 5.3.1

The production of IL-10 by unseparated Th1 and Th2 polarised cells was measured by stimulating polarized cells with α-CD3 for 48 hours, and the level of IL-10 in the supernatant was measured by standard ELISA. In parallel, Th2 polarised cells depleted either of APCs or APCs and CD8 by any of the following methods respectively: 1) Th2 polarised cells were incubated on a mouse anti-rat Ig treated plate (MaR) 2) Th2 cells treated with anti-classII and anti-CD8 before adding onto a MaR treated plate and the non-adherant cells were removed and stimulated with anti-CD3 and the level of IL-10 in the supernatant was measured after 48 hours by standard ELISA. Data are shown for 1 experiment and are representative of 3 independent experiments (see appendix (table 5.3.1)). Data are means ± SEM (n=3). Number of mice used per experiment was 12. *, $p \leq 0.05$ compared with Th2 cells depleted of CD8+ T-cells and APCs.

5.3.2 Th2 cells driven either in IL-10 or anti-IL-10 with or without indomethacin produced different levels of IL-10

We polarised OVA-specific DO11.10 lymph node cells *in vitro*, in the presence of either IL-10, IL-10+INDO, anti-IL-10 or anti-IL-10 +INDO (all 4 groups received IL-4+OVA+anti-IFN-γ in addition). On day 8, cells were stimulated with α-CD3 for 48 hours and IL-10 in the supernatant was measured by standard ELISA. The results indicate that Th2 cells polarised in anti-IL-10 produced virtually no IL-10 (figure 5.3.2; table 5.3.2 in the appendix), presumably because the availability of IL-10 required for the development of IL-10 producing Th2 cells was restricted. The amount of IL-10 produced by Th2 cells polarised in exogenous IL-10 showed no significant difference to that produced by Th2 cells polarised in the absence of exogenous IL-10. APCs are known to be a major source of IL-10, our inability to show any effect of exogenous IL-10 may therefore be because the Th2- polarised cells are already receiving sufficient IL-10 from APCs to stimulate maximal production of IL-10.

Th2 cells polarised in the presence of indomethacin produced about 75% less IL-10. This suggests that prostaglandins may influence the production of IL-10 by stimulating IL-10 producing cells and this effect was inhibited by indomethacin.

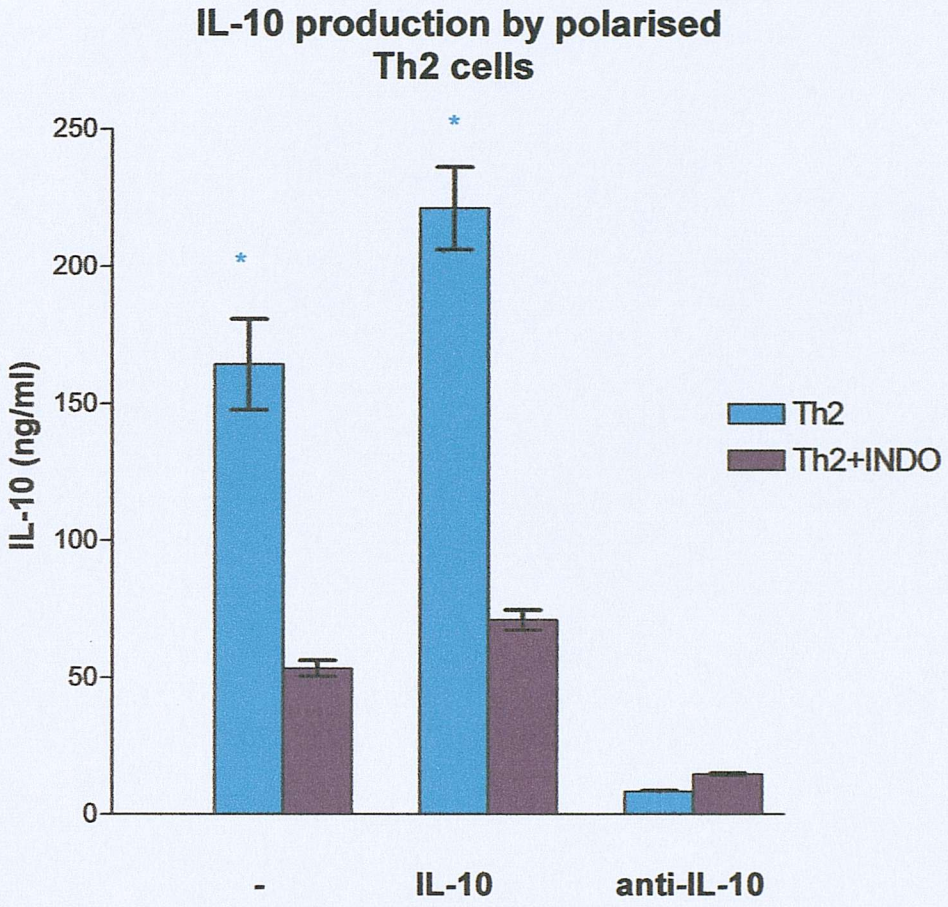


Figure 5.3.2

The level of IL-10 production by Th2 polarised cells driven in IL-10 (10ng/ml) or IL-10+INDO (2μg/ml) or anti-IL-10 (5μg/ml) or anti-IL-10+INDO was measured by stimulating polarised Th2 cells with anti-CD3 (2μg/ml) for 48 hours. All groups received IL-4(2ng/ml), OVA (1μg/ml) and α-IFN-γ (5μg/ml), in addition, during the polarisation process. The level of IL-10 in the supernatant was measured by standard ELISA. Data are shown for 1 experiment and are representative of 2 independent experiments (see appendix (table 5.3.2)). Data are means ± SEM (n=3). Number of mice used per experiment was 14. *, $p \leq 0.05$ compared with eitherTh2 cells driven in indomethacin or Th2 cells driven in IL-10+indomethacin.

5.4 SUMMARY

The production of regulatory T cell cytokine IL-10 was assessed in Th1 and Th2-polarised cell populations. The effect of prostaglandins and the availability of exogenous IL-10 on the production IL-10 by Th2-polarised cells was also assessed. The principal observations were:

1. Th2- polarised cells produced approximately 10 times more IL-10 than Th1-polarised cells and the Th2- polarised cells depleted of APCs led to a reduced IL-10 production in response to α-CD3.

2. The analysis of the effect of prostaglandins and the availability of exogenous IL-10 on the production of IL-10 by Th2- polarised cells resulted in, that DO11.10 lymph node cells polarised in the presence of indomethacin produced 75% less IL-10. The amount of IL-10 produced by DO11.10 lymph node cells polarised in Th2-favouring environment in the presence of exogenous IL-10 showed no significant difference to that produced by Th2 cells polarised in the absence of exogenous IL-10.

Chapter 6

The effect of exogenous IL-4 on the production of IFN-γ by PLN cells.

6.1 INTRODUCTION

It has been shown by others that exogenous IL-4 decreases the production of IFN-γ by PLN cells. IFNγ has several functions which include, promoting phagocytosis and upregulating microbial killing by macrophages. In mice, IFNγ promotes immunoglobulin isotype switching to IgG_{2a}, known to be important for the opsonization of bacteria (Heinzel, F.P. *et al.*, 1995). In addition, IFNγ promotes the expression of FcγRI receptors which are important in phagocytosis. It also upregulates the production of NO, hydrogen peroxide and superoxide in cells actively participating in phagocytosis (Boehm, U. *et al.*, 1997).

We therefore investigated the effect of a range of concentration of exogenous IL-4 on the production of IFN-γ by unpolarised DO11.10 PLN cells. In parallel, as it has been proposed that prostaglandins influence IL-4 production, the experiment was also performed in the presence of indomethacin.

6.2 AIM:

Assess the effect of exogenous IL-4 on the production of IFN-γ by PLN cells.

6.3 Results

6.3.1 IFN-γ produced by unpolarised PLN cells is influenced by exogenous cytokine IL-4.

OVA-specific unpolarised DO11.10 peripheral lymph node cells were stimulated with OVA or with OVA+IL-4 or with OVA+IL-4+INDO for 48 hours. The IFN-γ produced by PLN cells in the supernatant was assessed by standard ELISA. PLN cells produced about 25% more IFN-γ in the absence of exogenous IL-4 (10ng/ml) (figure 6.3.1; table 6.3.1 in the appendix). The addition of indomethacin appears to have produced no effect.

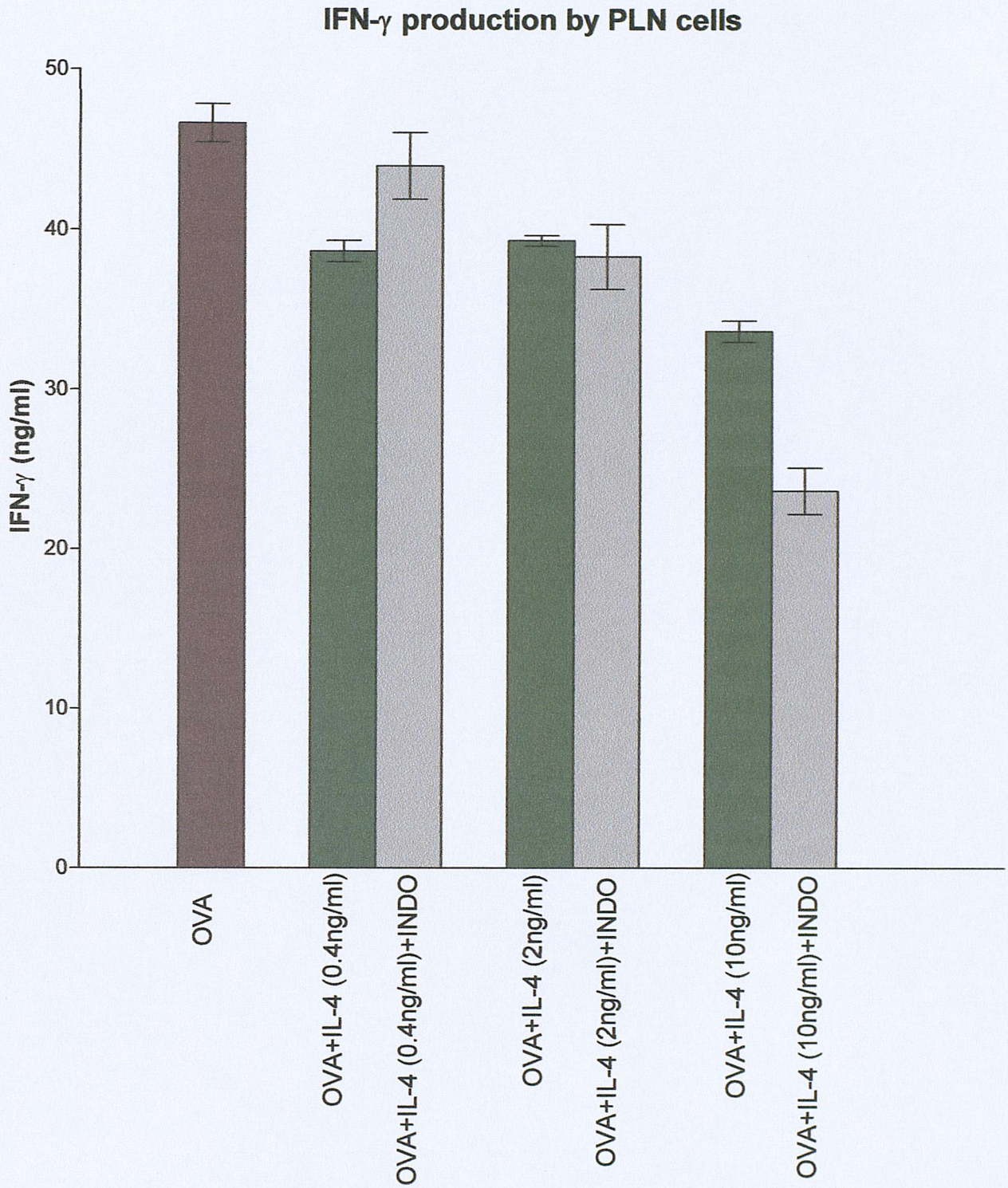


Figure 6.3.1

Peripheral lymph node cells were stimulated with OVA (1μg/ml) or OVA+IL-4 or OVA+IL-4+INDO (2μg/ml) for 48 hours. The level of IFN-γ in the supernatant was measured by standard ELISA. Data are shown for 1 experiment and are representative of two independent experiments (see appendix (table 6.3.1)). Data are means ± SEM (n=3). Number of mice used per experiment was 10.

6.4 Summary

The effect of exogenous IL-4 and the prostaglandins on the production of IFN-γ was analysed by stimulating DO11.10 PLN cells with IL-4+OVA or IL-4 +OVA+indomethacin. The principal observations were:

1. DO11.10 PLN cells produced about 25% more IFN-γ in the absence of exogenous IL-4 (10ng/ml).

2. Stimulation of DO11.10 cells in the presence of indomethacin appeared to have produced no effect.

Chapter 7

The percentage of CD4+CD25+ T-cells in PLN of DO11.10 mice.

Purification of CD4+CD25+ T-cells from PLN of DO11.10 mice.

7.1 INTRODUCTION

A distinct population of CD4+ T cells that constitutively express the IL-2 receptor (IL-2R) α chain (CD25) has recently been identified in mice (Sakaguchi, S., 2000. ; Asseman, C. *et al.*, 1999). CD4+CD25+ T cells are regulatory cells which constitute about 4-10% of lymphocytes in mice and in humans. These cells play an essential role in the induction and maintenance of peripheral self-tolerance.

Murine CD4+CD25+ T cells appear to mediate the suppression of effector T cell function both *in vitro* and *in vivo* via several mechanisms requiring either cell-cell contact or the production of immunosuppressive cytokines such as IL-10 and TGF-β (Sakaguchi, S., 2000. ; Asseman, C. *et al.*, 1999.). It has been well established that CD4+CD25+ Regulatory T cells control inflammation in autoimmune diseases and transplantation. I hypothesised that these cells might also have a role in regulating allergic inflammation in the lung. To address this, a series of experiments was conducted starting by, determining the percentage of CD4+CD25+ T cells in PLN of DO11.10 mice.

In the first phase of these experiments, the proportion of PLN T cells which express the regulatory T cell phenotype was assessed. This was performed by FACS staining for CD4+CD25+ T cells. In parallel, PLN T cells were stained with antibodies for KJ1-26 +CD25 and analysed by FACS to determine the proportion of CD25+ T cells expressing KJ1-26 (OVA specific receptor).

In order to work with the CD4+CD25+ T cells, it was first necessary to purify them, since total unpolarised lymphocytes from DO11.10 mice constitute only about 4-7% of CD4+CD25+T cells. Although it proved possible to deplete CD4+CD25+ T cells

by panning, it was not possible to achieve sufficient purity for functional experiments. Instead, a magnetic bead separation column was used to obtain

CD25+ and CD25- cell populations. This approach was effective at both depleting and enriching CD4+CD25+ T cells. Magnetic bead separation with a single anti-CD25 antibody was inadequate, but effective separation was achieved when two CD25 monoclonal antibodies were used. The efficiency of the purification process was assessed by FACS.

In this experiment, Tr cells were purified and enriched on the basis of their constitutive expression of CD25. CD25 is the α-chain of the high affinity IL-2 receptor, which is also induced on conventional T cells after activation (Sakaguchi, S. *et al.*, 1995). In an attempt to find novel Treg markers, two groups have independently found that freshly isolated CD4+CD25+ Treg cells but not CD4+CD25- T cells expressed uniformly high levels of GITR (Shimizu, J. *et al.*, 2002.; McHugh, R.S. *et al.*,2002). Activation of the CD4+CD25- T cells *in vitro* rapidly increased GITR surface expression to levels comparable to those observed on CD4+CD25+ T reg cells. Therefore, although IL-2R expression is not a perfect way to identify T reg cells, at present there are no better markers for distinguishing Tregs from activated conventional T cells.

7.2 AIMS:

1). Determine the percentage of CD4+CD25+ T-cells in PLN of DO11.10 mice.

2). Purify CD4+CD25+ T-cells from PLN of DO11.10 mice.

7.3 Results

7.3.1 CD4+CD25+ T cells constitute about 6.8 % of the total lymphocytes in DO11.10 mice

In order to characterize CD4+CD25+T regulatory cells phenotypically and functionally, we first assessed the proportion of these cells in the PLN of DO11.10 mice.

Unpolarised DO11.10 PLN cells were stained with CD4PE and CD25FITC and analysed by FACS. The FACS results indicate that about 4- 6.8% of PLN lymphocytes were CD4+CD25+ T cells (figure 7.3.1; table 7.3.1 in the appendix). Separately, we assessed the proportion of CD25+T cells that expressed OVA-specific T cell receptors. Unpolarised DO11.10 PLN cells were stained with CD25-PE and KJ1-26-FITC and analysed by FACS. About 72% of CD25+T cells expressed the OVA specific TCR (figure 7.3.1).

Frequency of CD4+CD25+ T cells in DO11.10 mice

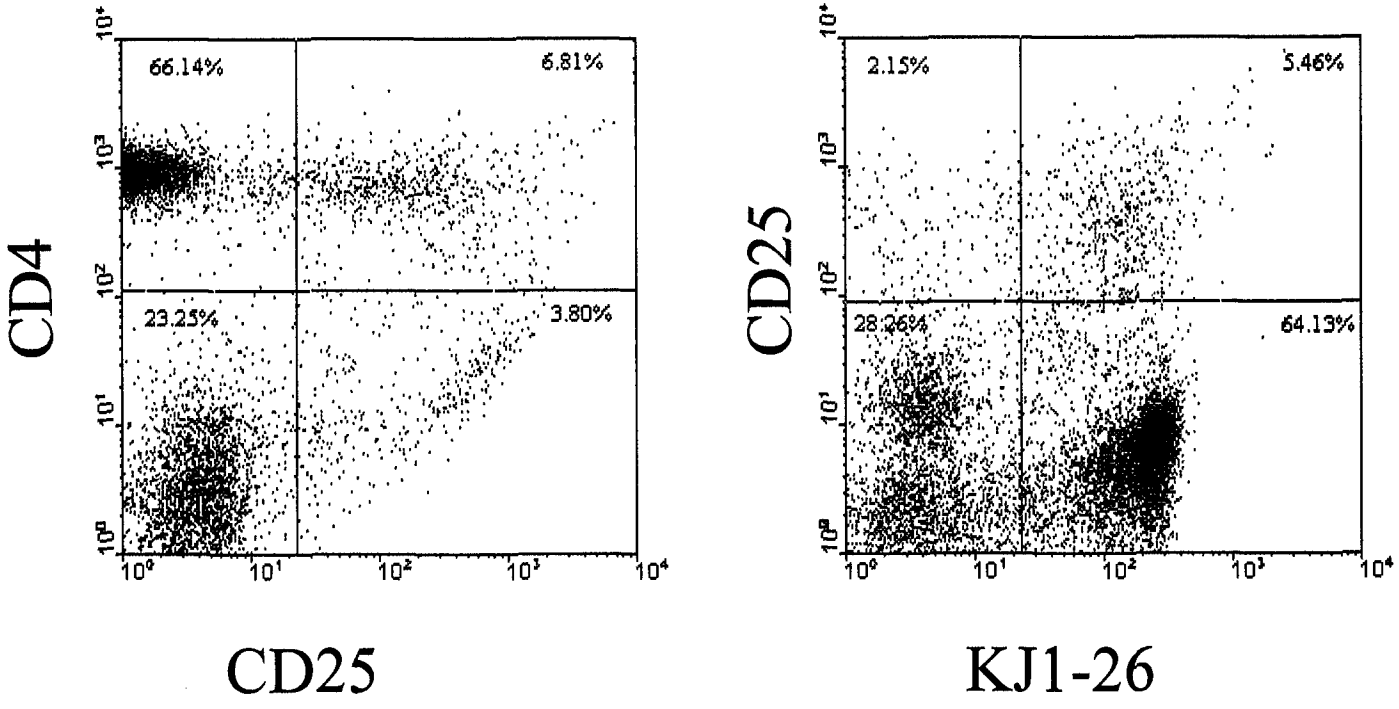


Figure 7.3.1

Lymph node cells (unpolarised) from DO11.10 mice were stained with either CD25-FITC+ CD4-PE or KJ1-26- FITC +CD25-PE. The proportion of cells which expressed either CD4+CD25 or KJ1-26+CD25 was analysed by FACS. The figure is representative of 4 independent experiments. Number of mice used per experiment was16.

