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THE ROLE OF TUMOR NECROSIS FACTOR ALPHA (TNF-α) IN ASTHMA

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ABSTRACT

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

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Doctor of Medicine

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By Dr. Kesavan Suresh Babu

Asthma is an airway inflammatory disorder characterised by variable airflow obstruction and airway hyperresponsiveness on a background of diffuse airway inflammation. The pathophysiological features are complex involving various inflammatory cells and cytokines orchestrating the inflammatory process. TNF-α is a pro-inflammatory cytokine implicated in the modulation of inflammation in various diseases including asthma. While TNF-α blocking strategies have been an effective therapeutic modality in diseases like rheumatoid arthritis its role in asthma and the effects of its blockade in asthma is poorly understood. This thesis looks at the role of TNF-α in asthma and the effects of blocking TNF-α as a possible therapeutic option in patients with severe corticosteroid dependent asthma.

Allergen challenge induces the proinflammatory cytokines and has been used to study the pathogenic mechanisms in asthma. The study presented in Chapter 3 used a repeated low dose allergen challenge model which is more likely to simulate natural allergen exposure to investigate the effects of allergen on bronchial biopsies from mild allergic asthmatics. Low dose allergen exposure results in up regulation of TNF-α in the bronchial biopsies. This increase was associated with a parallel increase in mast cell numbers suggesting the possible source of TNF-α as the mast cells. This was associated with an associated increase in adhesion molecules ICAM-1 and VCAM-1.

In the *ex vivo* study on bronchial biopsies of moderately severe asthmatics the results were similar to the low dose allergen exposure where TNF-α levels were increased following exposure to *Der p* and this was suppressed in the presence of CDP 870- a TNF-α blocking monoclonal antibody. CDP 870 was also able to suppress the levels of IL-8 and adhesion molecules.

Having seen a positive response with CDP 870 we had the opportunity of observing the effects of blocking TNF-α with a soluble fusion protein-p75 receptor (etanercept) on patients with chronic severe corticosteroid refractory asthma. Administration of 25 mg of etanercept twice a week for 12 weeks produced improvements in lung function seen as improvements in FEV_1, FVC and both morning and evening PEF. There was a marked improvement in asthma control and a 2.5 fold doubling dose increase in methacholine airway hyperresponsiveness. Treatment with etanercept markedly reduced the need for rescue medications as all the subjects completely withdrew from their nebulised salbutamol by the end of the study. The medication was well tolerated with minimal side effects.

This thesis provided evidence for an important role for TNF-α in asthma pathophysiology especially those with severe disease and a possible therapeutic option in such patients.

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PUBLICATIONS

The following publications have arisen from the research work presented in this thesis and from studies not formally included in this thesis

1. **Suresh Babu K**, Howarth PH, Arshad SH, Lau L, Buckley M, McConnel W, Beckett P, Al Ali M, Chauhan AJ, Wilson SJ, Reynolds A, Davies DE, Holgate ST. Tumour Necrosis Factor (TNF-α) as a Novel Therapeutic Target in Symptomatic Corticosteroid-Dependent Asthma. *Thorax 2005; 60(12): 1012-1018.*
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ABBREVIATIONS

ADAM	:	proteins containing a disintegrin and metalloproteinase domain
AHR	:	Airway Hyperresponsiveness
AIDS	:	Acquired Immune Deficiency Syndrome
ANA	:	Anti Nuclear Antibody
AP-1	:	Activated Protein-1
APC	:	Antigen Presenting Cell
AQLQ	:	Asthma Quality of Life Questionnaire
ASM	:	Airway Smooth Muscle
AUC	:	Area under the Curve
BAL	:	Broncho-alveolar-lavage
BALF	:	Broncho-alveolar-lavage fluid
BECS	:	Bronchial Epithelial Cell Culture System
BHR	:	Bronchial Hyperresponsiveness
CCR3	:	Chemokine receptor 3
CTLA-4	:	Cytotoxic T Lymphocyte Antigen 4
DC	:	Dendritic Cell
DD	:	Death Domain
Der p	:	*Dermatophagoides pteronyssinus*
DNA	:	Deoxy Ribo Nucleic Acid
DTE	:	Dithioerythritol
EAR	:	Early Asthmatic Response
ECP	:	Eosinophil Cationic Protein
EGF	:	Epidermal Growth Factor
EGFR	:	Epidermal Growth Factor Receptor
ELISA	:	Enzyme Linked Immunosorbent Assay
EMTU	:	Epithelial Mesenchymal Trophic Unit
ENFUMOSA	:	European Network For Understanding Mechanisms Of Severe Asthma
EPN	:	Eosinophil Derived Neurotoxin
EPX	:	Eosinophil protein X
FADD	:	Fas-associated death domain protein
FBC	:	Full Blood Count

FCεRI	:	High Affinity IgE receptor
FCεRII	:	Low affinity IgE receptor
FEV1	:	Forced Expiratory Volume in 1 second
FGF	:	Fibroblast Growth Factor
FVC	:	Forced Vital Capacity
GINA	:	Global Initiative for Asthma
GMA	:	Glycol Methacrylate resin
GM-CSF	:	Granulocyte Monocyte Colony Stimulating Factor
HDM	:	House dust mite
HIV	:	Human Immunodeficiency Virus
ICAM-1	:	Intercellular Adhesion Molecule 1
ICOSL	:	Inducible Co-Stimulatory Molecule Ligand
IFN-γ	:	Interferon gamma
IgE	:	Immunoglobulin E
IgM	:	Immunoglobulin M
IL	:	Interleukin
IL-1Ra	:	Interleukin-1 Receptor Antagonist
LAR	:	Late Asthmatic Response
LTC4	:	Leukotriene C4
mAB	:	monoclonal Antibody
MAPK	:	Mitogen Activated Protein Kinase
MCCT	:	Chymase positive tryptase positive Mast Cell
MCP-1	:	Monocyte Chemotactic Peptide-1
MCT	:	Mast Cell tryptase only phenotype
MHC	:	Major Histocompatibility Complex
MIP-1α	:	Monocyte Inflammatory Protein 1 alpha
MMP	:	Matrix Metalloproteinase
MNC	:	Mono Nuclear Cell
mRNA	:	messenger Ribonucleic acid
MUC5AC	:	Mucin 5 subtypes A and C
NHLBI	:	National Heart Lung and Blood Institute
PAR	:	Protease Activated Receptors
PBMCs	:	Peripheral Blood Monocytes
PC20	:	Provocating Concentration to produce a 20% fall in FEV1

PD20	:	Provocation Dose to produce a 20% fall in FEV1
PEF	:	Peak Expiratory Flow
PEG	:	Poly ethylene glycol
PGD2	:	Prostaglandin D2
RA	:	Rheumatoid Arthritis
RANTES	:	Regulated on Activation, Normal T Expressed and Secreted
RAST	:	Radio Allergen Sorbent Test
RNA	:	Ribo Nucleic Acid
SCF	:	Stem Cell Factor
SLE	:	Systemic Lupus Erythematosis
TACA	:	Tumour Necrosis Factor Alpha converting activity
TACE	:	Tumour Necrosis Factor Alpha converting enzyme
TB	:	Tuberculosis
TBS	:	Tris Buffered Saline
TGF-β	:	Transforming Growth Factor Beta
Th1	:	T Helper 1 lymphocytes
Th2	:	T Helper 2 lymphocytes
TNFR	:	Tumour Necrosis Factor Alpha Receptors
TNF-α	:	Tumour Necrosis Factor alpha
TR	:	T regulatory cells
TRADD	:	Tumour Necrosis Factor Alpha Receptors I associated death domain protein
TRAF 2	:	Tumour Necrosis Factor receptor associating factor 2
VCAM-1	:	Vascular Cell Adhesion Molecule-1

1. Chapter 1

1.1. Introduction

Bronchial asthma is an inflammatory airway disorder characterised by variable airflow obstruction and airway hyperresponsiveness. Asthma is a heterogeneous disease with various factors like the environment, genetics, levels of hygiene and atopic status playing a role in its development and progression. The prevalence of asthma has increased to epidemic proportions and the current health care expenditure for asthma in the industrialised countries is enormous. In Western Europe, asthma has doubled in the last 10 years and in the US it has increased by over 60% since the 1980's (1, 2). The human and economic burden associated with asthma is large and the economic cost is estimated to exceed those of TB and HIV/AIDS combined together. It is an irony that 43% of the cost of asthma care is related to the use of emergency departments in hospitals. The medical resource utilisations are increased three fold among high users of inhaled β_2 agonists and the hospital costs are dependent upon disease severity (3).The two important factors that are fundamental in determining asthma burden are the severity of asthma and the levels of control. While asthma severity can be considered as an intrinsic stable characteristic of the disease the levels of control is mainly influenced by the patient, the environment and therapy. Sub-optimal therapy and poor compliance with medications places the patient at risk for asthma exacerbations, unscheduled visits and hospitalisations.

1.2. Pathological features of asthma

The airway pathology in asthma includes macroscopic evidence of over inflation of the lung and obstruction of the small and large airways by mucus plugs which are composed of mucus, serum proteins, inflammatory cells and cell debris. Airway wall thickness is increased and this is related to the severity of asthma (4). The increase in thickness is due to increase in most tissue compartments including smooth muscle, epithelium, sub-mucosa, adventitia and mucosal glands. The inflammatory edema involves the whole airways particularly the sub-mucosal layer with marked hypertrophy and hyperplasia of the sub-mucosal glands and goblet cell hyperplasia. Goblet cell hyperplasia and hypertrophy is a non-specific response to the loss of

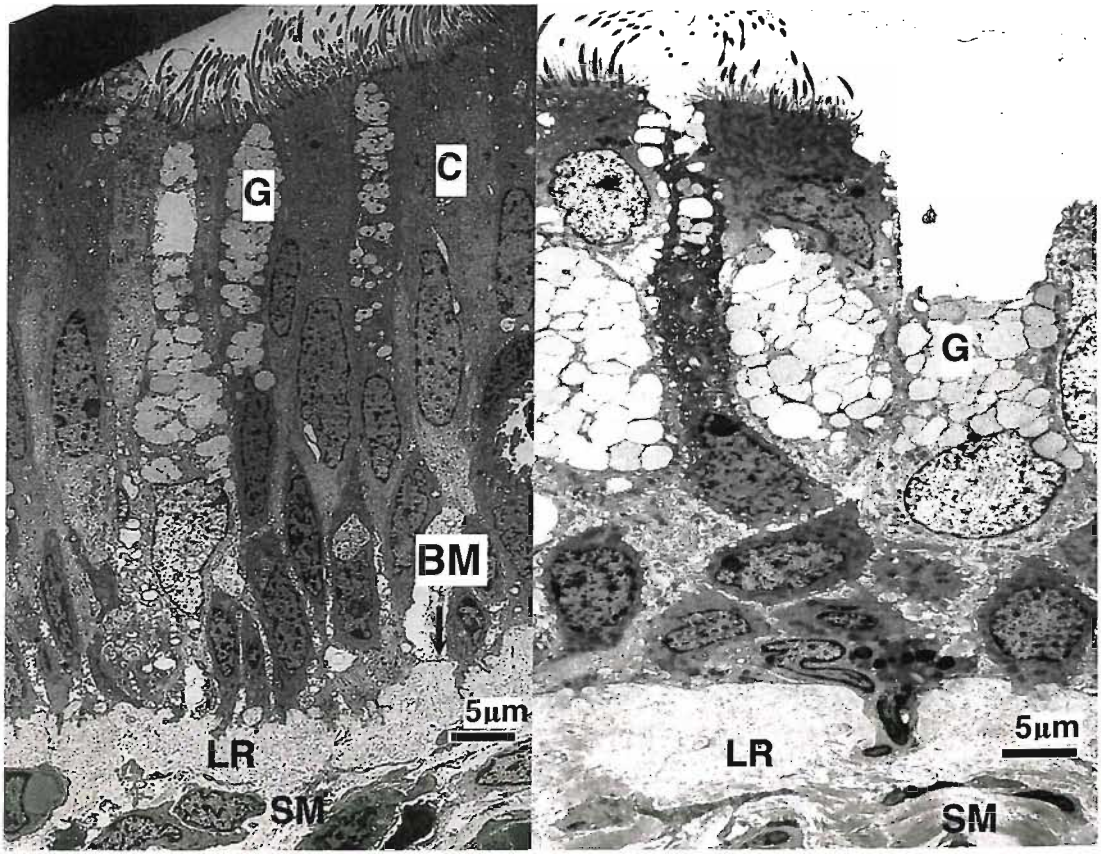
epithelial cells and this is particularly evident in the smaller airways. There is hyperplasia of the muscularis layer and micro vascular vasodilatation in the mucosal and adventitial layers of the airways (5).

Microscopically there is an intense inflammatory and immunological cell infiltration of the airways accompanied by microvascular leakage and epithelial disruption. Epithelial damage and shedding occurs as a consequence of separation of the columnar cells from the basal cells which are more resistant to detachment in asthma even in the presence of extensive inflammation (6). Damage to the airways leads to a reparative process which is characterised by sub epithelial basement membrane thickening, fibrosis and smooth muscle hypertrophy (7, 8). The basement membrane thickening occurs due to the deposition of collagen and is confined to the *lamina reticularis* (Fig 1.1). The sub-epithelial collagen is produced by myofibroblasts lying beneath the epithelium their numbers correlating with the duration of the disease (9). Smooth muscle hypertrophy characterized by an increased thickness of the smooth muscle layer is present throughout the airways down to and including small membranous bronchiole with an internal perimeter of less than 2mm (10).

1.3. Th1 vs. Th2 differentiation in asthma

At a simple level, naïve T (Th0) cells can differentiate into one of two phenotypes, T helper1 (Th1) or T helper2 (Th2) cells. While Th1 cells drive the immune response towards cell-mediated immunity, Th2 cells promote a humoral or allergic response (11). The differentiation between these two groups is based on the cytokine profile of these two cells. Specifically, IL-4 promotes Th2 development and antagonises Th1 differentiation. Conversely, IL-12 and IFN-γ promote Th1 differentiation and antagonise Th2 development. A number of transcription factors including GATA-3 and c-MAF regulate IL-4 production and Th2 differentiation while, T-bet promotes Th1 differentiation (12, 13).

Figure 1-1 Comparison of Normal and asthmatic airways.



Normal Asthmatic

C- Ciliated columnar cells; G- Goblet cells; SM- Smooth muscle; LR- Lamina reticularis, BM- Basement membrane

Th1 responses are thought to protect against allergic disorders by dampening the activity of Th2 responses (14). In experimental animal models of infection a Th2 response can down regulate a Th1 response (15, 16). However in reality Th1 cells can exacerbate asthma and allergy. Allergen specific Th1 cells when adoptively transferred to naïve recipients migrate to the lung but fail to counterbalance Th2 induced airway hyperresponsiveness (17). Asthma is generally perceived as a Th2 disease with their signature cytokines IL-4, IL-5, IL-9 and IL-13 having key pathogenic roles. The ability of Th2 cells to induce the characteristic features of asthma was shown in animal models by transferring Th2 cells into naïve animals before antigen challenge. These animals developed airway eosinophilia, bronchial hyperresponsiveness and mucus hyper-secretion (18, 19).

1.4. T cell tolerance and asthma

The immune mechanism that regulates the development of asthma and is affected significantly by changes in the environment is immune tolerance induced by mucosal exposure to the antigen. The peripheral $CD4^+$ T cell tolerance induced by respiratory exposure to allergens prevents the development of Th2 bias and allergen induced BHR (20). The degree of allergen exposure affects the induction of tolerance with higher exposure inducing greater tolerance (21). The mechanisms include clonal deletion, anergy or active suppression mediated by cells secreting IL-10 or TGF-β. Allergen specific $CD4^+$ regulatory T (T_R) cells mediate in part the T cell tolerance that inhibits airway inflammation and BHR (22). The production of IL-10 by the dendritic cells plays a major role in the development of T_R cells and IL-10 blocking antibody blocks this process (22). The addition of large amounts of exogenous IL-10 to T cell cultures generates T_R cells (23, 24).

The inducible co-stimulatory molecule ligand (ICOSL) plays an important role in the activation and function of the effector Th2 cells and induces CD28 independent T cell proliferation and cytokine production (25, 26). Mice deficient in ICOS show reduced IgE production, Th2 cytokine production and development of BHR indicating a key role for ICOS in the development of allergic responses (27, 28). ICOS stimulation promotes preferential IL-10 production and induction of T_R cells that inhibit the

function of antigen specific T cells and the development of BHR (22). The *in vivo* and *in vitro* inhibitory capacities of T_R cells on BHR and inflammation can be abrogated by neutralisation of IL-10 or by interrupting the ICOS-ICOSL interactions with an ICOSL mAB. Furthermore, interference with the ICOS-ICOSL signalling pathway not only abrogates the inhibitory capacity of T_R effector cells but also blocks the IL-10 production and induction of T cell tolerance. Therefore, T_R cells play an important role in mediating respiratory tolerance and protection against asthma through specific pathways that involve IL-10, ICOS-ICOSL interactions and B7-2 which preferentially costimulates IL-10 production (28, 29).

Although Th2 cells play a critical role in asthma pathogenesis the binary Th1-Th2 paradigm cannot explain all the immunological processes. The development of Th2 cells and allergic diseases may represent an aberration of T_R cells development possibly due to inadequate IL-10 (Fig 1.2). Thus Th2 cells may develop as a consequence of limited IL-10 and enhanced IL-4 and IL-13 production and from the failure to develop allergen specific T_R cells rather than a failure to develop Th1 cells (Fig 1.2). However, the specific signals that preferentially induce the development of T_R cells are not entirely clear but may involve IL-10 production by DCs. The T_R cells that develop at the mucosal sites inhibit both the development of Th2 biased inflammation in allergic diseases and also inhibit Th1 biased inflammation as in auto immune disorders. Th1 cells may be involved in inhibiting the development of Th2 cells but this more likely occurs in the lymphoid organs where the pro-inflammatory effects of Th1 cells cause little tissue damage. But the major anti-inflammatory effector mechanism in the mucosa involves T_R cells (30)

1.5. Cellular involvement in asthma

The inflammatory infiltrate in asthma is multi-cellular in nature and characteristically involves T-cells, eosinophils, macrophages/monocytes and mast cells. Recently neutrophils too have been implicated in the pathogenesis of severe asthma (31).These cellular events are a consequence of activation of resident cells and recruitment of inflammatory cells and their infiltration into airways (32).

1.5.1. CD4 T cells

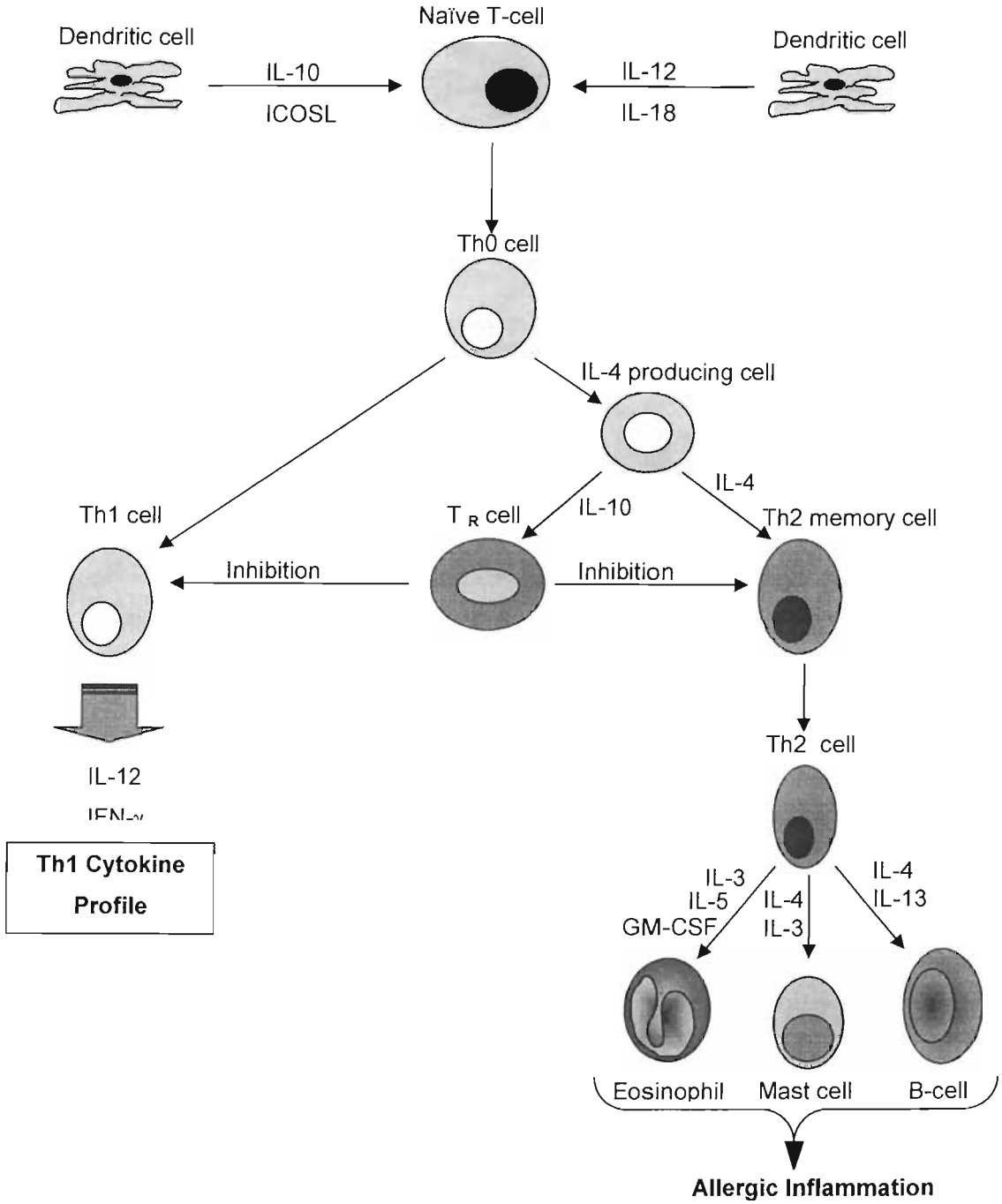
Th2 cells are an important driving force for orchestrating the inflammatory process in asthma. On contact with an allergen, the antigen presenting cells (APCs) process the allergen and present it to the Th2 cells via MHC class II molecules. This interaction is followed by activation of a second pathway involving costimulatory molecules CD28 on the T cells and B7-1 (CD80), B7-2 (CD86) located on the APCs and B cells (29). Another molecule that is closely related to CD28 and located on the T cells surface is CTLA-4. While activation of CD28 results in transcription of Th2 cytokines CTLA-4 effectively inhibits this process. Culture of asthmatic but not normal bronchial biopsies *ex vivo* with allergen resulted in transcription of IL-4, IL-5 and IL-13 but not of IFN-γ. This process was attenuated upon co-incubation with anti-CD80 or anti-CD86 and inhibited by the addition of CTLA-4Ig reflecting the importance of CD80/86-CD28 pathway for the expression of Th2 cytokines (33, 34). However, in subjects with moderately severe asthma the pro-inflammatory cytokines IL-5 and GM-CSF production in response to allergen was insensitive to suppression by CTLA-4Ig suggesting that the airway cytokine production in the more severe asthma may be less amenable to modulation by antagonists of CD28 mediated costimulation (35) Unstimulated bronchial explant tissue from healthy, mild and moderately severe asthmatics released significant amounts of IL-16 but *Der p* stimulation induced a significant increase in IL-16 release only from mild asthmatics but not from healthy controls or moderately severe asthmatics (36). Furthermore, allergen induced release of IL-16 from mild asthmatic bronchial biopsies was inhibited with CTLA-4Ig. In contrast CTLA-4Ig did not have any effect on moderately sever asthmatic bronchial biopsies (36, 37). This study suggests that IL-16 activity in response to allergen in asthmatic airways is a prominent feature in both mild and moderately severe asthma, but the mechanisms underlying this activity is different.

Following allergen challenge there is an increase in cells exhibiting the Th2 phenotype, IL-4, IL-5 and IL-13 (38). In-situ hybridisation techniques have co-localised a large number of cells expressing IL-4 and IL-5 as CD4 T cells, confirming their source. Allergen challenge also results in an average 63% and 37% reduction in the proportion of BAL T cells producing IFN-γ and IL-12, but no changes were detectable in the Th2 phenotype (39). Broadly, severity of asthma and BHR have been

related to the numbers of activated eosinophils and T cells expressing IL-5 mRNA in asthmatic airways, underscoring the role of T cells in asthma pathogenesis (40).

The type 2 cytokine IL-4 is crucial for the production of IgE in B-cells. IL-4 and IL-13 produced by the Th2 cells induce B cells expressing surface IgM and IgD to produce IgE by a process of isotype switching. IgE bound to the high and low affinity receptors mediate activation and degranulation of other metachromatic cells leading to airway obstruction and recruitment of inflammatory cells. The role of Th2 cells in allergic inflammation is not limited to their ability to induce the production of allergen-specific IgE antibodies by B cells and to promote the eosinophilic infiltration in target tissues. Indeed, B-cell, IgE, CD40, and mast cell gene deficient mice can develop allergic airway inflammation whereas CD4 T-cell, IL-4 and IL-5 gene deficient mice cannot (41).

Figure 1-2 T cell differentiation in asthma



Dendritic cells (DCs) producing IL-12 and IL-18 enhance development of Th1 cells while DCs expressing ICOSL enhance the development of IL-4 producing cells. The IL-4 producing cells differentiate into either Th2 cells or T_R cells which produce high concentrations of IL-10. T_R cells inhibit the function of both Th1 and Th2 cells.

1.5.2. Eosinophils

Eosinophils differentiate within the bone marrow under the influence of GM-CSF, IL-3 and IL-5 with IL-5 having the most cell-specific effects. The preferential expression of Th2 phenotype results in an increased amount of IL-4, IL-5 and IL-13 which influences eosinophil recruitment, survival and chemotaxis into the asthmatic airways. Eotaxin is a key chemokines belonging to the CC family that is involved in the cellular activation and inflammatory mediator release from the eosinophils (42, 43). Two other CC chemokines were later discovered and these functional homologues were termed as eotaxin-2 and 3. IL-4, IL-13 and TNF-α stimulate the generation of eotaxin by airways epithelial cells, smooth muscle cells and fibroblasts (44). The eosinophil is predominantly a secretory cell containing preformed mediators like eosinophil cationic protein (ECP), eosinophil protein X/eosinophil derived neurotoxin (EPX/EPN), reactive oxygen species, lipid mediators like LTC_4, PGE_2 and platelet activating factor (PAF) and enzymes like histaminase and phospholipase. Studies have shown that eosinophils are also recognised sources of certain cytokines including IL-3, IL-4, IL-5, IL-6, GM-CSF, IL-8, TNF-α, TGF-α and TGF-β (45).

Peripheral blood and BAL eosinophilia has been reported in patients with asthma and suggests a causal role for eosinophils in the severity of asthma (46). Twenty four hours following allergen challenge BAL eosinophilia occurred only in subjects who developed late asthmatic response (LAR) supporting a role for eosinophils in the LAR. This was also associated with elevated levels of ECP a marker of eosinophil activation (47). However recent evidence suggest that there is a dissociation between airway eosinophilia and BHR (48). This was also confirmed by the anti IL-5 trial in asthmatics which reduced both airway and peripheral blood eosinophils but did not alter the BHR (49, 50). It is thought that eosinophils interact with the airway epithelium causing epithelial damage in asthmatics. Metalloproteases (e.g. MMPs), oxidants and arginine rich protein secretions from activated eosinophils probably contribute to epithelial damage making it more susceptible to exogenous noxious agents such as allergens and pollutants (51).

1.5.3. Mast Cells

Mast cells are important effector cells in asthma and have a key role in the early phase asthmatic response. Mast cells constitutively express the high affinity IgE receptors, FcεRI on their surface (>130,000/cell). Cross-linking of the cell-bound IgE molecules by polyvalent allergens results in activation of mast cell which releases a range of preformed and newly generated pro-inflammatory mediators (Table 1.1) (52). Mast cells require stem cell factor (SCF) for their survival. Two major types of mast cells are recognized, the MC_T type with contain only tryptase alone and the MC_{TC} contains tryptase, chymase and carboxypeptidase (53). Tissue mast cells can be further classified as reactive mast cells and constitutive mast cells. Reactive mast cells are found in the epithelial compartment in mucosal inflammation and require Th2 cytokines from T cells for their development (54). It is therefore possible that alteration in the mast cell phenotype by the Th2 cytokine milieu in allergic individuals' permits otherwise trivial infections and chemical signals to initiate harmful inflammatory cascade. It has been shown that mast cell tryptase can induce eosinophil and neutrophil influx in a dose dependent manner (55). The target receptor for mast cell tryptase is the protease activated receptors (PAR) (56). Four PARs have been discovered and are denoted as PAR1-4. Mast cell tryptase is an activator of PAR2 receptors (57). Cells expressing cleavable PAR2 are activated by tryptase. Mast cell tryptase is a mitogen for epithelial cells, stimulates IL-8 production and ICAM-1 expression which play a significant role in recruitment of eosinophils and neutrophils (58). Mast cells also contain IL-4 which is required for Th2 cell differentiation; hence it is possible that mast cell activation can have an amplifying effect on allergic inflammation.

In asthmatic lung, mast cell numbers are increased and bronchial hyperresponsiveness correlates with mast cell number mostly consisting of the MC_T subtype in both asthmatic adults and children (59, 60). In addition, *in vivo* evidence from endobronchial biopsies in asthma show that mast cell degranulation is greater in asthmatic patients compared with non-asthmatic subjects (61-63). Mast cells are also important sources of cytokine including IL-4, IL-5, IL-6, IL-8, IL-13, GM-CSF and TNF-α. The mRNA for these cytokines are induced by IgE dependent activation of mast cells (64, 65). Furthermore, the number of IL-4 containing mast cells was increased in the mucosa of patients with allergic diseases (66). Mast cells but

interestingly not eosinophils are also increased in the airway smooth muscle compartment in asthmatic individuals providing evidence that the interactions of airway smooth muscle and infiltrating mast cells is a key element in the development of disordered airway function in asthma (67). It is important to note that TNF-α containing mast cells were increased only in asthma and not in allergic rhinitis (66, 68). Therefore the mast cells play a role not only in the early phase allergic response by their release of preformed mediators but also in the development of chronic mucosal inflammation by recruitment of T cells and eosinophils through the mediators released during the early phase reaction.

Table 1-1 Preformed and newly generated Mast cell mediators

Mediators	Biological effects
Histamine	Bronchoconstriction, tissue edema, mucus secretion, fibroblast and endothelial proliferation
Heparin	Anti-coagulant, storage matrix for mast cell mediators, fibroblast activation
Tryptase	Generated c3a and bradykinin, increases BHR, activates collagenase, fibroblast proliferation
Chymase	Mucus secretion, extra cellular matrix degradation
PGD_2	Bronchoconstriction, tissue edema, mucus secretion
LTC_4	Bronchoconstriction, tissue edema, mucus secretion
TNF-α	Neutrophil chemotaxis, MHC class II expression, increased expression of adhesion molecules, mucus secretion, increases IL-8 and IL-6 synthesis in fibroblasts
IL-4, IL-13	B cell proliferation, increase IgE synthesis, activation of eosinophils
IL-5, GM-CSF	Eosinophil recruitment, activation and survival
IL-6	IgE synthesis, differentiation of T cells, mucus secretion
IL-8	Neutrophil chemotaxis
IL-16	T cell chemotaxis
Stem cell factor (SCF)	Growth, differentiation and survival of mast cells
Basic FGF	Angiogenesis, fibroblast proliferation
MCP-1	Monocyte and T cell chemotaxis
$MIP\text{-}1^{\alpha}$	Macrophage differentiation, neutrophil chemotaxis

1.5.4. Neutrophils

Chronic severe persistent asthma is characterized by certain features that separate them from the well controlled disease. In addition to female predominance, association with aspirin intolerance and reduced association with allergy, these sub groups of asthmatics have elevated neutrophil levels in the sputum and the blood. Acute severe asthma is associated with neutrophilic inflammation of the airways and this neutrophil recruitment has been implicated in early asthmatic deaths following an acute asthma attack (69). Increased neutrophil numbers in airway biopsies and BAL has been described in chronic severe asthma (70). Although airway neutrophilia is a common denominator in severe corticosteroid dependent asthma there are wide variations in cellular inflammation and structural changes. Wenzel et al found that severe corticosteroid dependent asthmatics could be divided into those with and without airway eosinophils with both the groups showing neutrophilia. However, the group with eosinophils demonstrated increased sub-basement membrane thickening an altered slow vital capacity to forced vital capacity ratio and an increased occurrence of near fatal events (71).

Given the profile of mediators generated by neutrophils which include IL-8, TGF-β and MMP-9 it is unlikely that the neutrophil plays a key role in acute bronchospasm associated with asthma (72). However, it is much more likely to be involved in chronic inflammation, wound repair and remodeling process in asthma. Increased neutrophils in asthma are associated with increased IL-8 levels, a neutrophil chemoattractant (73, 74). Sputum neutrophilia correlated with IL-8 and neutrophil myeloperoxidase levels (75). In bronchial biopsies, epithelial IL-8, MIP-1α, EGFR and submucosal neutrophils were all significantly increased in severe compared to mild disease and there was a strong correlation between EGFR and IL-8 expression which suggests that in severe asthma, epithelial damage has the potential to contribute to neutrophilic inflammation through enhanced production of IL-8 via EGFR dependent mechanisms (76). Recent evidence suggests that neutrophilic infiltration in asthmatic airways may not only be confined to the severe end of the spectrum but also in those with mild to moderate asthma (31). Animal studies support a role for neutrophils and neutrophil elastase in the up regulation of MUC5AC RNA and protein and in degranulation of goblet cells suggesting a role in mucus production and secretion (77, 78). It is believed that human neutrophil elastase-induced MUC5AC

mucin production in airway epithelial cells occur via its proteolytic activation of EGFR signalling cascade involving TGF-alpha (79). As mucus production and secretion are prominent issues up-regulation of MUC5AC RNA could be critical in this particular group of asthmatics.

The common pathological features of neutrophilic asthma involve IL-8 mediated neutrophil influx and subsequent activation of the neutrophils which is a potent stimulus for airway hyperresponsiveness (80). Neutrophils are important sources of proinflammatory cytokines and proteolytic enzymes and sustained release of these mediators can lead to airway injury and remodeling in chronic severe asthma (81). Neutrophil elastase and reactive oxygen species have been shown to increase with asthma severity (82, 83). It appears that neutrophilic inflammation is a feature of chronic severe asthma and this is relatively insensitive to corticosteroids which may account for the high doses of steroids needed to control these patients.

1.6. Role of Th1 cells in airway inflammation

Based on the Th1-Th2 paradigm for allergic diseases a Th1 response is believed to protect against allergic diseases by dampening the activity of Th2 responses, as has been shown in models of parasitic infection (14). Th1 cells inhibit the proliferation and development of Th2 cells and IFN-γ inhibits IgE synthesis and eosinophilia (84-86). However, in reality Th1 cells may exacerbate asthma as human asthma is associated with increased levels of IFN-γ (87). Segmental allergen challenge also induces increases in IFN-γ along with IL-4 (88). The IFN-γ producing T cells were significantly higher in BAL fluid from atopic asthmatic children than either atopic non-asthmatic subjects or normal controls suggesting that the pro-inflammatory effect of a Th1 cytokine could play an important role in asthma pathogenesis indicating that asthma is not simply a Th2 mediated phenomenon (89). In experimental asthma adoptive transfer of antigen specific Th1 cells to mice with an established Th2 response to the same antigen resulted in a worsening of airway inflammation and increased BAL eosinophilia (90, 91). In this study the authors suggested that a Th1 cells instead of being counter regulatory augment a Th2 response. The Th1 dependent increase in TNF-α and VCAM-1 can provide a setting for enhanced allergen

responsiveness. Depending on the genotype it is possible that Th1 stimulation may increase rather than dampen the Th2 response and promote airway inflammation.