7.3.2 The purification of CD4+CD25+ T cells from lymph node cells by magnetic bead separation.

Although CD4+CD25+ cells were present in DO11.10 mice it was important to determine whether these cells actually mediated regulatory function. This required developing approaches to either deplete or purify these cells. The relatively low numbers of CD4+CD25+ T cells present in DO11.10 mice made it difficult to purify this cell type. Initially CD25+ cells were depleted by panning, however, while this approach was effective at depleting cells it proved unsuitable for purifying CD25+ cells. More recently sorting cells by magnetic bead separation (MACS) was used. This approach proved effective at both depleting and enriching CD4+CD25+ T cells. To purify CD4+CD25+ T cells 200 x 10^6 lymph nodes cells were depleted of B, CD8+ and Ia+ cells by panning, and the resulting CD4+ cells fractionated into CD25+ and CD25- populations by magnetic bead separation (MACS) and analysed by FACS. The yield of CD4+CD25+ T cells was 0.5-3.0 x 10^6 (0.25-1.5% of starting population). The purity of CD4+CD25- T cells was about 99% i.e virtually all CD25+ cells were removed, while CD4+CD25+ population was 70% pure, representing a marked enrichment compared to the baseline proportion (about 6%) (figure 7.3.2). It was considered that this degree of enrichment would be sufficient for most practical purposes.

Purification of regulatory T cells

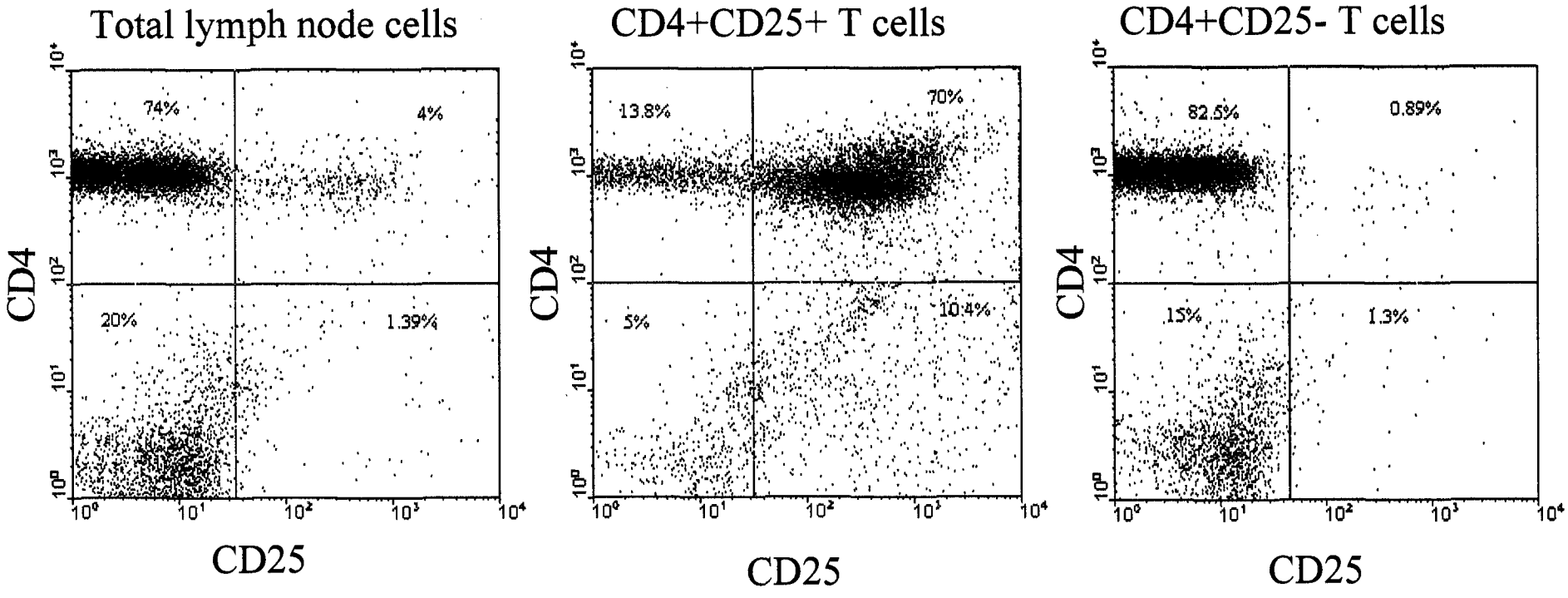


Figure 7.3.2

CD4+CD25+ T regulatory cells were purified from peripheral lymph node cells by magnetic bead separation (MACS) using the antibody, anti-CD25, streptavidin-FITC and anti-FITC magnetic beads. Total (unseparated) lymph node cells, CD4+CD25+ T cells and CD4+CD25- T cells were analysed by FACS. The figure is representative of 3 separate experiments. Number of mice used per experiment was 16.

7.4 Summary

The proportion of CD4+CD25+ T cells in the PLN of DO11.10 mice was analysed by FACS. In parallel, DO11.10 PLN cells depleted of B, CD8+ and Ia+ cells by panning and fractionated into CD4+CD25+ and CD4+CD25- T cells by magnetic bead separation and the percentage purity was determined by FACS. The principal observations were:

1. 4-6.8% of PLN lymphocytes were CD4+CD25+ T cells.
2. After fractionation, the purity of CD4+CD25+ T cells was 70%, while the purity of CD4+CD25- T cells was about 99%.

Chapter 8

Characterisation of unpolarised CD4+CD25+ T cells and CD4+CD25- T cells with respect to cytokine production.

Functional properties of unpolarised CD4+CD25+ T cells, specifically, in terms of inhibition of CD4+CD25- T cells proliferation in response to α-CD3 stimulation.

8.1 INTRODUCTION

It has been shown that the ability of Tr1 cells to downregulating immune responses *in vitro* and *in vivo*, mediated by the production of the immunosuppressive cytokines IL-10 and TGF-β (Roncarolo, M.G. *et al.*, 2001). Characteristically, Tr cells produce high levels of IL-10 and TGF-β, moderate amounts of IFN-γ and IL-5, but little or no IL-2 or IL-4 (Roncarolo, M.G. *et al.*, 2001).

To investigate whether difference in the nature of stimulants influence cytokine production, unpolarised CD25+ or CD25- T cells were stimulated with either α-CD3 or OVA in the presence or absence of APCs. The characterisation of unpolarised cells in terms of cytokine production prior to polarisation may assist to demonstrate the importance of polarisation process to produce proinflammatory cytokines.

Existence of regulatory CD4+ T cells subsets, CD25+, CD62L+ or $CD45RB^{low}$ has been reported recently. However, the bulk of suppressor cells inhibiting the proliferation of CD4+CD25- T cells in coculture is concentrated within the CD25+ but not the CD62L+ or $CD45RB^{low}$ (Alyanakian, M.A. *et al.*, 2003). Similarly, cytokine production patterns are significantly different for each regulatory T cell subset. Using nonobese diabetic (NOD) mice, depending on the regulatory T cells that are depleted, distinct immune diseases appear after transfer into NOD severe combined immunodeficiency recipients, i.e reconstitution of NOD SCID mice with CD25- T cells induces major gastritis and late –onset diabetes, but no or mild colitis. Reconstitution with CD62L- T cells induces fulminant diabetes with no colitis or gastritis. Reconstitution with $CD45RB^{high}$ T cells induces major colitis with wasting disease and no or very moderate gastritis and diabetes. This observation presents evidence for the existence of different subsets of regulatory CD4+ T cells.

8.2 AIMS:

1). Characterise unpolarised CD4+CD25+ T-cells and CD4+CD25- T-cells with respect to cytokine production.

2). Assess the functional properties of unpolarised CD4+CD25+ T cells, specifically, in terms of inhibition of CD4+CD25- T cells proliferation in response to α-CD3 stimulation.

8.3 Results

8.3.1 Cytokine production by unpolarised CD4+CD25+ and CD4+CD25- T cells.

Unpolarised CD4+CD25+ or CD4+CD25- cells were stimulated either with α-CD3 or α-CD3+APC or APC+OVA. CD4+CD25+ cells produced large amounts of IL-5 and little IFN-γ when stimulated with α-CD3 in the presence of APCs (figure 8.3.1; table 8.3.1 in the appendix). The CD4+CD25- population was unresponsive to this stimulus. No IL-4 was detected from either cell population with any of the 3 stimuli. The absence of IL-4 production by CD4+CD25+ or CD4+CD25- unpolarised cells suggests that IL-4 production is induced only when cells become polarized. These data show that in the unpolarised state, anti-CD3- responsive cells are confined to the CD25+ population and they require the presence of APCs to become activated.
To date, there are no other published studies addressing the APC requirements of unpolarised CD4+CD25+ T cells to be activated with α-CD3.

CYTOKINE PRODUCTION BY UNPOLARISED T-CELLS

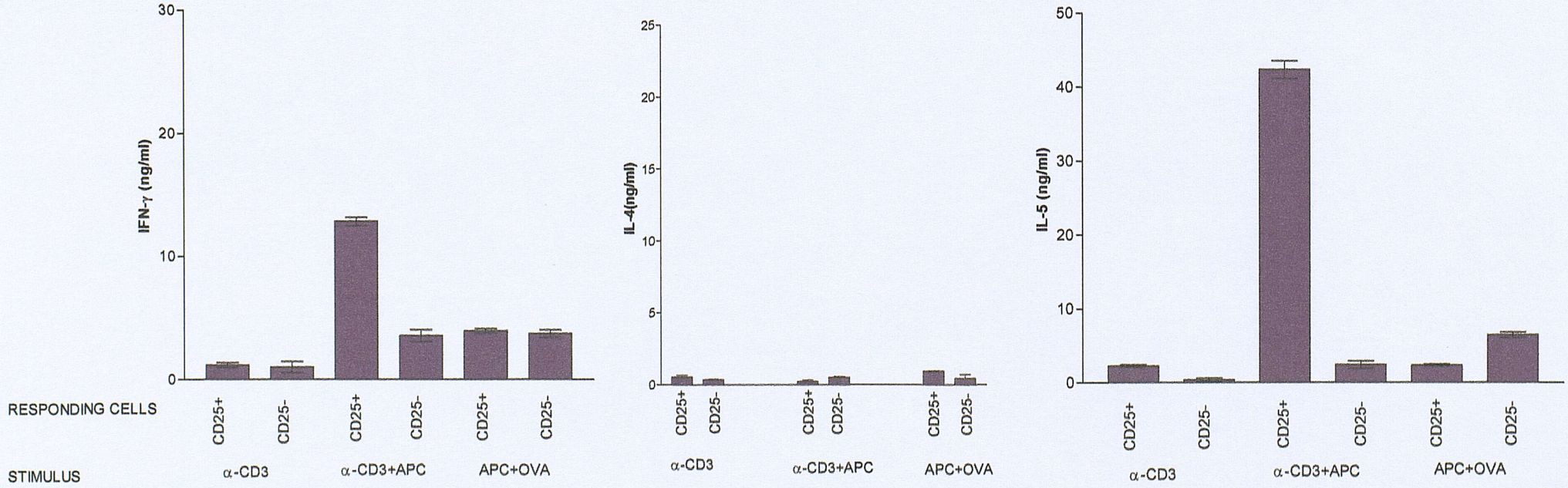


Figure 8.3.1 Unpolarised CD25+ or CD25- cells were stimulated with α-CD3 or α-CD3+APC or APC+OVA for 48 hours. The level of cytokines (IFN-γ, IL-4 and IL-5) produced was measured by standard ELISA. Data are shown for 1 experiment and are representative of 2 independent experiments (see appendix (table 8.3.1)). Data are means ± SEM (n=3). Number of mice used per experiment was 13.

8.3.2 CD4+CD25+ regulatory T cells from DO11.10 mice inhibit T cell proliferation.

CD4+CD25+ regulatory T cells have been shown by others to suppress T cell proliferation. This functional property of CD4+CD25+ unpolarised T cells was assessed here by adding increasing numbers of CD4+CD25+ T cells to unseparated, unpolarised DO11.10 lymph node cells. The proliferative response to anti-CD3 was measured by ^{3}H-thymidine incorporation after 3 days. CD4+CD25+ T-cells inhibited the proliferation of DO11.10 T cells in a dose-dependant manner (figure 8.3.2; table 8.3.2 in the appendix).

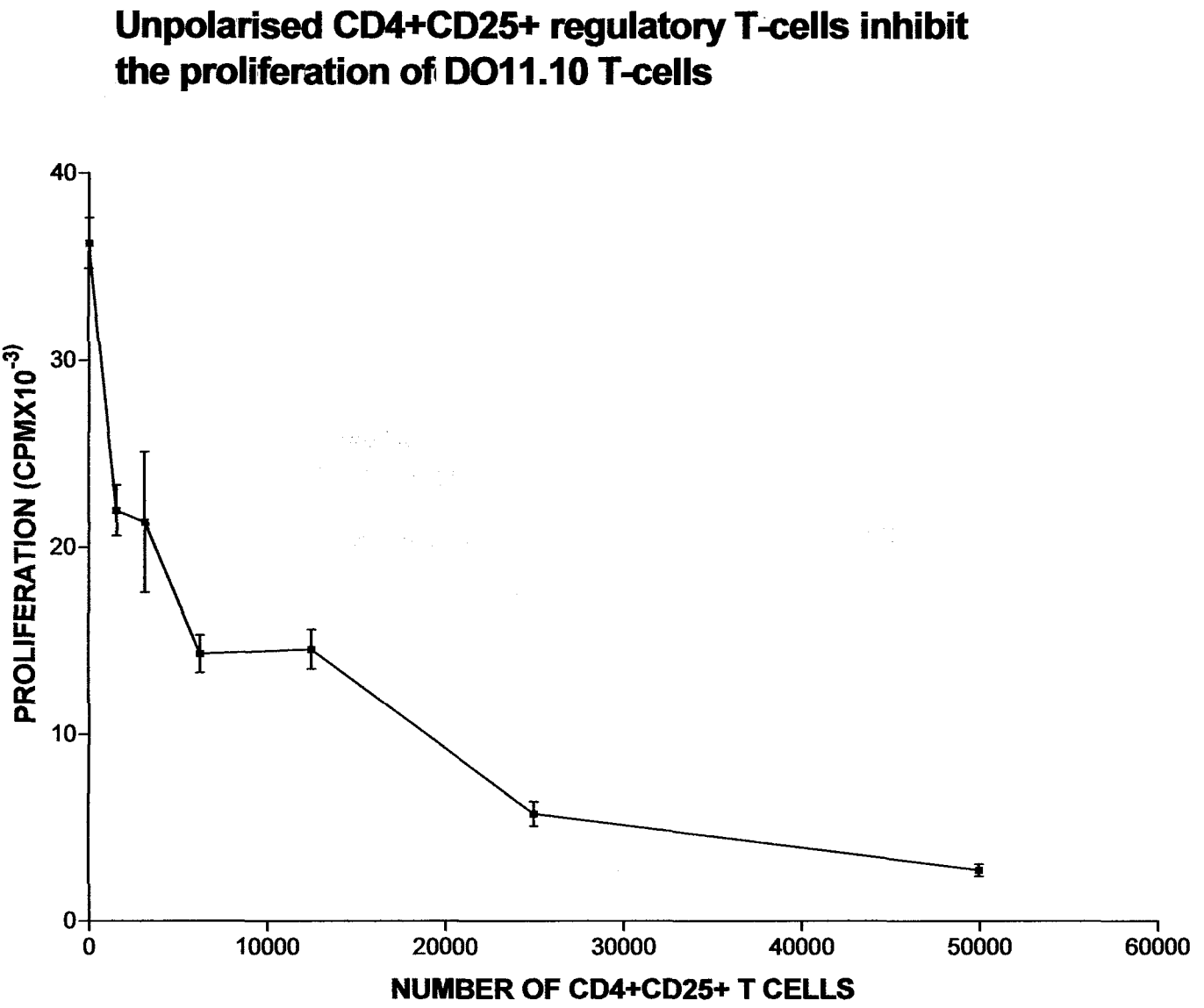


Figure 8.3.2

To examine whether CD4+CD25+ T cells inhibit proliferation, $5x10^4$ DO11.10 lymph node cells were cultured in the presence of different numbers of CD4+CD25+ T-cells. The proliferative response to soluble anti-CD3 was measured by the level of ^{3}H-thymidine incorporation after 3 days. Data are shown for 1 experiment and are representative of 2 independent experiments (see appendix (table 8.3.2). Data are means ± SEM (n=3). Number of mice used per experiment was 12.

8.4 Summary

Unpolarised CD4+CD25+ and CD4+CD25- T cells were characterised with respect to cytokine production in response to α-CD3 or α-CD3+APCs or APCs+OVA stimulation. The functional property of unpolarised CD4+CD25+ T cells was also analysed in terms of inhibition of DO11.10 PLN cells proliferation in response to anti-CD3. The principal observations were:

1. CD4+CD25+ T cells produced large amounts of IL-5 and little IFN-γ when stimulated with α-CD3 in the presence of APCs.

2.CD4+CD25- T cells were unresponsive to this stimulus.

3.Neither CD4+CD25+ nor CD4+CD25- T cells produced IL-4 with any of the 3 stimuli.

4. The assessment of functional property of CD4+CD25+ T cells showed that these cells were able to inhibit the proliferation of unseparated, unpolarised DO11.10 lymph node cells in response to α-CD3 in a dose-dependent manner.

Chapter 9

Polarisation and expansion of CD4+CD25+ and CD4+CD25- T- cells *in vitro* in Th2 favouring culture conditions.

9.1 INTRODUCTION

Ex vivo expansion of regulatory T cells without loss of suppressor function is an important prerequisite in using these cells for immunotherapy. In recent years it has been reported that human anergic regulatory T cells could be expanded by Ag-specific stimulation in the presence of IL-2 (Koenen, H.J. *et al.*, 2003). Both IL-15 and IL-2, are T cell growth factors, but, in contrast to IL-2, IL-15 stimulates the survival of T cells. It has been observed that compared to IL-2-expanded T reg cells, IL-15- expanded regulatory CD4+ T cells were more effective at suppressing both naïve and memory T cells. The possibility of using regulatory T cells as a new therapeutic for the control of GVHD was recently proposed and has been demonstrated in mice by several groups (Cohen, J.L. *et al.*, 2002.; Hoffmann, P. *et al.*, 2002.; Taylor, P.A. *et al.* 2002). *Ex vivo* expansion of regulatory T cells is also possible by stimulation with allogenic APCs, which has the additional effect of producing alloantigen- specific regulatory T cells (Trenado, A. *et al.*, 2003).

9.2 AIM:

Polarise and expand CD4+CD25+ and CD4+CD25- T cells *in vitro* in Th2 favouring culture conditions.

9.3 Results

9.3.1 CD4+CD25+ T cells and CD4+CD25- T cells can be polarised and expanded *in vitro* in Th2 favouring culture conditions.

Before using these cells in functional assays and in adoptive transfer experiments, we polarised and expanded them *in vitro* using the same Th2 favouring culture conditions used earlier. The Th2 polarised cells were then stained with KJ1-26 to assess how many of the polarised cells recognise OVA peptide. About 97% of polarised CD4+CD25+ T cells and about 98% of polarised CD4+CD25- cells stained with KJ1-26 (figure 9.3.1).

Consistent with the observation that CD4+CD25+ T cells stained with the KJ1-26 antibody was the finding that these cells could be expanded in the presence of OVA peptide. The purity of the expanded regulatory T cells following polarization is difficult to determine because all expanded cells expressed CD25. Collectively, these observations confirm that CD4+CD25+ T cells are present in DO11.10 mice and that these cells can be expanded and polarised in culture in response to OVA.

Characteristics of "Polarised" T cells

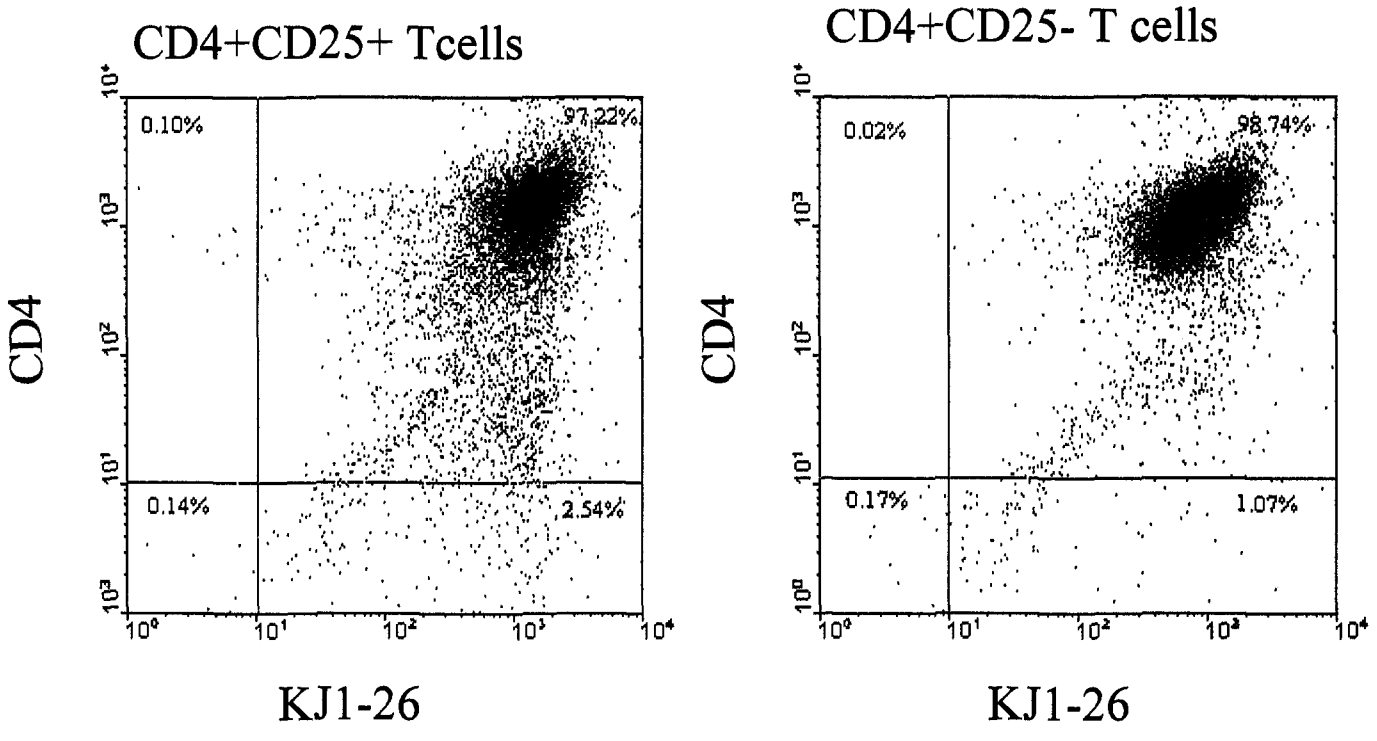


Figure 9.3.1

Purified CD25+ or CD25- T cells were driven *in vitro* in IL-4 (2ng/ml), OVA(1μg/ml), α-IFN-γ (5μg/ml), APCs ($5x10^5$/ml) and IL-2 (100U/ml)for 8 days. The polarised cells were then stained with KJ1-26 FITC and CD4 PE and the proportion of cells expressing KJ1-26 and CD4 receptors were analysed by FACS on day 8. The figure is representative of 3 independent experiments. Number of mice used per experiment was 16.

9.4 Summary

CD4+CD25+ and CD4+CD25- T cells were polarised *in vitro* in Th2 favouring culture conditions and analysed by FACS for the expression of KJ1-26 (OVA specific receptor) + CD4. The principal observations were:

1. About 97% of polarised CD4+CD25+ T cells and about 98% of polarised CD4+CD25- T cells stained with KJ1-26.
2. Consistent with this observation was the finding that these cells could be expanded in the presence of OVA peptide.

Chapter 10

Characterisation of polarised CD4+CD25+ regulatory T cells and CD4+CD25- T-cells *in vitro* in terms of adhesion molecule expression and cytokine production.

The ability of CD4+CD25+ regulatory T cells to affect T- cell proliferation and cytokine production by polarised unseparated DO11.10 PLN cells *in vitro.*

10.1 INTRODUCTION

Polarised subsets were assessed in terms of adhesion molecule expression and cytokine production, as well as their ability to affect T cell proliferation and cytokine production by polarised unseparated DO11.10 PLN cells *in vitro*.

It has been reported that induction of mucosal tolerance to E-selectin, a cytokine-inducible adhesion molecule restricted to activating blood vessels, prevents ischemic and hemorrhagic stroke in spontaneously hypertensive, genetically stroke-prone rats (Chen, Y. *et al.*, 2003). It has also been demonstrated that E-selectin-specific DTH responses were significantly suppressed in E-selectin-tolerized animals. Suppression of the Th1-mediated DTH reaction to E-selectin provides evidence for the generation of antigen-specific regulatory T cells in rats tolerized to E-selectin.

CD62L (L-selectin) is a unique molecule expressed by naïve regulatory T cell subset, which confer distinct regulatory functional property to these population (Alyanakian, M.A. *et al.*, 2003). Therefore, differential expression of adhesion molecules was analysed in distinct polarised cell populations, as these may be implicated in later stages in inflammatory process.

Expression of CD25 by naïve Tr cells is associated with low production of IFN-γ and IL-4 (Alyanakian, M.A. *et al.*, 2003) consistent with our observation presented earlier for unpolarised Tr cells. But, the data presented here indicates that cytokine production pattern by polarised CD4+CD25+ regulatory T cells is reversed in response to α-CD3 stimulation i.e enhanced production of IL-4 and IL-5.

Although it has been reported that CD4+CD25- T cells produced larger amounts of IFN-γ (Alyanakian, M.A. *et al.*, 2003), unpolarised CD4+CD25- T cells from DO11.10 mice produced virtually no cytokines. The cytokine production by CD25

depleted CD4+ T cells driven in Th2 favouring environment was greatly reduced, suggesting that CD25- T cells require the assistance of CD4+CD25+ regulatory T cells to differentiate towards a Th2 cytokine profile.

We had already demonstrated that unpolarised CD4+CD25+ regulatory T cells inhibited the proliferation of freshly isolated DO11.10 lymph node cells, polarised CD4+CD25+ regulatory T cells also inhibited the proliferation of freshly isolated DO11.10 lymph node cells. The evidence presented by Alyanakian, M.A. *et al.* supports our observation that CD4+CD25+ regulatory T cells are capable of inhibiting the proliferation of CD4+CD25- T cells.

10.2 AIMS:

1). Characterise polarised CD4+CD25+ regulatory T cells and CD4+CD25- T cells *in vitro* in terms of :

a). adhesion molecule expression
b). cytokine production

2). Assess the ability of CD4+CD25+ regulatory T cells to affect T cell proliferation and cytokine production by polarised unseparated DO11.10 PLN cells *in vitro*.

10.3 Results

10.3.1 Total, CD25+ and CD25- CD4+ T cells were analysed for the expression of different adhesion molecules.

Polarised total, CD25+ and CD25- T cells were stained with monoclonal antibodies to identify the expression of different adhesion molecules and analysed by FACS. CD44 was the predominant adhesion molecule expressed by all 3 groups (figure 10.3.1). Total, CD25+ and CD25- T cells also expressed β7 chain and CD62L but the level of expression was lower than for CD44. CD31 is the only adhesion molecule expressed predominantly by CD4+CD25- T cells. The only possible explanation for this discrepancy is that the polarisation of CD4+CD25- T cells in the absence of CD4+CD25+ T cells may have influenced the expression of CD31 molecule by CD4+CD25- T cells. The expression of the different adhesion molecules may influence the migration of lymphocytes into the inflammed airways.

The expression of adhesion molecules by unseparated, CD25+ and CD25- T-cells

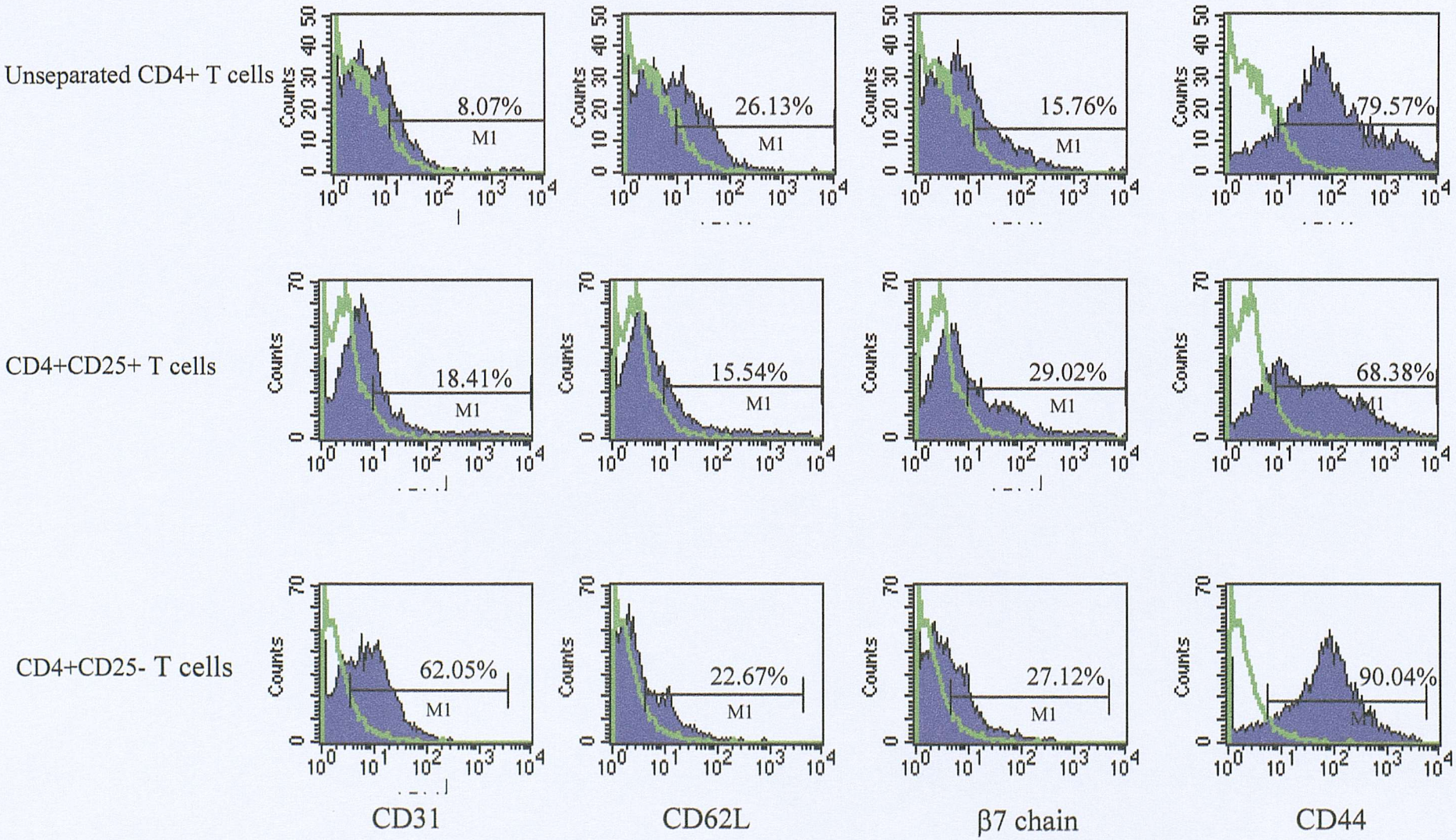


Figure 10.3.1 CD4+ T cells from DO11.10 mice were separated on the basis of CD25 expression and then polarised with OVA peptide, IL-4, IL-2 and anti-IFN- γ for 8 days. Polarised cells were then stained with FITC -labelled monoclonal antibodies specific for different adhesion molecules and then analysed by FACS. The figure is representative of 2 independent experiments. Number of mice used per experiment was 14.

10.3.2 CD4+CD25- T cells following Th2 differentiation produce only low levels of Th2 cytokines.

Th2 polarised (total or CD25+ or CD25-) cells were stimulated with either α-CD3 or KJ1-26 and cytokine production was measured after 48 hours. When stimulated with KJ1-26, polarised CD4+CD25+ cells produced IFN-γ but when stimulated with anti-CD3 they produced both IL-4 and IFN-γ (figure 10.3.2; table 10.3.2 in the appendix). This suggests that IL-4 production is only induced when the cells receive higher level of signal i.e via CD3 ligation, while for IFN-γ production, single receptor stimulation would be sufficient. Th2 polarised unseparated CD4+ T cells produced both IL-4 and IL-5, which was reproducible and consistent with the previous data. In contrast, polarised CD4+CD25- cells consistently produced reduced levels of IL-4 and IL-5, as compared to unfractionated CD4+ T cells (figure 10.3.3). This difference is much more marked for IL-5, suggesting that the CD25- population requires the assistance of the CD25+ population in order to be stimulated to produce IL-5 (figure 10.3.2; table 10.3.2 in the appendix).