Further evidences from human studies that have addressed therapeutic interventions with Th1 enhancing activity (92) or anti Th2 cytokine administration (49) demonstrated significant decreases in blood and sputum eosinophils but this was not accompanied by changes in the early and late phase asthmatic response or to measures of airway hyperreactivity (93). Cytokine modulation with monoclonal antibodies directed against IL-5 demonstrated reduction in the sputum and blood eosinophils implying a biological response however, this fall in eosinophils was not accompanied by changes in the early or late asthmatic responses or airway reactivity to histamine (49). This was also associated with a reduction in the airway eosinophils and and bone marrow eosinophils (50). Similarly sub-cutaneous administration of human recombinant IL-12 in patients with mild atopic asthma lowered the blood and sputum eosinophils with no change in the airway hyperresponsiveness or late phase reaction (92). On a similar note, administration of IFN-γ both by the sub-cutaneous and nebulised route in patients with asthma resulted in the reduction of eosinophil numbers with no change in the lung functions or clinical improvements (94, 95). These studies suggest that deviating T cell responses away from Th2 can improve the inflammatory cell population in asthma but may not affect asthma outcomes. Thus although Th2 cells play a critical role in the pathogenesis of asthma, the binary Th1-Th2 paradigm where Th1 cells balance Th2 cells cannot explain all the immunological process that occur in asthma.

Th1 cells induce neutrophilic inflammation without airway hyperresponsiveness in mouse models of allergen induced lung inflammation (19). Furthermore Th1 diseases like rheumatoid arthritis and inflammatory bowel disease are associated with neutrophilic inflammation (96, 97). The ENFUMOSA study and other studies have shown that severe asthma is associated with airway and sputum neutrophilia in contrast to the eosinophilic inflammation in mild asthma (75, 98). It is therefore believed that severe asthma could possibly involve other mechanisms different from the now well established Th2 mediated inflammatory mechanisms observed in mild asthma.

1.7. Cytokine Networks in asthma

Cytokines play a critical role in orchestrating the airway inflammation in asthma by promoting the development, differentiation, recruitment, priming, activation and survival of inflammatory cells. Individual cytokines may have overlapping cell regulatory action and function through complex cytokine networks. This accounts for the complexity of cytokine expression where depending upon the local environment cytokines can induce or inhibit each other. They interact with specific receptors on the cell surface and through unique signaling pathways activate specific transcription of genes involved in the inflammatory response (Table 1.2).

Although a strict Th1-Th2 dichotomy dose not exist in humans, it is widely accepted that the histopathological and functional abnormalities characterizing asthma are largely mediated by the cytokines IL-3, IL-4, IL-5, IL-9, IL-13 and GM-CSF produced primarily, but not exclusively, by Th2 cells (99). In mice, targeted pulmonary expression of IL-13 causes mucus hyper secretion induces metaplasia of mucus cells, deposition of Charcot-Leyden like crystals, airway fibrosis, eotaxin production, airways obstruction, and non-specific BHR(100).IL-13 blockade inhibited most of these characteristics in allergen immunised wild type mice suggesting a critical and non-redundant role for IL-13 in allergen induced BHR (101). Studies with IL-13 transgenic mice have provided insights into the mechanisms of IL-13 induced airway inflammation and fibrosis (102). IL-13 stimulates the expression of chemokines and matrix metalloproteinases (MMPs). The chemokine receptor CCR2, MMP-9 and MMP-12 play a crucial role in these responses (103, 104). The fibrotic responses results from the ability of IL-13 to stimulate the production and activation of transforming growth factor beta1 (TGF-β_1) which is activated by MMP-9 and plasmin dependent pathways (105).

Table 1-2 Cellular source and actions of cytokines involved in asthma

Cytokine	Cellular Source	Signalling	Gene	Actions
IL-1 (IL-1α, IL-1β, IL-1RA)	Monocytes, Macrophages, Smooth Muscle cells, Endo-thelium, B and T cells	IRAK, TRAF6, NF-κB	2q13-21	Activation of T cells, Proliferation of CD4 and CD 8 cells, coupling of β_2 adrenergic receptors to adenylyl cyclase
IL-2	T cells, Eosinophils, Airway epithelial cells	JAK1 JAK3 STAT5	4q26-27	Growth and differentiation of T, B and NK cells, eosinophil chemotaxis
IL-3	Activated helper T cells, Mast cells	JAK2 STAT 5	5q23-31	Eosinophilia, growth factor for neutrophils, eosinophils, basophils, mast cells and monocytes
IL-4	T cells, Eosinophils, Basophils, Mast cells	JAK1 JAK3 STAT6	5q23-31	Th2 differentiation, B cell activation, IgE class switching, Class II MHC expression, Up regulate adhesion molecules, mucus hyper secretion
IL-5	T cells, Eosinophils, Airway epithelium	JAK2 STAT5	5q23-31	Promotes growth, differentiation and activation of eosinophils, inhibit apoptosis, promote BHR
IL-6	Monocytes, Macrophages, T and B cells, Fibroblasts	JAK1 STAT3	7p21	B and T cell growth factor, promotes IL-4 dependent IgE synthesis
IL-8	Macrophages, T cells, Mast cells, Eosinophils, Airway epithelial cells	MAPK	4q12-13	Activation, differentiation and chemoattractant for neutrophils
IL-9	CD4 T cells	JAK1 JAK3 STAT9	5q31	T cell growth factor, , regulates BHR
IL-10	T cells, Macrophages, Monocytes	JAK1 TYK2 STAT3	1q31-32	Inhibits Th1 and Th2 cells, down regulates pro-inflammatory cytokines
IL-12	B cells, Monocytes, Macrophages, Dendritic cells	JAK2 TYK2 STAT4	5q31	Inhibits Th2 cell development, suppresses IgE production, promotes Th1 phenotype
IL-13	T cells	JAK1 TYK2 STAT6	5q31	Activates eosinophils, increases IgE production
IL-18	Airway epithelium,	IRAK STAT6 NF-κB	11q22	Th1 cell differentiation, inducer of IFN-γ production
GM-CSF	Macrophages, eosinophils, T cells, Fibroblasts, Airway epithelial and smooth muscle cells	JAK2 STAT5	5q31	Eosinophil apoptosis and activation, release of leukotrienes, modulates BHR
IFN-γ	CD4, CD8 T cells, NK cells	JAK1 JAK2 STAT1	12q24	Inhibits Th2 cells, inhibits eosinophilia, IgE synthesis and BHR, increases TNF-α release
TNF-α	Macrophages, T cells, mast cells, neutrophils and epithelial cells	TRAF TRADD FADD NF-κB	6p21	Activates epithelium, endothelium, antigen presenting cells, macrophages, increases adhesion molecules expression and BHR

1.8. Chemokines in asthma

Chemokines or chemotactic cytokines are a family of about 40 small distinct, but structurally related, molecules that mainly regulate trafficking of leukocytes through interaction with its receptors. These are classified as CXC, CC, CX_3C and C subgroups based on a standard nomenclature (Table 1.3) (106). Chemokines are produced by a variety of cell types including airway epithelium, endothelium, smooth muscle cells, alveolar macrophages and for eotaxin, Th2 cells. Chemokines are relevant in asthma not only for their role in regulating leukocyte recruitment, but also for other activities, such as cellular activation, inflammatory mediator release, promotion of Th2 inflammatory responses, and regulation of IgE synthesis (107). Of particular interest is the role of CC chemokines, including eotaxin-1, -2, and -3, regulated on activation normal T-cell expressed and secreted (RANTES), MIP-1α, and MCP-2, -3, and -4, in the context of asthma, as these are potent chemoattractants for eosinophils, basophils, monocytes, and T lymphocytes.

Chemokines activate a family of seven transmembrane spanning G-protein-coupled receptors, of which at least 11 have been cloned. Of particular interest in the context of airway inflammation is chemokine receptor CCR-3, which is expressed on T lymphocytes, basophils, and eosinophils (108). In addition, certain members of the chemokine family have the capacity to modulate T cell differentiation towards a Th1 or Th2 phenotype. In particular, MCP-1 can drive naïve T cells towards a Th2 phenotype whereas MIP-1α promotes a Th1 type response by enhancing IFN-γ and decreasing IL-4 production (109). Furthermore, RANTES and MIP-1 alpha can enhance IgE production by directly stimulating B cells (110).

The presence of RANTES, eotaxin, MCP-1, MCP-3, MCP-4, and MIP-1α has been demonstrated at the mRNA or protein level in bronchial biopsy specimens or BAL of asthmatic patients (111). RANTES, MCP-3, MCP-1, and MIP-1α have been found to be significantly elevated in asthmatic airways in comparison with non-asthmatic subjects (112). Chemokines blocking strategies in mouse models of airway inflammation has proven to be useful in abrogating the airway hyperresponsiveness and eosinophil accumulation in the airways. Blocking CCR3 using a decoy ligand met-RANTES significantly reduced eosinophil accumulation in the BAL and the

interstitium. Similarly eotaxin neutralisation and MCP-5 neutralisation resulted in the abrogation of BHR in mouse models of airway inflammation (113, 114).

In asthmatic airways, eotaxin expression was found to be highest in the airway epithelium and least in the eosinophils (115). The expression of CC chemokines can be up-regulated relatively quickly following acute exposure to antigen. Thus, 4–6 hr following endobronchial allergen challenge of mild asthmatic subjects, there is a significant increase in the levels of MIP-1α, MCP-1, and RANTES in lavage fluid (116). Inflammatory cytokines, including IL-1β and TNF-α, can induce the synthesis of various chemokines, including eotaxin and RANTES while IFN-γ does not directly stimulate the production of eotaxin from human lung epithelial cells, but acts in synergy with TNF-α and IL-1β (117, 118). It is therefore clear that cytokines and chemokines mediate the asthma phenotype, however no single cytokine or chemokine will explain or account for all the processes defined by the asthma phenotype.

Table 1-3: Chemokine receptor family (106)

Name	Functions
CXC subgroup	
CXCR1	Neutrophil migration; innate immunity; acute inflammation
CXCR2	Neutrophil migration; innate immunity; acute inflammation; angiogenesis
CXCR3	T cell migration; adaptive immunity; Th1 inflammation
CXCR4	B cell lymphopoiesis; bone marrow myelopoiesis;
CXCR5	B cell trafficking; lymphoid development
CXCR6	T cell migration
CC subgroup	
CCR1	T cell and monocyte migration; innate and adaptive immunity; inflammation
CCR2	T cell and monocyte migration; innate and adaptive immunity; Th1 inflammation
CCR3	Eosinophil, basophil, and T cell migration; allergic inflammation
CCR4	T cell and monocyte migration; allergic inflammation
CCR5	T cell and monocyte migration; innate and adaptive immunity
CCR6	Dendritic cell migration
CCR7	T cell and dendritic cell migration; lymphoid development; primary immune response
CCR8	T cell trafficking
CCR9	T cell homing to gut
CCR10	T cell homing to skin
CX_3C and C subgroups	
CX_3CR1	T cell and NK cell trafficking and adhesion; innate and adaptive immunity; Th1 inflammation
XCR1	T cell trafficking

1.9. Severity of asthma

Bronchial asthma has a wide clinical spectrum ranging from mild intermittent disease to the severe persistent difficult to treat asthma. In characterising the severity of asthma various factors need to be considered including symptoms, medication use, pulmonary function, bronchial hyperresponsiveness (BHR), features of airway inflammation, hospitalisations, frequency and severity of exacerbations, response to treatment and the impact on health. A number of criteria have been adopted by the Global Initiative for Asthma (GINA) and the National Heart Lung and Blood Institute (NHLBI) to define subgroup of asthmatics in terms of asthma severity (119)(Table 1.3). The inclusion of lung function parameters are useful in the assessment of asthma severity since the symptoms are assessed subjectively both by the patient and the physician.

Table 1-4: Classification of asthma severity based on GINA guidelines

	Clinical features before treatment	**Lung function**
STEP 1 (Mild intermittent)	▪ Symptoms < 2 times a week ▪ Asymptomatic and normal PEFR between exacerbations ▪ Intensity of exacerbations brief ▪ Night time asthma symptoms ≤2 times a month	▪ FEV_1 or PEF ≥ 80% predicted ▪ PEFR variability < 20%
STEP 2 (Mild persistent)	▪ Symptoms > 2times a week but < 1 a day ▪ Exacerbation may affect activity ▪ Night time asthma symptoms >2 times a month	▪ FEV_1 or PEF ≥ 80% predicted ▪ PEFR variability 20- 30%
STEP 3 (Moderate persistent)	▪ Daily symptoms ▪ Daily use of short acting β_2-agonists ▪ Exacerbations affecting sleep and activity ▪ Night time asthma symptoms >1 a week	▪ FEV_1 or PEF ≥ 60% but ≤ 80% predicted ▪ PEFR variability > 30%
STEP 4 (Severe persistent)	▪ Continuous symptoms ▪ Frequent exacerbations ▪ Frequent night time asthma symptoms ▪ Limited physical activities	▪ FEV_1 or PEF ≤ 60% predicted ▪ PEFR variability > 30%

1.9.1. Chronic severe asthma

The NIH guidelines for the Diagnosis and Treatment of Asthma has characterised severe persistent asthma by symptoms that tend to be continual, limits physical activity and associated with frequent and severe exacerbations (120). These patients also have frequent night time symptoms and persistent airflow obstruction with an FEV_1< 60% predicted before treatment. The American Thoracic Society workshop modified this description of severe asthma to account for the appropriate use of medications, specifically high dose inhaled corticosteroids with or with out systemic corticosteroids. Based on this workshop the term "refractory asthma" was agreed upon (121). There were two major and seven minor criteria's (Table 1.4) and refractory asthma was defined as the presence of one or both major criteria and at least two minor criteria's. Chronic severe asthma is characterised by ongoing airflow limitation with incomplete reversibility. The physiology of chronic severe asthma includes air trapping with increased residual volume, altered collapsibility of the airways, shift of the pressure/volume curves and loss of compliance.

Table 1-5: Diagnosis of Refractory asthma {, 2000 #220}

Major Criteria
• Treatment with oral corticosteroids ≥ 50% of the time • High doses of inhaled corticosteroids (≥ 1200 μg beclomethasone or equivalent)
Minor Criteria
• Requirement for daily treatment with long acting β2 agonists, theophyllines or leukotriene modifiers • Daily asthma symptoms requiring rescue medications • Persistent airflow obstruction (FEV_1 < 80%); diurnal PEF variation > 20% • One or more emergency visits for asthma per year • Three or more oral steroid bursts per year • Prompt deterioration with ≥ 25% reduction in oral or inhaled corticosteroid dose • Near fatal asthma event in the past

1.9.2. Relationship of airway inflammation to asthma severity

The airway pathophysiology of severe asthmatics are not entirely due to a direct extrapolation of the inflammatory process seen in mild asthmatics. Certain features are unique to patients with severe asthma. The dimensions of large and small airways in both fatal and non fatal asthma was studied and it was found that the large airway walls were thickened along with an increase in the inner wall area in the fatal asthmatics when compared to nonfatal asthmatics. Furthermore the smooth muscle area and the mucus gland area were significantly higher in the larger airways of fatal asthmatics when compared to the non-fatal group (10). However the non-fatal group had a higher large and small airway dimension and smooth muscle area when compared to the control group. This increased wall thickness will amplify the effects of smooth muscle shortening on airway narrowing and the effect is in direct relation to the degree of the airway wall thickness. A 50-230% and 25-150% increase in the area of the smooth muscle has been shown in fatal and non-fatal asthma (10). The percentage of smooth muscles in the normal airways increases from 5% of the wall tissue in the central bronchi to about 20% of the total wall thickness in the bronchioles. Therefore for the same degree of muscle shortening there is a greater effect on the calibre of the airways in the distal bronchi and bronchioles than on the larger airways (8, 122). Studies show that the increase in thickness of the reticular basement membrane correlates with the severity of asthma (123) and this thickening is due to the deposition of collagen types I, III and V (7).

TGF-β released from the inflammatory cells promotes airway remodelling and induces the transformation of fibroblasts into myofibroblast (124). In a study by Minshall et al the expression of TGF-β immunoreactivity in the bronchial mucosa of mild to severe asthmatics was increased and this was directly related to the severity of the disease and correlated with the decline in FEV_1 (125). Following segmental allergen challenge, concentrations of TGF- β1 was significantly elevated after 24 h but were no different after 10 minutes suggesting that basal TGF-β1 levels in the airways are elevated in response to allergen exposure (126). Furthermore thickening of the sub epithelial basement membrane correlated with the number of fibroblasts in the submucosa in asthmatic subjects with a significant correlation between the number of fibroblasts and the expression of TGF-β1 (127). The subepithelial basement membrane thickening seen in asthma also correlated with the number of cells

expressing TGF-β while no such correlation was found with EGF and GM-CSF (128). These findings are consistent with the hypothesis that TGF-β is involved in airway wall remodelling in asthma. Epithelial injury in asthma leads to the release of TGF-β which induces the transformation of fibroblasts to myofibroblasts which in turn are functionally more active in producing and deposition of collagen in the sub epithelial basement membrane leading to airway wall remodelling. Levels of TGF-β were elevated in the bronchial biopsies of patients with severe asthma along with IL-11 and IL-17 (129). Since TGF-β plays a role in airway remodelling and in the phenotypic transformation of fibroblasts to myofibroblasts, severe asthmatics are likely to have unyielding airways with poor reversibility.

There is also evidence to suggest that TNF-α is an important element in determining the severity of asthma. Sputum and biopsy samples from patients with severe persistent asthma have been shown to contain increased numbers of neutrophils (70, 75). One of the major stimuli for neutrophil recruitment is exposure to endotoxin. The severity of asthma symptoms has been related to the endotoxin content of house dust, rather than to the allergen load in these samples (130). That the endotoxin-induced effects are largely mediated through the endogenous release of TNF-α is illustrated in *in vivo* rat models of airway inflammation. Exogenous administration of TNF-α was shown to induce airway neutrophilia and hyperresponsiveness, whereas pre-treatment with anti-TNF-α antibodies profoundly reduced the endotoxin-induced airway changes (131). Bronchoalveolar lavage fluid from subjects who require ventilation due to status asthmaticus contain increased numbers of neutrophils and levels of pro-inflammatory cytokines including TNF-α (132). Therefore, certain features of severe asthma are distinctive and could prove to be a therapeutic target in devising newer therapies.

1.10. Experimental models of asthma

Models are used to study the pathophysiological mechanisms and to assess the therapeutic efficacy of therapeutic agents in the treatment of asthma. The inflammatory events underlying asthma have largely been unravelled by using challenges with provocative stimuli of the airways.

1.10.1. Standard allergen challenge model

Allergen challenge testing of the airways has been used extensively to study the mechanisms of allergen induced airway inflammation in asthma. Standard methodologies include acute allergen challenge, chronic administration of low doses of allergen and natural allergen exposure (133). Standard bronchial provocation tests in allergen sensitive patients results in an early response starting within minutes after allergen challenge and reaching a maximum 15-20 minutes after challenge. In 50-80% of patients this is followed by a late response usually starting 3-4 hours after the challenge and reaching a maximum by 7-10 hours (134, 135). The early reaction is thought to be a reflection of mast cell degranulation and histamine release while the late response reflects the multicellular events more analogous to the inflammatory response seen in asthma (136). The histopathological changes after allergen challenge resembles the changes observed in symptomatic disease but the presence of a positive reaction does not always coincide with clinical manifestations. Muller et al demonstrated that bronchial hyperresponsiveness to allergen challenge in allergic subjects had little relationship to the presence or absence of clinical asthma caused by natural environmental challenge (137). Although the acute allergen challenge model of asthma is repeatable and reproducible it has its drawbacks too (138, 139). From the safety perspective acute allergen challenge can cause significant bronchoconstriction. Standard allergen challenge models induce reversible bronchoconstriction, airway inflammation and bronchial hyperresponsiveness the three cardinal features of asthma, however its relevance to clinical asthma are questioned because the doses required to produce an EAR and LAR are not often encountered in daily life. Standard allergen challenge lacks chronicity of the natural allergen exposure however is an excellent tool to assess the complex cellular and molecular mechanisms of inflammation and also to evaluate the efficacy of new medications for the treatment of allergic diseases.

1.10.2. Repeated low dose allergen challenge model

Chronic administration of low doses of allergen over a period of time is more likely to create a cellular milieu seen in clinical disease and reflect the events that occur in natural asthma. Here the chronicity of allergen exposure is imitated, while the antigen load is standardised. Ihre and colleagues showed that isolated late reactions can be provoked by inhalation of low doses of allergen (140). In a study by Sulakvelidze and

colleagues, repeated low dose allergen exposure resulted in steady worsening of airway hyperresponsiveness with an increase in the sputum eosinophils, eosinophil cationic protein (ECP) and IL-5 (141). Other studies have also shown that low dose allergen provocation models are safe and well tolerated (142, 143). These models produce a significant increase in methacholine sensitivity while the FEV_1 was not significantly reduced. This is also a useful model to evaluate the effects of therapeutic agents. A repeated allergen challenge model was used to evaluate the effects of budesonide on airway hyperresponsiveness and sputum eosinophilia which found that a higher dose of budesonide reduces both airway hyperresponsiveness and sputum eosinophilia (144). This indicates that chronic low dose allergen exposure is safe, well tolerated and a useful investigational model for asthma not only to study the pathophysiological mechanisms but also to evaluate therapeutic efficacy of novel agents.

1.10.3. Bronchial explant models of asthma

The development of a bronchial explant culture system has been extremely useful to assess the involvement of particular cytokines in the inflammatory response in asthma. Initial studies with explanted nasal polyps derived from atopic and non-atopic subjects showed increase in IL-8, GM-CSF and RANTES in the supernatants after stimulation with allergen (145, 146). Unlike isolated cell culture systems, explant culture is an integrated system using biopsies obtained from the lower airways or nasal tissues. This tissue includes structural elements such as airway epithelium and fibroblasts as well as resident inflammatory cells including T cells, mast cells, macrophages, eosinophils and neutrophils. The methodology for the explant cultures were standardised in previous studies from our department. As this is a closed system the cell coults are not liely to change and variations in cellular population could be due to the cells being shed from the explant biopsies into the culture medicum. Earlier studies have shown the release of IL-5 and IL-13 by bronchial explants from mild allergic asthmatics is increased following *in vitro* exposure to *Der p* allergen and IL-5 production in the airways of moderately severe asthmatics is largely independent of CD28 mediated costimulation (34, 35). Jaffar et al has shown that allergen specific T cells are resident in asthmatic bronchial tissue and costimulation by both CD80 and CD86 is essential for allergen induced cytokine production (33). Bronchial biopsy explants from moderately severe asthmatics show that IL-16 release is not dependent

on CD28/B7 costimulation in contrast to mild asthmatics (36). The advantages of the explant models are that the sampling is performed on one occasion and challenge performed *ex vivo*. These models are viable and informative and allow the testing of unapproved substances, which could, otherwise, not be delivered, *in vivo*.

1.11. Tumour Necrosis Factor Alpha

Tumour necrosis factor belongs to the TNF ligand family which consists of 18 genes encoding 20 type II trans-membrane proteins characterised by a trimeric domain that is responsible for receptor binding and sequence identity (147). The trimeric domain is responsible for the receptor binding and the sequence identity between family members is 20-30%. This similarity between TNF ligands is largely confined to the internal aromatic residues responsible for the trimer assembly (148). Most ligands are synthesised as membrane bound proteins however, soluble forms are generated by proteolysis. The proteases involved include ADAM (proteins containing **a** **d**isintegrin **a**nd **m**etalloproteinase domain), matrilysin (which acts on Fas ligand) and members of the subtilisin-like furin family (149-151). The shedding of these ligands inhibits their activity and acts as a control mechanism for down regulating the activity of these mediators. The ligands of the TNF family are listed in Table 1-6.

Table 1-6 TNF Ligand Family

Ligand	Standardised names	Other names
EDA		EDA-1
CD40L	TNFSF5	CD154, TRAP, HIGM1
FasL	TNFSF6	APT1LG1
OX40L	TNFSF4	TXGP1, gp34
AITRL	TNFSF18	
CD30L	TNFSF8	
VEG1	TNFSF15	TL1
LIGHT	TNFSF14	
4-1BBL	TNFSF9	
CD27L	TNFSF7	CD70
LTα	TNFSF1	TNF-β, LT
TNF	TNFSF2	TNF-α, Cachectin, TNFA
LTβ	TNFSF3	TNFC, p33
TWEAK	TNFSF12	DR3L APO3L
APRIL	TNFSF13	
BLYS	TNFSF13B	THANK, BAFF
RANKL	TNFSF11	TRANCE, OPGL, ODF
TRAIL	TNFSF10	Apo 2L

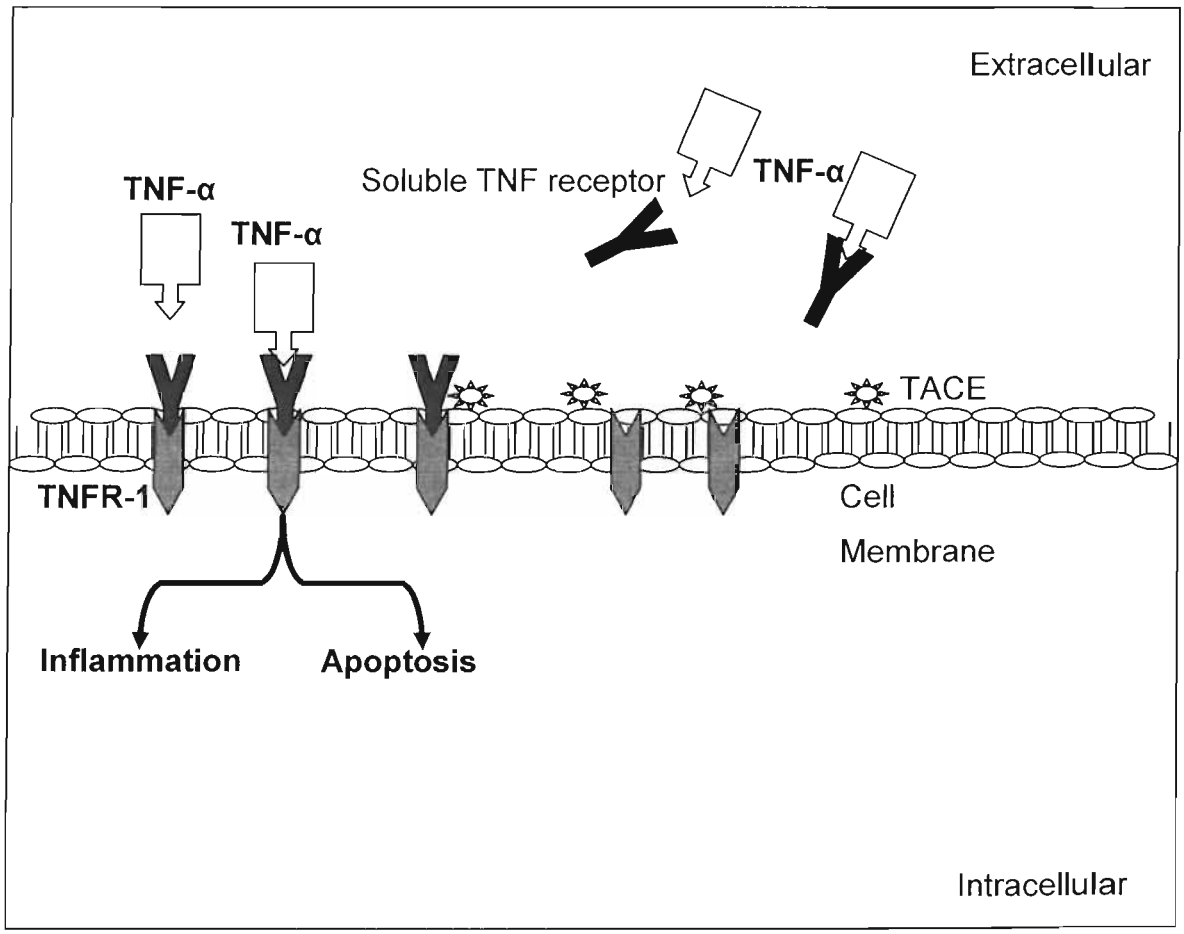
The TNF ligand is structurally related to lymphotoxin-α, which is secreted from activated T cells (152). Tumour Necrosis Factor (TNF) exists in two forms, TNF-α and TNF-β. These two forms share similar inflammatory activities. TNF-α and TNF-β are the prototype member of a large family of related proteins that include CD30L, CD 40L, Fas ligand and TRAIL ligand. Although these proteins are related they exhibit marked differences in tissue expression, ligand specificity and biological functions. In general, both TNF-α and TNF-β display similar spectra of biological activities in *in vitro* systems. TNF-β is a larger molecule, not as abundant as TNF-α, less potent and is predominantly produced by the T cells.

Human TNF-α is a type II membrane, non-glycosylated protein of 17kDa with 157 amino acids (153). This is the soluble cleaved for of TNF-α. It shares 30% homology with TNF-β. Members of the TNF family have an intracellular tail, a single trans-membrane domain and an extracellular ligand binding part. The 17kDA soluble form of TNF-α is produced by processing of a 233 amino acid precursor protein by TNF-α converting enzyme (TACE) or TNF-α converting activity (TACA). TNF-α contains a single di-sulphide double bond that can be destroyed without altering the biological activity of the factor. The human gene for TNF-α and TNF-β maps to chromosome 6p21.1-21.3 within the class III region of the human major histocompatibility complex (154). The gene encoding TNF-β is 1.2kb downstream of the TNF-α gene and both the genes are regulated independently.

TACE is also known as ADAM 17 and is part of a larger ADAM family. TACE is a multi-domain type I trans-membrane protein, which includes a zinc dependent catalytic domain and a disintegrin-cysteine rich sequence (155). TACE is responsible for cleaving the 26kDa trans-membrane TNF-α to the soluble bio-active 17 kDa TNF-α. Both soluble and membrane bound forms of TNF-α are biologically active, although they have different affinities for the two receptors. After separating from the cell membrane, the soluble TNF-α molecules aggregate into trimolecular complexes (51 kDa homotrimers) that subsequently bind to the receptors. TACE also cleaves the extracellular domain of its complimentary ligand of TNF-α forming soluble TNF-α receptors (sTNFRs). This process results in diminished cellular signalling of TNF. The shed sTNFRs are cleared in the extracellular space, where it they are free to bind

to the trimolecular TNF-α rendering them biologically inactive (Fig 1.3). Thus sTNFRs function as natural inhibitors of TNF-α mediated inflammation. It is postulated that in patients with the TNF-receptor–associated periodic syndrome, the TNF receptor is not shed from the cell surface and in the absence of shedding, there is continuous signalling by TNF-α and, consequently, an inflammatory response (156).

Figure 1-3 TNF-α interaction with its receptors



Activation of the TNF-α receptor by TNF-α causes cleavage and shedding of the extracellular portion of the receptors by TACE. These soluble TNF-α receptors act as natural inhibitors of TNF-α. This system prevents the continuous activity of the inflammatory cycle as a consequence of the TNFRI activation by TNF-α.

1.11.1. Cellular Sources of TNF-α

The main source of TNF-α are the macrophages, although other cells including mast cells, eosinophils, neutrophils, smooth muscle cells, fibroblasts and epithelial cells also secrete TNF-α. CD4 cells secrete TNF-α while CD8 cells secrete little or no TNF-α. Furthermore, TNF itself can act to induce TNF production. Mast cells release

and respond to TNF-α indicating that there is a positive autocrine loop leading to augmentation of mast cell activation (157). The most potent stimulus for the production of TNF is lipopolysaccharides (LPS). In addition, all potentially noxious stimuli ranging from physical, chemical to immunological can rapidly induce TNF-α production and release.

1.11.2. Receptors

Approximately 500-100,000 high affinity receptors for TNF-α are expressed on all somatic cell types excluding erythrocytes. Tumour necrosis factor-α acts via two related receptors which are designated as p55 (CD 120a, TNF-RI, TNF receptor super family member 1A) and p75 (CD 120b, TNFRII, TNF receptor super family member 1B) (158). The p55 receptor is expressed on cells susceptible to the cytotoxic action of TNF while p75 is strongly expressed on stimulated B cells and T cells. TNF-α receptor subtypes are type I trans-membrane glycoproteins with 28% homology mostly in the extra-cellular domain with both containing four tandemly repeated cysteine-rich motifs. Their intra cellular sequences are largely unrelated. The cytoplasmic domains of these receptors lack any intrinsic enzymatic activity. Hence, signal transduction is achieved by recruitment and activation of adaptor proteins that recognise specific sequences in the cytoplasmic domains of these receptors. The recruitment of these adaptor molecules result in activation of many signalling process that lead to a remarkably diverse set of cellular responses, including differentiation, activation, release of pro-inflammatory mediators and apoptosis (159).

1.11.3. TNF signalling pathways

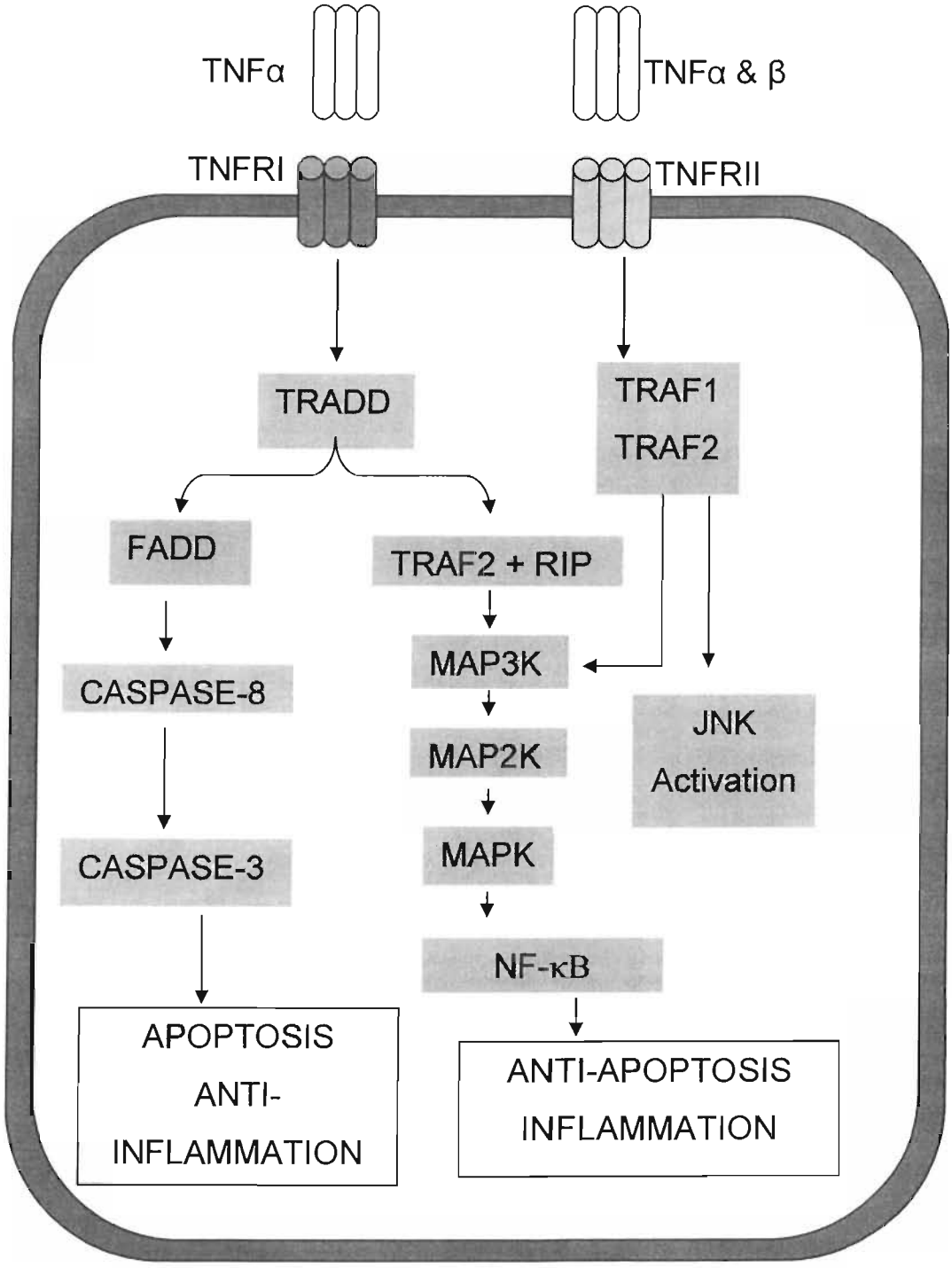
Binding of the trimeric TNF-α to TNFRI and TNFRII induces recruitment of several signalling proteins to the cytoplasmic domains of the receptors (Fig 1.3). TNFRI contains a conserved ~80 amino acid motif called "Death Domain" (DD) which is involved in signalling process leading to programmed cell death or apoptosis. This is required for the recruitment of DD containing adaptor molecules that are involved in initiation of apoptotic cell death. The binding of TNF-α to TNFRI activates TNFRI associated death domain protein (TRADD), which recruits three other proteins, receptor interacting protein 1 (RIP1), Fas-associated death domain protein (FADD) and TNF receptor associating factor 2 (TRAF2). Occupancy of TNFR2 results in direct recruitment of TRAF2 which in turn recruits TRAF1. TRAF2 plays a central

role in early events common to TNFR1 and it is these adaptor proteins that transduce the TNF signal from biochemically inert receptors to dramatic changes of signalling molecules within target cells (160).

TNFRI can induce apoptosis by the interaction of TRADD with FADD. This results in recruitment and activation of caspase 8 which promotes apoptosis by subsequently activating caspase 3 which in turn cleaves multiple cellular proteins resulting in cell death (161). The inability of TNFRII to recruit FADD could be a possible explanation for its inability to induce apoptosis. Furthermore, Fas does not engage effectors like TRADD, RIP1 and TRAF2 and is a poor activator of NF-κB and AP-1 (162).

Interaction of TRADD with TRAF2 and RIP1 will produce the opposite effect leading to the activation of IκB kinase complex (IKK) and mitogen activated protein kinase (MAPK). This results in activation of two major transcription factors AP-1 and NF-κB which in turn induce genes involved in acute and chronic inflammatory responses to TNF-α (163).

Figure 1-4 Signalling pathways for TNF-α



1.11.4. Biological Activities of TNF-α

Studies in animals have helped us understand the importance of TNF-α in immunocompetence. TNF-α knock out mice were viable but succumbed to infections by *Candida albicans* and *Listeria monocytogenes* that normal mice could control (164). Furthermore, these mice lacked primary B cell follicles and germinal centres in the spleen and had a reduced ability to form granulomas. They also have impaired IgG and IgE antibody responses and problems with antigen presentation because of poorly sustained antibody responses. Complementation of TNF-α functioning by the expression of either human or murine TNF alpha transgenes was sufficient to reconstitute these defects, establishing a physiological role for TNF-α in regulating the development and organization of splenic follicular architecture and in the maturation of the humoral immune response (165). TNF-α is also believed to protect against tuberculosis by promoting apoptosis of infected cells (166)

In humans, TNF-α has a wide spectrum of activity causing cytolysis and cytostasis of many tumour cell lines *in vitro*. TNF-α in conjunction with IL-1, causes alterations in the endothelium and exerts a pro-coagulant activity. It also promotes angiogenesis. TNF-α is also a strong chemoattractant for neutrophils. TNF-α is a growth factor for fibroblasts and promotes the synthesis of collagenase and prostaglandin (PG) E_2 in fibroblasts. TNF-α induces the synthesis of IL-1 and PGE_2 in resting macrophages. TNF-α exerts a proliferative response on T cells in the absence of IL-2. In leukocyte and lymphocyte progenitors TNF-α stimulates expression of class I and II HLA antigens and the production of IL-1 and colony stimulating factors. In the presence of IL-2 TNF-α promotes the proliferation of B cells. TNF-α is a major mediator of cachexia and acts by blocking the action of lipoprotein lipase. TNF-α also mediates part of cell mediated immunity against parasites, obligate and facultative bacteria.

1.12. Tumour necrosis factor Alpha in Asthma

Asthma is perceived as a Th2 disease with a particular cytokine profile characterised by IL-4, IL-5 and IL-13. There are however evidence to suggest that other cytokines which are considered to be associated with a Th1 type profile are associated with the

inflammatory response in asthma. TNF-α is a Th1 cytokine which has been implicated in asthmatic airway inflammation in both *in vitro* and *in vivo* studies.

There is evidence of increased expression of TNF-α in the airways of asthmatics when compared to normal subjects (66, 167). Mast cell granules have been shown to contain TNF-α and this is released in response to allergen challenge (168). Human lung mast cells produce up to 150pg/10^6 cells/24h of TNF-α and this is stimulated by both IgE dependent activation and by stem cell factor (c-kit ligand) a mast cell growth and survival factor. Allergen cross-linking of the IgE in sensitised mast cells release preformed TNF-α within minutes (169). Lung mast cells both release and respond to TNF-α with a positive autocrine loop that augments the activation of mast cells. This also augments NF-κB activation not only of mast cells but also other inflammatory cells promoting a pro-inflammatory effect on the local environment (170). In addition other cells in the airways have the ability to generate TNF-α namely, eosinophils, epithelial cells and airway macrophages (171-173).

Tumour necrosis factor -α stimulates the production of IL-8, RANTES and GM-CSF by the airway epithelial cells and also increases the expression of adhesion molecules including ICAM-1 and VCAM-1 involved in the recruitment of inflammatory cells to the airways. The up regulation of adhesion molecules on the pulmonary endothelium is important for eosinophil recruitment (174, 175). *In vitro,* both TNF-α and IL-1β increase the expression of ICAM-1 and VCAM-1 on respiratory epithelial cells (176). Airway smooth muscle (ASM) cells express ICAM-1 and VCAM-1, and TNF-α aids in the binding of activated T lymphocytes to ASMs (177). Inflammatory cells bind to adhesion molecules and transmigrate into airway interstitium.

Tumour necrosis factor-α may have an important amplifying effect on asthmatic inflammation as it increases the trans-epithelial migration of neutrophils mediated through IL-8, promotes chemotaxis of eosinophils/monocytes and plays a role in T-cell activation. Airway neutrophilia is associated with the more severe forms of asthma and administration of TNF-α in rats was associated with airway neutrophilia (131). Asthmatic subjects peripheral blood monocytes (PBMCs) when stimulated with LPS, showed an increase in the production of TNF-α, IL-8 and GM-CSF compared to normal subjects (178). Moreover, BAL leukocytes from asthmatic subjects when

cultured with PHA and PMA produced significantly higher levels of TNF-α and IFN-γ (87).

There is evidence that TNF-α also plays a role in tissue remodelling with its powerful growth promoting effects on fibroblasts. Tumour necrosis factor-α promotes myofibroblast proliferation as well as increasing the mitogenicity of fibroblasts (179). Additional to these direct effects, TNF-α has indirect effects on airway remodelling through its ability to induce eosinophils to release matrix metalloproteinase-MMP-9 and to stimulate glycosaminoglycan synthesis in human lung fibroblasts (180). Airway epithelial cell also secrete mucus when stimulated with TNF-α (181).

Allergen challenge is considered to be a good experimental tool to mimic some aspects of asthma in controlled settings. In allergic asthmatics, increased sputum and serum TNF-α and IL-5 levels were noted 24 hours after allergen challenge (182). Allergen challenge in asthmatic increases the generation of TNF-α by macrophages and peripheral blood mononuclear cells (PBMCs) along with increasing the levels of TNF-α mRNA cells present in the airway lavage fluid (183, 184). Alveolar macrophages from allergic asthmatics with a documented late response had an elevated TNF-α secretion when challenged with inhaled extracts of *Der p* (185). TNF-α levels also increase during asthma exacerbations and this is associated with increased expression of ICAM-1, VCAM-1 and E-selectin (186, 187). Increased exhaled nitric oxide is associated with asthmatic airway inflammation and it is believed that TNF-α could increase inducible nitric oxide synthase which in turn increases exhaled nitric oxide levels (188).

1.13. TNF-α and Airway Responsiveness

Airway smooth muscles are an essential element in the pathophysiology of asthma. Exaggerated airway responsiveness to contractile agonists and a decrease in β-adrenoceptor mediated airway relaxation is the recognizable cause for the morbidity and mortality in asthma. Airway smooth muscle cells when cultured with TNF-α show markedly increase synthetic functions defined as secretion of cytokines and chemokine and expression of adhesion molecules (189). Animal studies have shown that administration of recombinant TNF-α can induce airway hyperresponsiveness in

murine models (131). Furthermore, this airway hyperresponsiveness was significantly abrogated by the administration of anti-TNF-α antibodies. In human studies TNF-α has been shown to cause an increase in the airway reactivity (190). In a study on eight non-asthmatic subjects' administration of inhaled TNF-α caused a 3 fold shift in the methacholine concentration response curves at 24 hours compared to control subjects (191). *In vitro* studies have revealed that TNF-α potentates the contractile response of human airway smooth muscles to acetylcholine (192).

The mechanisms involved in the TNF-α modulation of ASM responsiveness could be either due to the recruitment of inflammatory cells or through a direct effect on the airway smooth muscle (193). Intra-cellular calcium plays an important role in mediating contractile responses of smooth muscle cells. Tumour necrosis factor-α can increase cytosolic calcium influx by direct activation of membrane associated calcium channels (194). Furthermore, ASM exposed to TNF-α *in vitro* or *in vivo* become hyper responsive to many contractile agonists (195). The modulation of airway hyperresponsiveness by TNF-α is through the p55 receptor (TNFRI) coupled to the TRAF2-NF-κB pathway.

1.14. TNF-α blocking strategies as novel therapies

The central role for TNF-α in the initiation and perpetuation of the inflammatory process in diseases like rheumatoid arthritis (RA) suggests a role for TNF-α blocking strategies as an exciting therapeutic option. Etanercept (Enbrel®) and Infliximab (Remicade®) have been approved for use in RA. TNF-α inhibition provides rapid control of synovial inflammation and retards cartilage and bone destruction in RA. TNF-α blockade is also effective in treating juvenile RA, psoriasis, psoriatic arthritis and Crohn's disease (196-200). Infliximab is approved for use in Crohn's disease while Etanercept has been approved for use in juvenile RA and psoriatic arthritis. Other conditions where TNF-α has been evaluated are sarcoidosis, ankylosing spondylitis, adult and childhood uveitis, Sjogren's syndrome and Wegener's granulomatosis (201).

Studies with infliximab and etanercept in patients with ankylosing spondylitis and psoriatic arthropathy had shown improvements in the disease activity scores,

functional quality of life and inflammatory markers (199, 202-204). Bechets' disease is a multi system disorder and TNF-α blocking strategies have been found to be effective in inducing short term remissions of all the all manifestations of the disease, including acute, sight threatening panuveitis. Interestingly Infliximab was found to be more effective than etanercept in Bechets disease (205). Sarcoidosis, a chronic inflammatory multi-system illness characterised by non-caseating granulomas had responded to TNF-α blocking strategies with infliximab and etanercept, however the responses were not uniform suggesting that a particular group may benefit more from this intervention (206). Further controlled trials are required to assess whether TNF-α blocking therapy can be safely and efficaciously applied to other inflammatory disorders other than rheumatoid arthritis and inflammatory bowel disease.

Various molecules are available for blocking the effects of TNF-α. These include a monoclonal antibody that targets TNF-α (Infliximab), a soluble TNF-α receptor (p75) (Etanercept) which are in clinical use. The newer TNF-α targeting immunobiologicals that are being developed are a polyethylene glycol bound p55 TNF-receptor (PEG-TNFRI), PEGylated TNF-α antibody fragments (CDP-870), D2E7 (adalizumab)- a fully human anti-TNF-α antibody and TACE inhibitors (207).

1.14.1. CDP 870

CDP870 (Celltech Research and Development, Slough, Berkshire, UK) is a novel compound that neutralizes TNF-α *in vitro*. It comprises an engineered human anti-TNF-α antibody Fab fragment that is linked chemically to polyethylene glycol (PEG). The Fab fragment is made by microbial fermentation in *E.coli* rather than in mammalian cell culture. The attachment of PEG increases the circulating half-life of Fab to approximately 14 days. Initial phase 2 trials in rheumatoid arthritis showed CDP870 to be effective, well tolerated and possesses an extended duration of action (208).

1.14.2. Anti TNF-α monoclonal antibody (Infliximab)

Infliximab (Remicade ®) is a genetically engineered human-mouse chimeric monoclonal antibody that targets TNF-α (Fig 1-5). The murine antigen binding Fv domain is linked to the Fc portion of the human IgG1 (Fig 1.4). The approximate

molecular weight of Infliximab is 149 kDa and is produced from a recombinant mammalian cell line. The human moiety confers the effector function, while the mouse portion contains a variable region binding site (209). The murine monoclonal antibody has a high affinity to TNF-α while the human IgG_1 reduced the immunogenicity. Infliximab neutralises the biological activity of TNF-α by binding with high affinity to the soluble and trans-membrane forms of TNF-α and thereby inhibits the binding of TNF-α with its receptors (210, 211). *In vivo* Infliximab rapidly forms stable complexes with human TNF-α which is associated with loss of TNF-α bioactivity. Infliximab does not neutralise TNF-β, a similar cytokine that uses the same receptors as TNF-α and has 30% homology with TNF-α.

Administration of infliximab has powerful effects on the inflammatory cascade with an early reduction in serum IL-6 levels followed by C-reactive protein levels (212). In RA treatment with infliximab reduced the magnitude of T cell infiltrate and the expression of VCAM-1 on endothelial cells in the synovial tissue (213). This was also associated with a reduction in serum MMP-1, MMP-3 and VEGF (214-216). The disease activity returned to pre-treatment levels within several weeks to a few months following a single injection it was clear that maintaining disease control would require repeated infusions. There are evidences to suggest that infliximab can prevent or inhibit progression of structural damage in patients with rheumatoid arthritis (217).

1.14.3. Soluble TNF-α receptor (Etanercept)

Etanercept is a dimeric fusion protein consisting of 2 identical chains of the extracellular ligand-binding portion of the human 75 kDa p75 tumour necrosis factor receptor (TNFRII) linked to the Fc portion of human IgG1. The Fc component of etanercept contains the CH2 domain, the CH3 domain and hinge region, but not the CH1 domain of IgG1 (Fig 1-6). Etanercept is produced by recombinant DNA technology in a Chinese hamster ovary mammalian cell expression system. It consists of 934 amino acids and has an apparent molecular weight of approximately 150 kDa (218, 219). Etanercept binds specifically to both TNF-α and TNF-β and blocks its interaction with cell surface TNF receptors. However in contrast to infliximab etanercept forms less stable complexes with cell bound TNF-α and monomeric TNF-α. As TNF-α is a trimer, a TNF-R dimer would more effectively compete with binding of TNF-α to the membrane receptors than a monomer and prevent cell

signalling. Due to its unique dimeric nature, the binding affinity is increased 1000 fold compared to the native TNF monomer, while the Fc portion increases the half-life 5-8 fold (220).

After sub-cutaneous injection of etanercept, serum TNF-α levels are increased but TNF-α activity is reduced because once bound to etanercept TNF-α molecules are no longer biologically active (221). Etanercept modulates biological responses that are induced or regulated by TNF, including expression of adhesion molecules responsible for leukocyte migration viz. E-selectin and ICAM-1, serum levels of IL-6 and serum levels of matrix MMP-3 or stromelysin. In a randomised placebo controlled trial treatment with Etanercept produced rapid improvement in disease activity in patients with RA. However, akin to Infliximab the disease relapsed within 1-2 months following cessation of treatment (222).

1.14.4. Infliximab Vs Etanercept

Despite the improvements produced by infliximab and etanercept in patients with rheumatoid disease and other connective tissue disorders the side effects of the medications has been a cause of concern. The most significant is a causal link between the use of infliximab and tuberculosis. The estimated tuberculosis rate in all European recipients of infliximab calculated from the data presented by Keane and colleagues is 173 cases per 100000—several times higher than the background rate in European countries. (223). While etanercept is also associated with tuberculosis the approximate crude world tuberculosis rate is 20·7 cases per 100000 (224). The difference is attributed to many causes including the use of infliximab for Crohns disease, the differences in the study population, the method of administration (infliximab is administered intravenously while etanercept is administered subcutaneously) and the wider use of infliximab in Europe. Hence, comparisons of the rates of tuberculosis reported with infliximab and with etanercept need cautious interpretation.

On a scientific note infliximab and etanercept both have high binding affinities to TNF. Etanercept in addition, binds to lymphotoxin α and in vitro studies show that infliximab forms highly stable complexes (Fig 1-5, 1-6). The high avidity binding of infliximab to trans membrane TNF-α results in cytolysis of TNF-α expressing cells

(211). Etanercept, on the other hand, forms complexes with TNF and lymphotoxin that dissociate readily in the presence of free TNF or receptors. Moreover, no cell lysis is reported. Although both agents interact with TNF-α in various forms, there are subtle differences in their mechanisms of action. In particular, drug-mediated apoptosis and monocytopenia appear to be unique to infliximab. On the basis of these differences, infliximab might be predicted to have a more significant effect on the host's ability to suppress *M tuberculosis* infection. The effect of bolus dosing of infliximab versus more steady dosing of etanercept may also affect the host's ability to control *M tuberculosis* infection. It is possible that differences in stability of complexes with TNF, antigen specificity, and cell lysis properties may contribute to differences in their overall safety profiles in addition to differences in clinical outcomes.

Figure 1-5 Schematic representation of infliximab and Infliximab- TNF-α complex

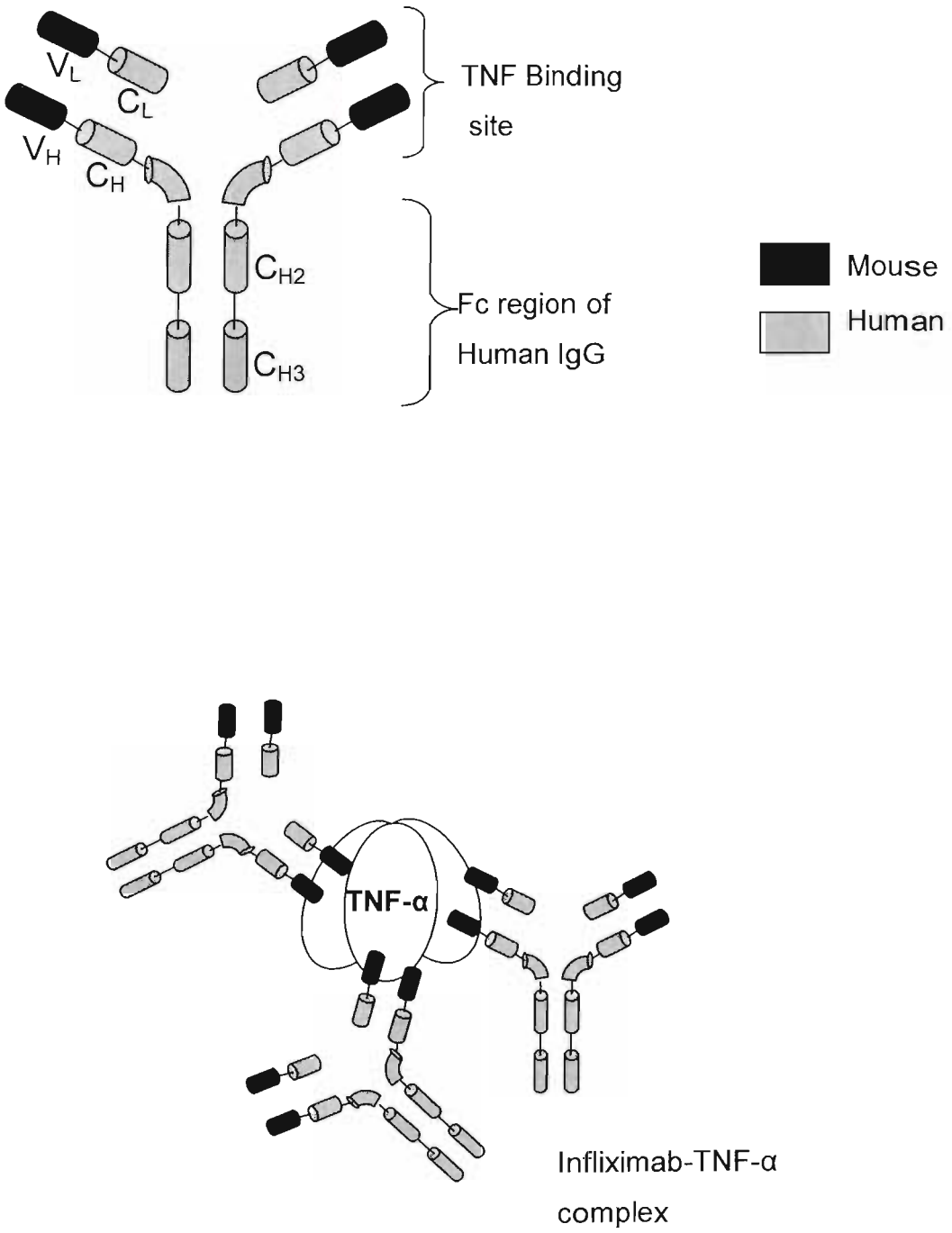
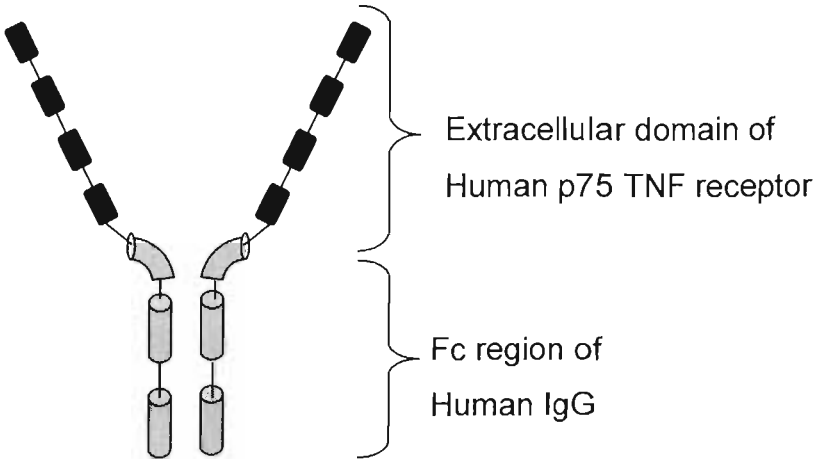
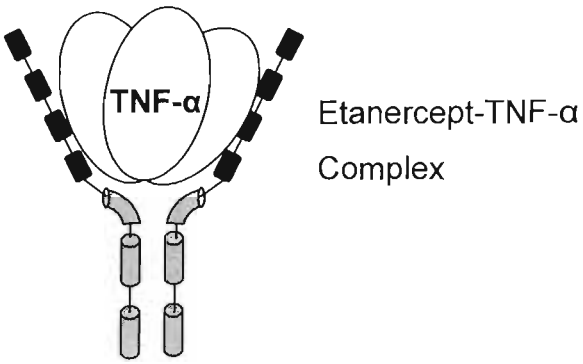


Figure 1-6 Schematic representation of etanercept and etanercept- TNF-α complex





1.15. Specific aims and objectives

1. To assess the expression of TNF-α and adhesion molecules ICAM-1 and VCAM after repeated low dose allergen exposure in bronchial biopsies of patients with mild atopic asthma

2. To assess the expression of adhesion molecule ICAM-1 and TNF-α in the presence of a blocking monoclonal antibody against TNF-α (CDP 870) on bronchial tissues from moderately severe asthmatics in an explant culture system

3. To assess the ability of the TNF-α blocking monoclonal antibody (CDP 870) to inhibit the production of cytokines in response to stimulation with *Der p* allergen in an explant culture system

4. To evaluate the efficacy of a soluble TNF-α receptor (Etanercept) on lung function, airway hyperresponsiveness and asthma control in patients with chronic severe asthma

5. To assess the sputum cells and cytokines in patients with chronic severe asthma before and after treatment with a soluble TNF-α receptor (Etanercept)

Overall these studies will contribute to our current understanding of the pathophysiology of asthma and a role for TNF-α in asthma. This would also provide insight into the prospects of TNF-α blockade as a novel therapeutic strategy in patients with chronic severe asthma.

2. Materials and Methods

2.1. Subjects

Mild, moderate and severe asthmatics were recruited from the Southampton conurbation, either from the departmental database or from chest clinics. Asthmatic subjects had a physician based diagnosis of asthma and were characterised according to symptoms, pulmonary function, asthma medication requirement and on going treatment for β_2 agonists. Asthma severity was assessed in accordance with the GINA guidelines on the diagnosis and management of asthma (119). Subjects with a current smoking history or with a smoking history of more than 10 pack years were excluded. Written informed consent was obtained from all volunteers prior to participation and ethical approval obtained from the Joint Ethics Committee of Southampton University and General Hospital.

Serum IgE levels were measured by standard enzyme linked immunosorbant assay (ELISA) by the regional immunology laboratory, Southampton General Hospital, UK.

2.2. Spirometry

Spirometry is widely accepted both for diagnosis of asthma in clinical practice, and for classification of disease severity. The following parameters were measured.

FEV_1 (forced expiratory volume in one second, measured in litres): The maximal volume of air that can be exhaled in one second from the point of maximum inhalation. A value of < 80% predicted is agreed to indicate airflow obstruction (119). The severity of FEV_1 impairment is a useful indicator of asthma severity

FVC (forced vital capacity): The total volume that can be forcibly exhaled from maximal inhalation until the point where no further air can be expired (i.e. residual volume has been reached).

FEV_1/ FVC ratio: Expression of the forced expiratory volume in one second as a percentage of the forced vital capacity is sensitive for the detection of airways obstruction than FEV_1 alone. A value of < 70% it suggestive of airflow obstruction.

Spirometry was performed according to standardised guidelines, outlined by the American Thoracic Society (225). Subjects took the maximum possible inspiration through the mouth and then exhaled forcibly into the spirometer mouthpiece, blowing as hard as possible for at least the first second and continuing until there was no breath left when the residual volume had been reached. When three technically satisfactory blows had been obtained, the highest values of both FEV_1 and FVC were recorded.

2.3. Bronchodilator reversibility

Response to bronchodilator was measured by recording FEV_1 following administration of 200μgm of salbutamol through a spacer device. Twenty minutes later spirometry was repeated in an identical fashion and the result of the test was expressed as percentage change in FEV_1 from the original value.

2.4. Allergen skin prick testing

Skin prick test sensitivities to a panel of common aeroallergens were determined for each subject. The following allergen extracts were used (ALK; *Dermatophagoides pteronyssinus*, mixed grass pollens, mixed tree pollens, feathers, cat dander, dog fur, aspergillus spores and Candida spores using a standard lancet. Saline (0.9% sodium chloride) and histamine (1mg/ml) were used to exclude non-specific responses and to provide a positive control respectively. Tests were considered positive if a wheal response of 3mm or greater than the negative control was observed.

2.5. Bronchial provocation tests

Bronchial provocation tests are employed to determine the sensitivity of the airways to a variety of stimuli. Non-specific airway hyperresponsiveness is usually assessed by inhalation of either histamine or methacholine which constrict the airways by their direct action on airway smooth muscle via H_1 and M_3 receptors respectively. Specific bronchial provocation tests employ allergens as the provocation stimuli to determine airway reactivity. The results are expressed as the provocating dose (PD_{25}) or provocation concentration (PC_{25}) required to produce a 25% decrease in the FEV_1 from the baseline levels.

2.5.1. Methacholine bronchial provocation challenge

Non-specific bronchial hyper-responsiveness was assessed using the methacholine bronchial provocation test. This test was carried out in all subjects whose FEV_1 was greater than 60% predicted. The method described used is a technique modified from Chai H, et al., (226). Subjects were asked not to take their salbutamol (or other rescue bronchodilators) within at least 4 hours prior to the methacholine challenge. Nebulised methacholine (Sigma Co, Poole, Dorset, UK) dissolved in saline was administered through a dosimeter (Spira electro 2, Spira, Finland) in doubling dilutions. The procedure is stopped if the FEV_1 drops by at least 20% or if the patient becomes symptomatic and is unable to continue the procedure. The degree of airways responsiveness to methacholine was expressed as the cumulative PC_{20} – the concentration producing a 20% fall in FEV_1.

2.5.2. Allergen Bronchial Provocation tests

Standard allergen bronchial provocation tests were performed with house dust mite allergen in patients who had a positive skin prick test reaction to house dust mite. Spirometry was recorded to establish a baseline FEV_1 which should be >70% predicted. After ensuring that the patient is suitable to continue, the subjects inhaled 5 breaths of saline through a dosimeter (Spira electro 2, Spira, Finland). Each breath delivered a fixed volume of saline (3.67 µl). The FEV_1 was measured and this value was used to calculate the percentage fall from the post saline baseline. Following saline inhalation the subjects inhale varying concentrations of house dust mite allergen (Aquagen® SQ, ALK-Abello A/S, Horsholm, Denmark) starting with the least concentration. After each concentration of allergen, a 4 fold increment is administered providing the FEV_1 has not fallen by more than 10% from the post-saline value. If the fall in FEV_1 is between 10 and 15% a 2 fold increment is administered. If the fall is greater than 15% but less than 25% from the post saline baseline a further five breaths of the same concentration is administered. The challenge is terminated when a fall in FEV_1 of > 25% from the post saline baseline FEV_1 measurement is achieved. After the final concentration of allergen, FEV_1 measurements are taken at 5, 10, 15, 30, 45, 60 minutes and thereafter every 30 minutes up to 10 hours.

An early asthmatic response (EAR) is defined as a fall in FEV_1 of at least 25% within 15 minutes of allergen inhalation and the late asthmatic response (LAR) is defined as a fall in FEV_1 of at least 15% between 3 and 10 h. The cumulative PD20 (provocation dose to cause a 20% fall in FEV1) is usually expressed. This can be calculated by linear interpolation on a plot of log-dose versus response or by using the following equation

$$PD20 = \text{anti log}\left(\log D1 / \frac{(\log D2 - \log D1)(25 - R1)}{(R2 - R1)}\right)$$

Where: D1 = Cumulative dose immediately before the 20% fall in FEV1

D2 = Cumulative dose immediately after the 20% fall in FEV1

R1 = % fall in FEV1 after D1

R2 = % fall in FEV1 after D2

2.5.3. Repeated low dose allergen challenge

This concept is based on the notion that a repeated low dose allergen administration is more likely to simulate natural allergen exposure in contrast to the standard allergen bronchial provocation which is more likely to result in an acute inflammatory response (227).

Low doses of allergen were administered on alternate days three days a week for 4 weeks on subjects after the standard bronchial provocation testing. The standard bronchial provocation tests were used to calculate the PD25 and 10% of the PD25 was administered on a regular basis. Spirometry was performed to detect changes in lung function. Patients were issued diary cards to monitor their symptoms and any deterioration in their lung function. After administration of the allergen for 4 weeks bronchoscopy was performed and bronchial biopsies were obtained for immunohistochemistry.

2.6. Sputum induction and processing

Sputum induction was carried out as described by Pizzichini et al (228) using inhaled aerosolized hypertonic saline (4.5%). Subjects were seated in an induction chamber, 400 μg of inhaled salbutamol was administered via a spacer device, and the peak expiratory flow (PEF) was recorded. Patients with a post bronchodilator FEV_1 of <50% of predicted and < 1.0 L were excluded from the induction procedure. Hypertonic saline was administered via an ultrasonic nebuliser (Devilbiss Ultraneb 2000, PA, USA). After every five minutes the nebulisation was stopped and PEF noted. The procedure was discontinued when there was a fall in PEF of >15% or if there were troublesome symptoms. Sputum was expectorated into a Petri dish. The induction period was for a maximum of 20 minutes or until a satisfactory quality of the sample was obtained.

Unselected sputum was processed. An equal weight of 0.01M DTE (dithioerythritol) was added to the sputum and placed on a rocker bench for 30 minutes. The contents were then filtered through a 70 micron filter to remove mucus. The filtrate was centrifuged for 10 minutes at 1500rpm (400g) at 4^0C to pellet the cells. The supernatants were aliquoted into labelled eppendorfs and stored at -80^0C for future analysis. The cell pellet was resuspended in 1ml of tris-buffered saline (TBS) and cells were counted in a Neubauer's chamber after staining with trypan blue. Cytospins were obtained based on the total cell counts and the cells were stained by Rapi-Diff stain. Differential cell counting was performed by counting 600 cells in each cytospin in a blinded fashion. The mean of the two scores was used for analysis. The differential cell counts were expressed as percentage of the total cells or in absolute numbers.

The co-efficient of variation between the two counts was less than 10% which is acceptable. Co-efficient of variation is an expression of intra-operator variability and is defined as the ratio of standard deviation and mean expressed as percentage.

2.7. Fiberoptic Bronchoscopy

Bronchoscopy was performed using a fibreoptic bronchoscope (Olympus, FB-XT20, Tokyo, Japan) in accordance with the standard published guidelines (229, 230). Patients with moderately severe asthma were advised to stop their inhaled

corticosteroids for a minimum of one week prior to the bronchoscopy. Subjects who are on a long acting β_2 adrenoceptor agonists were asked to withhold their medication for at least 48 hours prior to bronchoscopy. The subjects were monitored closely for any deterioration of their asthma. They were issued with a diary card and a Wright's mini peak flow meter to assess their symptoms, asthma control and rescue medication use. A doctor and a research nurse were available over the telephone 24/7 for the patient to contact if there was any change in their asthma control, symptoms or rescue medication use. If the patients PEF dropped by more than 15% or if the patients' use of rescue medications is significantly higher then the subject was deemed not fit to continue in the study and was withdrawn. Subjects fasted for 5 hours prior to bronchoscopy and were pre-medicated with nebulised salbutamol (2.5 mg) and intravenous atropine (0.6 mg). Light sedation was achieved with intravenous midazolam (0-6 mg). Topical 10% lignocaine spray was used for local anaesthesia (total maximum lignocaine dose < 300mg). Oxygen saturation was monitored throughout the procedure by pulse oximetry. Bronchial biopsies were taken from the sub-carinae of the 2nd and 3rd generation bronchi of the right lower lobe using FB15 alligator forceps and were placed in tissue culture medium for subsequent culture studies or in ice-cold acetone with a protease inhibitor (2mM phenylmethylsulphonyl fluoride and 20mM iodoacetamide) for processing into glycol methacrylate (GMA) resin for immunohistochemistry analysis.

2.8. Bronchial Explant culture

This is a useful integrated cell system to study inflammatory immune response in complex tissues using bronchial biopsies obtained from lower airways. Bronchial biopsies were cultured for 24 hours in 24 well culture plates with 500µl of culture media in each well. The explant cultures used three environments; a serum free medium alone (500 µl, AIMV, Life technologies, Paisley, UK); serum free medium plus *Der p* allergen (5000 SQ U/ml, ALK, Horsholm, Denmark); serum free medium plus *Der p* plus anti TNF-α (CDP-870, 10 ng/ml, Celltech Immunex Corporation, USA). The medium was supplemented with HEPES buffer (10mM), L-glutamine (1mM), sodium pyruvate and 2-mercaptoethanol (2µM). Two biopsies were used for each culture condition to minimise the effects due to tissue heterogeneity. After 24 hours the cultures were stopped, the biopsies dried and weighed and placed in ice-cold

acetone with a protease inhibitor (2mM phenyl methyl sulphonyl fluoride and 20mM iodoacetamide) for processing into GMA for immunohistochemistry analysis. The supernatants were centrifuged and stored at -80^0 pending analysis. The doses of allergen and culture duration were based on conditions previously determined to be optimal for explant derived from mild and moderately severe asthmatics (33-35). A 24 hour culture period was chosen for monitoring cytokine production based on preliminary time course experiments by Jaffar *et al* on IL-5 production in bronchial explants. IL-5 production commenced at 12 hours and peaked at 24 and 48 hours. Due to the small number of biopsies that could be taken from moderately severe asthmatics for ethical reasons it was not possible to repeat these control experiments in this study.