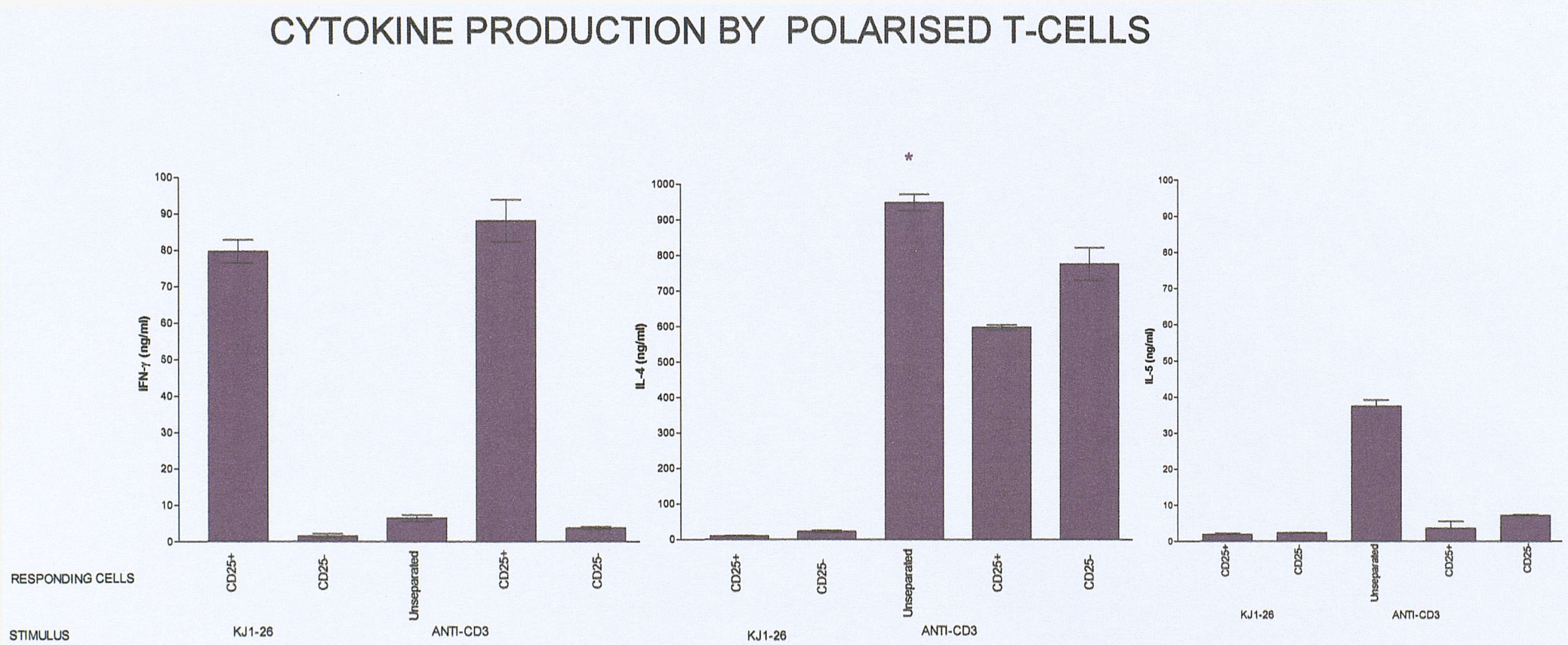


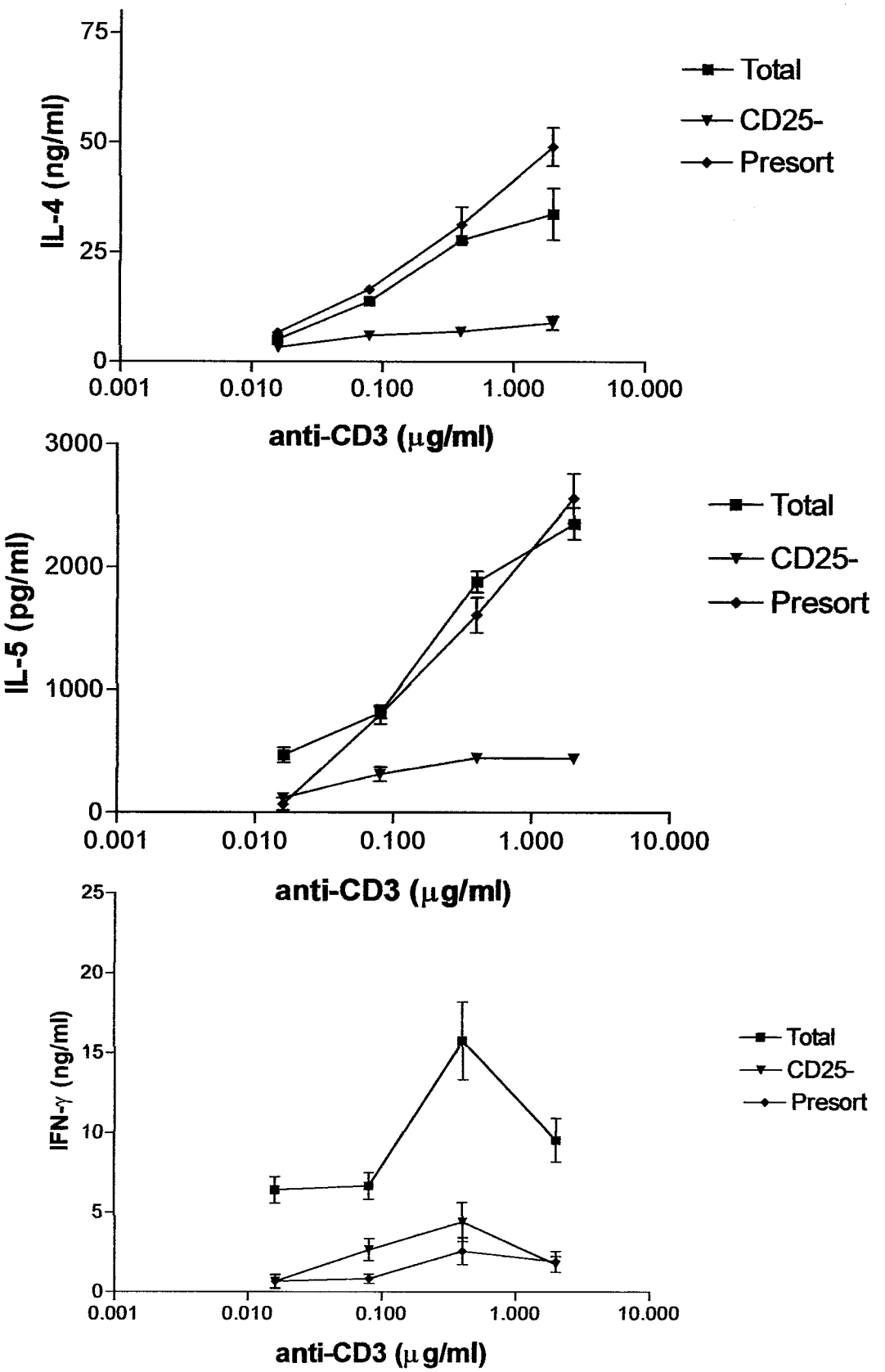
Figure 10.3.2

Total (unseparated) or CD25+ or CD25- cells were driven *in vitro* in OVA peptide (1μg/ml), IL-4 (2ng/ml), IL-2 (100 U/ml) and α- IFN -γ (5μg/ml) for 8 days. The polarised cells were then stimulated with anti-CD3 or KJ1-26 for 48 hours and the level of cytokines (IFN-γ, IL-4 and IL-5) produced was measured by standard ELISA. Data are shown for 1 experiment and are representative of 2 independent experiments (see appendix (table 10.3.2)). Data are means ± SEM (n=3). Number of mice used per experiment was 14. *, $p \leq 0.05$ compared with CD25- T-cells stimulated with α-CD3.

10.3.3 Depletion of CD4+CD25+ T cells prior to Th2 polarization results in reduced cytokine production

In order to assess whether the low production of cytokines was due to the separation procedure , I compared unfractionated CD4+ T cells with cells that had been prepared for CD25 separation but had not yet been sorted (presort sample). Sorted CD25- T cells were used as a comparator. As in the previous experiment, the level of IL-4 and IL-5 produced by CD25- T cells was low compared to unfractionated cells (figure 10.3.3). The presort cells behaved similarly to the total cells in terms of IL-4 and IL-5 production. Only low levels of IFN-γ were produced by all three samples. This helps to confirm that CD25- T cells produce cytokines only if the favourable environment for polarization is provided, i.e the presence of CD25+ T cells is important.

Depletion of CD4+CD25+ T cells prior to Th2 polarization results in reduced cytokine production



gure 10.3.3 DO11.10 T cells were depleted of CD25+ T cells and polarized into Th2 cells over 8 days in Th2 favouring culture conditions used earlier. Control comprised of either unfractionated PLN cells (total) or unfractionated CD4+ T cells (presort) were also polarised into Th2 cells. After 8 days, cells were washed and stimulated with anti-CD3 coated plates. After 48 hours, supernatants were harvested and the cytokine levels was determined by standard ELISA. Data are shown for 1 experiment and are representative of 2 independent experiments. Data are means ± SEM (*n*=3). Number of mice used per experiment was 16.

10.3.4 Th2 polarised CD4+CD25+ DO11.10 regulatory T cells inhibit the proliferation of T cells.

I have already shown that unpolarised CD25+ T cells inhibited the proliferation of DO11.10 lymph node cells in response to anti-CD3 (figure 8.3.2; table 8.3.2 in the appendix). Th2 –polarised CD4+CD25+ T cells also inhibited proliferation in response to OVA or anti-CD3. At low number of T regulatory cells, this inhibition was much more effective against stimulation with anti-CD3 (figure 10.3.4). This may reflect the different nature of the two stimuli : with OVA, signalling is transduced through the TCR, while anti-CD3 directly triggers the CD3 transduction mechanism, bypassing the TCR.

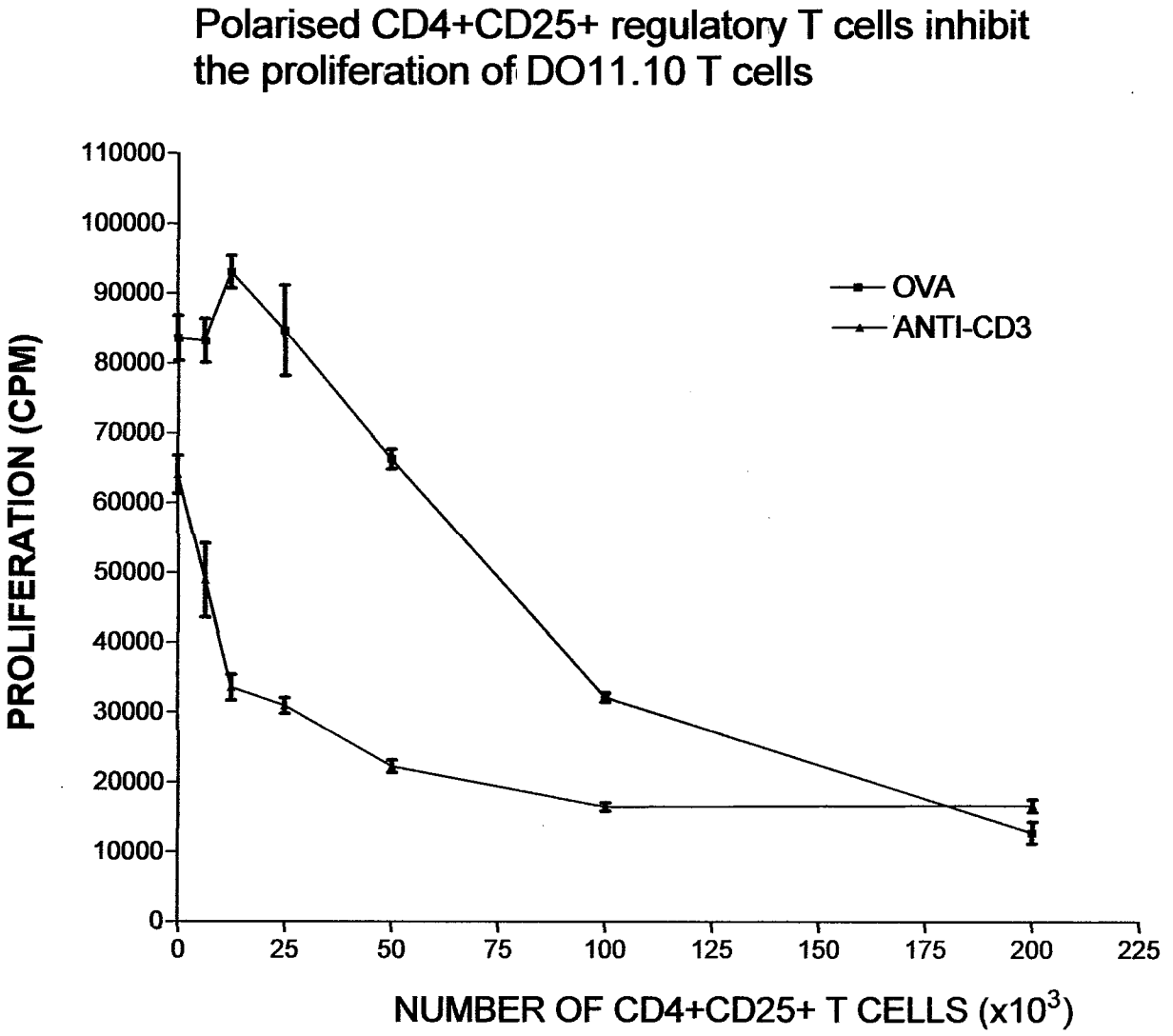


Figure 10.3.4

To examine whether polarised CD4+CD25+ T cells inhibit proliferation, $1x10^5$ DO11.10 lymph node cells were cultured in the presence of different numbers of polarised CD4+CD25+ T- cells. The proliferative response to soluble anti-CD3 or OVA was measured by the level of ^{3}H-thymidine incorporation after 3 days. Data are shown for 1 experiment and are representative of 2 independent experiments. Data are means ± SEM (n=3). Number of mice used per experiment was 12.

10.3.5 Th2 polarised CD4+ CD25+ regulatory T cells inhibit IL-4 but not IL-5 production by Th2 cells

Next, I assessed the influence of Th2 –polarised CD4+CD25+ T regulatory cells on cytokine production by Th2 cells. Polarised Th2 cells were stimulated with anti-CD3 in the presence or absence of Th2 polarised T regulatory cells. As a control I used T regulatory cells that had been polarised in the absence of IL-4. Th2 polarised T regulatory cells were able to inhibit the anti-CD3 driven production of IL-4 by Th2 polarised cells but had no effect on IL-5 production (figure 10.3.5; table 10.3.5 in the appendix). The specificity of this effect was confirmed by the demonstration that CD4+CD25+ T cells polarised in the absence of IL-4 had no effect on IL-4 production by Th2 polarised cells.

An alternative explanation is that the Th2-polarised cells may consume any IL-4 that is produced in response to anti-CD3. We know that the Th2-polarising conditions are likely to induce IL-4 receptors which may then bind any IL-4 that is produced.

It was observed that stimulation of the CD4+CD25+ T cells with irradiated accessory cells, $OVA_{323-339}$ peptide, α-IFN-γ and either IL-2 or IL-2 + IL-4 resulted in a marked expansion (10 and 18 fold respectively). Reproducibly, cell yields from CD4+CD25+ T cells expanded in IL-2 + IL-4 exceeded those obtained using IL-2 alone. CD4+CD25+ T cells could be polarised and expanded in the presence of irradiated accessory cells, OVA peptide, α-IFN-γ, IL-4 and IL-2, however, in the absence of IL-4, cells could be expanded but not polarised.

Polarised regulatory T cells (Tr) inhibit IL-4 but not IL-5 production by polarised Th2 cells

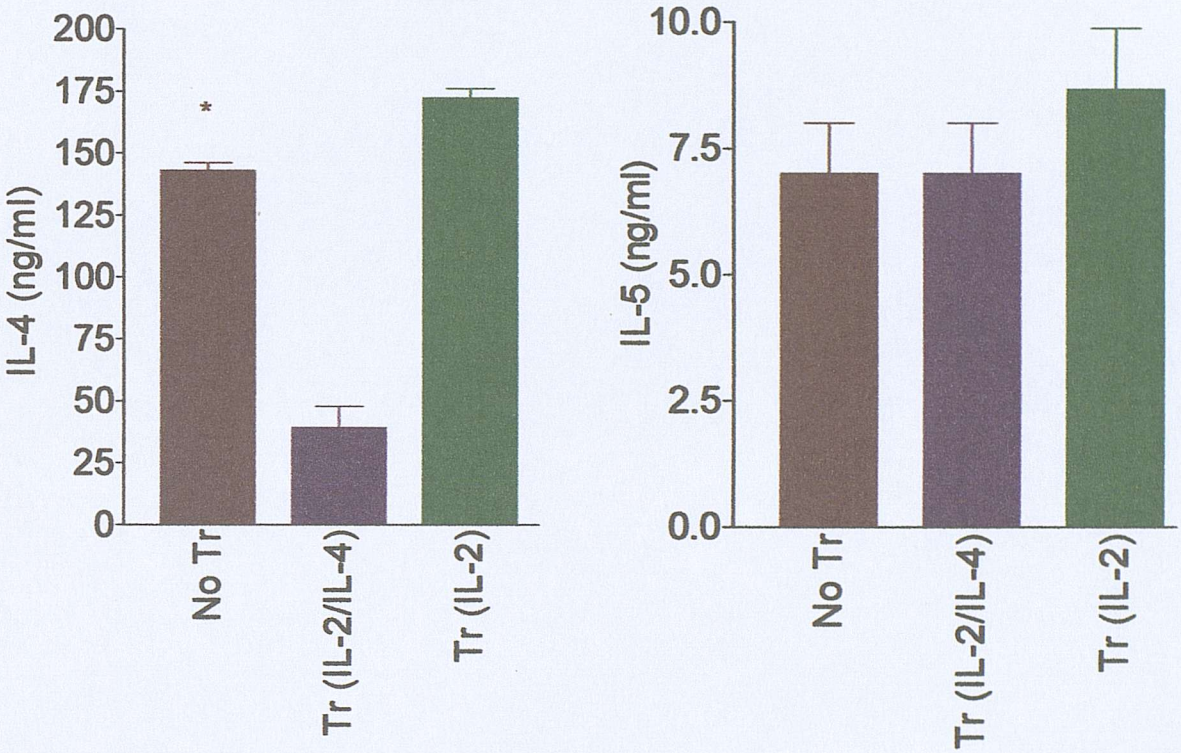


Figure 10.3.5

Purified CD4+CD25+ Tr cells were polarised in vitro in the presence of APCs ($5x10^5$/ml)+OVA (1mg/mλ) +IL-4 (2ng/ml)+IL-2 (100 U/ml)+ a -IΦN -g (5μg/mλ) or APC+OVA+ IL-2+aIFN–g. Iv parallel, PLN cells were polarised with APC+OVA+IL-4 +IL-2+a-IFN-g. The concentration of reagents used in all culture conditions are same, unless stated otherwise. After 8 days, polarised Tr cells were added to polarised Th2 cells (1:1) which were then stimulated with anti-CD3 for 48 hours. The level of cytokines produced by polarised Th2 cells was measured by standard ELISA. Data are shown for 1 experiment and are representative of 2 independent experiments (see appendix (table 10.3.5)). Data are means ±SEM (n=3). Number of mice used per experiment was 12. *, $p \leq 0.05$ compared with Th2 cells stimulated in the presence of Tr cells driven in IL-2+IL-4.

10.4 Summary

Th2 polarised (unseparated, CD4+CD25+ and CD4+CD25-) T cells were characterised *in vitro* in terms of adhesion molecule expression and cytokine production. The ability of polarised CD4+CD25+ T cells to affect the proliferation of DO11.10 PLN cells in response to α-CD3 or OVA and the cytokine production by Th2 -polarised unseparated DO11.10 PLN cells was also assessed *in vitro*. The principal observations were:

1. Analysis of adhesion molecule expression by FACS showed that CD44 was the predominant adhesion molecule expressed by all 3 groups. Unseparated , CD4+CD25+ and CD4+CD25- T cells also expressed β7 chain and CD62L but the level of expression was lower than for CD44. CD31 is the only adhesion molecule expressed predominantly by CD4+CD25- T cells.
2. Analysis of cytokine production showed that Th2 polarised CD4+CD25+ T cells when stimulated with KJ1-26 produced IFN-γ, but when stimulated with anti-CD3 they produced both IL-4 and IFN-γ. Th2 polarised unseparated T cells produced both IL-4 and IL-5 when stimulated with anti-CD3. In contrast, CD4+CD25- T cells consistently produced reduced levels of IL-4 and IL-5, as compared to unfractionated CD4+ T cells.
3. Examining the effect of Th2 polarised CD4+CD25+ T cells on the proliferation of DO11.10 PLN cells showed that these cells inhibited the proliferation of DO11.10 PLN cells in response to α-CD3 or OVA. At low number of CD4+C25+ T cells, the inhibition was much more effective against stimulation with α-CD3.

4. Assessing the effect of Th2- polarised CD4+CD25+ T cells on cytokine production by polarised- Th2 cells showed that Th2-polarised CD4+CD25+ T cells were able to inhibit the anti-CD3 driven production of IL-4 by Th2-polarised cells but had no effect on IL-5 production.

Chapter 11

The ability of Th2- polarised CD4+CD25+ T-cells to modify the effect of Th2 cells in the adoptive transfer model of allergic lung inflammation.

11.1INTRODUCTION

In the final part of this work, Th2- polarised CD4+CD25+ T cells were assessed for their ability to modify the effect of Th2 cells in the adoptive transfer model of allergic lung inflammation.

SCID mice developed severe colitis when adoptively transferred with naïve CD4+CD25- T cells and infected with the protozoan parasite Leishmania major (Liu, H. *et al.*, 2003), but the development of disease could be completely suppressed by transferring freshly isolated or activated CD4+CD25+T cells from syngeneic donors. Furthermore, the role of CD4+CD25+ T cells in transplantation immunology has also been demonstrated (Wood, K.J. *et al.*, 2003): donor alloantigen-specific CD4+CD25+ regulatory T cells can control aggressive CD4+ as well as CD8+ T cells thereby preventing rejection and can mediate MHC- linked unresponsiveness.

The DO11.10 mouse model has been successfully used by others to study the induction of regulatory T cells in the nose-draining cervical lymph node (Unger, W.W. *et al.* 2003).

The cytokines IL-4, IL-5 and IL-13 released from activated Th2 cells are associated with airway hyperreactivity, airway inflammation and mucus hyperproduction (Wills-Karp, M., 1999.; Taube, C. *et al.* 2003). In our mouse model, the role of CD4+CD25+ T cells in lung inflammation was investigated by assessing airway eosinophilia and airway hyperreactivity and by analysing the proportion of KJ1-26+ CD4+ T cells in bronchoalveolar fluid and in the lung tissue of BALB/c mice that had received either polarised total Th2 cells or polarised Th2 cells depleted of CD4+CD25+ T cells.

11.2 AIM:

Assess the ability of Th2- polarised CD4+CD25+ T-cells to modify the effect of Th2 cells in the adoptive transfer model of allergic lung inflammation.

11.3 Results

11.3.1 Th2 cells generated from CD4+CD25 – cells elicit an increased levels of eosinophilia in aerosol challenged BALB/c mice.

To examine the role of CD4+CD25+T cells *in vivo*, Th2 polarised T cell subsets were prepared and injected into naive BALB/c mice, which were then challenged on 5 consecutive days with ovalbumin. The mice were then sacrificed and airway eosinophilia was assessed by EPO assay of BALF. Somewhat unexpectedly, mice that received unseparated Th2 polarised CD4+ T cells showed a marked airway eosinophilia as measured by EPO in BALF. Mice that received Th2 –polarised CD4+CD25- T cells showed only a small degree of eosinophilia after OVA challenge, and there was no difference when a mixture of Th2 polarised CD25- and CD25+ cells were given to the mice (figure 11.3.1; table 11.3.1 in the appendix).

In contrast, when the same experiment was performed over 7 days, the animals that received Th2 –polarised CD4+CD25- cells showed an enhanced airway eosinophilia as shown by increased EPO in BALF (figure 11.3.2; table 11.3.2 in the appendix), increased number of eosinophils in the BALF (figure 11.3.3; table 11.3.3 in the appendix) and a significantly higher level of AHR in response to methacholine (figure 11.3.4).

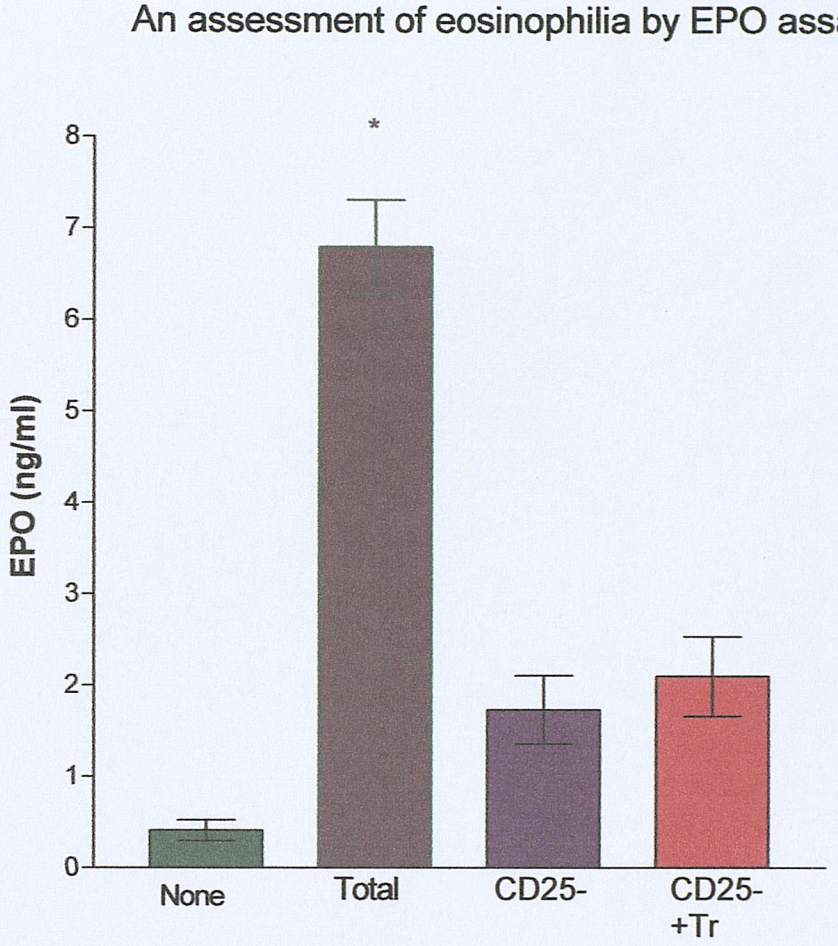
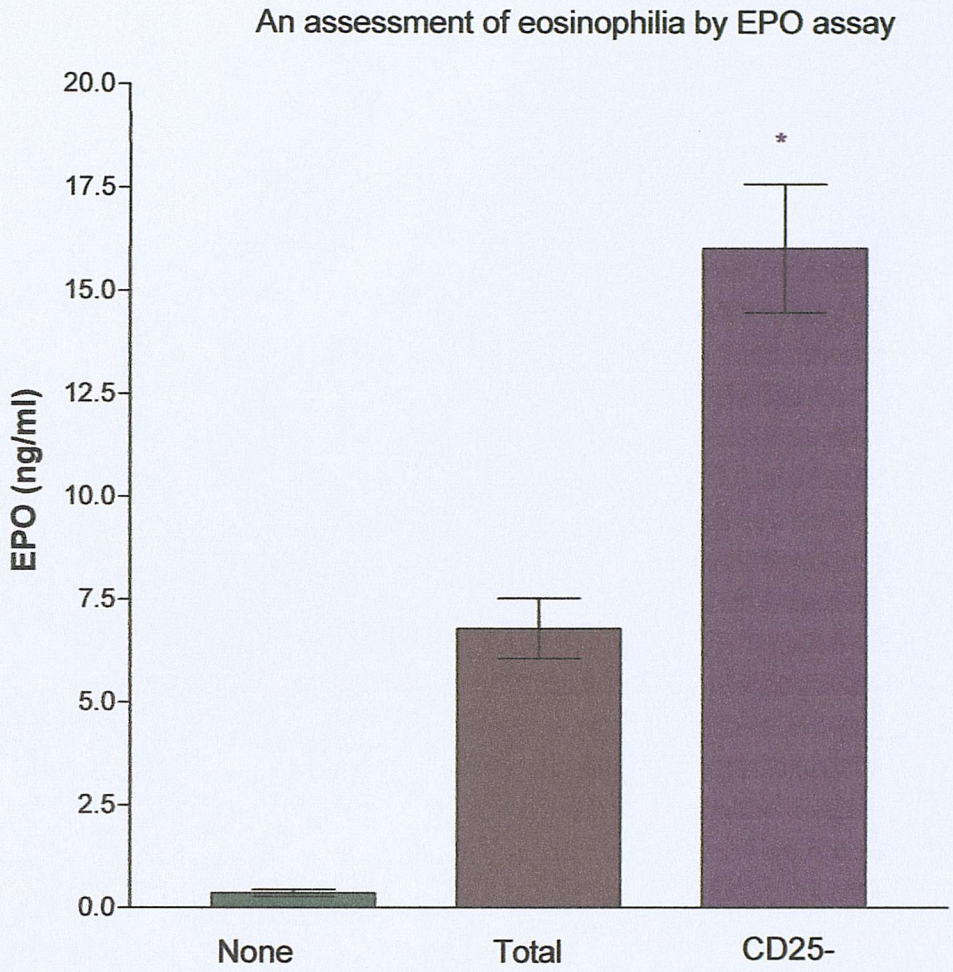


Figure 11.3.1 The level of eosinophilia in the bronchoalveolar lavage was measured by EPO assay. BALB/c mice were given either $10x10^6$ total (unseparated) or CD25- or CD25- + Tr cells (CD25-:Tr 1:1). Recipients were exposed to OVA aerosol and the level of EPO in the broncho alveolar lavage fluid determined at day 5. Control mice did not receive Th2 cells (none). Data are shown for 1 experiment and are representative of 3 independent experiments (see appendix (table 11.3.1)). Data are means ±SEM (n=3). Number of mice used in each group per experiment was 6. *, $p \leq 0.05$ compared with recipients of CD25- T cells.



gure 11.3.2

The level of eosinophilia in the broncho alveolar lavage was measured by EPO assay. BALB/c mice were given Th2 cells ($10x10^6$) prepared from either total (unseparated) or CD25-. Receipients were exposed to OVA aerosol and the level of EPO in the broncho alveolar lavage fluid determined at day 7. Control mice did not receive Th2 cells. Data are shown for 1 experiment and are representative of 3 independent experiments (see appendix (table 11.3.2)). Data are means ± SEM (n=3). Number of mice used in each group per experiment was 6. *, $p \leq 0.05$ compared with recipients of total Th2 cells.

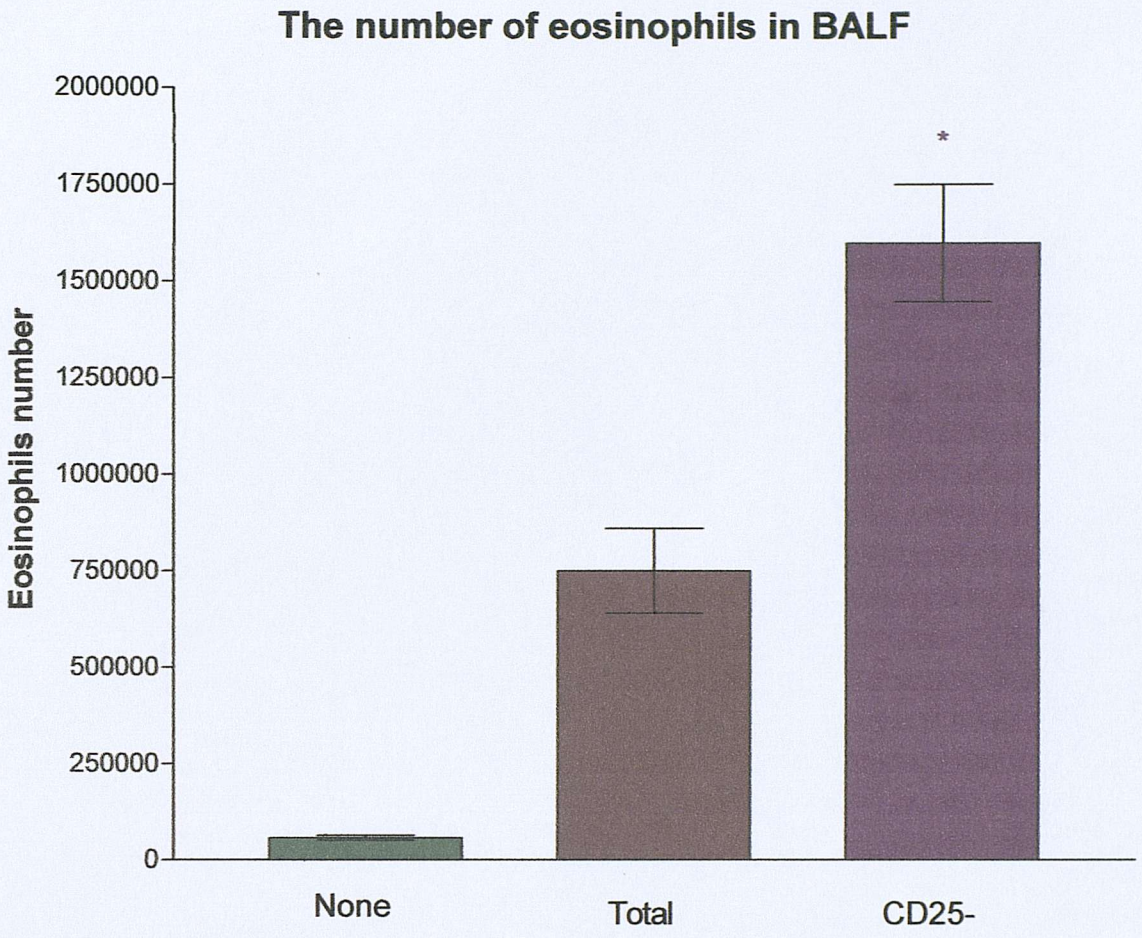


Figure 11.3.3

The number of eosinophils in the broncho alveolar lavage was counted. BALB/c mice were given Th2 cells ($10x10^6$) prepared from either total (unseparated) or CD25-. Recipients were exposed to OVA aerosols and the number of eosinophils in the broncho alveolar lavage fluid determined at day 7. Control mice did not receive Th2 cells. Data are shown for 1 experiment and are representative of 3 independent experiments (see appendix (table 11.3.3)). Data are means ± SEM (n=3). Number of mice used in each group per experiment was 6. *, $p \leq 0.05$ compared with recipients of total Th2 cells.

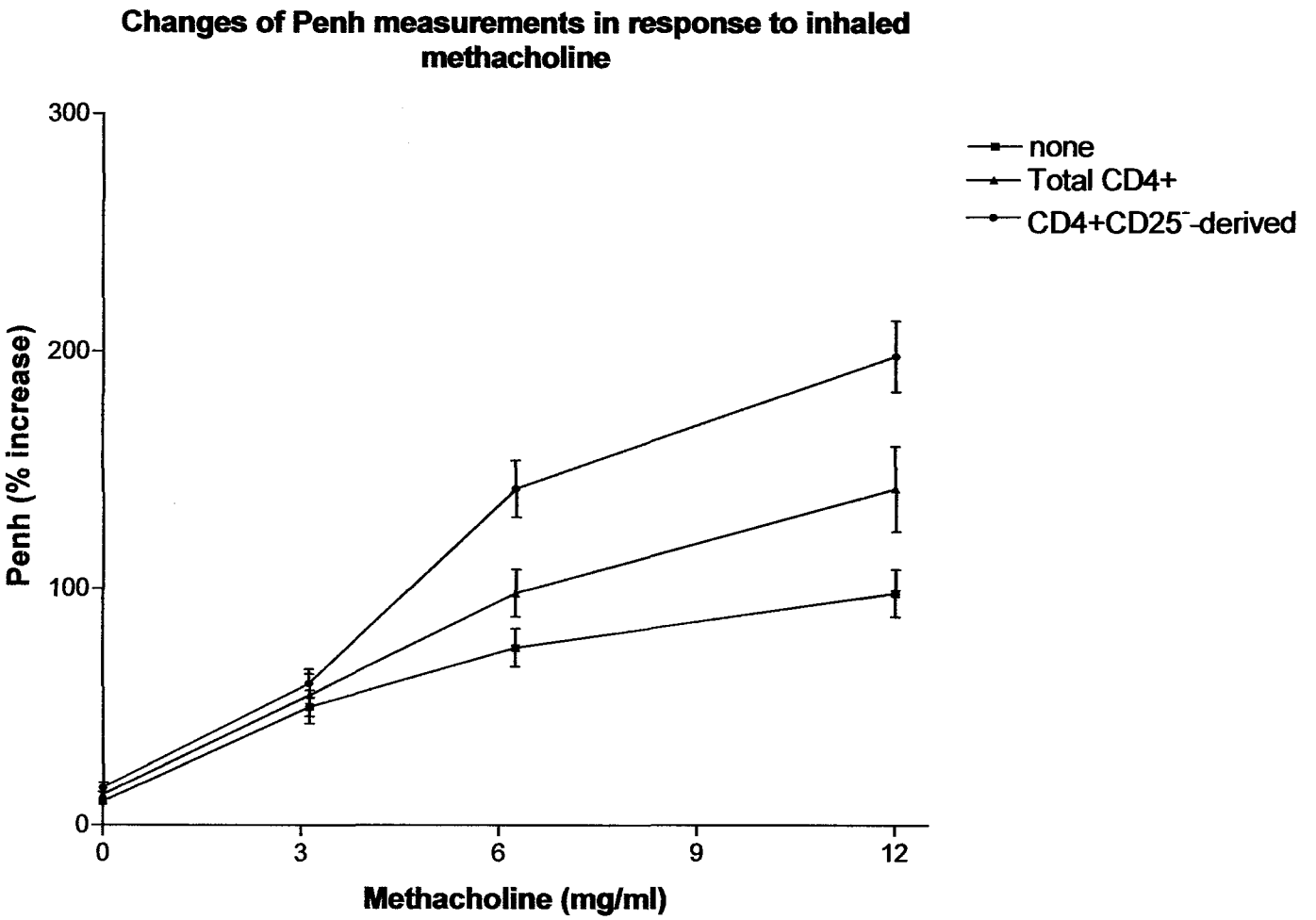


Figure 11.3.4

Airway hyperreactivity was measured in response to methacholine inhalation by whole-body plethysmography. BALB/c mice were given Th2 cells ($10x10^6$) prepared from either total (unseparated) or CD25-. Receipients were exposed to OVA aerosols for 7 consecutive days. Control mice did not receive Th2 cells (none). On day 7, animals were placed in chambers and exposed to nebulized PBS (baseline) followed by increasing concentrations of methacholine. Enhanced pause (Penh) was measured after each 3-min exposure. Data are shown for 1 experiment and are representative of 2 independent experiments. Data are means ±SEM (n=3). Number of mice used in each group per experiment was 6.

11.3.2 BALB/c mice that received Th2 cells generated from CD4+CD25- T cells expressed lower levels of CD4+KJ1-26+ T cells in the BALF.

Having shown that CD4+CD25+ T cells influenced airway eosinophilia, we next assessed the proportion of CD4+ T cells that expressed the OVA specific T cell receptors in the BAL of BALB/c mice that were used in these adoptive transfer experiments.