2.9. Immunohistochemistry

2.9.1. GMA Processing of Bronchial biopsies

The biopsies were processed and cut based on the departmental protocols. The bronchial biopsies were processed into glycol methacrylate resin prior to sectioning and immunostaining. The biopsies were placed in ice-cooled acetone plus protease inhibitors (phenylmethylsulfonyl fluoride [2M] and iodoacetamide [2M]) and stored overnight at -20^0C. The following day the biopsies were placed in acetone at room temperature for 15 minutes and then in methylbenzoyl for 15 minutes. The biopsies were then immersed in glycol methacrylate JB4 solution A (Polysciences, Northampton, UK) for 6 hours, the solution being changed every 2 hours. Then the tissue was embedded in GMA resin prepared by mixing GMA monomer, N-N-dimethylaniline PEG-400 and benzoyl peroxide and was polymerised overnight at 4^0C (231). The resin blocks were then stored at -20^0C in air tight containers until sectioning for immunostaining. Two micron thick sections were cut using a microtome (Supercut 2065, Leica, Germany), floated onto ammonia water (1:500) and picked on to 0.01% poly-L-lysine coated glass slides and air dried at room temperature. Biopsies were initially stained with toludine blue to assess morphology of the epithelium and sub-mucosa, and biopsies with the best morphology from each subject was chosen for sectioning and immunostaining for the various inflammatory cells and bio-markers relevant to asthma.

2.9.2. Immunostaining

Sodium azide (0.1%) and hydrogen peroxide (0.3%) were applied to the sections to block endogenous peroxidases. This was followed by 3, five minute rinses with TRIS buffered saline (TBS) adjusted to pH 6. Blocking medium was applied for 30 minutes subsequently drained and primary antibodies (Table 2.1) applied and incubated overnight at room temperature. After 3, 5 minute rinses with TBS, biotinated rabbit anti-mouse IgG Fab (Dako Ltd, High Wycombe, UK) secondary antibodies were applied for 2 hours followed by streptavidin-horse radish peroxidase complex (Dako) for a further 2 hours. After rinsing with TBS, amino-ethyl carbazole (AEC) in acetate buffer (pH 5.2) and hydrogen peroxide was used as a substrate to develop a peroxidase dependent red colour reaction. The sections were then counter stained with Meyer's haematoxylin, dried and mounted in DPX. As a negative control for each biopsy 2 sections had TBS and isotype matched controls with a mouse IgGκ (MOPC 21) antibody applied at appropriate concentrations to the primary monoclonal antibody.

Table 2-1: Cell Markers used for immunohistochemistry

Name	Cells	Marker	Dilution	Source
AA1	Mast Cells	Tryptase	1:100	DAKO, High Wycombe, UK
EG2	Eosinophils	ECP	1:200	Pharmacia Upjohn, Milton Keynes, UK
NOE	Neutrophils	Elastase	1:1000	DAKO, High Wycombe, UK
CD3	T cells	CD3	1:100	DAKO, High Wycombe, UK
CD4	T cells	CD4	1:10	DAKO, High Wycombe, UK
CD8	T cells	CD8	1:100	DAKO, High Wycombe, UK
CD68	Macrophages	CD68	1:40	Becton Dickinson, UK
ICAM-1	Endothelium	CD54	1:600	Serotech, Oxford, UK
VCAM-1	Endothelium	CD106	1:10	Serotech, Oxford, UK
EN4	Endothelium	CD31	1:200	Monosan, Uden, Netherland
TNF-α	-	TNF-α	1:100	HyCult Biotechnology
Biotinated Anti-mouse	-	Secondary antibody	1:300	Uden, The Netherlands DAKO, High Wycombe, UK
Streptavidin Biotin horse Radish peroxidase	-	Third stage antibody	1:200	DAKO, High Wycombe, UK
IgG_1	-	Isotype control	1:60	Sigma, Poole, Dorset, UK

2.9.3. Quantification of Inflammatory cells in biopsy samples

All measurements were performed by an observer blinded to the coded sample. Positively stained inflammatory cells were counted in the epithelium and submucosa using a light microscope (Leitz Labourlux S, Leica Ltd, Milton Keynes, UK) with the aid of an eyepiece graticule. The results were expressed per mm^2 submucosal area and mm epithelial length. Computerised image analysis (Apple Macintosh Quadra 700, colour vision 1.6 software, Improvision, Coventry, UK) was employed to measure area of the submucosa and length of the epithelium. Sections were visualised on a computer screen using a microscope linked, via digital camera, to the computer, at a pre-set magnification. Focus and colour balance was adjusted on the microscope to achieve the best possible image on the computer screen. The area of interest was delineated on the image by a mouse operated cursor. The areas of the bronchial biopsies were calculated by delineating the biopsy while separating and excluding all areas of non-submucosa (eg glands, smooth muscle, cartilage). The area was then quantified automatically. In the epithelium, the length of the intact, well orientated epithelium was measured..

2.10. Cytokine assay by ELISA

The levels of cytokine protein in the supernatants of bronchial explant cultures and sputum supernatants were determined using commercially available **E**nzyme **L**inked **I**mmuno-**S**orbent **A**ssay (ELISA) kits for the various cytokines in accordance with the manufacturers' instructions (Table 2.2). In the text of this thesis cytokine production or release refers to cytokine protein measurements in the supernatants.

In view of the large volume of sample required for the measurement of TNF-α with the kit provided by R & D systems (200μl), sputum TNF-α was assayed with the kits obtained from BioSource, Nivelles, Belgium as the sputum supernatants available for cytokine assay was limited. These required only 100μl of the sample for estimation of TNF-α levels.

Table 2-2 ELISA kits used for cytokine assay

Cytokine	Sensitivity	Source
IL-1β	0.1pg/ml	BioSource United Kingdom Ltd, Nivelles, Belgium
IL-5	<4 pg/ml	BioSource United Kingdom Ltd, Nivelles, Belgium
IL-8	<5 pg/ml	BioSource United Kingdom Ltd, Nivelles, Belgium
TNF-α	0.12pg/ml	BioSource United Kingdom Ltd, Nivelles, Belgium
	<0.18pg/ml	R & D Systems, Minneapolis, MN, USA

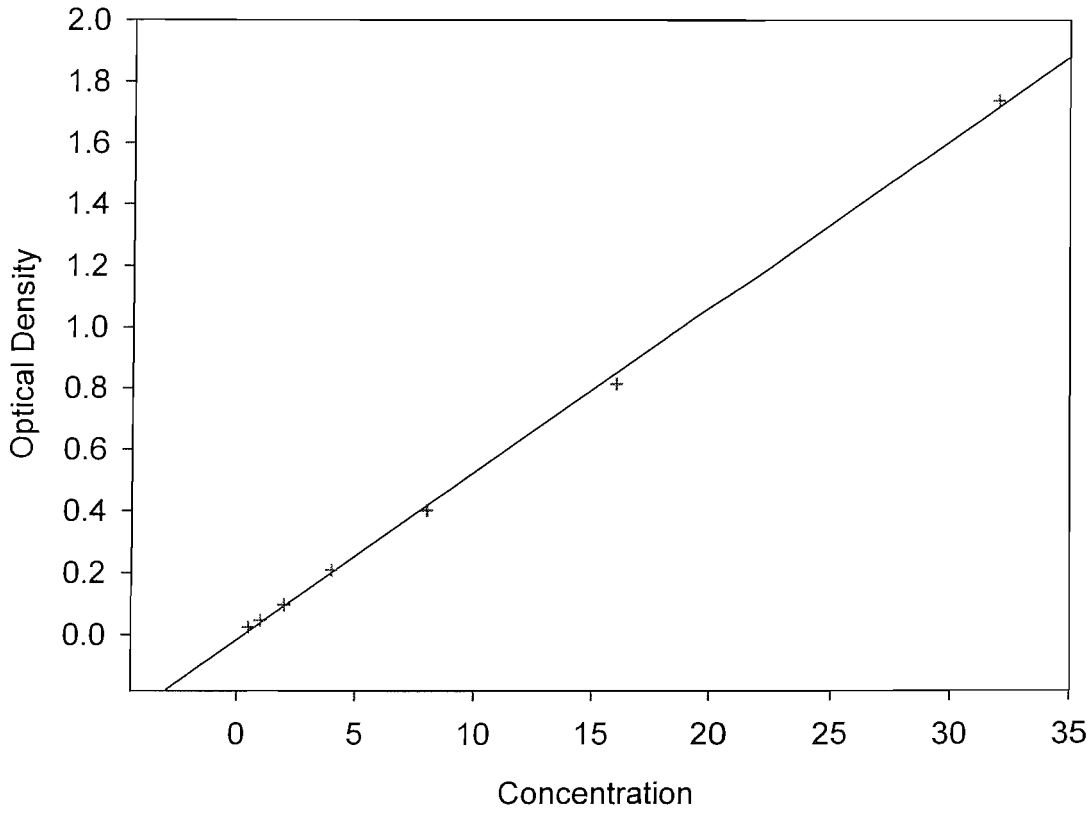
2.10.1. TNF-α ELISA Protocol

All the reagents were brought to room temperature before use. All the samples and standards were assayed in duplicates. Standards were made by serial dilutions in accordance with the manufacturers' instructions. 50 µl of the assay diluent was added to a 96 well microtiter plate precoated with the antibody for the appropriate cytokine (murine monoclonal antibody against human TNF-α in this case) to which 100 µl of the standards, controls or sample was added. 50µl of biotinated anti- TNF-α is then added to each well. The plate was sealed with the adhesive strip and incubated for 3 hours at room temperature. During the first incubation the human TNF-α antigen binds to the immobilised antibody (capture antibody) on one site and to the solution phase of the biotinated antibody on the second site. After a series of 6 washes, to remove the excess antibody 100 µl of streptavidin horse radish peroxidase was added to each well and incubated for 30 minutes. This binds to the biotinated antibody to complete the four member sandwich. Following incubation and 6 washes to remove the unbound enzyme, a substrate solution is added (chromogen) and incubated for 30 minutes. This is acted upon by the bound enzyme to produce colour. The colour development was then stopped by 50 µl of the stop solution. The plates were then read at 490 nm within 30 minutes using an ELISA plate reader. A standard curve was plotted and the cytokine concentration (pg/ml) of the samples was determined (Fig 2.1). The intensity of the coloured product is directly proportional to the concentration of human TNF-α present in the original specimen. The minimum detectable dose of

human TNF-α is < 0.09 pg/ml. The coefficient of variation for inter assay precision was 8.2 ± 0.5.

Figure 2-1: Standard curve for TNF-α

Representative standard curve obtained with serial dilutions of TNF-α over the range 32pg/ml to 0.5pg/ml using high sensitive TNF-α ELISA (R & D Systems, Minneapolis, USA). Plot represents mean of duplicate samples and line of best fit using linear regression



3. Effects of Low dose allergen challenge on TNF-α and adhesion molecules in patients with mild asthma

3.1. Introduction

Airway inflammation characterised by Th2 type inflammation coupled with airway remodelling is a feature of asthma. While asthma is an inflammatory disorder of the airways, inflammation alone does not explain the chronic and relapsing nature of this disease. It has been proposed that Th2 inflammation *per* se is insufficient to cause asthma, rather the asthmatic state results from a combination of increased susceptibility of the bronchial epithelium to tissue injury and prolonged tissue repair mechanisms involving EGF, TGF-β and Th2 cytokines (232). According to this new paradigm, the interaction between the Th2 cells and the epithelial-mesenchymal trophic unit (EMTU) leads to induction of stress injury and repair responses leading to myofibroblast activation and propagation of remodelling responses. In asthma the airway epithelium shows evidence of injury, activation and goblet cell metaplasia. Epithelial stress is seen in the form of widespread activation of NF-κB (233), activator protein 1 (AP-1) (234), STAT-1 (235) and cyclin dependent kinase inhibitor $P21^{waf}$ (236). It is believed that epithelial damage and Th2 cytokines can act in concert to cause a functional disturbance of the EMTU, which leads to myofibroblast activation and induction of inflammatory and remodelling responses characteristic of chronic asthma.

Experimental allergen challenge of the airways is an excellent tool to study the inflammatory events in patients with asthma. Bolus allergen challenge has provided a starting point for understanding the cellular events in asthma, but is far from physiological. The huge dose and abrupt delivery of allergen to the airways do not reflect natural exposure which even in mild disease is evident as minimal persistent inflammation. Bolus allergen challenge models are invariably based on acute flare-ups of airway inflammation. These models do not assess the development and consequences of chronicity of the inflammatory response which is the feature of asthma. Chronic exposure to an allergen could prime the response to acutely encountered pro-inflammatory stimuli like allergens and virus infections potentially facilitating asthma exacerbations (237, 238). Understanding the chronicity of airway

inflammation is therefore essential for developing newer and specific therapeutic targets in asthma.

Repeated small doses of allergen resemble more closely the natural asthmatic state and the low dose sub-clinical allergen exposure is more relevant to the pathogenesis of chronic allergic asthma (239). Repeated low dose allergen inhalation can induce a state of chronicity and could serve as an experimental tool to assess airway inflammation in asthma. The initial studies of repeated low dose allergen exposure were from nasal challenges in ragweed allergic patients. It was suggested that lower doses of pollen were required on successive days to increase the nasal airway resistance (240, 241).

There are only a limited number of studies in the lower airways using repeated low dose allergen challenge (140, 142, 143). Protagonists of this method suggest that chronic administration of allergen at low doses creates a cellular milieu similar to that seen in clinical disease and this could serve as a model to determine the pathological basis of the disease and the response to new therapies. One reservation about this approach is the ethical issue. While the safety of this procedure has been proven in many studies (142, 242, 243), there are questions as to whether this approach can precipitate and worsen asthma and whether this approach could make a subject more sensitised to house dust mite allergen which is associated with an increased risk of development/persistence of asthma. In the study by Sulakvilidze and colleagues, all the changes induced by low dose allergen exposure appear to be reversible (141). Therefore it is unlikely to have major ethical restrictions on this kind of research.

Sensitisation to house dust mite (HDM) is an important factor associated with an increased risk and persistence of asthma. The effect of exposure to house dust mite allergen seems to be most pronounced in children with atopic predisposition and with exposure during the first months/years of life. There seems to be a synergistic effect of several co-existing environmental factors (244). In some series as much as 85% of asthmatics have a positive skin prick test reaction to dust mite (245, 246). Epidemiological studies report a causal association between asthma and HDM but direct evidence comes from bronchial challenges with incremental doses of HDM extract. The relevance of these studies are difficult to spell out as the relatively large

doses required to produce immediate and late responses do not mimic natural exposure (247). The consequence of low dose sub-clinical exposure is more relevant to study the cellular and molecular pathways for the development of airway remodelling. It is likely that significant HDM allergen exposure occurs in the night in the bedroom because of the time spent in the bed and the proximity of the reservoir during sleep. Therefore, in this present study the effects of sub-bronchoconstrictive doses of HDM allergen was evaluated in patients with mild asthma.

3.2. Hypothesis

i. That repeated inhalation of small doses of allergen in patients with mild asthma induces inflammatory responses in the airways

3.3. Objectives

i. To evaluate the airway inflammatory responses in bronchial biopsies of mild atopic HDM sensitive asthmatics after repeated small doses of allergen inhalation
ii. To evaluate the expression of TNF-α and adhesion molecules in bronchial biopsies of mild atopic HDM sensitive asthmatics after repeated small doses of allergen inhalation
iii. To correlate airway hyperresponsiveness with the expression of TNF-α in patients with asthma

3.4. Methods

3.4.1. Subjects

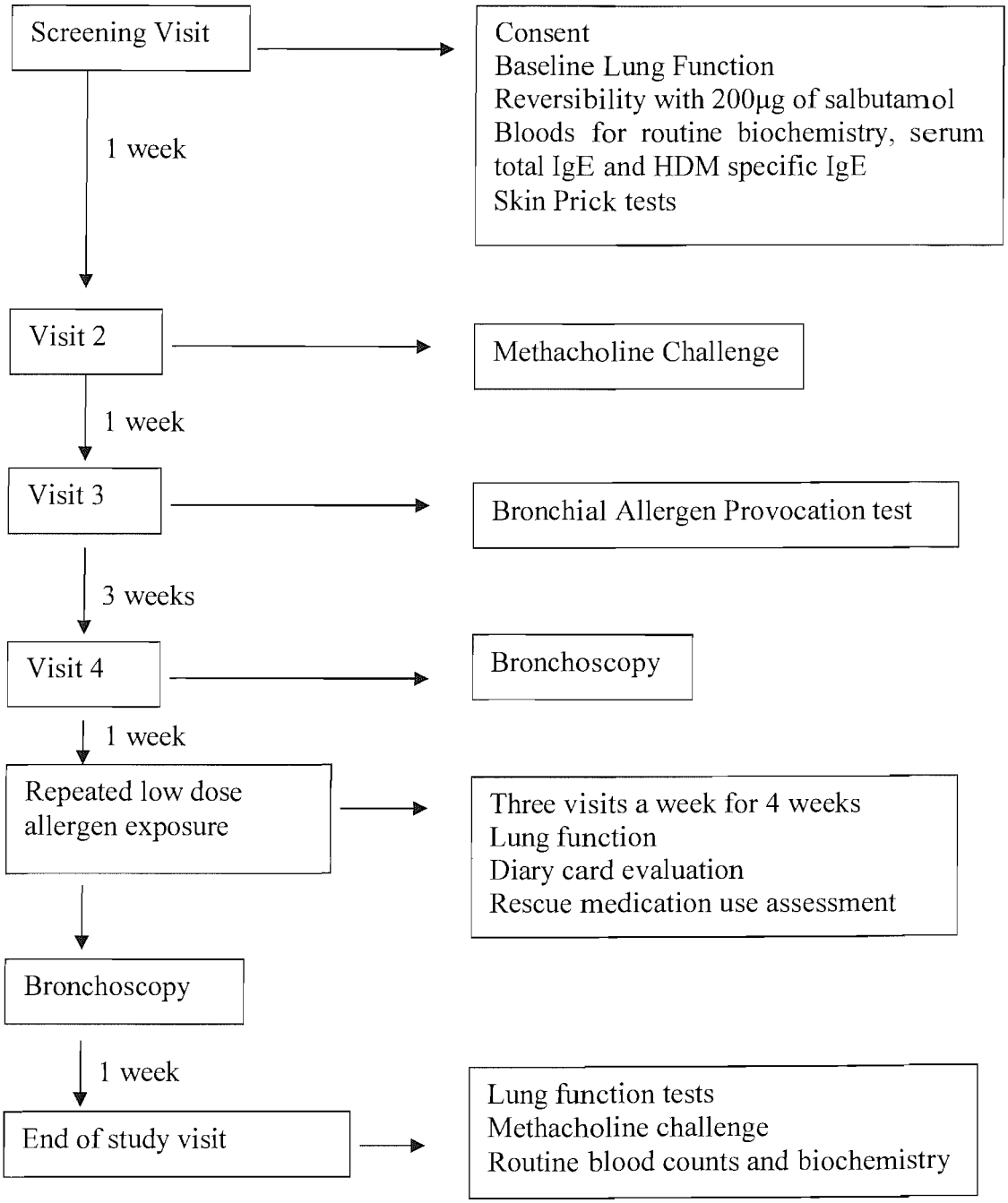
Adults aged between 18-55 years with a history of physician diagnosed asthma for at least one year were enrolled into this study from the departmental database. This study was approved by the local ethics research committee and informed written consent was obtained from all the participating subjects.

Table 3-1: Baseline characteristics of mild asthmatics recruited for repeated low dose allergen challenge

Subject	Age	Sex	FEV_1 Litres/ min	FEV_1 % pred	FEV_1 / FVC	SPT Mean diameter	PC_{20} mg/ml	IgE IU/ml	RAST
TBC	21	M	4.79	102.4	82.9	13	2.49	124	4
JMC	30	M	3.13	96.3	82.8	8.5	1.81	45	4
DLM	29	M	4.00	91.7	76.3	10.5	0.22	108	3
KS	37	M	3.95	99.7	79.3	13	0.9	5	3
MO	47	M	3.93	104.1	81.0	12.5	3.22	75	3
NZ	20	M	6.17	103.7	100.0	8.5	7.32	269	4
LH	32	M	3.68	88.7	75.1	12	1.97	120	4
MFR	22	M	4.34	104.3	86.1	15	5.38	-	-
KH	23	F	3.32	106.8	109.2	6.5	2.74	-	-
Mean	29.6	8M		99.7	85.9	11.1	2.04*	68.8*	
SEM	2.8	1F		2.1	3.8	0.9			

*Geometric mean

Figure 3-1: Study Plan



3.4.2. Study Design

The study design is outlined in Fig 3.1. Informed written consent was obtained on the screening visit. Baseline lung function measurements including FEV_1, FVC and flow/volume loop were performed. Bronchodilator reversibility was assessed with 200μg of salbutamol administered through a metered dose inhaler. Bloods were sent off for routine biochemistry, full blood counts, serum total IgE levels and HDM specific IgE levels. The HDM specific IgE levels were assessed by radio-allergen sorbant test (RAST). The inclusion criteria were physician diagnosed asthma of more than one year duration, asthma symptoms less than once per day on average, FEV_1 of more than 80% predicted, as needed (prn) bronchodilator as the only treatment required and an abnormal response (PC_{20}<8 mg/ml) on methacholine bronchial provocation test. Sensitivity to house dust-mite (HDM) was determined by skin prick test and a positive result was taken as at least 5mm average diameter above saline control. All the subjects were clinically stable and did not have any history of respiratory tract infection during the last 3 months.

3.4.2.1. Methacholine provocation test

Bronchial responsiveness was measured using methacholine challenge (FEV_1 response to incremental doses of inhaled methacholine). The method employed was modified from Chai et al (226) and discussed in section 2.5.1. The results were interpreted as methacholine provocative concentration 20 (PC_{20}) which was defined as the final cumulative methacholine dose required to produce a 20% decline in FEV_1 from the value after saline inhalation.

3.4.2.2. Allergen bronchial provocation tests

A standard bronchial allergen challenge test was performed with a standardised house-dust mite allergen extract as described in section 2.5.2. The cumulative PD20 (provocation dose to cause a 20% fall in FEV1) is expressed and calculated by using equation in chapter 2, section 2.5.2.

3.4.2.3. Fibreoptic Bronchoscopy

Bronchoscopy was performed three weeks after the allergen bronchial provocation test using a fibreoptic bronchoscope (Olympus, FB-XT20, Tokyo, Japan) in

accordance with the standard published guidelines (229, 230). Bronchoscopy was performed only if the subjects' baseline FEV_1 was ≥60% of predicted. Six bronchial biopsies were obtained from the sub-carinae of the 2nd and 3rd generation bronchi of the right lower lobe using FB15 alligator forceps. The biopsies were then placed in acetone for GMA processing as described in section 2.9.1.

3.4.2.4. Methodology

After the screening visit, subjects were asked to keep a home diary to monitor their PEF three times a day for a week and indicate any symptoms (Scale 0-5; appendix IV) of asthma and rescue medication use after their screening visit. Methacholine provocation test was performed to assess the BHR. At the end of this week a standard bronchial allergen challenge was performed with a standardised house-dust mite allergen extract. PD_{20} (The dose of allergen required to produce 20% fall in FEV_1) was calculated. After a week bronchoscopy was performed to obtain bronchial mucosal biopsies. The eligibility criteria included a methacholine response of < 8mg/ml and the presence of an early and late phase response to bronchial provocation test with HDM extract.

3.4.2.5. Inhalation visits

Once eligibility was established and baseline measurements obtained, the subjects attended the department for inhalation of a small dose of a standardised dust mite extract calculated to be one tenth of the allergen provocation dose (PD_{20} allergen) for that subject. This dose was chosen from earlier studies by Arshad and colleagues who had estimated the average dust mite allergen in air and in normal activity conditions and sleep (142). The extract was freshly diluted in 0.9% saline and administered through a nebuliser using a standard dosimeter (Spira, Electra 2, Spira Respiratory Care, Finland). Subjects were allowed to return home after 30 minutes. This procedure was repeated three times a week for a period of four weeks unless a clinically significant change in their asthma status was detected earlier. On each visit, spirometry was performed prior to the dust-mite inhalation dose of that day. At the end of the inhalation period methacholine provocation test was repeated followed by a repeat bronchoscopy.

3.4.2.6. Monitoring Patients

Subjects were monitored for any symptoms of asthma based on the peak flow readings, asthma symptom score and rescue medication use. They were provided contact numbers of the study doctor and the study nurse in case they noted any change in their asthma control. Each subject enrolled into the study maintained a home diary throughout the study. Subjects were provided with an asthma action plan based on their PEF readings and were also asked about their asthma control during each visit. This monitoring ensured that the risks due to repeated inhalation of allergen in the study subjects were kept to a minimum.

3.4.3. Statistical analyses

Data were analysed for significance using non-parametric tests. Results are expressed as median (inter quartile ranges). The data were entered into s spread sheet (Microsoft Excel, Office 2002) and analysed using SPSS 11.0 for Windows. The Wilcoxon's signed rank test was used for paired data comparisons. Values of <0.05 were accepted as statistically significant.

Correlations between physiological variables and immunohistochemistry data were performed using Pearson's correlation test. All tests were 2 sided and conducted at the 5% significance level.

3.5. Results

Nine subjects were recruited for the low dose allergen challenge study and comprised 8 male and 1 female. The mean age was 29.6 (2.8) and none of the subjects were on inhaled corticosteroids for their asthma control (Table 3-1).

3.5.1. Pulmonary function after repeated low dose allergen exposure

3.5.1.1. Changes in lung function

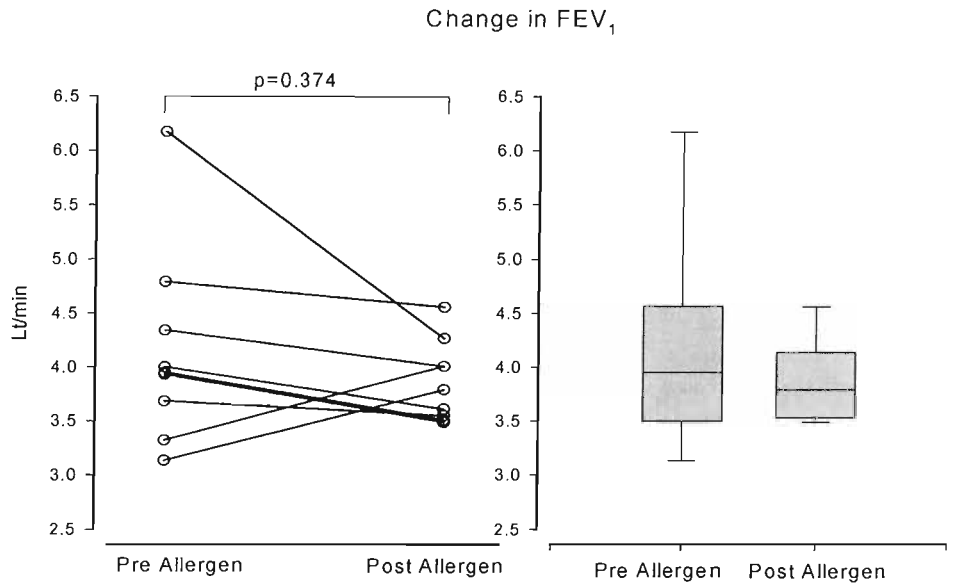
Prior to low dose allergen challenge the FEV_1 was 4.15L/min and after allergen exposure changed to 3.87 L/min (p=NS). Similarly, the PEF changed from 577 L/min to 527 Lt/min (p= NS) and the forced FVC from 4.86 L/min to 4.59 L/min (p= NS)

(Fig 3.2 a, b and c) following repeated low dose allergen inhalation. However, these changes failed to reach statistical significance.

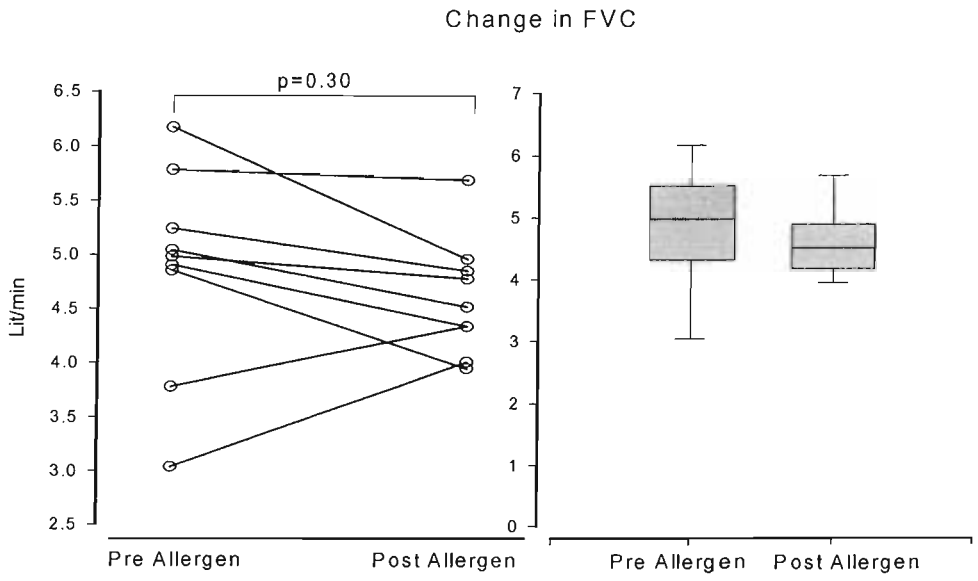
Figure 3-2: Changes in lung function before and after low dose allergen exposure. (a) Change in FEV1 (b) Change in FVC and (c) Change in PEF

FEV_1 and FVC are shown as litres/min and PEF is shown as litres. A p value of <0.05 was considered significant

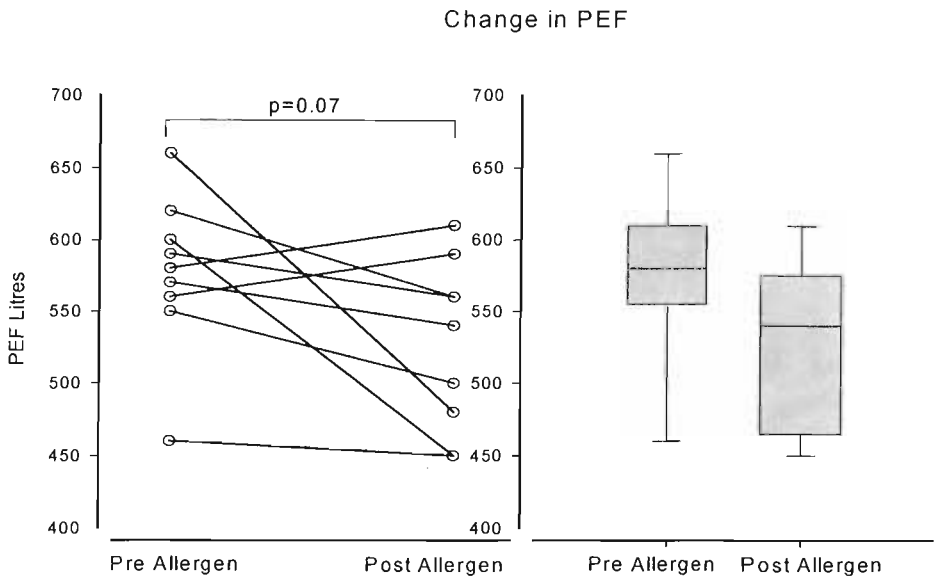
(a)



(b)



(c)



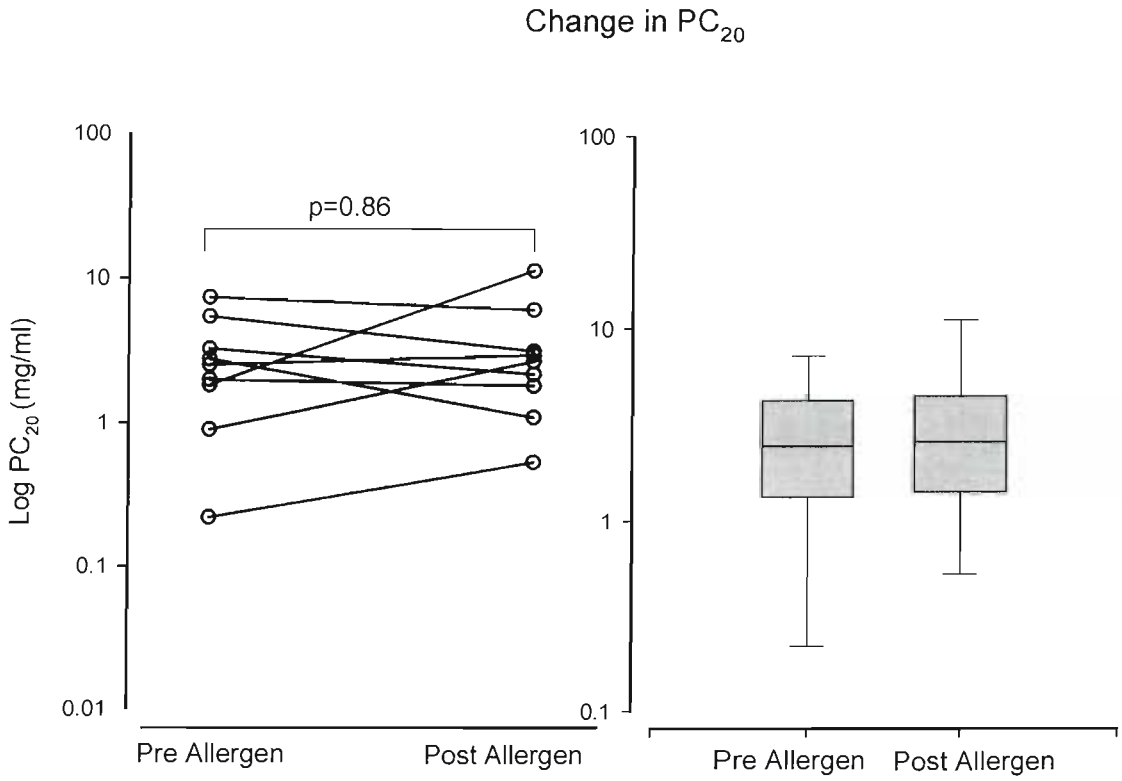
3.5.1.2. Changes in airway hyperresponsiveness

The methacholine PC_{20} did not show statistically significant changes following repeated low dose allergen inhalation (Fig 3.3). The methacholine PC_{20} changed from 2.49 mg/ml (1.36-4.3) to 2.63 mg/ml (1.44-4.52), p=0.86.

There was no increase in rescue medication use in any of the subjects enrolled in the study however bronchoscopy post allergen challenge could be performed only on 7/9 subjects. This was due to the upper airway sensitivity experienced by 2 subjects. Hence bronchial biopsies were obtained from seven subjects and the immunohistochemistry results discussed are for 7 subjects.

Figure 3-3: Change in methacholine PC_{20} following repeated low dose allergen exposure.

Values on the Y axis were log transformed. A p value of < 0.05 was considered significant



3.5.2. Immunohistochemistry staining of bronchial biopsies

To characterise the numbers of inflammatory cells in the bronchial mucosa of mild asthmatics before and after low dose allergen exposure, the bronchial biopsies were processed in GMA resin, and 2μm sections cut and stained with immunohistochemical markers for resident inflammatory cells as described in the methods section (Section 2.9).