In mice that received polarised unseparated Th2 cells, about 45% of CD4+ cells expressed KJ1-26. In contrast, in mice that received CD4+CD25- cells, about 30% of CD4+ cells expressed KJ1-26 (figure 11.3.5). One possible explanation is that regulatory T cells present in the unseparated cell population may have influenced the migration of OVA specific CD4+ T cells (KJ1-26+) from the lung into the airways where they are eliminated in BAL. In mice that received CD25- T cells, more OVA specific T cells may have been retained in the lung and therefore exacerbated the airway inflammation and eosinophilia shown in figure 11.3.2. Mice that received a mixture of equal number of CD25- and CD25+ T cells showed no significant difference to those that received CD4+CD25- T cells alone. This lack of effect of CD25+ cells may be a question of cell number and it would be interesting to repeat the experiment with a range of different proportion of CD25- and CD25+ T cells.

The expression of KJ1-26 and CD4 by lymphocytes in the BALF of BALB/c mice received adoptive cell transfer.

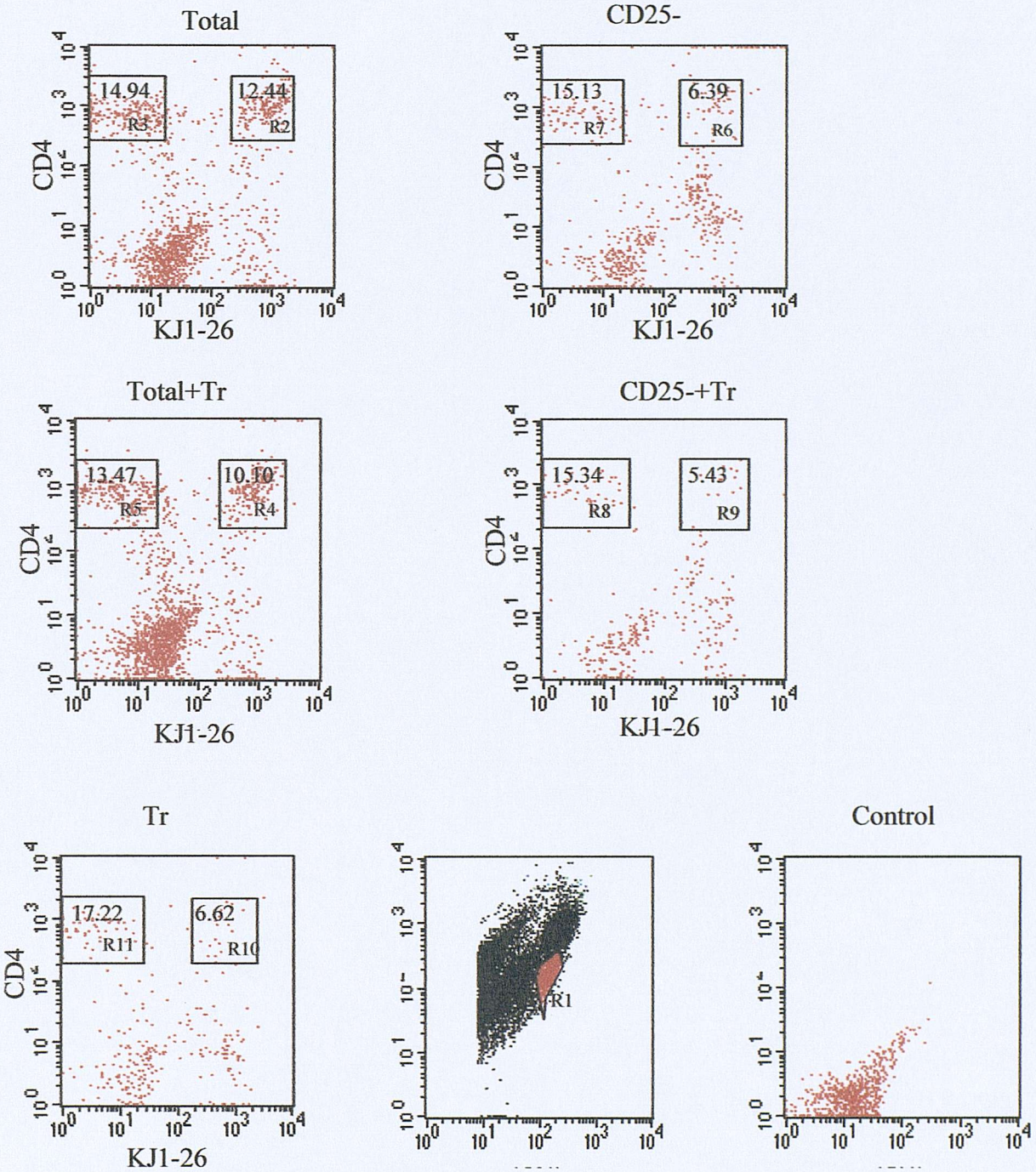


Figure 11.3.5

BALB/c mice were given either polarised total (unseparated ($10x10^6$)) or total +Tr (1:1) or CD25- ($10x10^6$)or CD25- + Tr (1:1) or Tr ($10x10^6$). Mice received adoptive cell transfer were exposed to OVA aerosol for 20 minutes for 7 consecutive days. On the last day, mice were sacrificed and the BALF was retrieved. The lymphocytes in the BALF of different groups were stained with KJ1-26 FITC+ CD4PE and analysed by FACS. The figure is representative of 2 independent experiments. Number of mice used in each group per experiment was 5.

11.3.3 More KJ1-26 expressing CD4+ T cells are retained in the lung of BALB/c mice given CD4+CD25- T cells.

As well as looking in BALF, we assessed KJ1-26 expression on T cells in the lung tissue of BALB/c mice that took part in these adoptive transfer experiments. In contrast to the BALF results, BALB/c mice which were given CD4+CD25- T cells had a higher proportion of KJ1-26+CD4+ in the lung than the mice that received unseparated CD4+Th2 polarised cells (figure 11.3.6). Taken together these results suggest that retention of CD4+CD25- T cells is much favoured in the lung in the absence of regulatory T cells. This observation further reinforces our hypothesis that CD4+CD25+ T cells control inflammation by eliminating T cells actively involved in mediating inflammation.

The level of expression of KJ1-26 and CD4 in the lung of BALB/c mice received adoptive cell transfer.

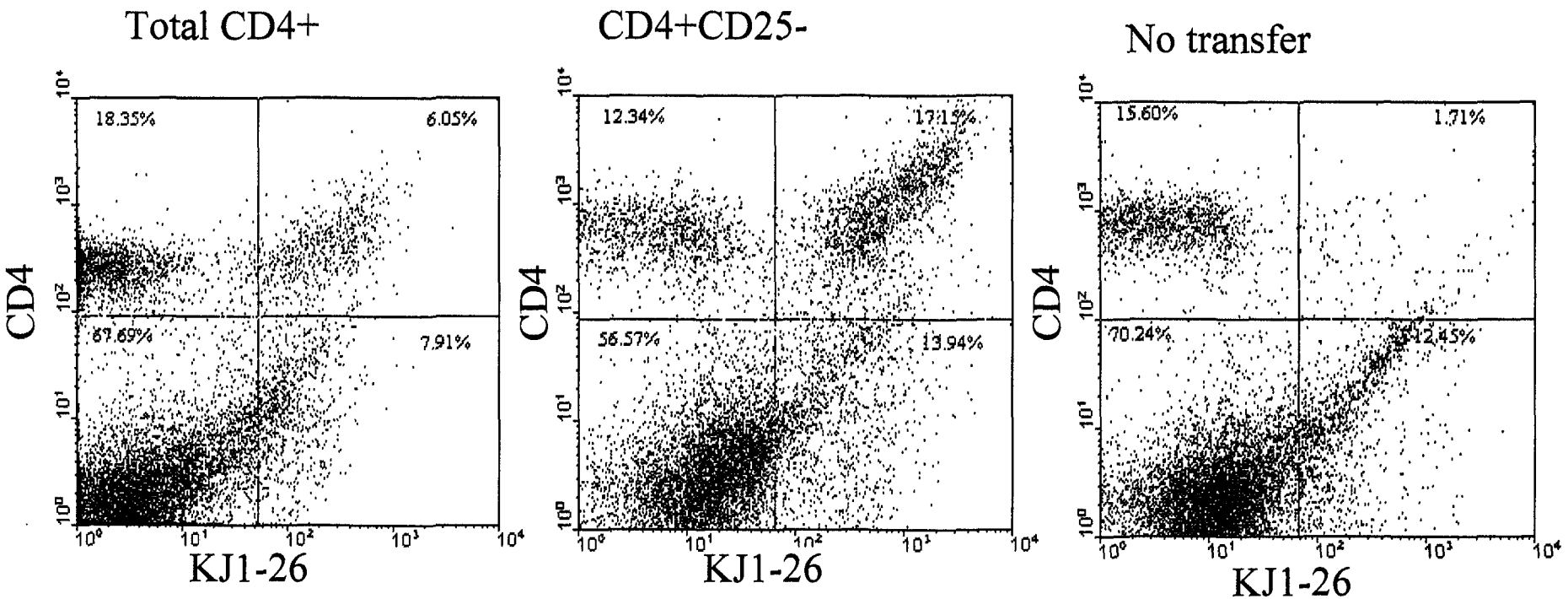


Figure 11.3.6

BALB/c mice were given either polarised total or CD25- T cells and exposed to OVA aerosol for 20 minutes for 7 consecutive days. On day 7, the mice were sacrificed and the lung was digested to concentrate T-lymphocytes. The samples from the 6 mice were pooled before staining for flow cytometry. Lymphocytes were stained with KJ1-26 FITC + CD4PE and the level of expression was analysed by FACS. The figure is representative of 3 independent experiments. Number of mice used in each group per experiment was 6.

11.4 Summary

The ability of Th2-polarised regulatory T-cells to modify the effect of Th2 cells in the adoptive transfer model of allergic lung inflammation was assessed. The principal observations were:

1. BALB/c mice that received unseparated Th2 -polarised CD4+ T cells showed a marked airway eosinophilia as measured by EPO in BALF at day 5. Mice that received Th2-polarised CD4+CD25- cells showed a small degree of eosinophilia after OVA challenge. In contrast, when the same experiment was performed over 7 days, mice that received Th2-polarised CD4+CD25- cells showed an enhanced airway eosinophilia as shown by increased EPO in BALF, increased number of eosinophils in the BALF and a significantly higher level of AHR in response to methacholine.

2. FACS analysis of the proportion of CD4+ T cells expressing OVA- specific T cell receptor (KJ1-26) in the BAL of BALB/c mice showed that in mice that received polarised unseparated Th2 cells, about 45% of CD4+ T cells expressed KJ1-26. In contrast, in mice that received CD4+CD25- cells, about 30% of CD4+ T cells expressed KJ1-26.

3. FACS analysis of the proportion of CD4+ T cells expressing OVA-specific T cell receptor in the lung tissue of BALB/c showed that mice which were given CD4+CD25- T cells had a higher proportion of KJ1-26+CD4+ in the lung than the mice that received unseparated Th2- polarised cells.

Chapter 12

The time course of recruitment of OVA- specific T- cells to the BAL and peripheral lymph nodes.

12.1 INTRODUCTION

In further analysis of this model, the time course of recruitment of OVA-specific T-cells to the BAL and peripheral lymph nodes was studied.

Previous studies by other groups show that long-term allergen exposure can attenuate inflammation and revert airway hyperreactivity to normal responsiveness (Cui, Z. *et al.*, 2003). This change in responsiveness and inflammation has been proposed to be associated with a transition from a Th2 phenotype to a Th1 cytokine-biased profile.

12.2 AIM:

Analyse the time course of recruitment of OVA-specific T-cells to the BAL and peripheral lymph nodes.

12.3 Results

12.3.1 The number of KJ1-26+CD4+ expressing T cells in BALF and peripheral lymph node varies with time.

Since the previous experiments indicated that BAL eosinophilia is time-dependent (figures 11.3.1 and 11.3.2; tables 11.3.1 and 11.3.2), we addressed the time course of migration of KJ1-26+CD4+ T cells in the BALF and peripheral lymph nodes of BALB/c mice that received polarised total T lymphocytes. The FACS data shows that the percentage of CD4+ T cells in BAL increased over the 3 time points and by day 6, 50% of the CD4+ T cells in BAL expressed KJ1-26. (figure 12.3.1). In peripheral lymph nodes, the proportion of CD4+ cells decreased over 6 days but the relative proportion of KJ1-26 cells was similar at all 3 timepoints. This suggests that the migration of inflammatory T cells from lung into the airways is time-dependent and we have showed earlier that it is dependent on exposure to OVA aerosol challenge.

The FACS plots also show a substantial number of of KJ1-26+CD4- cells. The identity of these cells remains obscure. They could conceivably be BALB/c host cells that have expanded but we have not pursued this aspect of the model in the present experiments.

The expression of KJ1-26 and CD4 by lymphocytes in BALF and peripheral lymph node cells at different time points.

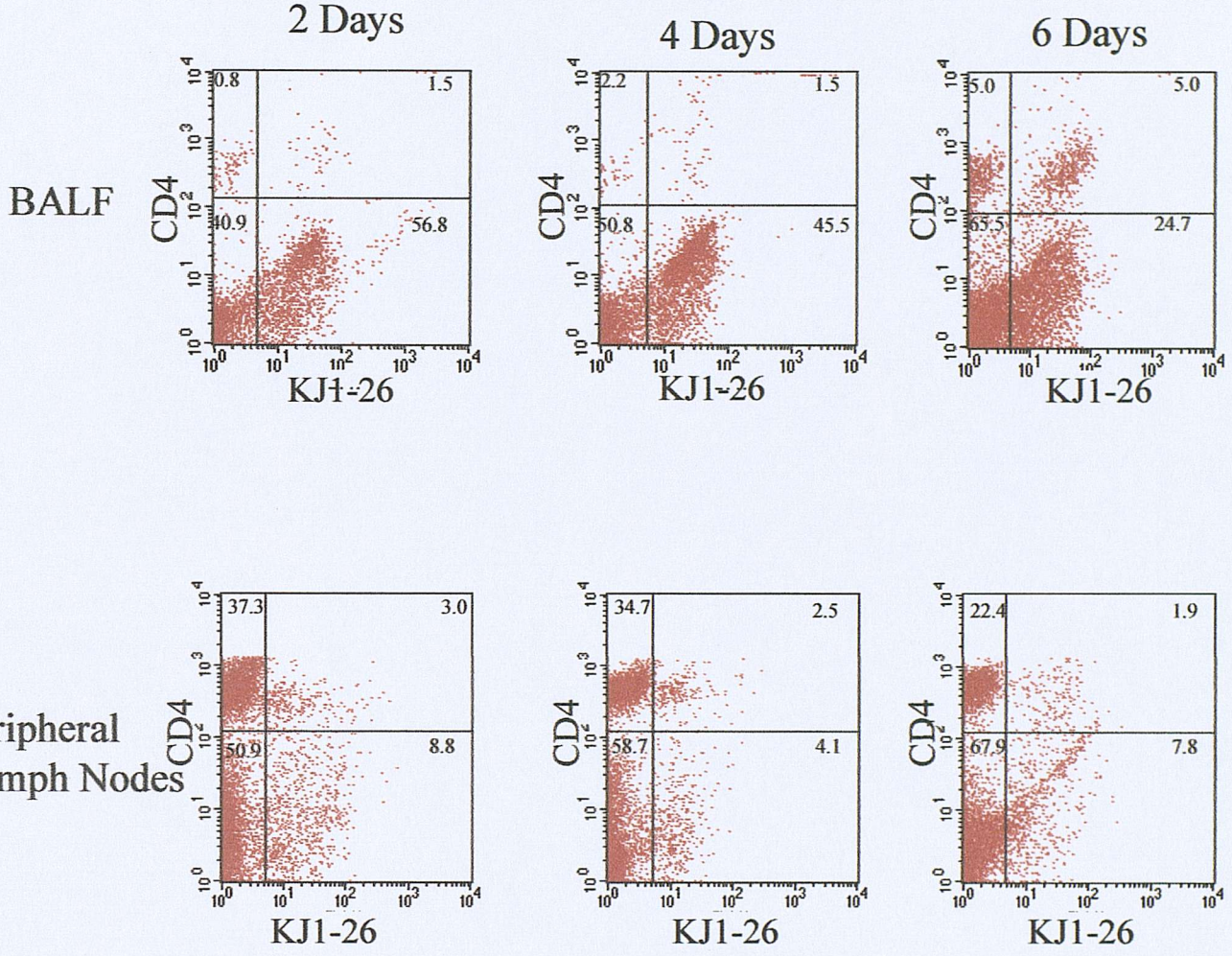


Figure 12.3.1

BALB/c mice were given polarised total (unseparated) T- Lymphocytes $10x10^6$ and exposed to OVA aerosol challenge for 6 consecutive days. Mice were sacrificed at different time points (2, 4, 6) and the expression of KJ1-26 and CD4 in BALF and peripheral lymph node cells were analysed by FACS. The figure is representative of 2 independent experiments. Number of mice used in each group per experiment was 5.

12.4 Summary

The time course of recruitment of OVA-specific T cells to the BAL and peripheral lymph nodes of BALB/c mice that received Th2 polarised total lymphocytes was assessed. The principal observations were :

1. FACS analysis showed that the percentage of CD4+ T cells in BAL increased over the 3 time points and by day 6, 50% of the CD4+ T cells in BAL expressed KJ1- 26.

2. In peripheral lymph nodes, the proportion of CD4+ T cells decreased over 6 days but the relative proportion of KJ1-26 cells was similar at all 3 timepoints.

Chapter 13

Coexpression of CD4 and CD103 by unpolarised and polarised lymph node cells.

13.1 INTRODUCTION

CD103 is an integrin expressed by intraepithelial lymphocytes, and is expressed on 20-30% CD4+CD25+ T cells but not on CD25- T cells. Both CD103+CD25+ and CD103- CD25+ were able to suppress anti-CD3 induced proliferation of CD4+CD25- T cells (McHugh, R.S. *et al.*, 2002), but CD103+CD25+ were more efficient on a per cell basis at suppressing the proliferation of the responders. Furthermore, it has been shown that the acquisition of suppressive activity was independent of CD103 expression, as CD25+CD103- cells were suppressive but did not express CD103 upon activation (McHugh, R.S. *et al.*, 2002).

There is some evidence that CD103 plays a role in the maintenance of lymphocytes in the intestine. I therefore thought it would be of interest to investigate its role in pulmonary inflammation.

13.2 AIM:

Analyse the coexpression of CD4 and CD103 by unpolarised and polarised lymph node cells.

13.3 Results

13.3.1 A population of unpolarised OVA specific lymph node cells coexpress CD4 and CD103.

In unpolarised lymph node cells, about 3.8% of CD4+ cells expressed CD103 (figure 13.3.1). However, after polarisation virtually no CD4+ cells expressed CD103 (figure 13.3.2). On the basis of this experiment, it does not appear that CD103 is relevant to the DO11.10 lung inflammation model. It remains unclear whether this reflects differences between gut and lung, or is a feature of the DO11.10 transgenic system.