3.5.2.1. Expression of inflammatory cells in Bronchial biopsies

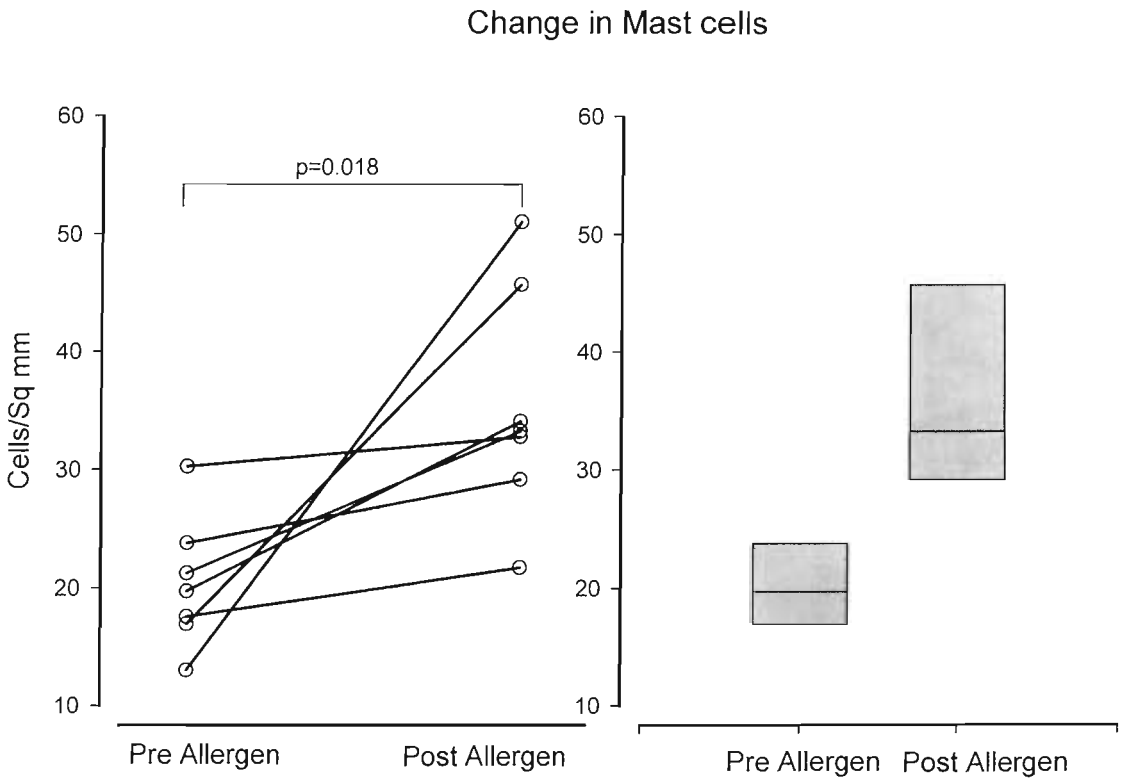
The studies confirmed the presence of CD3 T lymphocytes; neutrophil elastase (NE^+) neutrophils; $EG2^+$ eosinophils, and $AA1^+$ mast cells in the mucosa and submucosa of mild asthmatics. Following low dose allergen inhalation there were no significant changes in the cell numbers apart from the mast cell numbers which reached statistical significance (Table 3.2). The mast cell numbers increased from 19.8/sqmm (13.0-30.3) to 33.3/sqmm (21.8-51.1); p=0.018 (Fig 3.4)

Table 3-2: Changes in inflammatory cells before and after low dose allergen exposure. Results are expressed as medians (inter quartile ranges), n=7.

Inflammatory Cells	Submucosa [cells/sq mm] Median (range)		Mucosa [cells/sq mm] Median (range)	
	Pre Allergen	Post Allergen	Pre Allergen	Post Allergen
Lymphocytes ($CD3^+$)	39.0 (18.8-58.1)	64.0 (47.6-71.8)	0.00 (0-0.1)	0.00
Eosinophils ($EG2^+$)	10.5 (2.4-11.8)	3.3 (1.1-7.2)	0.00 (0-1.2)	0.00 (0-1.6)
Neutrophils (NE^+)	22.6 (16.7-61.3)	20.1 (10.4-59.5)	0.00	0.00 (0-0.3)
Mast Cells ($AA1^+$)	19.6 (17.3-22.6)	33.3* (31.0-39.9)	0.6 (0-4.4)	0.00

* $p=0.018$

Figure 3-4: Changes in sub-mucosal mast cell numbers in the bronchial biopsies before and after repeated low dose allergen exposure. A p value of < 0.05 was considered significant

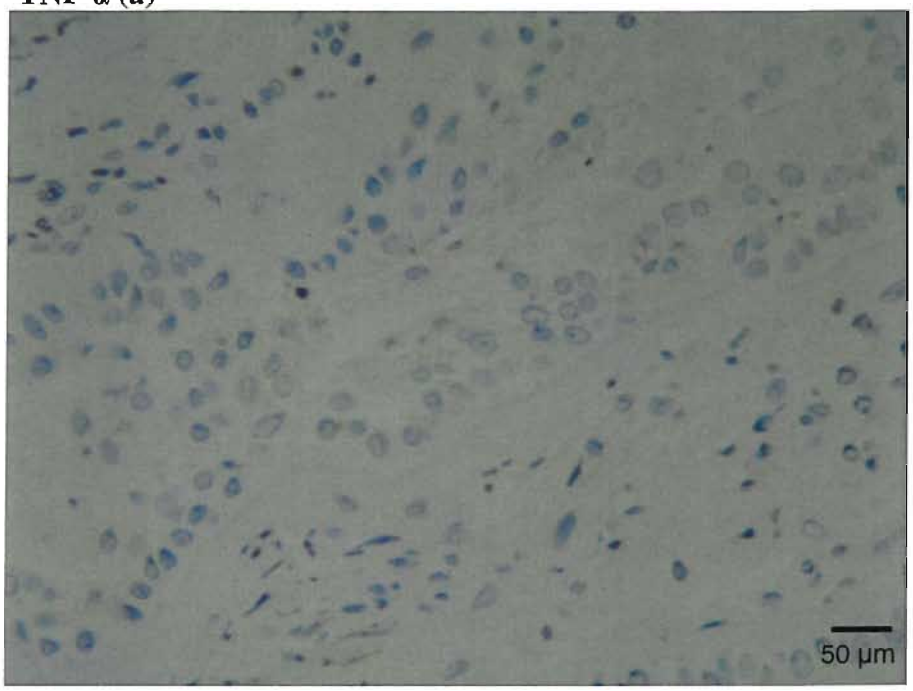


3.5.2.2. Expression of TNF-α in bronchial biopsies

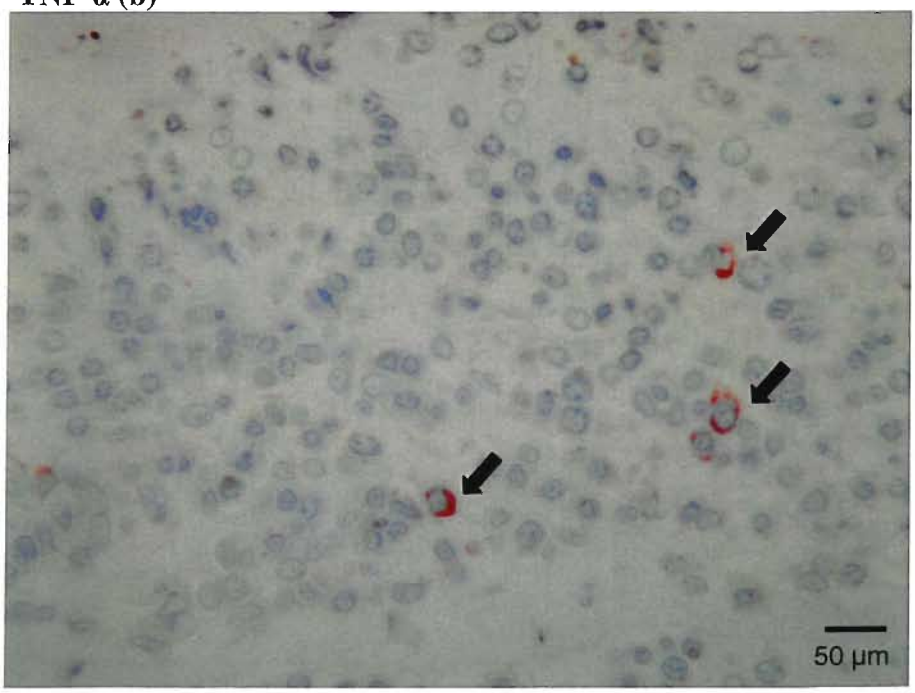
To assess the expression of TNF-α and adhesion molecules ICAM and VCAM, the bronchial biopsies of mild allergic asthmatic subjects were stained with monoclonal antibodies for TNF-α. TNF-α had a characteristic staining pattern on the surface of a subset of cells (Fig 3.5).

Figure 3-5: Immunostaining of bronchial biopsy section before (a) and after (b) low dose allergen exposure for TNF-α (magnification factor,x40). TNF-α positive cells stain pink and are shown by arrows.

TNF-α (a)

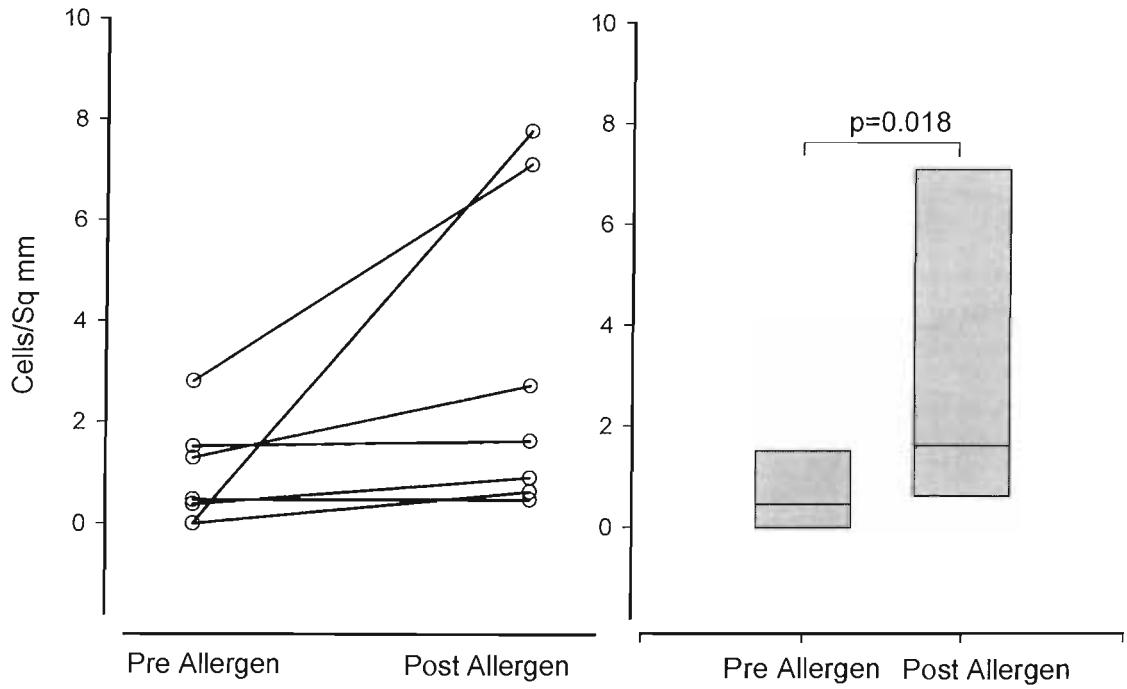


TNF-α (b)



Cells staining for TNF-α were up regulated following low dose allergen exposure. TNF-α positive cells increased from 0.46/sq mm (0.19-1.40) to 1.63/sqmm (0.76-4.91), p=0.018 following allergen exposure in the submucosa of the bronchial biopsies (Fig 3.6). There was no significant change in the TNF-α staining cells in the bronchial mucosa.

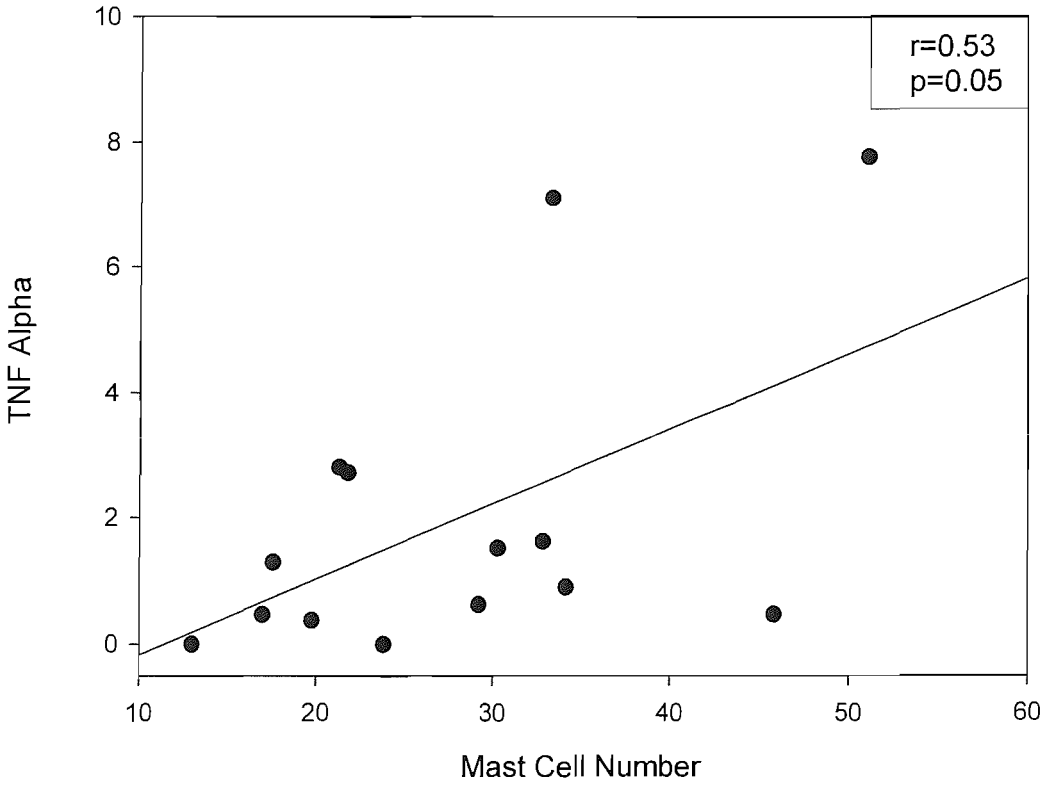
Figure 3-6: TNF-α expression in bronchial biopsies before and after low dose allergen exposure



3.5.2.3. Relationship between TNF-α and mast cells in bronchial biopsies

Co-localisation was not performed on the bronchial biopsy samples and hence it was not possible to get direct evidence for the source of TNF-α. As mast cells are the major source of TNF-α correlation were performed (Fig 3.7). TNF-α levels positively correlated with mast cell numbers in the bronchial biopsies with a Pearson's correlation of 0.53 (p= 0.05). While there was a weak positive correlation approaching statistical significance, in order to be able to show the cells were definitively mast cells, needs co localisation.

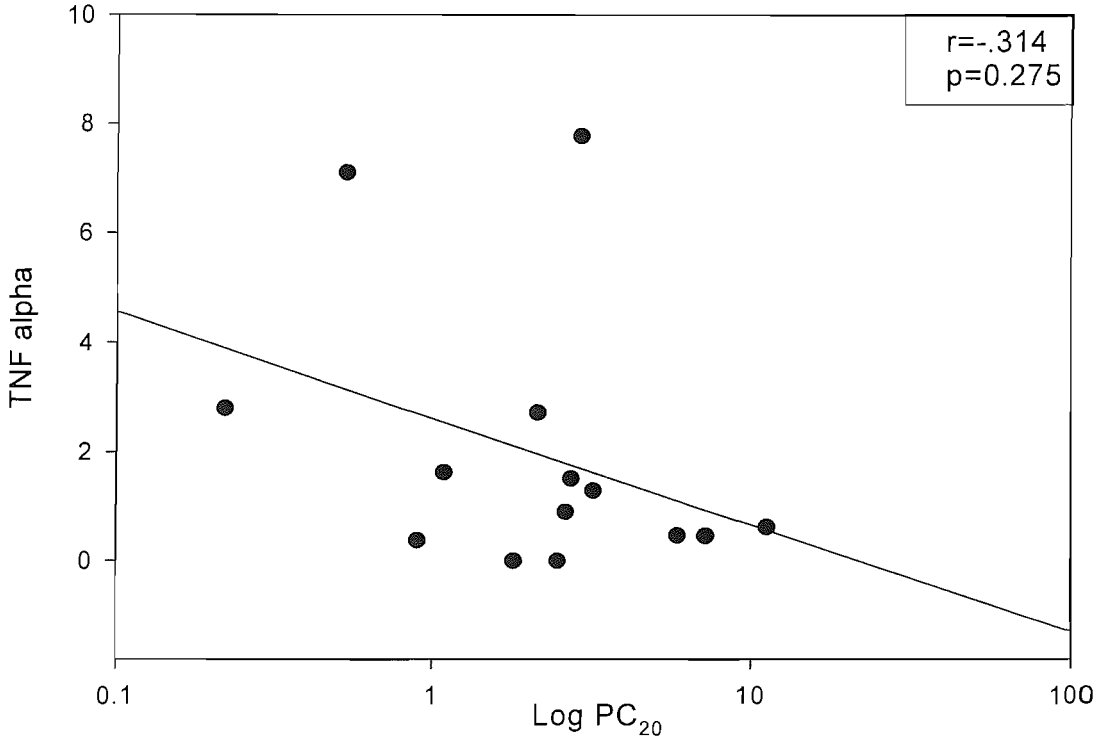
Figure 3-7 : Correlation between TNF-α and mast cell numbers in the bronchial biopsies of allergic asthmatics.



3.5.2.4. Relationship between TNF-α and BHR

There was no significant correlation with the TNF-α levels and BHR, Pearson coefficient 0.314, p=0.275 (Fig 3.8).

Figure 3-8 : Correlation between TNF-α and BHR



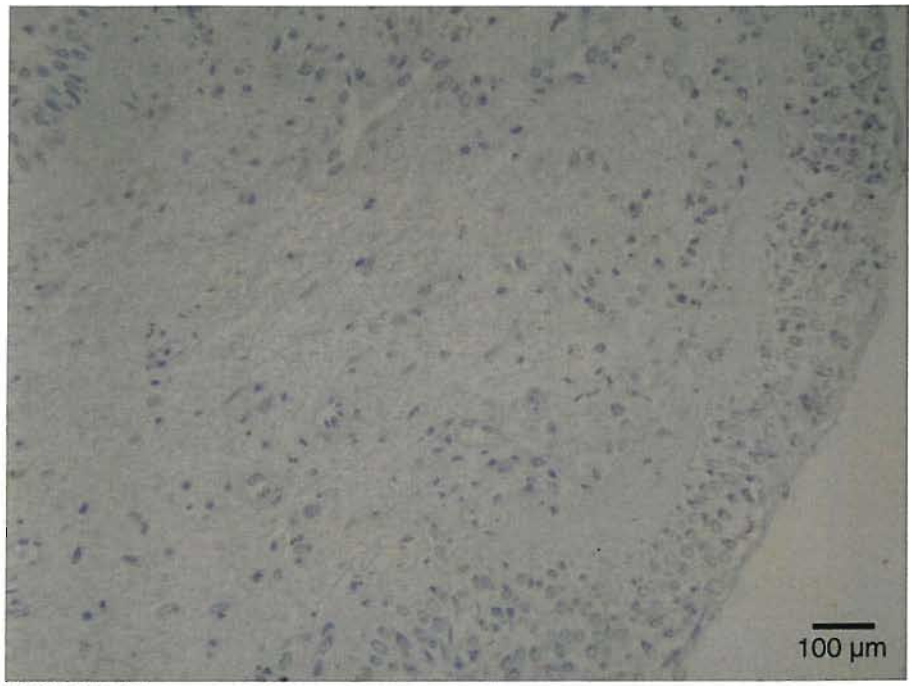
3.5.2.5. Expression of adhesion molecules VCAM-1 and ICAM-1

The expression of VCAM and ICAM was analysed as percentage staining of the endothelial cell marker EN4. While EN4 stains the entire vascular endothelium in the bronchial biopsies, VCAM and ICAM stain only components of endothelium where these adhesion molecules are expressed (Fig 3.9 and 3.10).

There was no significant difernce in the staining for the vascular endothelial marker EN4 (p= o.09) (Fig 3.11). The expression of adhesion molecules VCAM and ICAM was up regulated after low dose allergen exposure (Fig 3.11). VCAM was up regulated from 0% to 9.72% (5.33-71.28); p=0.046 and this attained statistical significance. ICAM levels increased from 48.49% (32.77-59.02) to 85.63% (67.31-100.0) in the submucosa of the bronchial biopsies. The results showed a trend towards statistical significance, p=0.07.

Figure 3-9: Immunostaining of bronchial biopsy section before (a) and after (b) low dose allergen exposure for VCAM (magnification factor x 20). Endothelium staining for VCAM is shown by arrows.

VCAM (a)



VCAM (b)

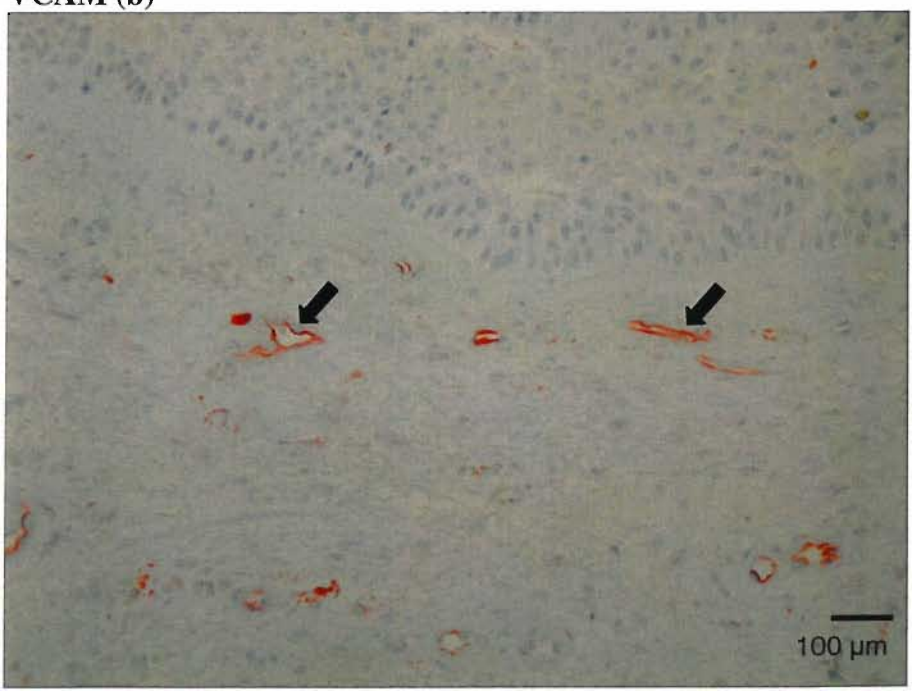
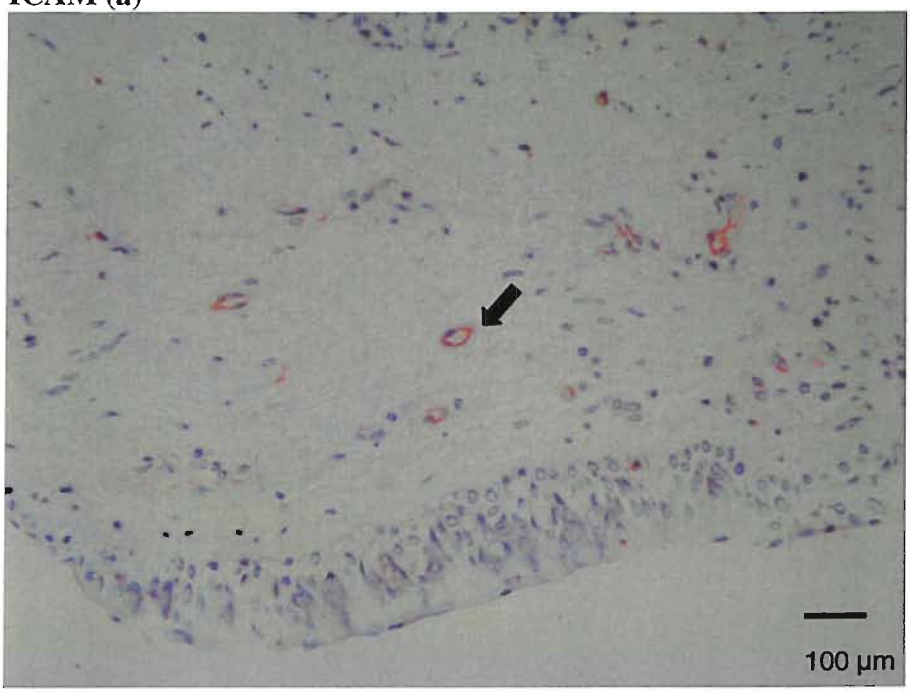


Figure 3-10: Immunostaining of bronchial biopsy section before (a) and after (b) low dose allergen exposure for ICAM (magnification factor x 20). Endothelium staining for ICAM is indicated by arrows

ICAM (a)



ICAM (b)

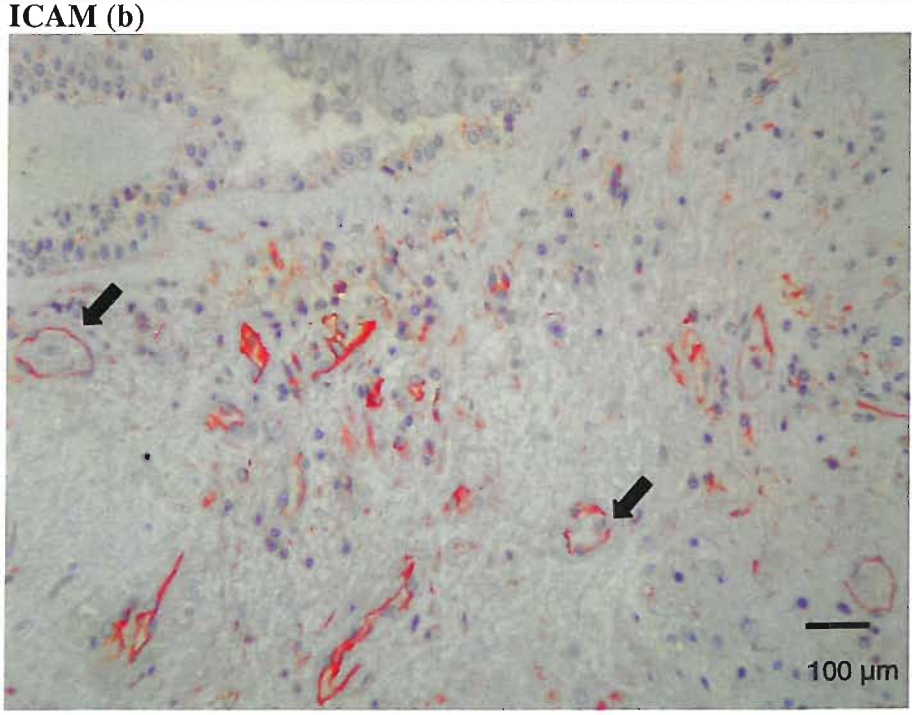
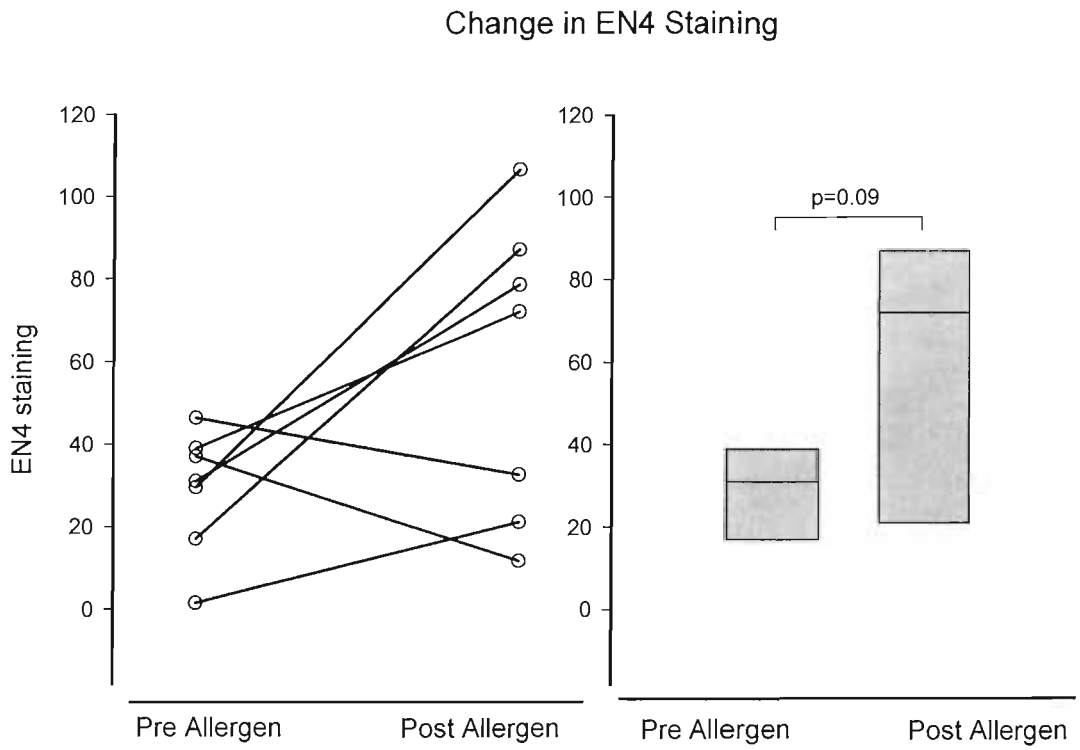
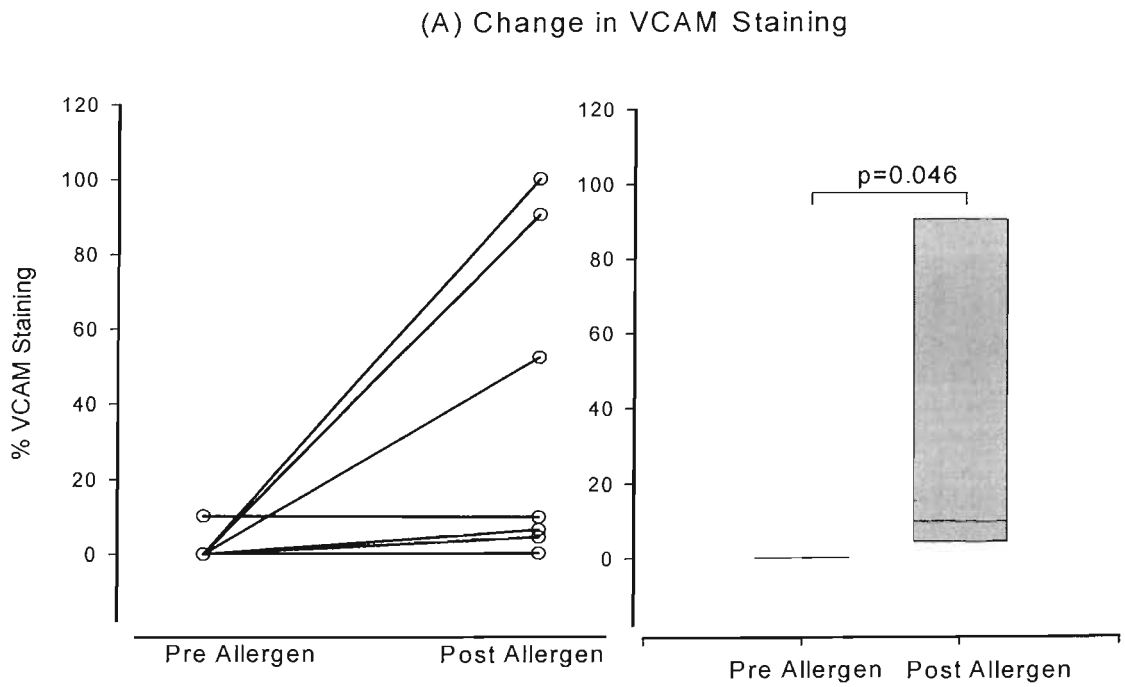
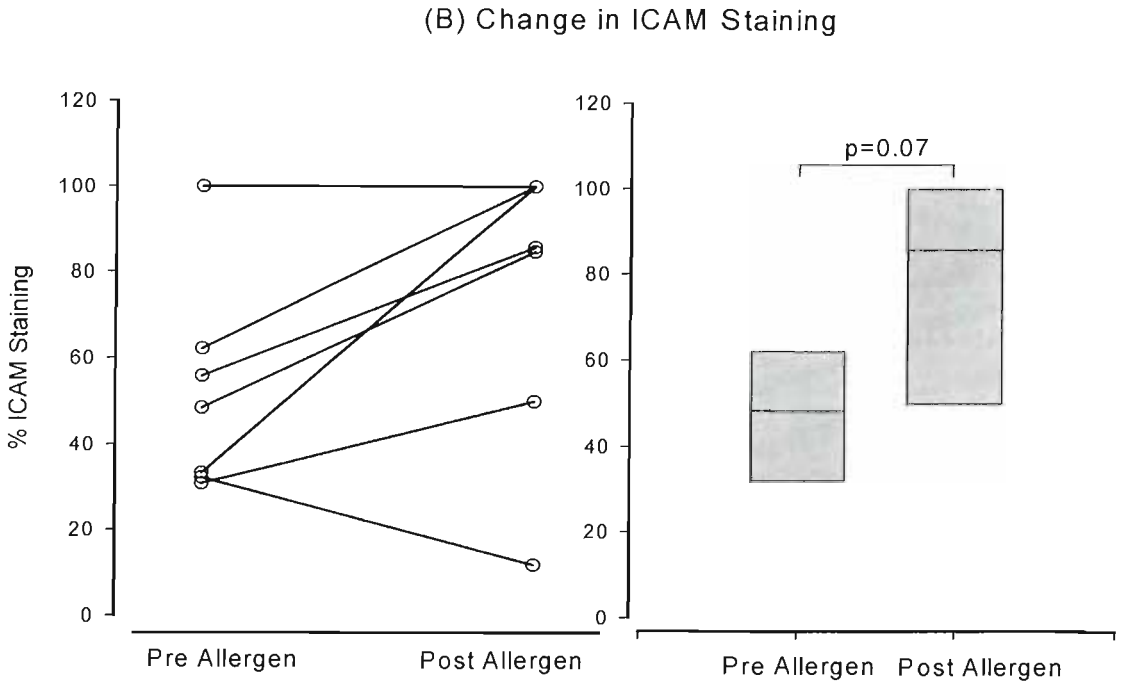


Figure 3-11: Staining for EN4 and adhesion molecules VCAM and ICAM before and after low dose allergen exposure



(A) % staining for VCAM and (B) % staining for ICAM-1





3.6. Discussion

Repeated low dose allergen exposure challenges tend to mimic patients natural exposure to environmental allergens in a controlled situation. An immediate response to allergen challenge that occurs naturally or during experimental conditions resolves rapidly and does not explain the pathophysiological features of chronic asthma. Late asthmatic responses with associated inflammatory sequelae are more relevant in the pathogenesis of chronic asthma. The likelihood of late asthmatic responses are primarily determined by the degree of sensitisation (248). In HDM sensitive asthmatics repeated administration of low doses of allergen are likely to induce a cellular milieu similar to that seen in clinical disease (133). This model could serve as an experimental method to assess therapeutic modalities in asthma (242).

The dosing regimen and the duration of allergen exposure have not been standardised for low dose allergen challenge. Various studies have used different dosing schedule for repeated low dose allergen challenge. In this study the dosing schedule was selected from the study by Arshad et al (142). However house dust could not be standardized for its allergen content and accurate estimation of the dose would be difficult and it was believed that this experimental model with repeated small dose inhalations mimics the natural exposure more closely than the acute bronchial

challenge. In the study by Arshad and colleagues, the authors found that following repeated low dose allergen exposure the subjects FEV_1 dropped by >10% and the PC_{20} reduced by 2-3 fold. While this study estimated the dose of allergen based on the mean airborne levels of HDM allergen other low dose allergen exposure studies have devised the dose based on the PD_{20}. The study by Palmqvist and colleagues administered 25% of the PD_{20} for 4 days in a double blind fashion and found that no overt or prolonged airflow obstruction was induced by low dose allergen in this group of asthmatics (143). Another study with low dose allergen provocation used a dosing schedule involving 10% of the PD_{20} of allergen daily for seven days (249). Due to the non-availability of a standardised protocol for low dose allergen exposure a dosing schedule of 10% of PD_{20} three times a week for 4 weeks to simulate chronicity of allergen exposure was chosen.

In the present study there was no change in the pulmonary function (FEV_1, FVC and PEF) following low dose allergen exposure. There was also no worsening of symptoms during the 4 week exposure period. This study demonstrates that repeated low dose allergen exposure can take place asymptomatically and yet promote features of airway inflammation as evidenced by changes in the mast cell numbers and expression of TNF-α and adhesion molecules. Many studies with low dose allergen exposure have reported mild worsening of asthma control with a reduction in the FEV_1 (143, 239). However, as noted by Palmqvist and colleagues, the baseline lung functions prior to each low dose allergen exposure did not significantly deteriorate during the study week, implying no obvious long term airflow obstruction. Low dose allergen exposure induces an increase in bronchial reactivity (142, 143, 239, 249-251). In the present study there was no change in the BHR following low dose allergen exposure. However, similar findings have been reported by other investigators (242, 243). This difference could be possible since different studies use differing allergen dosage and dosing schedule. The non-availability of a standardised low dose allergen challenge model results in diverse changes in the lung function. However the absence of a change in bronchial reactivity does not imply absence of deterioration in the asthmatic inflammation. Levels of exhaled NO were increased in patients after repeated low dose allergen exposure in the absence of changes in the clinical parameters or BHR (242). In the present study there was no change in the bronchial reactivity or pulmonary function, but the airway mast cells, TNF-α and

adhesion molecules were significantly increased pointing towards worsening airway inflammation.

This experimental protocol provides a model to study the events in the airways before the development of symptoms. During this stage, the inflammatory response is low as compared to the repair processes occurring after the asthmatic bronchoconstriction (252, 253). An increase in the number of mast cells is reported in the bronchus of asthmatic patients even at an early stage of the disease (66, 254, 255). The possible role for mast cells in asthma is confirmed by studies performed in mast cell deficient mice which when challenged with antigen had a lower increase in airway reactivity when compared to mast cell replete mice (256). In asthmatic subjects, provocative dose of allergen could induce bronchoconstriction and increase bronchial mast cell numbers (62, 257, 258). In this study, the increased number of infiltrating mast cells following low dose allergen exposure may be considered as an early event since the subjects did not develop asthma symptoms or BHR. This emphasises a possible early role for mast cells in the pathophysiology of asthma. However the absence of symptoms and changes in BHR following low dose allergen challenge despite an increase in mast cells in the bronchial biopsies could be due to the fact that asthma is a complex disease and a mere increase in mast cells numbers alone could not lead to the clinical manifestation of asthma. Then there is also the question as to whether inflammation and symptoms are independent of each other. It is possible that the mast cells are in the wrong place to induce BHR. It has been shown that mast cells infiltrate the airway smooth muscles in asthma and this is associated with disordered airway function (67). Furthermore, IL-4 and IL-13 were co-localised to mast cells with the airway smooth muscle in asthmatic bronchial biopsies (259). This suggests a role for IL-4 and IL-13 in mast cell- airway smooth muscle interactions. Mast cells, but not T cells or eosinophils, localize within the bronchial smooth muscle bundles in patients with asthma but not in normal individuals or those with eosinophilic bronchitis and smooth muscle mast cell density correlates significantly with indices of bronchial hyperresponsiveness, and is likely to be an important factor determining the asthmatic phenotype (260). In the present study the bronchial biopsies did not have any airways smooth muscles and the mast cells seen were in the sub-mucosal layer.

BHR was assessed with methacholine inhalation, which acts directly on the airway smooth muscles causing bronchoconstriction. In the present study there was no change in the PC_{20} following repeated low dose allergen inhalation. We would have predicted to see a change in the BHR if adenosine was used instead of methacholine to evaluate the airway responsiveness. The view that adenosine responsiveness could be used as a specific marker of disease activity with a closer relationship to allergic airway inflammation than histamine or methacholine has been addressed in various clinical studies (261). Adenosine is a purine nucleotide which markedly enhances the release of histamine and other preformed mediators from immunologically primed mast cells (262-264). It is likely that the repeated low dose allergen challenge had primed the mast cells and an indirect stimulus like adenosine that exerts its effect primarily on inflammatory cells would have lead to bronchoconstriction.

The increase in mast cell numbers in the bronchial biopsies after low dose allergen exposure was associated with a parallel increase in the expression of TNF-α and adhesion molecules ICAM and VCAM. There was no change in the staining for EN4 the vascular endothelial marker suggesting no increase in the blood vessels in the airways. In allergic asthmatics local endobronchial allergen challenge leads to an increased inflammatory cell infiltrate that involves up regulation of ICAM-1 (258). ICAM-1 expression was significantly higher in asthma than in controls and suggests an important role for this adhesion molecule in the migration of inflammatory cells in patients with asthma(265, 266). *In vitro* studies with cultured human epithelial cell lines have shown that TNF-α increases the release of both sICAM and ICAM-1 (267). Human bronchial epithelial cell lines also constitutively express ICAM and TNF-α up regulated ICAM-1 expression (268). In addition biopsies of asthmatic bronchial wall have shown VCAM to be up regulated (269). The present study looked at the expression of both VCAM and ICAM in the submucosa of asthmatic airways before and after allergen challenge and the results are consistent with other published studies. Studies have shown that the expression of integrins on the surface of the endothelium cell, leads in sequence to the adhesion, initially of the neutrophils and then the lymphocytes and monocytes, as a response to the antigenic stimulus (270).

Mast cells are the major source of TNF-α in allergic asthma. Earlier studies from this department had co-localised TNF-α to mast cells in the bronchial biopsies and a seven

fold increase in the number of mast cells immunoreactive to TNF-alpha in the asthmatic biopsies suggesting that this cytokine is also up-regulated in this disease (66). In the present study there was a significant increase in the mast cell numbers in the sub-mucosa after low dose allergen exposure. Although the baseline sub-mucosal mast cell numbers were lower than reported from previous studies from this department the heterogenicity of the population of asthmatics could likely explain this difference (66, 68). Furthermore, this increase in both the TNF-α and the mast cell numbers following low dose allergen exposure was a consistent finding in all the subjects (Fig 3.4 and Fig 3.6) suggesting the possible origin of TNF-α from mast cells. There was also a weak positive correlation between the sub-mucosal mast cell numbers and the staining for TNF-α (Fig 3.7) indicating mast cells to be a likely candidate for increased TNF-α expression following low dose allergen exposure. Previous studies with low dose allergen exposure have shown increased mast cells in the bronchial biopsies of asthmatic subjects (271). In view of the multiple parameters being examined there is a possibility that the increase in mast cells could be a chance finding but despite the sample size being 7, and all the subjects had an increase in the mast cell numbers in the bronchial biopsies over their baseline values this is unlikely. However, this is the first study to show an increase in TNF-α expression in the bronchial biopsies after low dose allergen exposure associated with a parallel increase in the mast cells.

TNF-α has been implicated in mediating BHR in patients with asthma (167). Thomas and colleagues showed a significant increase in methacholine responsiveness in normal subjects after inhaling nebulised TNF-α (272). In a recent study Halasz and colleagues evaluated the role of TNF-α in bronchial reactivity (273). They demonstrated significant elevation in serum concentrations of TNF-α, TNFRI, IL-4 and ECP levels in the hyper reactive group when compared to the non-reactive group. The airway reactivity also correlated with the serum TNF-α and ECP levels suggesting a role for this cytokine in the pathophysiology of BHR. In the present study, there was weak negative correlation between TNF-α levels and PC_{20} following low dose allergen exposure indicating TNF-α as a possible candidate mediating BHR. One possible explanation for the weak correlation could be the small sample size used for the evaluation and secondly the number of TNF-α positive cells were low in this group of subjects examined which could have had an effect on the correlation

This present model to look at the changes in airways in asthma has its own drawbacks. Chronic exposure to allergens could prime the response to acutely encountered pro-inflammatory stimuli potentially facilitating an asthma exacerbation but the duration of allergen exposure and the dose of allergen to induce the priming are not clear. Furthermore, this model cannot be tested in subjects at the severe end of the spectrum in view of the constraints, patient safety and ethical issues. This was a difficult study to conduct in view of the repeated visits to the hospital, multiple bronchoscopies, allergen provocation testing and the repeated low dose allergen exposure over a period of four weeks and the possibility of asthma exacerbations looming large following allergen inhalation. Hence, the sample size for this study was small. Exposure to other allergens during the study was also a potential confounding factor. Exposure to house dust mite on top of the experimental repeated low dose allergen challenge could make differences in the dose of allergen exposes and one way of getting around this issue would have been to perform the challenge with a non perennial allergen like grass. Furthermore bronchoscopies could not be performed in two subjects due to upper airway sensitivity preventing the second bronchoscopy to obtain tissue samples. This could have had an effect on the power of the study and hence played a significant role in the statistical significance of the data. We would ideally like to compare these results with a sham treated control group but the difficulty in recruiting patients, the demanding nature of the study, the multiple visits to the hospital and the multiple bronchoscopies involved in this study made it difficult and gruelling for us to enrol more subjects for this study. Despite these drawbacks the low dose allergen exposure model has provided some interesting information.

This study has shown that repeated low dose allergen exposure can increase airway inflammation without significant changes in airway responsiveness, causing airflow obstruction or inducing asthma symptoms in patients with mild atopic asthma. In the absence of a control group and the small sample size there is a possibility that the results of this study can be open to speculation. Despite these drawbacks, the findings suggest that allergen induced deterioration of airway inflammation can be silent which may possibly have implications in the monitoring and management of asthma. This study suggests that mast cells and TNF-α may have a role in the maintenance and deterioration of airway inflammation in patients with mild asthma. As this model

could not be followed up to evaluate the inflammatory responses in severe asthma an *in vitro* model involving bronchial explants would be more suitable to assess the effect of allergens and provide an opportunity to try newer therapeutic modalities which could be potentially harmful *in vivo*.

4. Effects of blocking TNF-α on bronchial biopsies of moderately severe asthmatics in an explant culture system

4.1. Introduction

Chronic airway inflammation is a crucial component of asthma and characteristically involves T-cells, eosinophils, macrophages/monocytes and mast cells. Various cytokines are involved in modulating the airway inflammation in asthma. These cytokines promote the recruitment, development, survival and activation of inflammatory cells into the asthmatic airways (274).

TNF-α is an essential cytokine in the development of an inflammatory response in various diseases including asthma. Acute allergen exposure is associated with increased TNF-α expression in the BAL and bronchial biopsies of asthmatics (185). Levels of TNF-α were elevated during asthma exacerbations and this was associated with increased expression of adhesion molecules VCAM and ICAM-1 (186, 187). We have earlier shown that repeated low dose allergen exposure was associated with up regulation of TNF-α in the bronchial biopsies. This was accompanied by an increase in the expression of adhesion molecules. While these changes in TNF-α occurred in the absence of any worsening of lung function it is possible that TNF-α levels are unrelated to symptoms or that inflammation precedes the development of symptoms in asthma. The latter explanation seems more likely as in mouse models of asthma the administration of TNF-α to the airways induces airway hyperreactivity and this effect was blocked by pre treatment with TNF-α blocking monoclonal antibodies (131). The former results have also been reproduced in normal human volunteers and in mild asthmatics (272) but the effects of TNF-α blockade in human subjects have not been evaluated. It is believed that TNF-α increases BHR by the recruitment of inflammatory cells into airway smooth muscle and via a direct effect involving calcium homeostasis (80, 272). Since TNF-α appears to play an important role as a mediator in asthma, it is important to evaluate the effects of blocking this molecule as a potential therapy and to determine mechanisms.

Blocking the effects of TNF-α by soluble receptor and monoclonal antibody has been used as a therapeutic option in patients with severe refractory rheumatoid arthritis and inflammatory bowel disease. Blocking the effects of TNF-α in mouse models of

toluene diisocyanate induced asthma prevented airway hyperreactivity to methacholine and reduced the neutrophil numbers in BAL fluid by 80% (275). The overall effect of inhibiting the effects of TNF-α in mouse models of asthma is a decrease in antigen presentation, T cell activation, inflammatory cytokine production and reduced recruitment of inflammatory cells (276).

Earlier studies from our department have shown the usefulness of explant culture methods. This has been evaluated and validated in previous studies. We had shown in explants from moderately sever asthmatics co-stimulation by both CD80 and CD86 is essential for allergen induced cytokine production and CD86 is the principal co-stimulatory molecule (33). Furthermore, T-Lymphocyte chemotactic activity release in moderate asthmatic airways, in contrast to mild asthmatic airways, is not dependent on CD28/B7 co-stimulation and does not involve IL-16 (36). We chose this model to evaluate and examine the effects of antagonising TNF-α as this has prooved its usefulness in earlier experiments. While the effects of blocking TNF-α in murine models have been studied, the effect of inhibiting TNF-α in a human model of allergic inflammation has not been investigated. We used *in vitro* bronchial explant cultures obtained as biopsies from moderately severe asthmatic patients to assess the role of TNF-α blocking strategies in asthma.

4.2. Objectives

1. To assess the expression of adhesion molecule ICAM-1 and cells staining for TNF-α in the presence or absence of a PEGylated blocking monoclonal antibody against TNF-α (CDP 870) in bronchial biopsies from moderately severe allergic asthmatics cultured in the presence of *Der p* in an *in vitro* explant tissue culture system

2. To assess the ability of CDP 870 to inhibit the production of cytokines TNF-α, IL-8, IL-5 and IL-1β in response to stimulation with *Der p* allergen in the explant culture system.

4.3. Methods

4.3.1. Subjects

Twelve moderately severe allergic asthmatic subjects (9 male and 3 female) with a mean (± SEM) age of 33.6 (± 3.7) years were recruited for bronchoscopy to obtain bronchial biopsies (Table 4.1). All subjects had a history compatible with asthma with a mean predicted FEV_1 of 87.4 (± 3.2) %, bronchodilator reversibility of at least 12% and a geometric mean PC_{20} methacholine of 3.65 (2.45-8.26) mg/ml. All the subjects were atopic as determined by positive skin prick testing to a panel of common aero-allergens (ALK, Abello, Horsholm, Denmark), and were sensitised to house dust mite allergens (*Der p*). They had a geometric mean skin prick test diameter to *Der p* of 6.47 mm. All the subjects were receiving regular inhaled corticosteroids (mean ± SEM daily dose of 658 ± 97 μg per day of beclomethasone dipropionate or equivalent) and short acting β2 agonists as required for symptom relief. One subject was on maintenance therapy with an inhaled long acting β2 agonist.

Table 4-1 Baseline Characteristics of the subjects

Subjects No	Age/Sex (yrs)	SPT to HDM (mm)	Dose of inhaled steroids (mcg)	Baseline FEV_1(L)	Predicted FEV_1(%)	Baseline FVC (L)	PC_{20} (mg/ml)
01	33/M	7	600	2.46	69.9	3.22	9.13
02	42/M	10.5	500	4.36	108.2	4.93	4.94
03	58/M	5.5	400†	2.6	77.6	5.41	2.51
04	57/M	5.5	1000	3.21	89.2	3.8	5.62
05	20/F	6.5	800	3.05	89.2	3.55	11.28
06	29/F	6	400	3.22	97.3	3.62	7.97
07	32/M	6	700	3.22	83.6	3.62	2.26
08	22/F	4.5	1500	2.25	74.8	2.81	0.42
09	26/M	5.5	400	4.64	97.7	6.02	3.97
10	21/M	8	400	4.23	91.2	5.02	7.97
11	28/M	7.5	800	2.91	91.5	3.5	0.29
12	35/M	7	400	2.92	78.2	4.08	11.14
Mean	**33.6**	**6.25***	**658**	**3.25**		**87.4**	**4.13**
3.65*							
SEM	**3.7**		**96**	**0.22**	**3.2**	**0.28**	

* Geometric mean

† On regular long acting β2 agonist 2puffs twice daily

Written informed consent was obtained prior to inclusion and the study was approved by the combined Southampton University and Hospitals ethics committee. Subjects withheld their inhaled corticosteroids for a minimum of one week prior to bronchoscopy and bronchial biopsy to optimise inflammatory responses in the bronchial tissue culture. If subjects were on long acting β2-agonists they were withheld for a minimum of 48 hours prior to bronchoscopy. The study plan is shown in Fig 4.1. Subjects were provided with a peak flow diary to monitor PEF for any deterioration of their asthma symptoms and rescue medication use. A physician was available 24/7 on the telephone to deal with any worsening of their asthma symptoms.

The methods section gives a detailed account of the bronchoscopy and endobronchial biopsy procedure (Section 2.7), the bronchial explant tissue culture (Section 2.8),

immunohistochemistry (Section 2.9) and ELISA analysis for cytokine production (Section 2.10). Briefly, bronchoscopy was performed under sedation (midazolam) and local anaesthesia (1% lignocaine). Six biopsies were obtained and transferred into a transport medium (AIMV, Life Technologies, Paisley, UK). The subjects were observed for 4 hours following bronchoscopy and later discharged home. They were provided with a contact number in case they had to speak to the doctor. Subjects were requested to resume their regular medications following bronchoscopy. The subjects were followed-up a week after the procedure to ensure that there is no worsening of their asthma and they did not have any complications.

Two bronchial biopsies were cultured under each condition to reduce interference due to tissue heterogeneity. The culture conditions were - Medium (500 µl; AIM V, Life Technologies, Paisley, UK); Medium + *Der p* extract (5,000 U/ml or 0.35 µg/ml; ALK, Horsholm, Denmark); Medium + *Der p* extract (5,000 U/ml or 0.35 µg/ml; ALK, Horsholm, Denmark) + CDP 870 (10 ng/ml) (Fig 4.2). CDP 870 (Celltech Laboratories, Slough, Berkshire, UK) is an engineered anti-TNF-α antibody fragment linked to a polyethylene glycol tail (Fig 4.3). CDP 870 comprises a Fab fragment of a humanized monoclonal antibody which is a potent neutralizer of TNF-α. The Fab fragment retains high affinity and potency, but lacks the Fc portion of the parent IgG_4 antibody. The site-specific addition of two molecules of polyethylene glycol to the antibody fragment increases the plasma half-life to approximately 2 weeks. CDP 870 was supplied by Celltech Laboratories, Berkshire, UK and has been evaluated in patients with rheumatoid arthritis and Crohn's disease (208, 277).

Figure 4-1: Study Plan

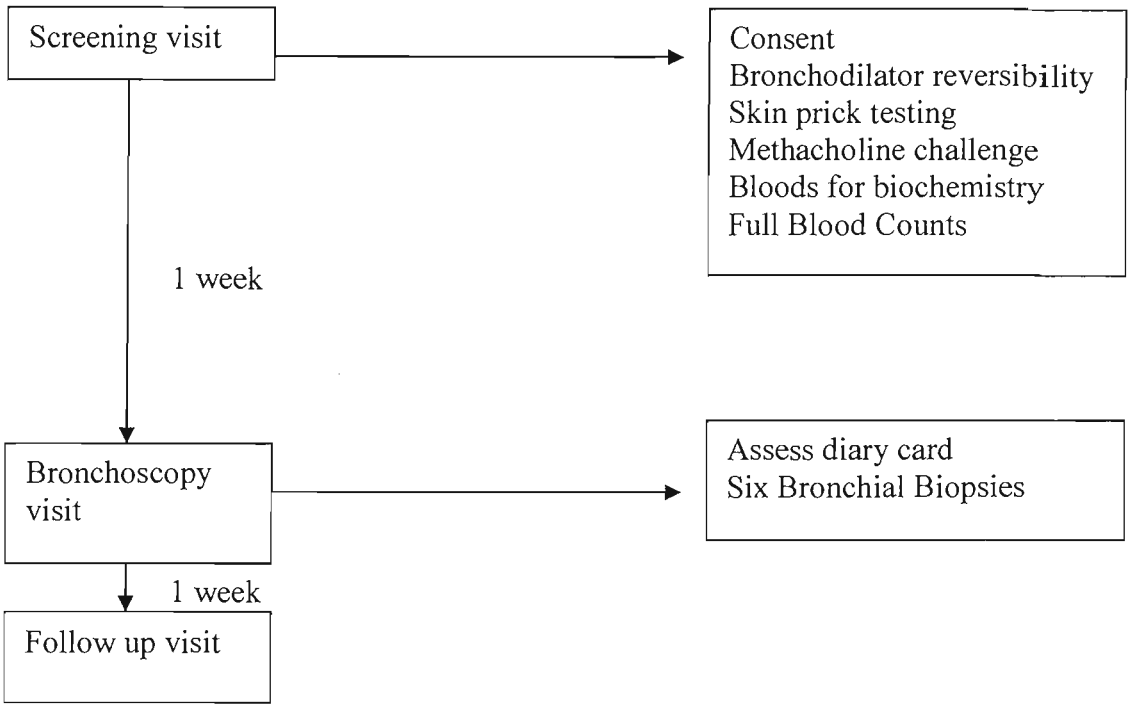


Figure 4-2: Explant culture plates

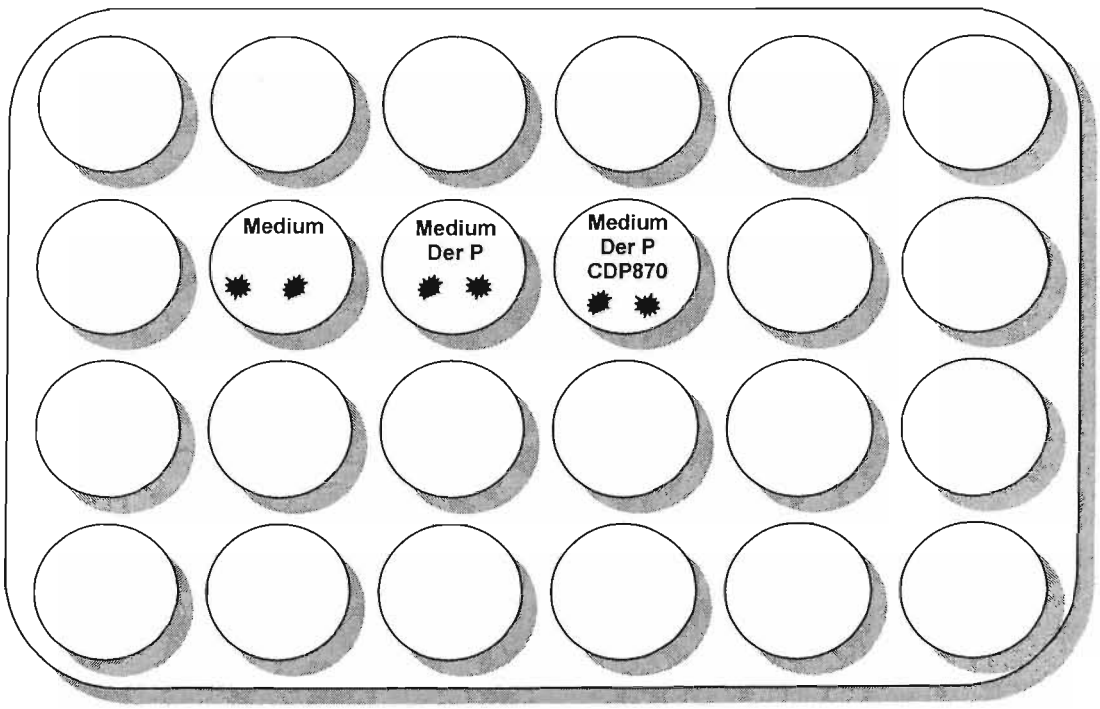


Figure 4-3: Model of CDP 870

The anti TNF-α antibody Fab fragment is linked to a polyethylene glycol tail

4.3.2. Statistical analyses

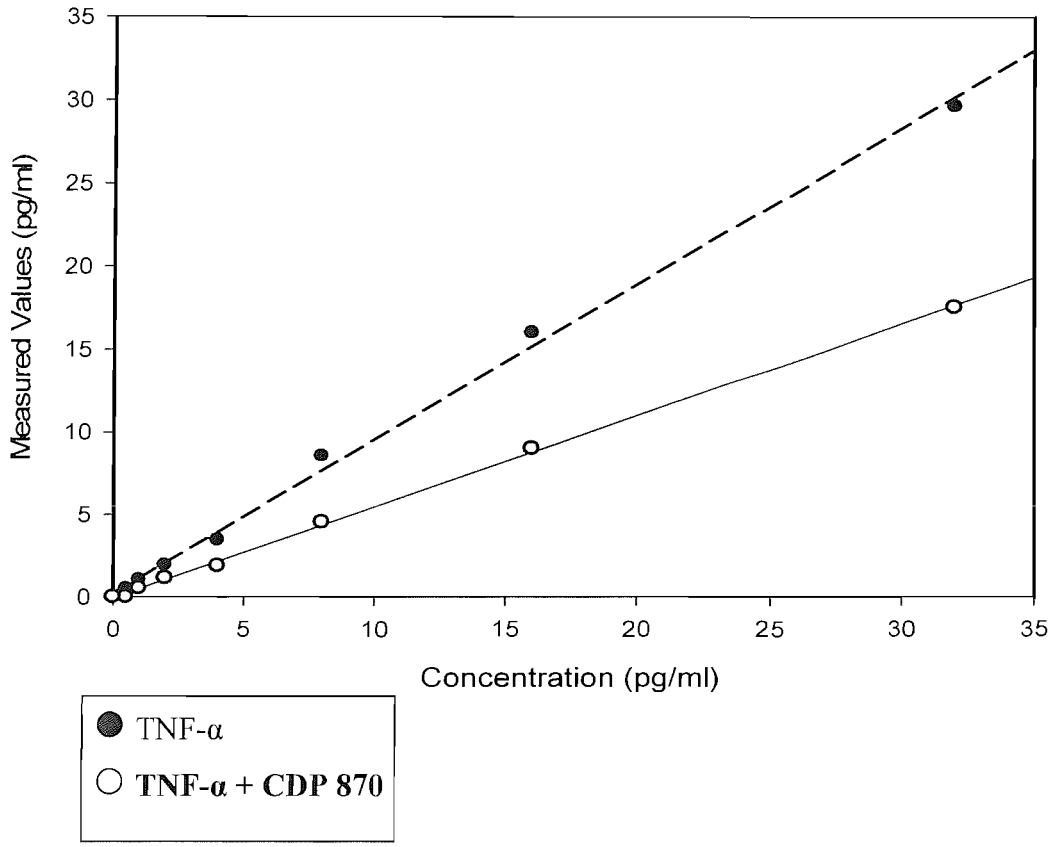
Although explant cultures were performed in all 12 subjects, there was only 450 μl of supernatant available for cytokine analysis by ELISA. As it was necessary to repeat the analysis in selected cases, this limited the number of assays that could be performed for each cytokine. Hence, the number of samples are different for the analysis of TNF-α, IL-8 and IL-1β. Intra class correlaions could not be performed to check the data due to the small numbers. Data were analysed for statistical significance using non-parametric tests. Results are expressed as median (interquartile ranges). The Wilcoxon's signed rank test for paired data was used for within-group comparisons of cytokine protein levels and immunohistochemistry cell counts, using SPSS 11.0 for Windows. Values of $p< 0.05$ were accepted as statistically significant.

4.4. Results

4.4.1. Effects of CDP 870 on the bio assay of TNF-α by ELISA

Initial experiments were conducted to assess whether the addition of CDP 870 would influence the assay of TNF-α in the supernatants. ELISA was performed with two standards, one with a known quantity of TNF-α and the other in the presence of 10 ng/ml of CDP 870 .It was found that the amount of TNF-α detected in the presence of CDP 870 fell by 33% (Fig. 4.4). Thus, the presence of CDP 870 in culture supernatants would to a certain extent be expected to mask the detection of TNF-α in specific explant experiments. Hence, a correction factor was used to calculate the levels of TNF-α in the supernatants containing CDP 870. This involved multiplication of the TNF-α values by 100/67 to achieve the corrected value.

Figure: 4-4 Effects of CDP 870 on the detection of TNF-α.

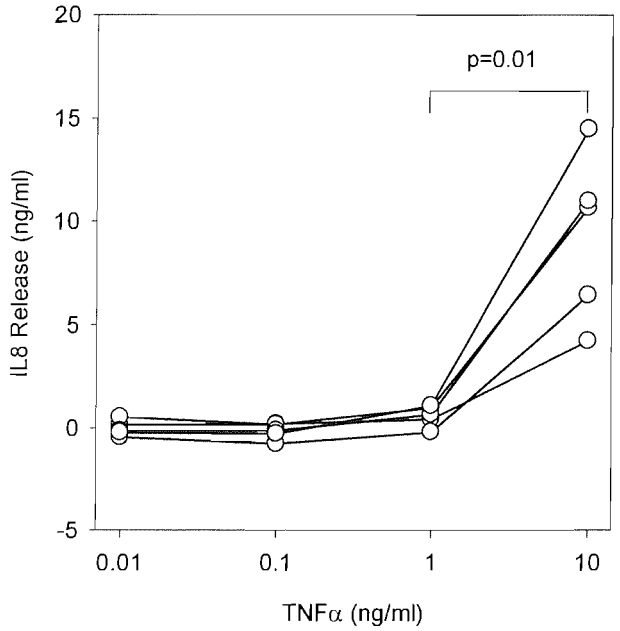


Detection of known quantities of TNF-α in the absence (dotted lines) and in the presence of CDP 870 (solid lines) was calculated by ELISA. In the presence of CDP870 a 33% reduction was detected in the levels of TNF-α

4.4.2. Optimisation of the dose of anti TNF-α (CDP 870) for explant cultures

Preliminary experiments were conducted to optimise the dose of CDP 870. Due to the small number of bronchial biopsies available for explant culture, airway epithelial cells obtained from the same study subjects were used to optimise the dose of CDP 870 to ensure complete neutralisation of any TNF-α produced in the explants. These experiments were performed by Dr. Puddicombe. IL-8 levels were used as readout of TNF-α activation of the primary bronchial epithelial cells. As shown in Fig 4.5 there was an increase in IL-8 levels with increasing doses of TNF-α with significant stimulation being observed at 10ng/ml of TNF-α.

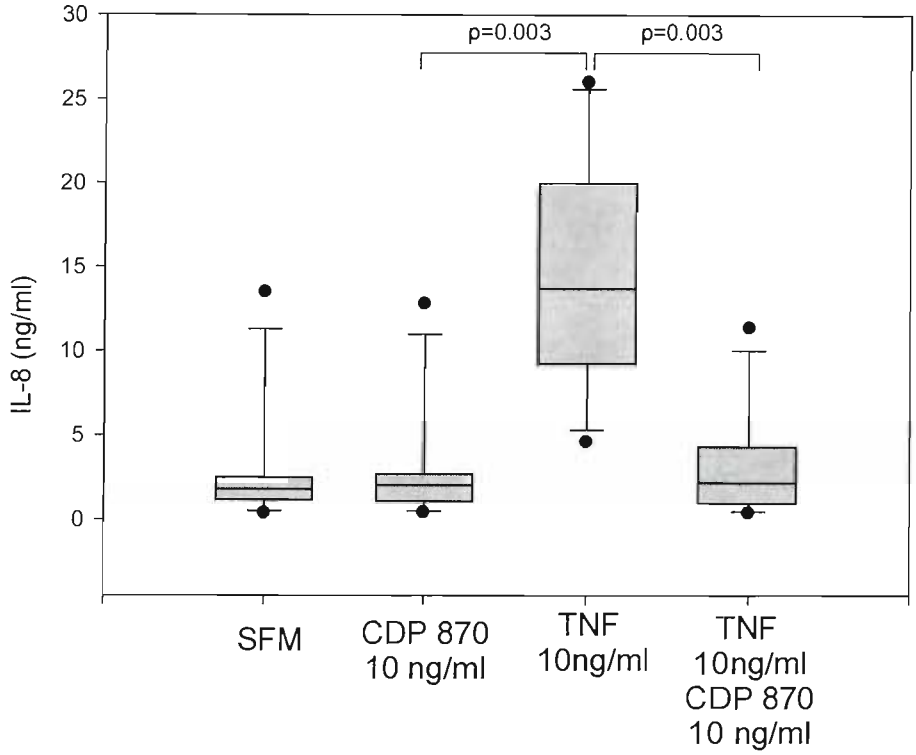
Figure 4-5 Dose response of IL-8 to TNF-α in primary bronchial epithelial monolayer cultures (BECS)



BECS exposed to increasing concentrations of TNF-α for 24 hours and IL-8 release into supernatants measured by ELISA. Data are from 5 subjects and were analysed using Wilcoxon's test.

A dose of 10 ng/ml of CDP 870 which was initially suggested by the suppliers and it was found to inhibit IL-8 release in the presence of 10ng/ml of TNF-α (Fig 4.6). CDP 870 had no effect on the basal IL-8 production by primary bronchial epithelial cells. Hence, a dose of 10ng/ml was chosen to block the effects of TNF-α in the explant culture system.

Figure 4-6: Response of BECS to TNF-α in the presence and absence of CDP 870



SFM- Serum Free Media; TNF-TNF-α

BECS were exposed to control media (SFM), CDP870 and TNF-α alone or in combination with CDP870 for 24 hours. IL-8 release into controlled media was measured by ELISA (Bio source). TNF-α increased IL-8 release and this was inhibited by CDP 870 (10ng/ml)

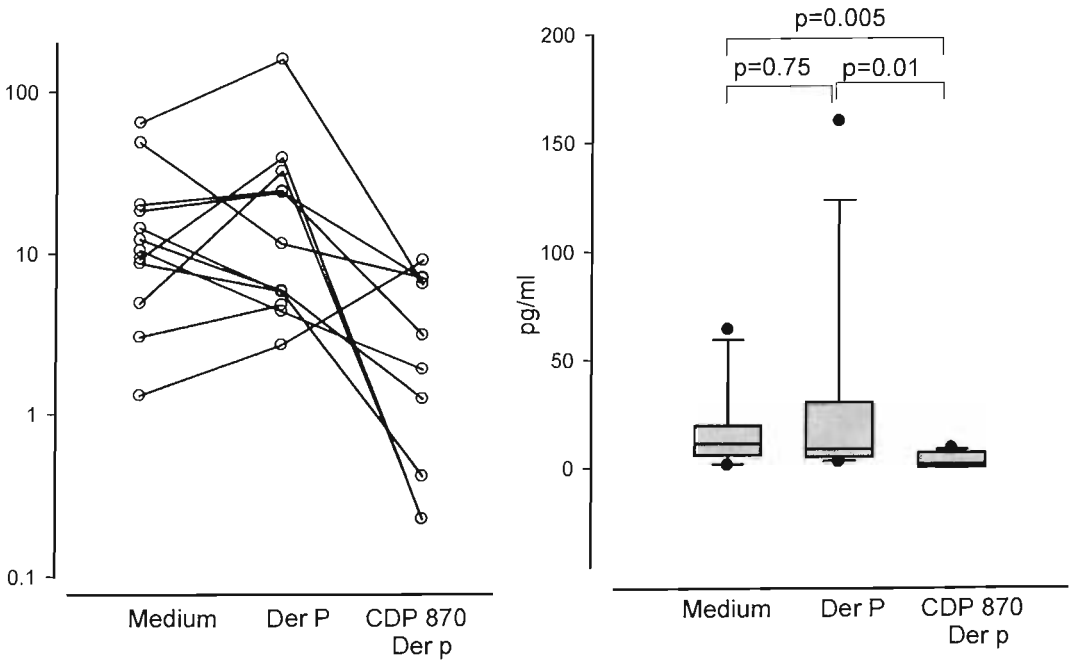
4.4.3. Safety issues of the study

None of the patients recruited for the study were withdrawn due to worsening of their asthma control. Furthermore, none of the subjects necessitated an unscheduled visit or a doctor consultation before or during withholding their inhaled corticosteroids and/or their long acting β_2 agonists or following the bronchoscopy. Therefore, this procedure was relatively safe in this group of asthmatics.