Receptor expression by unpolarised T-lymphocytes

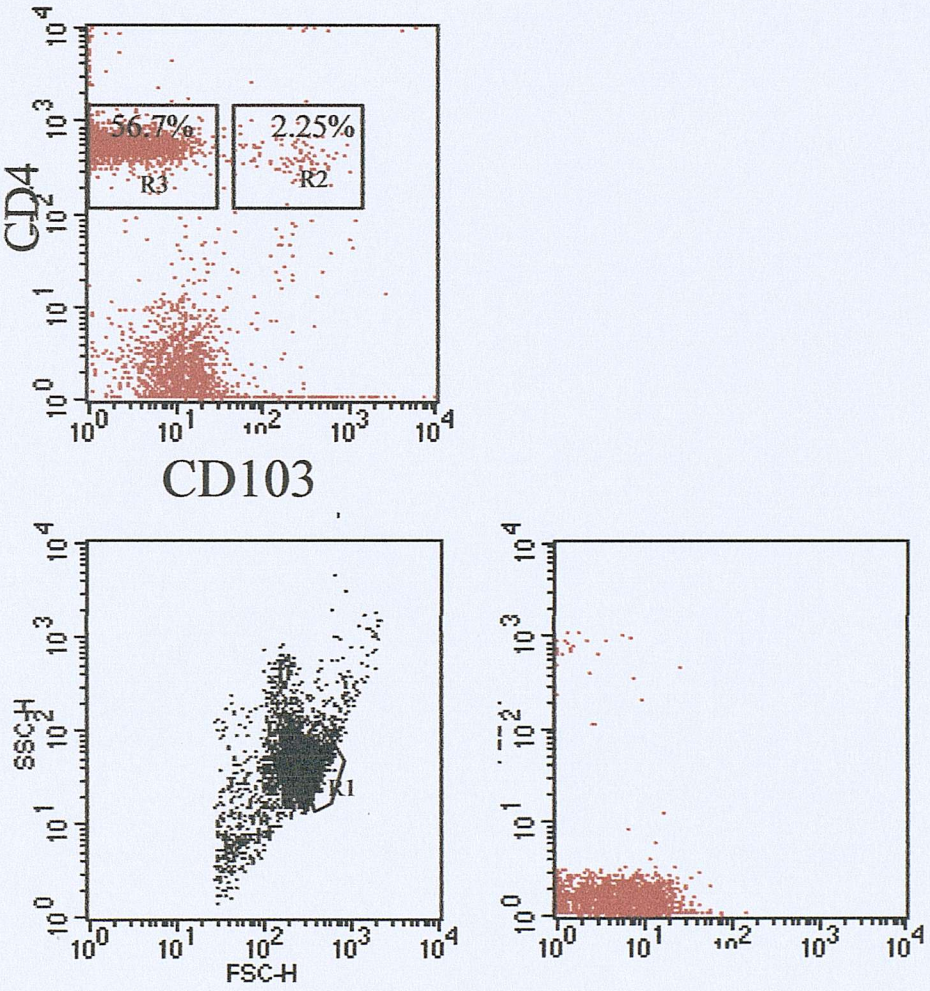


Figure 13.3.1

Lymph node cells (unpolarised)from DO11.10 mice were stained with CD103 FITC+CD4 PE. The level of expression was then analysed by FACS. The figure is representative of 3 independent experiments. Number of mice used per experiment was 6.

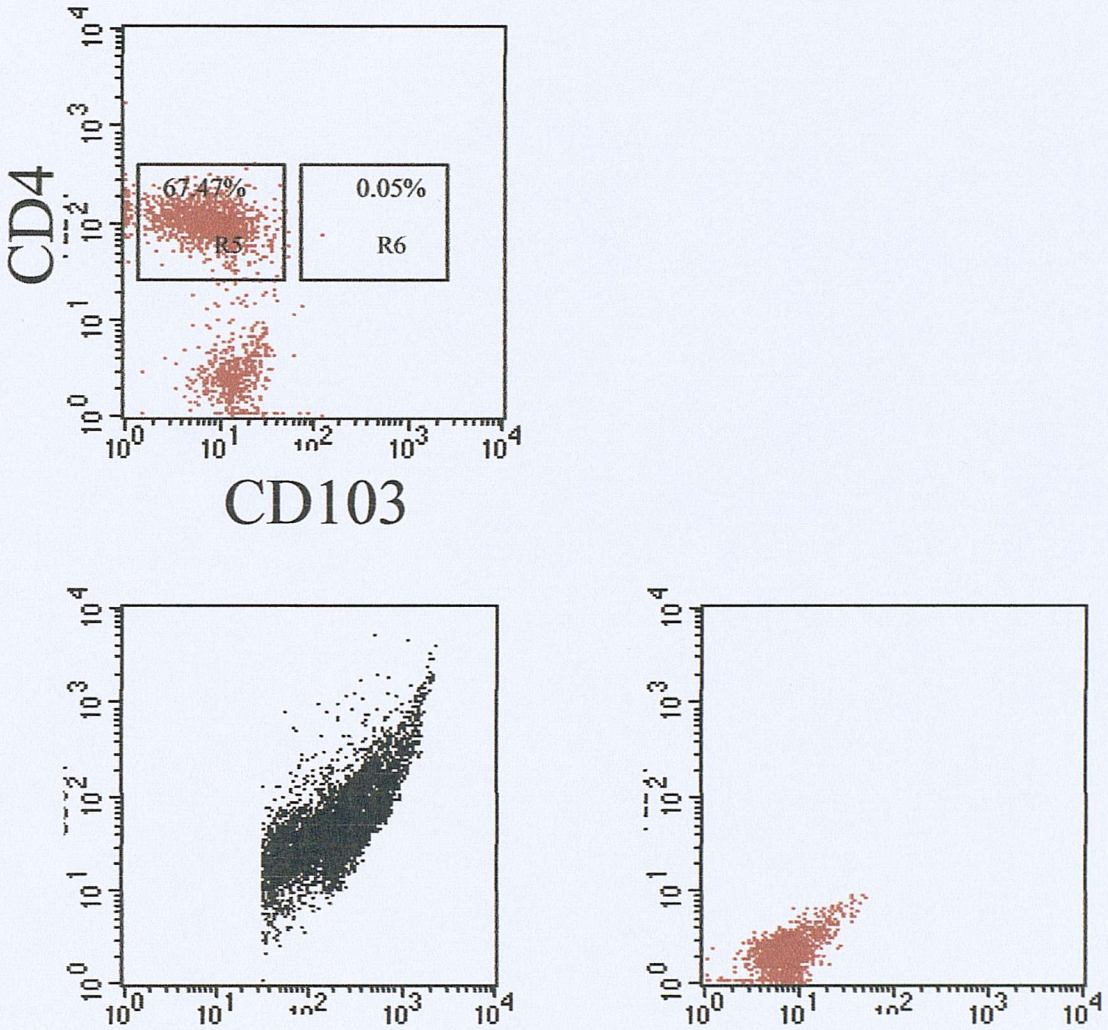


Figure 13.3.2

Lymph node cells from DO11.10 mice were driven *in vitro* in IL-4 (2ng/ml), IL-2 (100 U/ml), OVA (1μg/ml) and α- IFN-γ (5μg/ml) for 8 days. The polarised cells were stained with CD103 FITC+CD4PE. The level of expression was then analysed by FACS. Results are representative of 3 independent experiments. Number of mice used per experiment was 6.

13.4 Summary

Coexpression of CD4 and CD103 by unpolarised and polarised lymph node cells was analysed by FACS. The principal observations were:

1. In unpolarised lymph node cells, about 3.8% of CD4+ cells expressed CD103.
2. After polarisation virtually no CD4+ T cells expressed CD103.

Chapter 14

Discussion

Asthma is a common clinical condition that presents with episodes of wheezing and breathlessness. These symptoms are driven by physiological changes including variable airflow obstruction and airways irritability.

At the pathological level, asthma is characterised by mucosal inflammation, increased mucus production and a number of structural changes, loosely termed as airways remodelling. In many, but not all, patients, the airways are infiltrated by eosinophils (Kay, A.B. *et al.*, 1991; Haley, K.J. *et al.*, 1998.), a phenomenon that is thought to be driven by CD4+ T helper 2 cells (Umetsu, D.T and DeKruyff, R.H.,1997.; Wills-Karp, M., 1999.; Walter, D.M. *et al.*, 2001).

A key element in this process is the cytokine IL-5 which is produced by Th2 cells and promotes the differentiation/release of eosinophils from the bone marrow into the blood stream (Denburg, J.A., 1998). IL-5 is particularly important for the terminal differentiation of committed eosinophil precursors (Clutterbuck, E.J. *et al.*, 1989.; Welier, P.F., 1992.; Ogawa, M., 1994). It activates mature eosinophils and prolongs their survival in culture (Yamaguchi,Y., *et al.*, 1988), possibly by its ability to prevent apoptosis (Yamaguchi, Y., *et al.*,1991). In addition, it selectively enhances eosinophil degranulation, antibody-dependent cytotoxicity and adhesion to vascular endothelium (Lopez, A.F., *et al.*, 1988. Fujisawa, T., *et al.*, 1990). In contrast, activated Th1 cells characteristically produce IFN-γ, IL-2 and lymphotoxin (LT, TNF-β) (Abbas, A..K., *et al.*, 1996) and do not mediate eosinophilia in pulmonary inflammation but are associated with cell-mediated immunity (Modlin, R.L. and Nutman, T.B., 1993).

In this thesis, I have presented a series of experiments that have used the DO11.10 transgenic mouse to model allergic airway inflammation. I have focussed on the regulation of the response with particular reference to the role of CD4+CD25+ "regulatory" T cells.

The T cells of DO11.10 mice express a TCR specific for the $OVA_{323-339}$ peptide which is presented by the Ia^d class II MHC molecule. These mice enabled us to develop and study an adoptive transfer model of Th1- and Th2 –mediated pulmonary inflammation. The adoptive transfer of differentiated T cells into naïve hosts makes it possible to exert a high level of control over the nature of CD4 response elicited by antigen inhalation. This has marked advantages over models based on immunising mice before exposure to aerosolised antigens, since the immune response that develops following adoptive transfer is independent of antibody generated by the host. Certainly, in immunised animals the antibodies generated are likely to contribute to the inflammatory response developing to inhaled antigens. Using the adoptive transfer model, the contribution of immune complex formation and complement activation to the inflammatory process is obviated. In addition, the adoptive transfer approach allows a high level of control over the Th1- and Th2- balance of the infused cells. Finally, using the DO11.10 mice, antigen- specific T cells can be enumerated by flow cytometric techniques using the KJ1-26 monoclonal antibody.

DO11.10 lymph node cells can be polarised *in vitro* using the $OVA_{323-339}$ peptide under appropriate conditions to produce either Th1 (IFN-γ and IL-2) or Th2

cytokines (IL-4, IL-5, IL-13 and IL-10). Throughout these studies the culture conditions used to promote the differentiation of DO11.10 T cells into Th1 and Th2 effectors was as published previously by our group (Lee S.C. *et al.*, 1999). Th2 polarisation occurs in the presence of $OVA_{323-339}$ peptide and exogenous IL-4, whereas Th1 polarisation can be achieved with IL-12 and $OVA_{323-339}$. IL-4 powerfully inhibits the production of IL-2 and IFN-γ by naïve CD4+ T cells in response to accessory cell-dependent stimulation with soluble anti-CD3 (Tanaka, T. et al., 1993). In contrast, IL-12 is critical for the development of CD4+Th1 cells (Macatonia, S.E. *et al.*, 1995). An eight-day period of polarization was found to be sufficient for generating CD4+ Th1 and Th2 cells which retained their cytokine profiles when adoptively transferred into mice.

In recent years it has been demonstrated that immune responses are limited by the action of CD4+CD25+ regulatory T cells. To date there has been little evidence that such a suppressive mechanism acts during lung mucosal inflammation. However, it has been suggested that chronic inflammation evident in asthma may arise as a consequence of a failure of regulatory T cells to limit lung mucosal T cell responses (Umetsu, D.T. *et al.*, 2002). Using the DO11.10 mouse, we have examined whether CD4+CD25+ regulatory T cells inhibit Th2 polarization and/or the development of pulmonary eosinophilic inflammation.

The frequency of CD4+CD25+ regulatory T cells in DO11.10 mice: In normal mice, regulatory function has been shown to be mediated by a small population of CD4+ T cells (typically approx. 10%) which constitutively express the α chain (CD25) of the

IL-2R complex (Sakaguchi, S. *et al.*, 1995). Flow cytometry revealed that approximately 4-8 % of CD4+ T cells from DO11.10 mice were CD4+CD25+. Importantly, the majority of CD4+CD25+ T cells were found to stain with the KJ1-26 antibody and consequently would be expected to be OVA specific.

Having purified CD4+CD25+ and CD4+CD25- T cells, I assessed the effect of the separation procedure on cytokine production by comparing polarised unfractionated CD4+ Th2 cells with cells that had been prepared for CD25 separation but had not been separated and polarised in a Th2 favouring environment. We have found that the separation procedure did not have any significant effect on cytokine production, but the production of cytokines by polarised CD4+CD25- Th2 cells was very much reduced. This confirms that the reduced level of cytokine production by polarised CD4+CD25- Th2 cells was not due to the separation procedure but due to the depletion of CD4+CD25+ T cells. The reduced level of cytokine production by polarised CD4+CD25- Th2 cells suggests that these cells require the presence of CD4+CD25+ T cells during the polarisation process to produce typical Th2 cytokine profile.

Regulatory function mediated by DO11.10 CD4+CD25+ T cells: It was important to determine whether CD4+CD25+ T cells from DO11.10 mice mediated regulatory function. Previous *in vitro* work suggests that CD4+CD25+ T regulatory cells control inflammation via different mechanisms, among which suppressing the proliferation of inflammatory cells is likely to be the most important.

We found that CD4+CD25+ T regulatory cells polarised *in vitro* inhibited the proliferation of DO11.10 lymph node cells in response to either $OVA_{323-339}$ peptide or anti-CD3. Freshly isolated CD4+CD25+cells from DO11.10 mice also inhibited the T cell proliferative response to anti-CD3, but we did not obtain sufficient CD4+CD25+ T cells to determine whether they could inhibit the OVA response. Interestingly, inhibition of the anti-CD3 induced proliferation by polarised cells was much more effective than the inhibition of $OVA_{323-339}$ peptide induced proliferation. This may be related to the greater level of Tr cell activation that we observed with anti-CD3 compared with $OVA_{323-339}$ peptide. This is possibly because the DO11.10 T cells are able to express endogenous TCR α-chains in addition to the transgenic OVA specific TCR. α-CD3 interacts with both TCRs whereas, OVA peptide interacts only with the OVA specific TCRs. Collectively, these observations suggest that CD4+CD25+ T cells possess regulatory function prior to polarisation and retain it in culture. It has been demonstrated previously that CD4+CD25+ T cells when cocultured with CD4+CD25- cells markedly suppressed T-cell proliferation by inhibiting the production of IL-2 (Thornton, A.M. and Shevach, E.M., 1998). The inhibition was not cytokine mediated, but dependent on cell contact between the regulatory cells and the responders, and required activation of the suppressors via the TCR. Of course in such experiments it is important to distinguish between the effect of removing all activated (CD25+) T cells from the responding population, as opposed to the true effect of T reg cells or potential responders.

The effect of depleting CD4+CD25+ T cells on Th2 polarization: We next wanted to evaluate the effect of Tr cells on Th2 polarization and the development of pulmonary inflammation when Th2 cells were transferred into BALB/c mice. Since CD25 is

expressed by activated T cells, CD4+CD25+ T cells had to be removed prior to polarization of the DO11.10 T cells. Unexpectedly, their removal had a marked effect on both the level of cytokines produced by the Th2 cells generated by polarizing *in vitro* and the level of eosinophilia in BALF. Most notably, the level of IL-4, IL-5 and IL-13 production by Th2 cells generated in the absence of Tr cells was markedly lower than that of Th2-polarised unfractionated CD4+ T cells. This was irrespective of the concentration of anti-CD3 used to induce cytokine production. The mechanism by which Tr cells influence the level of cytokine production is unclear. It is unlikely that this arises as a consequence of Tr cells reducing IL-2 levels since exogenous IL-2 was added during polarization. It has been suggested that Tr cells favour Th2 polarization by inhibiting Th1 development (Suto, A.. *et al.*, 2001). However, in our experiments, the CD4+CD25- Th2 cells did not produce any detectable IFN-γ even after adoptive transfer into OVA- challenged mice. These results imply that the defect was not a consequence of incomplete polarization, but rather, results from reduced level of cytokine production.

Unpolarised T regulatory cells produced IL-5 and little IFN-γ and no IL-4. In contrast, T regulatory cells maintained in culture produced IL-4 and IFN-γ and no IL-5. In addition, cultured CD4+CD25+ T cells inhibited IL-4 but not IL-5 production by Th2 cells *in vitro*, suggesting that CD4+CD25+ T cells control inflammation by influencing IL-4 production by Th2 cells, thereby suppressing its development and proliferation. The level of cytokines produced by CD25- cells functionally distinguishes them from CD25+ cells, i.e. unpolarised CD25- cells produced virtually no cytokines while the polarised CD25- cells produced both IL-4 and little IL-5.

The effect of CD4+CD25+ regulatory T cells on pulmonary inflammation: Given that Th2 cells produced from CD4+CD25- cells released lower levels of cytokines, we next evaluated the level of inflammation that these cells could mediate *in vivo*. When transferred into BALB/c hosts, Th2- polarised CD4+CD25- cells were capable of eliciting a pronounced increase in the number of eosinophils present in the BALF on day 7, and this was notably greater than that observed when unfractionated CD4+T cells were used. This difference was associated with a more intense eosinophilic inflammation, evident in the histology, and a significantly higher level of AHR in response to methacholine. This appeared to be a consequence of more effective expansion of DO11.10 T cells in hosts in the absence of Tr cells since the number of KJ1-26+ T cells in the lung was significantly higher than in animals that received unfractionated CD4+ T cells. Moreover, restimulation of lung mononuclear cells with OVA peptide revealed that recipients of CD4+CD25- Th2 cells produced lower amounts of IL-4, IL-5 and IL-13. Collectively, these observations suggest that the reduced expression of Th2 cytokines was compensated by some additional proinflammatory attributes displayed by CD4+CD25- Th2 cells. Conceivably, Th2 cells generated in the absence of Tr cells produce an altered spectrum of proinflammatory cytokines, chemokines or prostanoids. Although, to date, there is no other evidence to support this observation, the data that we presented suggests that Tr cells influence the production of the inflammatory mediators mentioned earlier. In addition, the percentage of OVA- specific T cells (KJ1-26+CD4+) was lower in the BALF of BALB/c mice that received CD25- cells than in the mice that received unfractionated T cells. This further supports our hypothesis that CD25+ T cells appear to be involved in the elimination of the T cells that control lung inflammation as shown by the attenuated eosinophilia in the BALF of BALB/c mice that received

unseparated Th2 polarised cells. Since the level of eosinophilia is largely influenced by the time of exposure of mice to OVA aerosol challenge, the number of KJ1-26+CD4+ expressing T cells in BALF and peripheral lymph node of BALB/c mice received total Th2 cells was analysed at different time points and it was observed that the number of CD4+KJ1-26+ T cells present increased on day 6 of challenge.
Paradoxically, it has been reported by other groups the mice that had received CD4+CD25- DO11.10 T cells displayed an increased level of neutrophil and T cell recruitment in the airways of the mice (Suto, A. *et al.*, 2001). There is no immediate explanation for this discrepancy.