4.4.4. Allergen induced cytokine production and the effects of CDP 870 in bronchial explant cultures of moderately severe asthmatics

When bronchial explant cultures were incubated for 24 hours, the baseline levels of TNF-α were 11.3 (5.9-19.5) pg/ml and exposure to *Der p* produced no significant change in the levels of TNF-α (8.8 (5.1-30.2) pg/ml; p=NS). However, in the presence of CDP 870, the levels of TNF-α were significantly decreased (1.6 (0.2-6.9) pg/mL, (Fig 4-7) when compared both to the baseline levels (p=0.005) and following stimulation with *Der p* (p=0.01). The inhibitory effects of CDP 870 were even apparent after correcting for the masking of TNF-α by CDP 870.

Figure 4-7: TNF-α protein production by bronchial explant cultures.



Bronchial biopsies of moderately severe asthmatics after culture in medium alone, after *ex vivo* stimulation with house dust mite (*Der p*) allergen (5000SQ IU/ml) for 24 hours and in the presence of CDP 870 (10 ng/ml). Panel on left shows the levels of TNF-α under the different conditions. The values have been log transformed on the Y axis. Panel on right shows box plots with the error bars.

The spontaneous release of IL-8 in bronchial explants of moderately severe asthmatics was 28.1(13.0-39.0) pg/ml. Upon exposure to *Der p* extracts, there was considerable variability in IL-8 production ranging from 18.1-44.1 pg/ml with a mean of 38.1 pg/ml p=0.7. In the presence of CDP 870 the levels of IL-8 decreased to 26.4 (14.0-34.7) pg/ml; p=0.05 (*Der p* vs. CDP 870) (Fig 4-5[A]). There was no statistically significant difference between the levels of IL-8 following stimulation with *Der* p but the in the presence of CDP 870 the values did achieve statistical significance (p=0.05).

The cytokines IL-5 and IL-1β were also assayed in the supernatants from the bronchial explants by ELISA (Table 4-2). The levels of these cytokines did not change significantly following stimulation with *Der p* or in the presence of CDP 870 (Fig 4-5[B] and [C]).

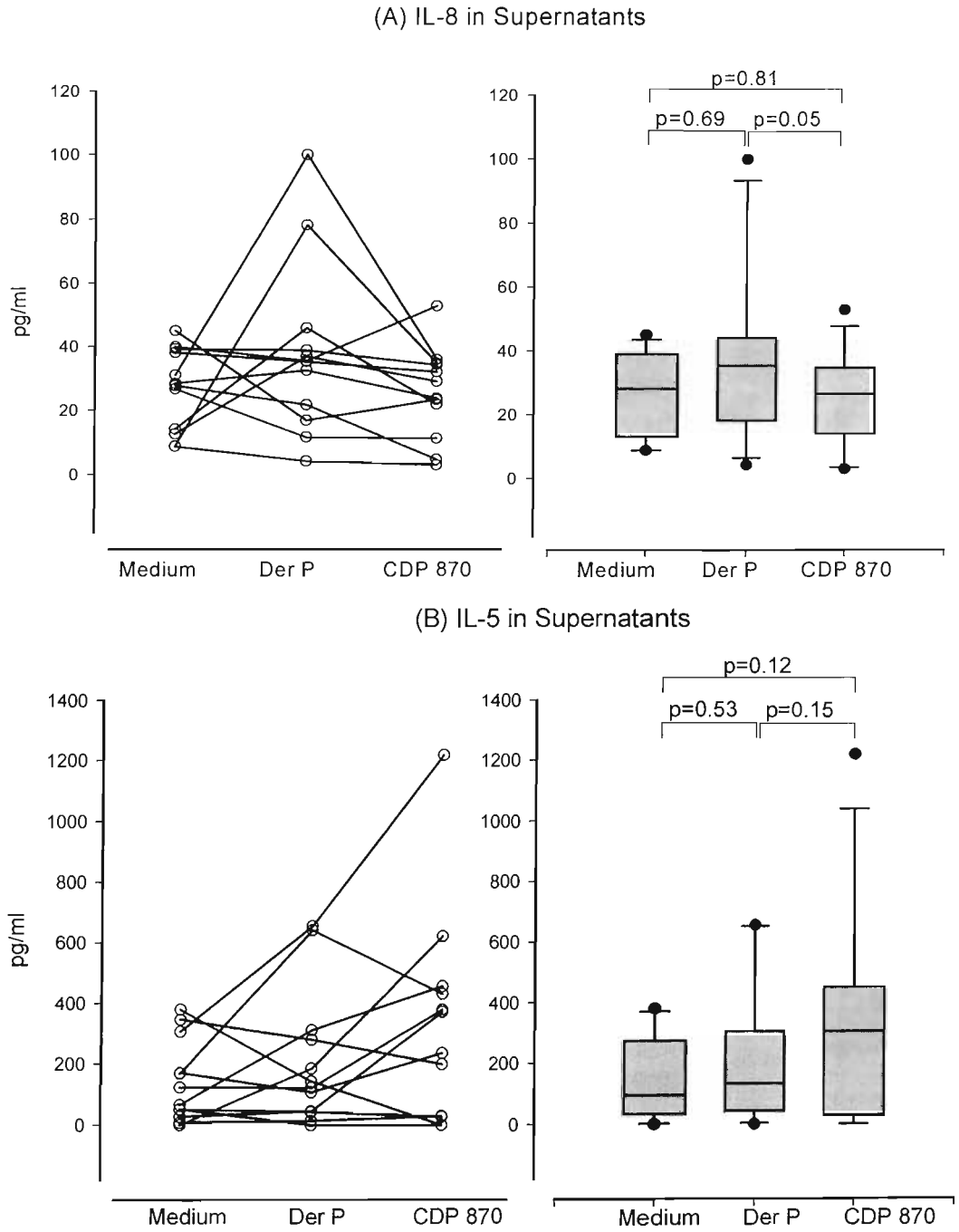
Table 4-2: Levels of cytokines in the supernatants from the bronchial explant cultures

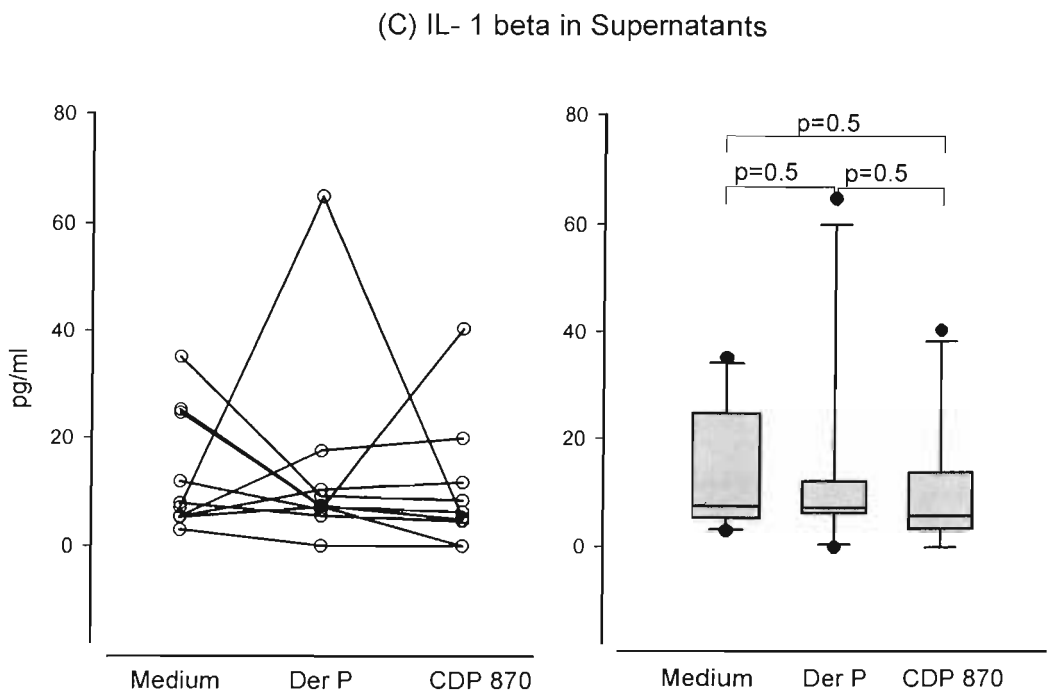
Cytokine (pg/ml)	**Medium**	**Medium + *Der p***	**Medium + *Der p* + CDP 870**
IL-8 (n=12)	28.1	35.4	26.5*
IL-5 (n=12)	93.9	131.4	302.9
IL-1β (n=10)	7.4	7.3	5.7

* p=0.05 (Medium + *Der p Vs* CDP 870)

Figure 4-8: Cytokine protein production by bronchial explant cultures

Bronchial biopsies from moderately severe asthmatics after culture in medium alone, after *ex vivo* stimulation with house dust mite antigen (*Der p*) allergen (5000SQ IU/ml) for 24 hours and in the presence of CDP 870 (10 ng/ml). (a) IL-8 production (n=12); (b) IL-5 production (n=12); (c) IL-1 beta production (n=10).





4.4.5. Immunohistochemistry staining of bronchial biopsies

To characterise the numbers of inflammatory cells in the bronchial mucosa the explant biopsies were processed in GMA resin after 24 hours in culture. Two 2μm sections were cut and stained with selective immunohistochemical markers for inflammatory cells as described in the methods section (Section 2.9).

4.4.5.1. Expression of inflammatory cells in Bronchial biopsies

The studies confirmed the presence in the explants of CD3, CD4, CD8 T lymphocytes; neutrophil elastase (NE^+) neutrophils; $EG2^+$ eosinophils, $AA1^+$ mast cells and $CD68^+$ macrophages in the submucosa of moderately severe asthmatics (Table 4-3). Following exposure to *Der* p and in the presence of CDP 870 there was no significant change in any of the cell numbers. The inflammatory cell population in the epithelium could not be assessed as the explant culture system denudes damages and alters the epithelial layer of the bronchial biopsies.

Table 4-3: Changes in inflammatory cells in the explant biopsies. Results are expressed as medians (inter quartile ranges), n=12.

Inflammatory Cells (cells/sq mm)	Medium	Medium + *Der p*	Medium + *Der p* + CDP 870
Eosinophils (EG^{2+})	0.8 (0.0-4.3)	3.9 (0.0-7.2)	1.2 (0.0-5.3)
Neutrophils (NE^{+})	4.5 (1.7-7.0)	5.3 (3.4-9.7)	5.6 (3.9-9.3)
Mast Cells (AA^{1})	7.5 (4.0-14.7)	11.9 (3.3-16.5)	9.1 (4.1-15.6)
$CD3^{+}$	13.7 (5.0-42.3)	20.8 (9.3-32.8)	11.1 (4.0-36.0)
$CD4^{+}$	1.3 (0.1-7.0)	1.6 (1.2-4.9)	1.3 (0.0-3.9)
$CD8^{+}$	2.2 (0.9-20.2)	10.8 (1.0-22.7)	3.8 (0.6-10.3)
Macrophages (CD68)	2.5 (1.1-6.2)	2.4 (0.7-13.0)	1.6 (0.8-4.0)

4.4.5.2. Expression of TNF-α in bronchial biopsies

To assess the expression of TNF-α and the adhesion molecule ICAM-1, the explant bronchial biopsies were stained with monoclonal antibodies for TNF-α and ICAM-1 (Fig 4-6). There was a significant up regulation of TNF-α following exposure to *Der p*. Cells staining for TNF-α increased from 1.1/sq mm (0.2-1.6) to 2.4/sqmm (0.9-7.1), p=0.02 following allergen exposure in the submucosa of the bronchial biopsies (Fig 3.6). In the presence of CDP 870 the cells staining for TNF-α decreased to 0.2/sq mm (0.0-0.5). This was statistically significant (Fig 4-7).

Figure 4-9: Immunostaining of bronchial biopsy section for TNF-α (magnification factor, x40). TNF-α positive cell stain pink and are shown by arrows.

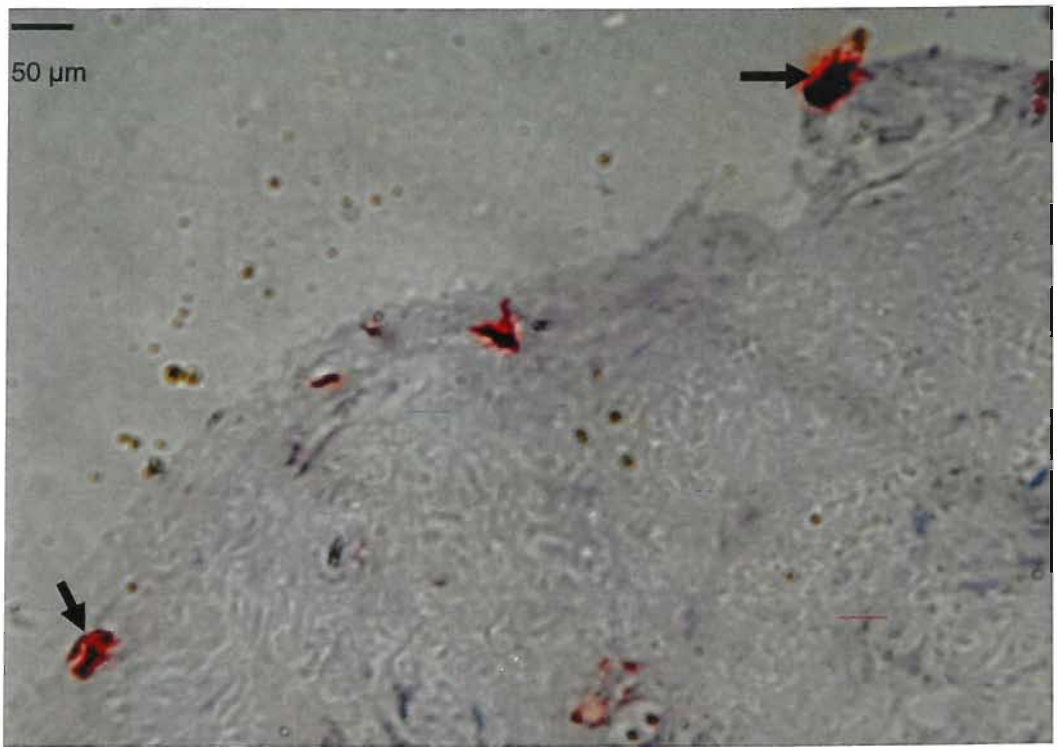
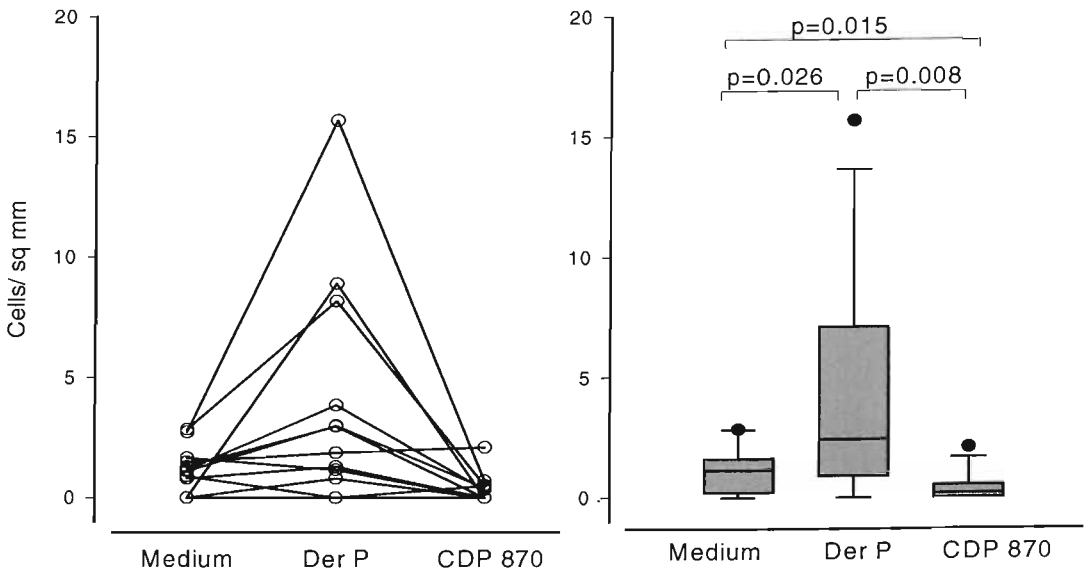


Figure 4-10: TNF-α expression in explant bronchial biopsies when cultured in medium; medium + *Der p* and medium + *Der p* + CDP 870

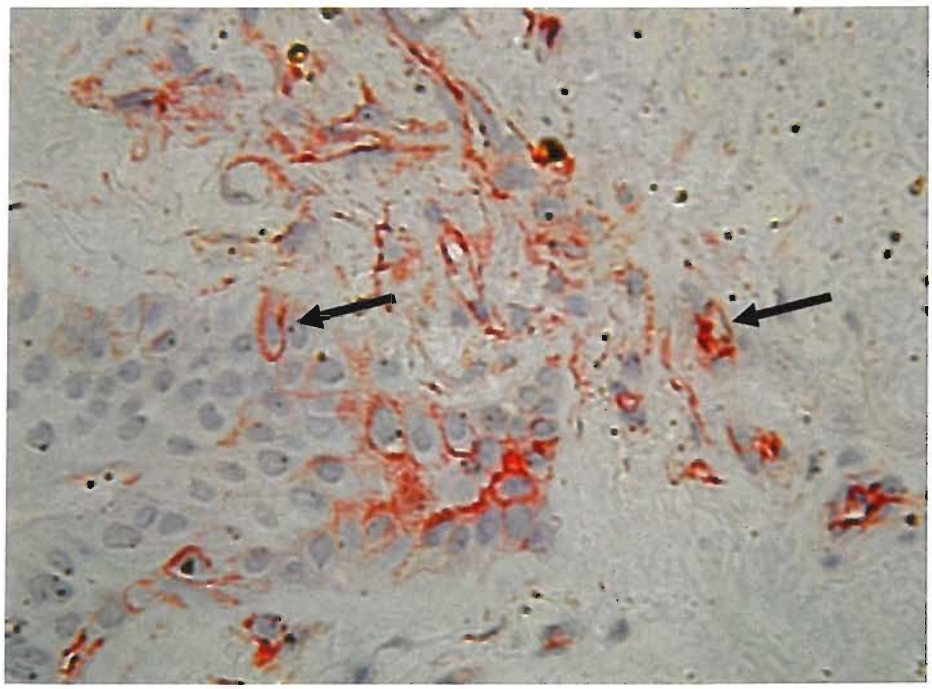


4.4.5.3 Expression of ICAM-1 in explant biopsies

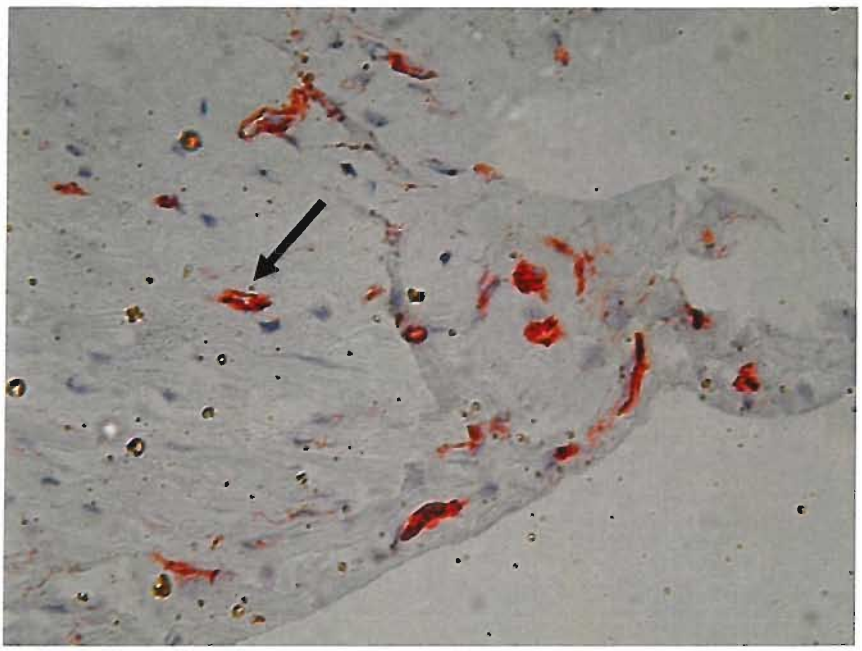
The expression of ICAM-1 was analysed as percentage staining of the endothelial cell marker EN4. While EN4 stains the entire vascular endothelium in the bronchial biopsies, ICAM-1 stains only components of endothelium where this adhesion molecule is expressed (Fig 4-8). The expression of adhesion molecule ICAM-1 in the presence of *Der p* increased from 79.03% (62.05-86.72) to 85.65% (75.49-99.63) in the submucosa of the explant biopsies (p=0.15). In the presence of CDP 870 the expression of ICAM-1 was significantly down regulated to 59.89% (36.52-80.12). These results were statistically significant (Fig 4-9); p=0.01.

Figure 4-11: Immunostaining of explant bronchial biopsy section for ICAM-1 when cultured in (A) medium; (B) medium + *Der p* and (C) medium + *Der p* + CDP 870;

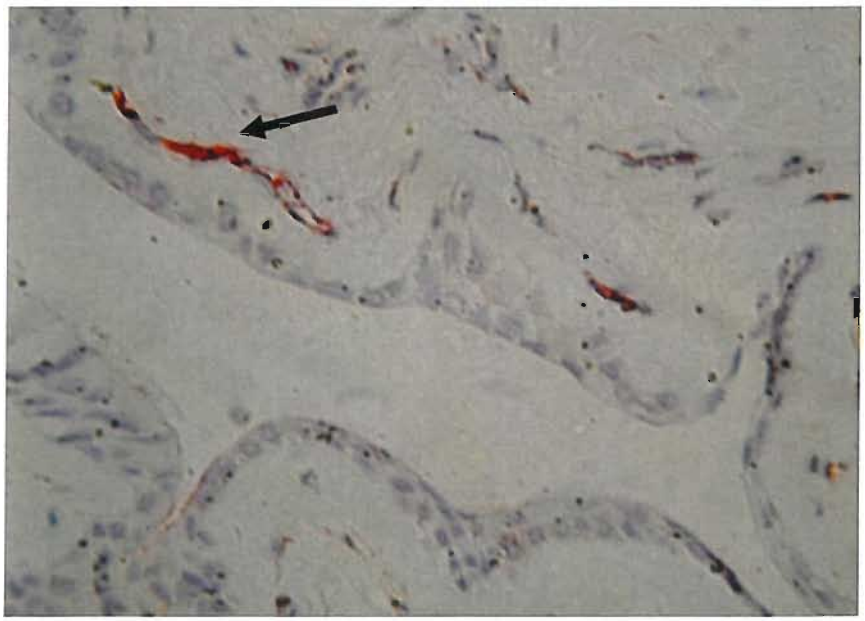
(A)



(B)

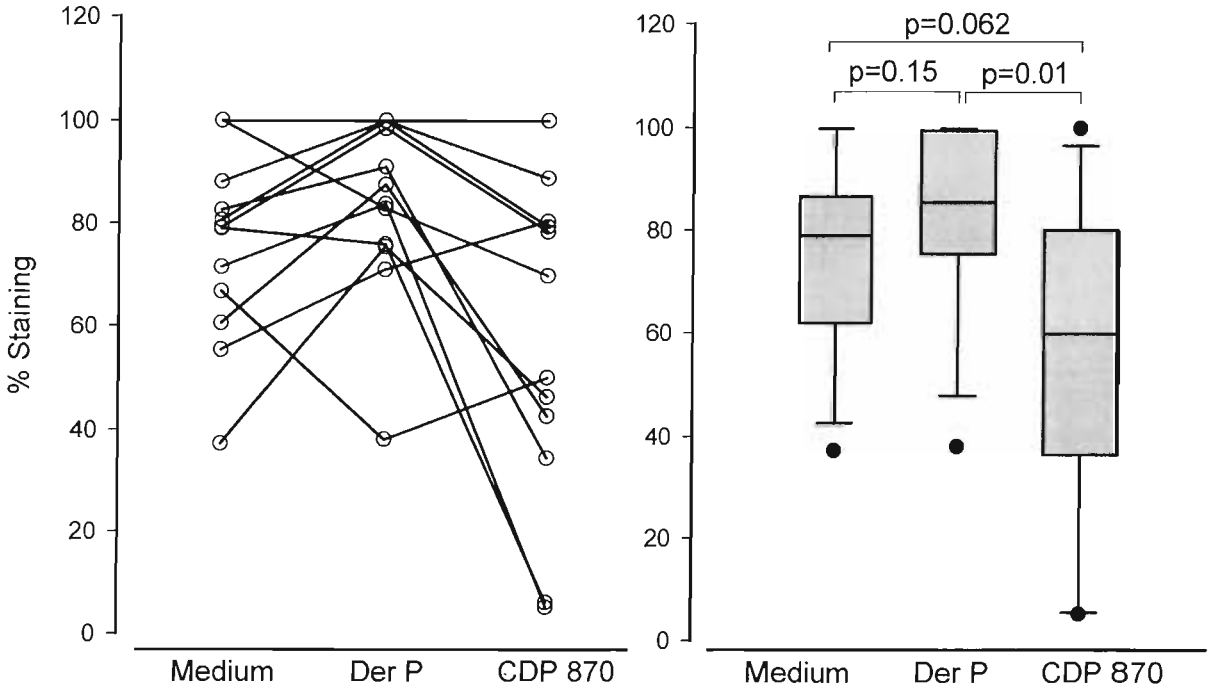


(C)



Endothelium staining for ICAM-1 is shown in pink by arrows. Magnification factor X 20

Figure 4-12: ICAM-1 expression in explant bronchial biopsies after culture in medium; medium + *Der p* and medium + *Der p* + CDP 870 for 24 hours



4.5. Discussion

The asthmatic bronchial explant culture system is a useful integrated cell culture system for assessing responses of the bronchial tissue to allergen and to evaluate the effects of newer therapeutic modalities (34). In contrast to isolated cell cultures the explanted bronchial tissue contains structural elements like airway epithelium, fibroblasts, blood vessels, resident airway inflammatory cells including T cell, eosinophils, mast cells, macrophages and neutrophils. This makes observations in this complex system particularly relevant to asthmatic airway inflammation. As two bronchial biopsies are necessary for each culture condition to optimise measurable cytokine production and reduce variability, this together with the limited number bronchial biopsies that could be obtained at bronchoscopy from individual subjects places a limitation to the conditions being examined. Hence we had to use primary bronchial epithelial cells from these subjects for the initial validations. We would ideally liked to have a positive control and culture the biopsies in the presence of

TNF-α however we could not perform these experiments due to the limitations of this culture system. Furthermore, in an integrated system like this it is also possible that differences in the composition of individual bronchial biopsies (e.g. size of biopsies, length of epithelium or thickness of the sub-mucosa in biopsy) may limit the widespread application of this system to study the contribution of individual cells such as the bronchial epithelium.

In this study, the bronchial tissue of moderately severe asthmatics were infiltrated by inflammatory cells including $CD3^+$, $CD4^+$, $CD8^+$ T lymphocytes, neutrophils, eosinophils, mast cells and macrophages. Following culture of the explant biopsies in the presence of *Der p* and in the presence of anti TNF-α monoclonal antibody (mAb) there was no significant changes in this cellular population. The absence of significant changes in the cellular *milieu* was not surprising. The explant culture technique being a closed static system, new inflammatory cells cannot infiltrate the tissues as this method does not provide an avenue for the influx of inflammatory cells. This is one of the drawbacks of this culture system as this does not provide a means of assessing inflammatory cell recruitment. We were also unable to find inflammatory cellular infiltration into the epithelium of the bronchial biopsies. The culture of the bronchial biopsies in an *in vitro* system has its limitations as the culture alters the morphology of the epithelium, damages the epithelium thereby precluding a reliable assessment.

Studies have shown an increase in the generation of TNF-α in isolated macrophages and PBMC's after allergen exposure (184). There is an increase in TNF-α production by macrophages after the late phase response following allergen challenge (278). In mild asthmatics there is an increased expression of TNF-α and this has been localised to the intracellular granules of the mast cells (66). In the present study we also observed that immunostaining for TNF-α in biopsies was significantly increased in the presence of *Der p* and in the presence of CDP 870 the staining for TNF-α positive cells were significantly lower both when compared to the baseline and to the cultures with *Der p*. Therefore both *in vivo* and *ex vivo* allergen exposure lead to up regulation of TNF-α in the bronchial biopsies of asthmatic subjects. In the *in vivo* study, BAL was not undertaken, so no measures of TNF-α release were available. In the explant culture system we did not see a corresponding increase in TNF-α release following exposure to *Der p* but, in the presence of the anti TNF-α mAb- CDP 870 the levels of

TNF-α were significantly reduced below the baseline levels. The concentration of CDP 870 used in the explant system was sufficiently high to neutralise the levels of TNF-α produced by the explants. This showed that the dose of CDP 870 used could block the effects of 10ng/ml of TNF-α which exceeded the levels observed in the explant model (maximum levels of TNF-α was 160 pg/ml).

The failure to detect an increase in TNF-α in the explant supernatant even though there was an increase in the expression of TNF-α in the bronchial biopsies may be due to the inability of the inflammatory cells in the bronchial biopsies to secrete TNF-α into the system or due to the entrapment of TNF-α by the tissue. Earlier studies have shown that TNF-α is predominantly secreted by mast cells in the airways (66). Sensitised mast cells release preformed TNF-α and the mast cell granules itself contain preformed TNF-α (279). Mast cells express the high-affinity receptor (FcεRI) for IgE. This receptor is of such high affinity that binding of IgE molecules is essentially irreversible. Although the subjects enrolled into the study were sensitive to *Der* p, it is possible that prior addition of allergen specific IgE to the system may have primed the mast cells to secrete TNF-α in the presence of *Der p.*

Alternatively, since CDP 870 suppressed TNF-α levels it is likely that the TNF-α that is released binds rapidly to its receptor and never accumulates in the medium. In the presence of CDP 870 TNF-α levels are suppressed but it is not possible to distinguish whether this suppression was on the basal levels or on the stimulated TNF-α by *Der p.* If TNF-α does accumulate and is used up in the autocrine loop system then by blocking the autocrine loop, we may see an increase in TNF-α following stimulation with *Der p.* It is also possible that TNF-α may be active as a membrane anchored precursor and hence is not detectable in the system (280).

TNF-α is known to up regulate adhesion molecule expression including ICAM-1 and VCAM (281, 282). Bronchial biopsies from asthmatic subjects have been shown to over express VCAM (269). When human bronchial tissue obtained was passively sensitized with serum from patients with atopic asthma who were sensitive to house dust mite, TNF-alpha and IL-1β increased the expression of ICAM-1, E-selectin, and VCAM-1. In addition, neutralizing antibody against TNF-alpha and IL-1beta partially blocked the up regulation of CAMs on passively sensitized bronchial tissue after

allergen exposure (283). In our earlier experiments with repeated low dose allergen exposure we have shown that in mild asthmatics there is up regulation of ICAM-1 and VCAM. In the present study, CDP 870 was able to reduce significantly the expression of ICAM-1 from the bronchial biopsies suggesting a possible role for TNF-α mediated ICAM expression and recruitment of inflammatory cells into the airways. We however did not look for the expression of VCAM-1 in the bronchial biopsies.

CDP 870 also reduced the levels of IL-8 in the explant supernatants. In 4 subjects there was an increase in IL-8 levels following stimulation with *Der p* and these were suppressed with CDP 870 but the IL-8 levels were unchanged in patients who did not have an increase following culture with *Der p*. As there was no medium + CDP 870 as a control it is difficult to know whether the suppression of IL-8 levels by CDP 870 is due to the blockade of basal TNF-α levels or due to the effect of *Der p* on IL-8. Due to the small sample numbers it was not possible to do a sub group analysis. It is well known that IL-8 is an important chemokine associated with the recruitment of neutrophils and by reducing the levels of IL-8, TNF-α blocking strategies could well inhibit the neutrophil influx into the airways of severe asthmatics. Studies have shown that the inflammatory cell population in patients with severe asthma has a predominance of neutrophils (72). Interestingly, we did not find significant changes in IL-5 and IL-1β levels in the explant supernatant. This was in contrast to previous studies from this department where there was an up regulation in the IL-5 levels following culture with *Der p* (35). The possible explanation for this difference could be the heterogeneity of the asthmatic population being studied. Secondly, the pre-treatment of these moderately severe asthmatics with inhaled corticosteroids may have lead to the lack of response. It is also possible that none of the TNF-α produced by the explants was responsible for IL-5 or IL-1β production in the bronchial biopsies of moderately severe asthmatics.

Coward and colleagues have shown important roles for TNF-α and NF-κB in the activation of human mast cells. They observed that mast cells both release and respond to TNF-α thereby suggesting that there is a positive autocrine loop which leads to augmentation of mast cell activation (157). If this is the case then it is likely that blocking the effects of TNF-α with a monoclonal antibody would not only reduce the release of TNF-α from mast cells but also block the autocrine loop system. This

could have a cascading effect on the inflammatory processes involving TNF-α since secretion of TNF-α from mast cells augments NF-κB activation not only of mast cells but also of surrounding inflammatory cells and in so doing has a considerable proinflammatory effect on its local microenvironment.

TNF-α antagonists have set new therapeutic standard for RA and their effectiveness in rheumatoid arthritis (RA) has been demonstrated in various clinical trials. They are extremely effective in controlling signs and symptoms of RA and also inhibiting progressive damage to articular surface (284-286). The pathophysiology of RA and the cytokine profile of RA has provided a therapeutic option which is now part of management for this crippling illness. Our studies have found that there is up regulation of TNF-α and this is associated with an associated increase in adhesion molecules. In the presence of CDP 870, a TNF-α blocking mAb there was significant down regulation of TNF-α, IL-8 and ICAM-1. Hence, this provides us an opportunity to assess TNF-α blocking strategy in patients with severe asthma. Severe asthma being associated with a different inflammatory profile in contrast to mild and moderate asthmatics and having a large unmet need could be the target study population to assess this new treatment option.

In this study every attempt was made to recruit patients with well characterised chronic severe asthma with sensitivity to house dust mite. However, some heterogeneity was apparent in the measures of lung function, airway hyperresponsiveness and medication use. This is a characteristic feature of moderately severe asthma where there is poor relationship between baseline spirometry, bronchial hyperresponsiveness, clinical manifestation of asthma and considerable independent variations in these indices over time (292, 293). It is therefore likely that various phenotypes exist in asthma and this could explain the heterogeneities of responses to various therapeutic interventions.

The explant culture method provides vital information to evaluate new molecules as a therapeutic option. However this model has its limitations. The ethical and safety issues limit the number of biopsies to six. This constraint imposed by the number of bronchial biopsies precludes assessment of various culture environments. We would have liked to assess the biopsies with a positive control namely TNF-α but as we were

limited by the number of biopsies we could not perform these experiments. The availability of approximately 500 µl of supernatant restricts the analysis of many cytokines following the culture of bronchial biopsies. Measurement of histamine levels in the culture supernatants would have helped us to show whether *Der p* was having an IgE dependent effect but the availability of 500 µl of supernatant limited our analysis of cytokines and mediators. The explant culture does not provide information on the recruitment of inflammatory cells. Although our data suggest that TNF-α may be involved in the recruitment of inflammatory cells via effects on cytokines and adhesion molecules, this approach does not address directly whether TNF-α is capable of recruiting inflammatory cell into the tissues and the addition of CDP 870 has any role in inhibiting the cellular influx. In addition, there is not enough data to look at the intraclass correlation which could be a limitation to detect meaningful differences between the various groups. While this system has its shortcomings it has been used in a number of studies and the model has been able to demonstrate the utility of CTLA-4 fusion protein and provide some insight into the cytokine response in mild and moderately severe asthmatics (35, 36). We have used this model to assess the role of TNF-α and the effects of blocking the effects with a monoclonal antibody in patients with moderately severe asthma. Despite this limitation we were still able to demonstrate significant changes in the cytokine responses and in the presence of CDP 870. Furthermore we were able to demonstrate consistent findings with the low dose allergen challenge model where in TNF-α was up regulated in the bronchial biopsies following allergen exposure.