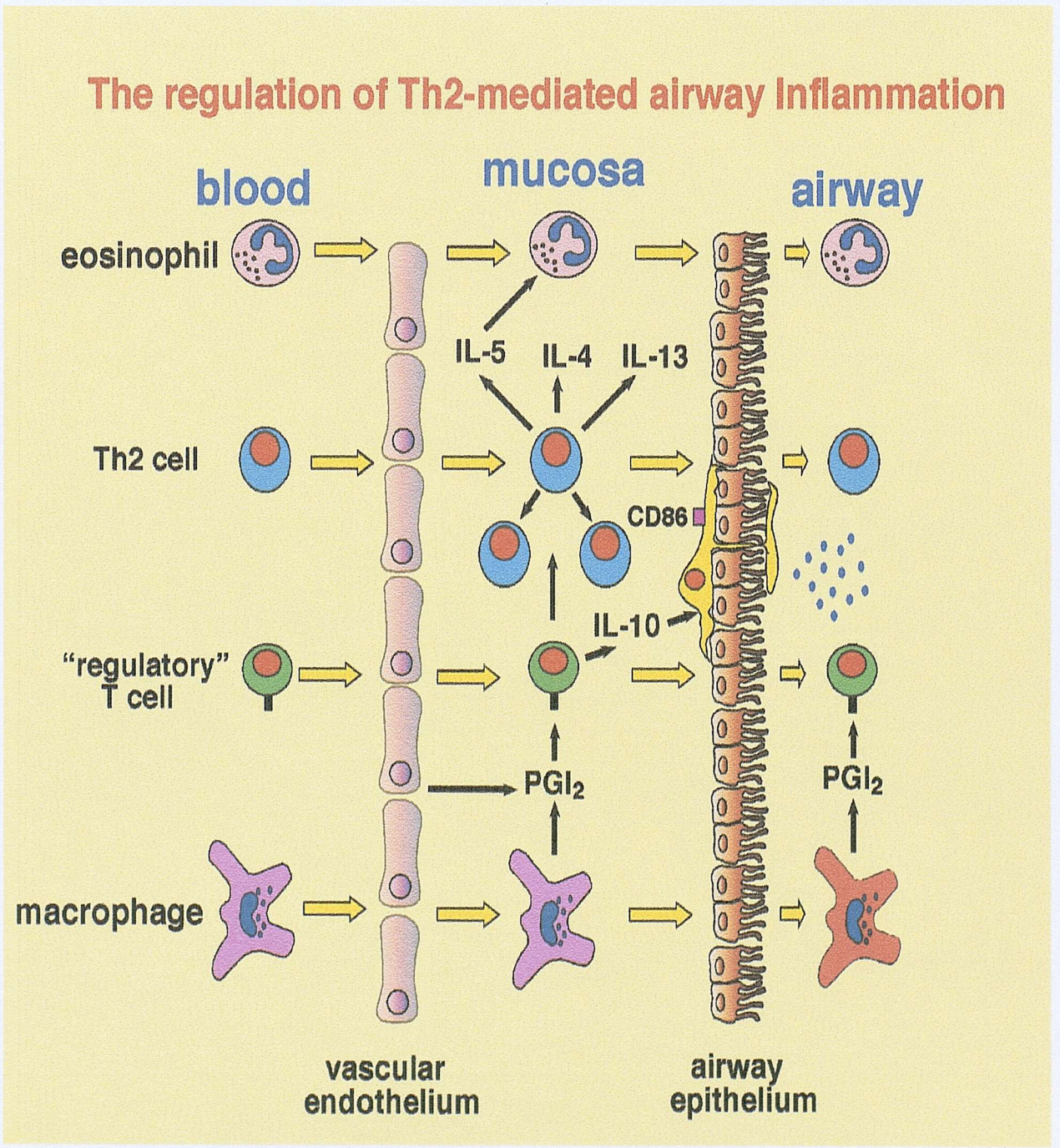
CD103 is an integrin, the ligand for epithelial cell E-cadherin has been reported to be expressed by sputum T lymphocytes in asthma (Leckie, M.J. *et al.*, 2003). It has been shown in our study that unpolarised DO11.10 lymph node cells express CD103, but its expression is lost in culture. The expression of CD103 could be implicated in the migration of T lymphocytes in pulmonary inflammation, but it needs to be examined in much greater detail in the lung as most previous work on CD103 has addressed its role in the maintenance of lymphocytes in the intestine (Schon, M.P. *et al.*, 1999). It has also been suggested that the expression of CD103 and its maintenance is influenced by the availability of TGF-β. TGF-β is produced by several cell types including eosinophils (Ohno, I. *et al.*, 1996) and has been implicated in the asthmatic process, especially in various features of airways remodelling (Duvernelle, C. *et al.*, 2003).

In conclusion, this work supports the proposed role of Tr cells in the regulation of allergen-specific airways inflammation. Although, there are several ways that Tr cells

may affect the process, the most likely roles are indicated in fig 14.1. These experiments show that CD4+CD25+ cells can be polarised in a Th2-favouring environment and characterised for their cytokine profile. This adoptive transfer system will allow further studies of the regulation and expression of allergic airways inflammation, and may be suitable for assessment of new therapeutic agents for asthma.

Fig: 14.1

Diagrammatic representation of how "regulatory" T-cells control Th2- mediated lung inflammation. The most likely mechanisms of regulation are: 1). Cell-Cell contact controlling the proliferation of T-cells that mediate inflammation; 2). Production of immunosuppressive cytokines such as IL-10 and TGF-β. In addition, PGI_2, as indicated, augments the production of IL-10 by the "regulatory" T- cell.



Aspects of the project that need clarification:

1) Depletion of CD4+CD25+ T cells prior to Th2 polarization resulted in reduced level of Th2 cytokine production. It is important to investigate the mechanism that results in reduced cytokine production. (1) Possibly reduced cytokine production is caused by ineffective T cell costimulation. The expression of CD28, ICOS, CD30 etc will be examined. The effect of adding irradiated APC on cytokine production will be addressed. (2) The effect of adding exogenous IL-2 from the start of the Th2 polarization process should be investigated. (3) It is important to determine the level of expression of cytokine mRNA to resolve whether this form of regulation occurs at a transcriptional level. (4) Ineffective cytokine production may arise if the TCR signalling is ineffective at eliciting an increase in intracellular Ca++. (5) Does depletion of CD4+CD25+ cells from human CD4+ cells result in reduced cytokine production by Th2 cells.

2) To gain an insight into the mechanism of action of regulatory T-cells in pulmonary inflammation ie. possible roles for IL-10 and TGF-β.

3) Characterizing CD4+CD25+ T cells from DO11.10 mice in more detail. For example, the CD4+CD25+ T cells expressed CD103 but this was rapidly lost in culture. It would be interesting to examine the requirements for CD103 expression by this population of T cells. One could study the possibility that TGF-β plays an important role in maintaining CD103 expression in mucosal sites and thereby facilitating the migration of CD4+CD25+ T lymphocytes to the lung mucosa.

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Appendix

Table 4.3.1 Characterisation of DO11.10 mouse model with particular reference to Th1+Th2 cells. Cytokines, IFN-γ, IL-4, and IL-5 were measured by standard ELISA. Data represent means ±SEM, attained in 3 independent experiments. Number of mice used per experiment was 10.

Experiment 1	IFN-γ (ng/ml)	IL-4 (ng/ml)	IL-5 (ng/ml)
Th1 cells + media	9.67± 0.33	0.06±0.01	0.13±0.02
Th1 cells+αCD3	277.33±39.27	0.06±0.01	0.27±0.13
Th2 cells+ media	4.67±0.310	0.59±0.26	0.45±0.25
Th2 cells+αCD3	5.0±0.577	3458.67±358.4	50.49±9.227
Experiment 2			
Th1 cells+ media	5.4±0.567	0.064±0.0078	0.360±0.058
Th1 cells+αCD3	71.33±6.96	0.060±0.0087	0.427±0.173
Th2 cells+media	3.33±0.333	0.515±0.237	0.537±0.123
Th2 cells+αCD3	4.33±0.88	1437.49±272.5	54.96±3.39
Experiment 3			
Th1 cells+media	4.67±0.882	0.041±0.0027	0.164±0.026
Th1cells+ αCD3	111.33±2.3	0.055±0.0069	0.430±0.128
Th2 cells+media	3.0±0.58	0.563±0.066	0.553±0.175
Th2 cells+ αCD3	3.4±0.52	800.58±34.57	62.3±3.46

Table 4.3.5. Validation of adoptive transfer experiments. The level of eosinophilia in the bronchoalveolar lavage was measured by EPO assay. Data represent means ±SEM, attained in 3 independent experiments. Number of mice used in each group per experiment was 5.

	Control (absorbance)	Mice received Th1 cells (absorbance)	Mice received Th2 cells (absorbance)
Experiment 1	0.061±0.0020	0.179±0.0035	0.625±0.033
Experiment 2	0.054±0.0013	0.129±0.013	0.495±0.011
Experiment 3	0.410±0.116	0.155±0.012	0.670±0.017

Table 5.3.1. An assessment of production of the regulatory T cell cytokine IL-10 in Th1 and Th2 polarised cell populations. Data represent means ±SEM, attained in 3 independent experiments. Number of mice used per experiment was 12.

	Th1 unseparated (ng/ml)	Th2 unseparated (ng/ml)	Th2 (MαR plate) (ng/ml)	Th2 (CD8+MHC II Depleted) (ng/ml)
Experiment 1	3.823±1.140	33.93±2.97	20.0±3.0	21.33±3.38
Experiment 2	3.03±0.77	29.8±0.79	16.66±2.028	19.33±1.45
Experiment 3	2.89±0.96	35.37±2.74	18.78±2.31	23.28±2.31

Table 5.3.2. An assessment of IL-10 production by polarised Th2 cells driven in IL-10 or α-IL-10 with or without indomethacin. Data represent means ±SEM, attained in 2 independent experiments. Number of mice used per experiment was 14.

	Th2 (ng/ml)	Th2+indo (ng/ml)	Th2+IL10 (ng/ml)	Th2+ IL-10+indo (ng/ml)	Th2+anti-IL-10 (ng/ml)	Th2+ anti-10+indo (ng/ml)
Experiment 1	164.33±16.58	53.33±2.9	221.33±15.06	71.0±3.61	8.33±0.33	14.67±0.33
Experiment 2	142.2±9.4	63.3±4.4	166.67±12.02	47.67±6.64	5.0±0.58	7.0±0.54

Table 6.3.1. An assessment of IFN-γ (expressed in ng/ml) production by PLN cells stimulated with different reagents. Data represent means ±SEM, attained in 2 independent experiments. Number of mice used per experiment was 10.

	PLN+ OVA	OVA+IL4 (0.4ng/ml	OVA+IL4 (0.4ng/ml) +indo	OVA+ IL-4 (2ng/ml)	OVA+IL-4 (2ng/ml)+ indo	OVA+ IL-4 (10ng/ml	OVA+IL-4 (10ng/ml)+ indo
Exp 1	46.67±1.2	38.67±0.67	44.0±2.08	39.33± 0.33	38.33±2.03	33.67± 0.67	23.67±1.45
Exp 2	35.0±1.73	25.67±1.86	31.0±3.60	28.0± 1.76	27.67±1.2	22.33± 1.86	16.0±1.16

Table: 7.3.1. Frequency of CD4+CD25+ T cells in DO11.10 mice, attained in 4 independent experiments. Number of mice used per experiment was 16.

Experiment 1	4.25%
Experiment 2	5.58%
Experiment 3	4.69%
Experiment 4	6.81%

Table 8.3.1. An assessment of cytokine (IFN-γ, IL-4, and IL-5) production by unpolarised T- cells. Data represent means ±SEM, attained in 2 independent experiments. Number of mice used per experiment was 13.

IFN-γ (ng/ml)

	CD25+ +α-CD3	CD25- +α-CD3	CD25+ +α-CD3+ APC	CD25- +αCD3+ APC	CD25+ +APC+OVA	CD25- +APC+OVA
Exp 1	1.16±0.19	1.01±0.45	12.83±0.34	3.56±0.49	3.9±0.19	3.7±0.31
Exp 2	1.07±0.17	0.78±0.36	10.37±0.47	2.67±0.59	4.7±1.09	2.52±0.49

IL-4 (ng/ml)

	CD25+ +α-CD3	CD25- +α-CD3	CD25+ +α-CD3+ APC	CD25- +αCD3+ APC	CD25+ +APC+OVA	CD25- +APC+OVA
Exp 1	0.54±0.12	0.35±0.03	0.24±0.076	0.510±0.06	0.940±0.02	0.443±0.26
Exp 2	0.46±0.11	0.29±0.02	0.16±0.06	0.44±0.07	0.76±0.04	0.31±0.22

IL-5 (ng/ml)

	CD25+ +α-CD3	CD25- +α-CD3	CD25+ +α-CD3+ APC	CD25- +αCD3+ APC	CD25+ +APC+OVA	CD25- +APC+OVA
Exp 1	2.24±0.167	0.367±0.21	42.33±1.2	2.42±0.49	2.33±0.136	6.45±0.37
Exp 2	1.78±0.074	0.337±0.17	38.33±3.5	1.78±0.09	2.03±0.09	4.96±0.46

Table 8.3.2. Functional properties of unpolarised CD4+CD25+ regulatory T cells was assessed *in vitro*: the proliferative response of DO11.10 T- cells, in the presence of different numbers of CD4+CD25+ T- cells, to immobilized α-CD3 was measured by the level of ^{3}H incorporation. Data represent means ± SEM, attained in 2 independent experiments. Number of mice used per experiment was 12.

CD25+ T-cells	0	1562	3125	6250	12500	25000	50000
Exp 1 CPMX10^{3})	36.25± 1.4	21.97±1.4	21.35± 3.8	14.3±1.0	14.55± 1.05	5.75±0.65	2.73±0.32
Exp 2 CPMX10^{3})	28.21± 4.12	22.57±0.99	20.47± 1.0	12.90±1.6	11.10± 1.4	7.37±0.82	3.9±0.55

Table 10.3.2. An assessment of cytokine (IFN-γ, IL-4, and IL-5) production by polarised T- cells. Data represent means ±SEM, attained in 2 independent experiments. Number of mice used per experiment was 14.

IFN-γ (ng/ml)

	CD25+ +KJ1-26	CD25- + KJ1-26	Unseparated +αCD3	CD25+ +αCD3	CD25- +αCD3
Exp 1	79.67±3.18	1.56±0.60	6.42±0.87	88.0±5.77	3.69±0.37
Exp 2	66.23±4.48	1.98±0.49	3.22±0.49	76.89±3.31	2.13±0.64

IL-4 (ng/ml)

	CD25+ +KJ1-26	CD25- + KJ1-26	Unseparated +αCD3	CD25+ +αCD3	CD25- +αCD3
Exp 1	9.51±1.03	22.33±2.96	947.33± 22.85	596.67± 6.67	774.33± 45.63
Exp 2	7.047±0.95	16.51±2.16	811.59± 25.35	499.26± 2.65	686.90± 64.13

IL-5 (ng/ml)

	CD25+ +KJ1-26	CD25- + KJ1-26	Unseparated +αCD3	CD25+ +αCD3	CD25- +αCD3
Exp 1	1.89±0.28	2.29±0.097	37.33±1.76	3.64±1.91	7.15±0.28
Exp 2	1.53±0.69	1.42±0.30	29.03±1.79	3.8±1.4	5.47±0.56

Table 10.3.5. An assessment of cytokine production by polarised Th2 cells in the presence of Tr cells (Tr cells were polarised in Th2 favouring culture condition, either in IL-2 or IL-2+IL-4). Data represent means ±SEM, attained in 2 independent experiments. Number of mice used per experiment was 12.

IL-4 (ng/ml)

	Th2	Th2+CD25+(IL-2)	Th2+CD25+(IL-2+ IL-4)
Exp 1	143.0±3.22	172.33±3.76	39.33±8.45
Exp 2	95.7±5.69	125.57±20.32	27.19±8.7

IL-5 (ng/ml)

	Th2	Th2+CD25+(IL-2)	Th2+CD25+(IL-2+ IL-4)
Exp 1	7.0±1.0	8.67±1.2	7.0±1.0
Exp 2	6.54±0.49	7.32±0.75	5.98±1.28

Table 11.3.1. Functional properties of CD4+CD25+ Regulatory T cells was assessed *in vivo*: the level of eosinophilia in the bronchoalveolar lavage was measured by EPO assay at day 5. Data represent means ± SEM, attained in 3 independent experiments. Number of mice used in each group per experiment was 6.

	Control (ng/ml)	Total (ng/ml)	CD25- (ng/ml)	CD25- +Tr (ng/ml)
Exp 1	0.410±0.116	6.8±0.513	1.73±0.38	2.10±0.44
Exp 2	0.36±0.21	5.2±1.51	1.28±0.33	1.62±0.24
Exp 3	0.58±0.15	4.94±1.5	1.39±0.48	1.46±0.31

Table 11.3.2. Functional properties of CD4+CD25+ Regulatory T cells was assessed *in vivo*: the level of eosinophilia in the bronchoalveolar lavage was measured by EPO assay at day 7. Data represent means ± SEM, attained in 3 independent experiments. Number of mice used in each group per experiment was 6.

	Control (ng/ml)	Total (ng/ml)	CD25- (ng/ml)
Exp 1	0.353±0.087	6.8±0.74	16.03±1.56
Exp 2	0.383±0.034	5.2±0.98	14.2±1.05
Exp 3	0.29±0.023	4.82±0.52	15.40±0.814

Table 11.3.3. The number of eosinophils in the bronchoalveolar lavage was counted at day 7. Data represent means ±SEM, attained in 3 independent experiments. Number of mice used in each group per experiment was 6.

	Control	Total	CD25-
Exp 1	57000±6700	750000±110000	1600000±152000
Exp 2	48000±5340	630000±90000	1400000±132000
Exp 3	51000±5120	690000±100000	1500000±142000