Another issue is whether the molecules that are present in the culture system are available to all the different cells in the biopsies or only to the superficial cells that are exposed to the culture medium. This can be a problem with larger molecules like monoclonal antibodies or proteins, which may not diffuse adequately into the tissue to bring about the expected effects. One way around this problem would be digest the tissue with collagenase and create a cell suspension with a mixture of various cells. This could then be cultured *in vitro* to provide a more standardised culture medium and culture technique. The second option would be to evaluate a TNF-α blocking medication in patients with severe asthma and look at both objective and subjective improvements in asthma control. As TNF-α blocking medications has been used in

the treatment of RA we are aware of the possible side effects and the safety profile of these medications.

4.6. Conclusions

This study has shown that the explant model is a useful technique that could be used to assess newer therapeutic modalities for asthma. Blocking the effects of TNF-α with a monoclonal antibody down regulates the expression of adhesion molecules, TNF-α and IL-8 in bronchial biopsies of moderately severe asthmatics. This could prove useful by reducing the recruitment of inflammatory cells into the asthmatic airways and possibly reduce airway inflammation and improvement in asthma symptoms and asthma control. The explant culture method, with its own limitations does not provide a mode for assessment of inflammatory cell recruitment. Hence a clinical trial with TNF-α blocking molecule could provide more useful information on the effectiveness of this strategy in patients with asthma.

5. Effects of blocking TNF-α on asthma control and lung functions in patients with severe chronic corticosteroid dependent asthma

5.1. Introduction

Chronic severe asthma imposes a huge burden both on the patient and the care provider due to the uncontrolled symptoms, hospital visits, emergency admissions and the consequence of the adverse-effects due to the medications. Both inhaled and oral corticosteroids are associated with significant side effects including easy bruising, reduction in the bone mineral density, alteration of the hypothalamo-pitutary axis, cataracts and impaired glucose tolerance. The aim of newer therapies is to find an alternative to corticosteroids or to find an acceptable and safe option that could effectively reduce the dose of corticosteroids required for the treatment of asthma.

Studies have shown that TNF-α is an important cytokine in inflammatory disorders and blocking the effects of TNF-α is an accepted therapeutic strategy for the treatment of rheumatoid arthritis, juvenile arthritis, ankylosing spondylitis, inflammatory bowel disease and psoriatic arthritis. Whilst airway dysfunction in mild-moderate asthma involves activation of Th2 inflammatory pathways that includes recruitment and activation of mast cells, basophils and eosinophils, as the disease becomes severe and less responsive to corticosteroids, neutrophils become more prominent compatible with a Th1 profile and/or tissue injury. In general, the disease becomes more chronic, aggressive, less dependent on environmental driven immunological pathways and characterised by an altered inflammatory response in favor of neutrophils.

Under such conditions TNF-α is a cytokine which serves to amplify signaling responses in many different cells including airway smooth muscle. Lung mast cells both release and respond to TNF-α with a positive autocrine loop that augments the activation of mast cells (157). Furthermore, TNF-α is released from eosinophils, macrophages and Th1-like T cells. Activation of TNF-α receptors in asthma leads to induction of other pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6 and IL-8, enhanced leukocyte migration and activation and stimulation of epithelial repair mechanisms associated with airway remodeling and expression of surface adhesion molecules (174-176).

Our earlier experiments have shown that TNF-α is up regulated after low dose allergen exposure and this was associated with enhanced expression of adhesion molecules ICAM-1 and VCAM in patients with mild asthma. Furthermore, blocking the effects of TNF-α in a bronchial explant system with a PEGylated antibody (CDP 870) directed against TNF-α resulted in the down regulation of TNF-α and adhesion molecules, ICAM-1 and the chemokine IL-8. TNF-α promotes neutrophil recruitment concomitantly in the lung with the appearance of bronchial hyperresponsiveness in normal subjects (191). We have recently shown that severe asthmatics had significantly higher concentrations of TNF-α in BALF than either the healthy controls or those with mild asthma. Evaluation of mRNA levels revealed that there was a significantly higher relative expression of TNF-α in biopsies from the severe asthmatic subjects compared to mild asthmatic subjects (294). Furthermore severe asthmatics have been associated with a different phenotype with a predominance of neutrophils in the airways (295). A recent study suggested that differences in corticosteroid responsiveness may exist between moderate to severe asthmatics with a neutrophilic process and those with persistent eosinophils. The neutrophil predominant pattern was associated with a much less robust response to corticosteroids than those with eosinophilic inflammation (296). It is therefore possible that other, non-Th2 factors are playing a role in the process.

Based on our experiments and the evidence suggesting severe asthmatics to belong to a different phenotype where there is a predominant neutrophil inflammatory response and the efficacy of TNF-α blocking strategy in Th1 diseases such as rheumatoid arthritis and inflammatory bowel disease there is a good case for evaluating the effects of TNF-α blockade in patients with chronic severe asthma. In view of our findings of elevated gene and protein expression for TNF-α in severe asthma, we undertook a proof-of-concept study with the soluble TNF-α receptor IgG1Fc fusion protein, etanercept (Enbrel®, Wyeth Laboratories, Berks, UK), which binds specifically to both TNF-α and TNF-β thereby preventing free cytokine binding to cell surface TNF receptors

5.2. Objectives

1. To evaluate the efficacy of a soluble TNF-α receptor (etanercept, Enbrel®, Wyeth Laboratories, Berks, UK) on lung functions (FEV_1, FVC, PEF, and

airway hyperresponsiveness), asthma control and rescue medication use in patients with chronic severe asthma.

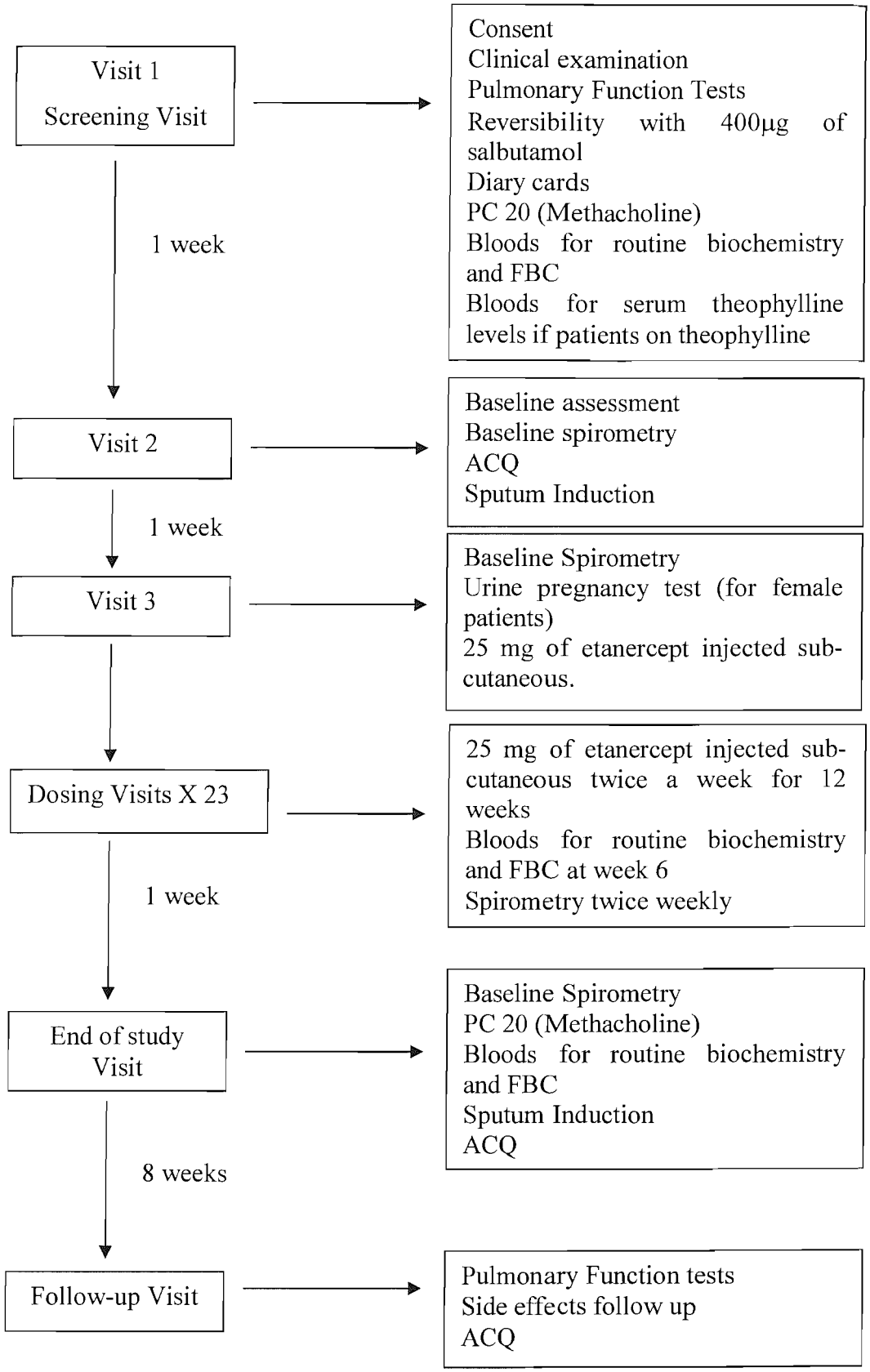
2. To assess the safety of etanercept in patients with chronic severe asthma
3. To assess sputum inflammatory markers before and after treatment with etanercept

5.3. Methods

5.3.1. Subjects

Patients with chronic severe asthma were recruited from the departmental database, asthma clinics at the Royal South Hants Hospital, Southampton and St. Mary's Hospital Portsmouth. This clinical study was approved by the local research ethics committee. This study was a single centre study conducted at Southampton General Hospital with patients being recruited from the Southampton General Hospital and St. Mary's Hospital. The study plan is outlined in Fig 5.1.

Figure 5-1 Study plan



5.3.2. Inclusion criteria

- Patients had a diagnosis of chronic severe asthma for at least one year with an FEV_1 of 50-80% of predicted at baseline and are on regular oral corticosteroids for at least 3 months prior to randomization, high dose inhaled corticosteroids (i.e. ≥ 1600 μg/day beclomethasone di propionate or equivalent) and/or long-acting β-agonists and/or theophylline for at least one year.
- They should have documented variable airflow obstruction as documented in the past 1 year by at least one of the following
    - Reversibility in FEV_1 of ≥ 9% predicted after 4 puffs of 100μg salbutamol from a metered dose inhaler and administered through a spacer
    - Mean diurnal variation in peak expiratory flow ≥ 15% on ≥ 4 days/week for at least 2 weeks
    - Diurnal variation = [(Highest PEF-Lowest PEF)/ Lowest PEF] X 100
    - Mean PEF
- For female patients:
    - Non-child-bearing potential (menopausal > 2 years) or
    - Childbearing potential and taking efficient oral or barrier contraception and having a negative pregnancy test.

5.3.3. Exclusion Criteria

- Current smokers and smokers who had smoked more than 10 cigarettes a day with a total smoking history of ≥ 10 pack years.
- Patient with any clinically significant cardiopulmonary abnormalities, either clinically diagnosed or documented on check X-ray or ECG that are not related to asthma
- Patient with a diagnosis or documented history of bronchopulmonary aspergillosis or other uncontrolled infections.
- Patients who had a diagnosis of systemic lupus erythematoses or had a positive anti nuclear factor antibody at screening visit
- Patients with a past history of treatment with tuberculosis or had a contact with an open case of tuberculosis
- Patient with a diagnosis of emphysema, chronic bronchitis or COPD.

- Patient who has had a respiratory or gastrointestinal parasitic infestation within 6 months prior to selection visit.
- Patient with clinically significant cardiovascular, neurological, renal, endocrine, gastrointestinal, hepatic, or hematological abnormalities that are uncontrolled with standard treatment.
- History of major psychiatric, medical or surgical disorders, which in the opinion of the investigator are likely to interfere with administration of the treatment.
- Alcohol abuse (history or signs of acute or chronic abuse: more than 45 g of alcohol per day).
- Patient who is known to be immunodeficient.
- Patient undergoing desensitization to a specific allergen who has been on a stable maintenance dose for < 3 months prior to selection visit.
- Regular use of sedatives, hypnotics, tranquillizers or other substances resulting in dependence.
- Blood donation in the 3 months prior to the start of the study or participant intends to donate blood during the study or within the 3 months following the study completion.
- Patient who has received live immunizations within 4 weeks of selection visit, or patients who are planning to be immunized at any point during the study.
- The patient has ongoing oxygen therapy.
- Patient unable to understand dosing directions, how to complete diaries, how to use a peak flow meter or how to use a metered dose inhaler, unlikely to co-operate in the study, and/or poor compliance anticipated by the investigator.
- Inability to be contacted in case of an emergency.
- Participation in another study at the same time, or within a prior 3-month period.

5.3.4. Evaluation of Patients

5.3.4.1. Initial evaluation

Patients gave written informed consent prior to participation in the clinical trial. The subjects then underwent a clinical examination. Their old medical records were checked to exclude any other chronic lung diseases. If they were found suitable to take part in the study bloods were sent off for the routine investigations which included a full blood count, renal function and liver function tests, an auto-immune

screen for anti nuclear antibody and antibody against double stranded DNA and serum IgE levels.

5.3.4.2. Lung Function Tests

- Spirometry was recorded as FEV_1 and FVC to establish a baseline using a Vitalograph compact spirometer (Vitalograph Ltd, Maids Moreton, Bucks, UK).
- Assessment of bronchodilator reversibility using inhaled salbutamol. All patients demonstrated a reversibility of at least 9% following inhalation of 400 mcg of salbutamol through a metered dose inhaler (297).
- Peak expiratory flow using a standard portable mini Wright peak flow meter. Patients were requested to perform three readings in the morning and in the evening prior to using their rescue medications (β2 agonists) and the best of the three readings was used for analysis. Each patient was provided with a peak flow meter and their technique were checked when they made the hospital visit.

5.3.4.3. Methacholine bronchial challenge

Methacholine challenge was performed according to the method described by Chai et al (226). Baseline spirometry was recorded and provided $FEV_1 \geq 60\%$ of predicted. The subject (using a nose clip) inhaled 5 breaths of nebulised 0.9% saline. Measurements of FEV_1 were made 1 and 3 minutes after each set of inhalation. If a fall in FEV_1 of greater than 10% occurs after saline, the challenge was aborted as the subject was considered to be too reactive. If there is no fall in FEV_1 (or less than 10%) after the saline, then the first dose of methacholine was delivered through a nebuliser with 0.03 mg/ml solution. FEV_1 was recorded after 1 minute and 3 minutes and the lower of the best readings at 1 and 3 minutes is used for calculation. The steps were repeated with doubling concentrations of methacholine (0.03 - 16 mg/ml) and FEV_1 recorded at 1 minute and 3 minutes after each exposure until a fall in FEV_1 from the post saline value of greater than 20% occurred, or the highest concentration of methacholine was reached. At the end of the procedure 2 puffs of ventolin were administered to the subject and spirometry performed to check that the subjects' FEV1 has returned to baseline before discharge. The PC_{20} was derived from percent change of FEV_1 from post saline baseline values plotted against log methacholine concentration and interpolation of the last two points. The degree of

airways responsiveness to methacholine was expressed as the cumulative PC_{20} – the concentration producing a 20% fall in FEV_1 using the formula

$$\text{Log PC20} = \log C1 + (\log C2 - \log C1)\,(20 - R1)/R2 - R1$$

Where

C1 is second to last concentration of methacholine

C2 is last concentration of methacholine

R1 is second to last FEV_1

R2 is last FEV_1

The airway responsiveness was also assessed by comparing the area under the curve (AUC) before and after treatment with the study drug. This was derived by plotting the FEV_1 against the methacholine concentration.

5.3.4.4. Questionnaire

Asthma control questionnaire (ACQ): This questionnaire is based on Elizabeth Juniper's Asthma Specific Quality of Life questionnaire (298). This questionnaire incorporates seven items looking at both subjective and objective measures of asthma control in the past one week (See Appendix III).

5.3.4.5. Induced Sputum

Sputum induction was performed according to the standard protocol as described in Chapter 2.6. The subjects initially inhaled 0.9% saline and then the concentration was increased to 1.5% and then to 3% saline. This was done as a precautionary measure to avoid broncho-constriction as the patients being investigated had severe asthma. The expectorated sputum was processed by adding an equal weight of 0.01M DTE (dithioerythritol) as described in Chapter 2.6. The cell pellet was resuspended in 1ml of tris-buffered saline (TBS) and cells were counted in a Neubauer's chamber after staining with trypan blue. Cytospins were obtained based on the total cell counts and the cells were stained by Rapi-Diff stain. Differential cell counting was performed by counting 600 cells in each cytospin in a blinded fashion. The mean of the two scores was used for analysis. The differential cell counts were expressed as percentage of the total cells. The supernatants were aliquoted into labeled eppendorfs and stored at -80^0C for analysis of TNF-α and IL-8 by ELISA.

5.3.5. Methodology

This was a proof-of-concept study and hence was planned as an open labeled study without a placebo control as this was the first time a TNF-α blocking medication was given to an asthmatic subject. The subjects recruited for this study were chronic severe asthmatics and hence it was thought not rational to conduct a placebo controlled study. This study was primarily conducted to observe the therapeutic response to TNF-α blocking strategy in patients with chronic severe asthma and also to assess the side effects of blocking TNF-α in this sub group of asthmatics.

Ethical approval was obtained from the local research ethics committee and the study was conducted at Southampton General Hospital, Southampton. The patients were recruited from the departmental database and also from asthma clinics at Southampton General Hospital and St. Mary's Hospital, Portsmouth after obtaining permission from the respective medical consultants of the patients.

This study was performed on 15 patients with chronic severe asthma. Each patient attended the hospital for a period of 32 weeks. The patient population included patients aged 18-65 years with a history of wheezing, breathlessness, chest tightness or cough, starting before the age of 45 years. Patients were taking regular oral corticosteroids, high dose inhaled corticosteroids, long acting inhaled β_2-agonists and/or theophylline. All medications were kept constant throughout the study.

On visit 1 written informed consent was obtained and the patients underwent a clinical examination and lung function tests including spirometry, reversibility with salbutamol and a PC_{20} methacholine. Bloods were taken for routine biochemistry (which included liver and renal function tests), full blood counts, auto immune screen and serum IgE levels. If patients were on theophylline serum theophylline levels were monitored.

Visit 2 included the asthma control questionnaire (ACQ), a baseline spirometry and sputum induction. Beginning with visit 3 the subjects had the study medication; 25 mg of etanercept twice a week subcutaneous for a period of 12 weeks (24 visits). During these visits spirometry was performed every week and bloods were taken for

routine biochemistry and full blood counts at 6 weeks. Diary cards were reviewed for any changes in lung functions and rescue medication use.

The last visit was a week after the final dose of the study medication and included spirometry, PC_{20} methacholine, bloods for routine biochemistry, full blood count and autoimmune screen, ACQ and a sputum induction.

The final follow-up visit was performed 8 weeks after the previous visit. Spirometry was performed to assess lung function and the subjects completed the asthma control questionnaire.

5.3.6. Monitoring adverse effects

Patients were asked about any side-effects they encountered during or after completing the study. If patients reported any side effects they were then fully assessed, investigated and treated if necessary. Patients were also provided a telephone number to contact the doctor and/or the study nurse 24/7 in case they have any side effects or adverse reactions to the medications. If any untoward adverse effects were encountered the medicine control agency (MCA) and the providers of etanercept were informed according to the protocol.

5.3.7. Statistical analyses

Students paired t test (mean ± SEM) was used to compare normally distributed paired data and non-parametric Wilcoxon's signed rank tests (median, IQR) were used for unpaired data in the statistical analyses. The data were entered into a spread sheet (Microsoft Excel, Office XP) and analyzed using SPSS for windows (version 11.5, Chicago, IL, USA). The data were plotted using Sigma plot for windows (Version 8).

The PC_{20} data was log transformed before analysis and the changes in PC_{20} following treatment was also analyzed as the number of doubling dilutions from the baseline to show the change.

5.4. Results

Seventeen subjects with chronic severe asthma were enrolled into this open-labeled trial. Two subjects were withdrawn as one developed a swelling in the left supraclavicular fossa which was diagnosed as a lipoma (biopsy proven) and the other subject withdrew for personal reasons as she was unable to visit the department twice a week for the dosing. The data presented are for 15 subjects who enrolled and completed the trial. Three measurements of FEV_1, FVC and PEF on different occasions were made prior to subjects taking part in the study. Subjects were assessed twice a week during the study period and the lung functions were measured along with their diary cards to evaluate changes in PEF. The baseline characteristics of the patients are shown in Table 5.1 and 5.2. All the subjects were on regular oral and inhaled corticosteroids and used nebulised salbutamol as their rescue medication in addition to the metered dose inhalers.

Table 5-1 Baseline characteristics of the subjects

Subjects enrolled	17
Subjects completed the study	15
Male: female	11:4
Mean age (years)	43 (± 10.6)
Mean daily dose of inhaled corticosteroids (mcg)	2540 (± 1600)
Mean daily dose of oral corticosteroid (mg)	12.1 (± 10.6)
Mean daily dose of nebulised salbutamol (mg)	8 (± 6.6)
Mean baseline predicted FEV_1 (%)	65.9 (± 18.3)
Mean baseline predicted FVC (%)	72.8 (± 18.9)
Mean morning baseline PEF (%)	54.8 (± 20.9)
Mean evening baseline PEF (%)	55.3 (± 18.9)

Data shown as mean (± SEM), FEV_1- Forced expiratory volume in 1 second; FVC- Forced vital capacity; PEF- Peak expiratory flow rate

Table 5-2 Baseline characteristics of each subject who completed the study

S. No	Age (yrs)	Sex	Duration of asthma (yrs)	Current daily medications	Dose of inhaled corticosteroids-beclomethasone equivalent (mcg)	Dose of oral prednisolone (mg)	Baseline FEV_1(L)	Baseline FVC(L)
1	46	M	42	Flixotide-1000 mcg, Zafirlukast-10mg, Terbutaline inhalers	2000	5	2.55	3.65
2	44	F	30	Theophylline 900 mg, Fluticasone 2000 mcg, salmeterol 100 mcg	4000	5	2.9	3.35
3	43	F	12	Aminophylline- 450mg, Montelukast 10 mg, Salmeterol 100mcg, Fluticasone 500 mcg, Combivent® inh	4000	5	1.96	3.27
4	32	F	31	Budesonide respules 2mg, Salmeterol 100 mcg, Theophylline 500 mg	1000	5	2.66	3.48
5	33	F	31	Fluticasone 2mg, Salmeterol 100 mcg, Montelukast 10 mg, Combivent inh, Neb Salbutamol	2000	5	1.83	2.57
6	30	F	16	Theophylline 450 mg, Fluticasone 2mg, Salmeterol 100 mcg Montelukast 10mg	4000	25	2.1	3.1
7	35	F	35	Theophylline 450mg, Fluticasone 1000 mcg, Salmeterol 100 mcg	2000	25	0.77	1.54
8	51	F	12	Qvar 600mcg, Budesonide respules 1mg, Montelukast 10 mg, Theophylline 750mg	2500	10	2.11	2.97
9	45	F	12	Flutiasone 2000mcg, Theophylline 450mg	4000	40	1.55	1.78
10	32	F	7	Aminophylline 250mg, Fluticasone 3000mcg, Montelukast 10mg, Salmetreol 100 mcg, Combivent® inh	6000	5	2.21	2.54
11	55	F	38	Theophylline 900 mg, Zafirlukast 40 mg, Fluticasone 2mg, Salmeterol 100mcg	4000	20	1.37	1.75
12	44	F	20	Theophylline 800mg	0	10	2.29	1.62
13	67	M	30	Ipratropium bromide 1mg, Budesonide respules 2mg	2000	10	1.03	1.87
14	58	M	6	Budesonide 1600mcg, Salmeterol 100mcg, Zafirlukast 40mg, Theophylline 400mg	1600	5	2.75	3.37
15	43	M	25	Budesonide Respules 2mg, Fluticasone 1000 mcg, Salmeterol 100mcg, Montelukast 10mg, Theophylline 600mg	4000	7	1.84	3.29

5.4.1. Lung function and symptoms

The mean baseline FEV_1 was 65.9% (± 18.3%) of the predicted while the mean FVC was 72.8 % (± 18.9%) of the predicted. The mean morning baseline PEF was 54.8% (± 20.9%) and the mean evening baseline PEF was 55.3% (± 18.9%) of predicted. During the treatment period the baseline spirometry measured as FEV_1, FVC and PEF improved significantly. The mean FEV_1 improved from 1.91 ± 0.56 L/min to 2.11 ± 0.59 L/min (p= 0.01). The mean FEV_1 percentage predicted improved from 65.9 % ± 4.7 to 75.4% ±4.7 (p=0.04) (Fig 5.2 a, b). The FVC improved from 2.55 ± 0.2 L/min to 2.88 ± 0.2 L/min (p=0.03) Fig 5.3. Due to the improvement in the FEV_1 and FVC the ratio of FEV_1 /FVC did not show statistically significant improvement (Fig 5.4). The FEV_1 /FVC ratio before treatment was 75.5 ±2.3% while after treatment with etanercept this was 75.4 ±3.4 % (p=0.98). The mean morning PEF improved from 280L/min to 311 L/min, an overall improvement of 31L/min (p= 0.023) while the evening PEF improved from 282L/min to 326 L/min, an improvement of 44L/min (p= 0.005) (Fig 5.5 a, b).

Bronchial responsiveness to methacholine markedly improved with the geometric mean PC_{20} increasing from 0.25mg/ml to 1.25mg/ml, (p=0.033) after treatment (Fig 5.6). One subject failed to demonstrate a fall in FEV_1 after the highest concentration of methacholine. Therefore, an arbitrary value of 32mg/ml was considered for statistical analysis. The methacholine responsiveness was also analyzed as area under the curve (AUC). The methacholine AUC improved from 1.45 (0.98-4.3) to 10.6 (3.74-42.61), p=0.022; following 12 weeks of treatment with etanercept. The change in methacholine PC_{20} was 2.5 (± 1.75) doubling dilutions following treatment with etanercept. One subject was unable to perform a methacholine challenge at the end of the study due to a respiratory tract infection.

The asthma control was assessed using asthma control questionnaire. This questionnaire includes both subjective and objective measurements of asthma and evaluates asthma control over a period of time. The baseline symptom scores were 26 (22-30) and following treatment with etanercept the symptom scores reduced to 12 (5-24), p<0.001, (Fig 5.7). All the patients were on inhaled salbutamol and nebulised salbutamol as rescue medications and all but one patient voluntarily withdrew

completely from the use of their regular nebulised salbutamol by the end of the study period.

Figure 5-2: Changes in FEV1 and FVC before and after 12 weeks of treatment with etanercept from baseline

(a) Changes in absolute FEV1 before and after 12 weeks of treatment with etanercept. Panel on left show the individual subjects with the mean values as bold bars and on the right represents the median (IQR). A p value of <0.05 was considered significant

(b) Changes in FEV1 predicted percentage

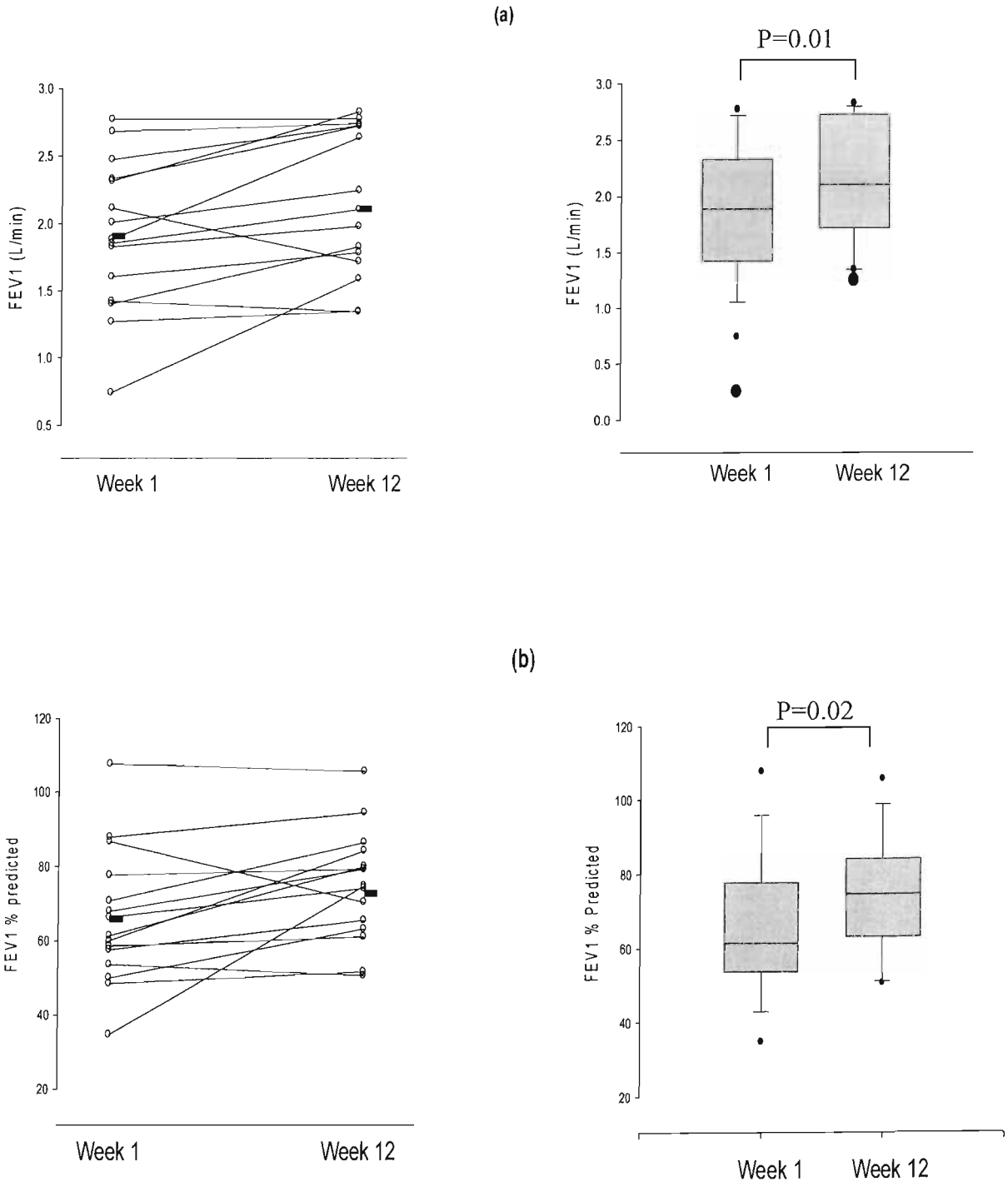


Figure 5-3 Changes in FVC before and after 12 weeks of treatment with etanercept from baseline

Panel on left show the individual subjects with the mean and the plots on the right represent the median (IQR). A p value of <0.05 was considered significant

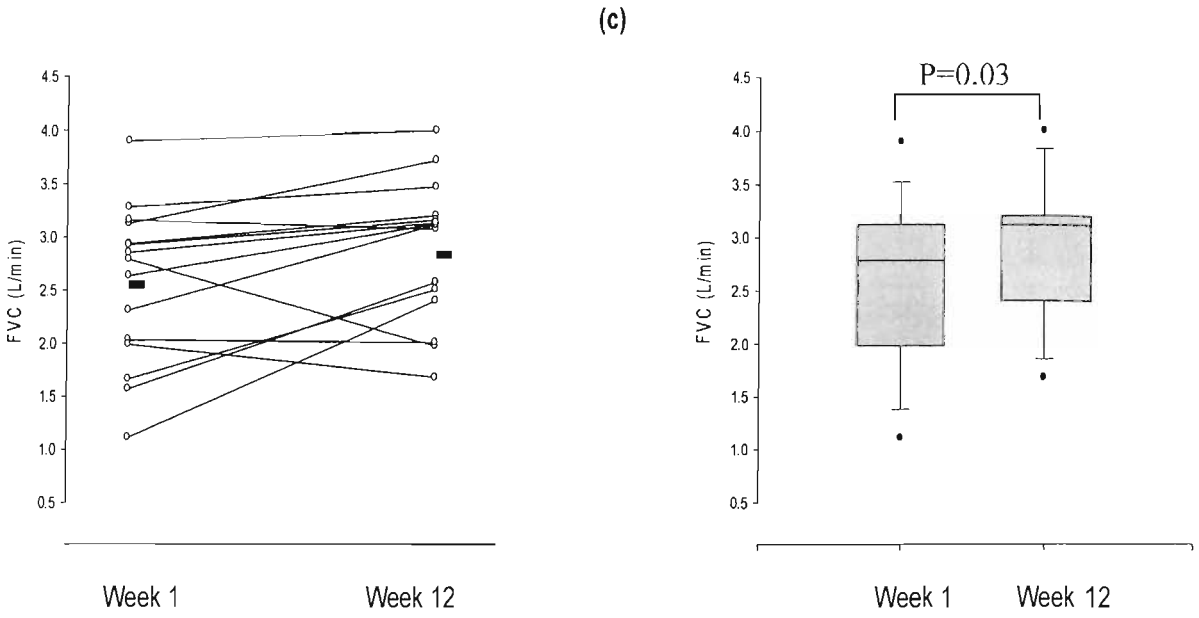


Figure 5-4 Changes in FEV1 /FVC ratio from baseline

Panel on the left shows the individual subjects with the mean and the plots on the right represents median (IQR) .The p value was not statistically significant due to improvements in both the FEV_1 and FVC.

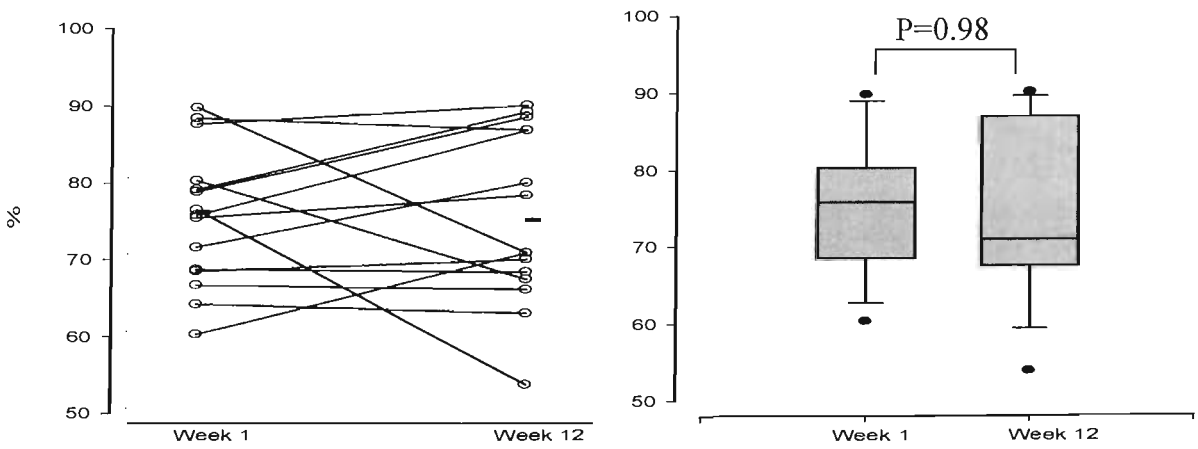


Figure 5-5:Changes in PEF before and after 12 weeks of treatment with etanercept from baseline (a) Changes in morning PEF (b) Changes in evening PEF.

Panel on left shows the individual subjects with their means and the plots on the right represent the median (IQR). A p value of <0.05 was considered significant

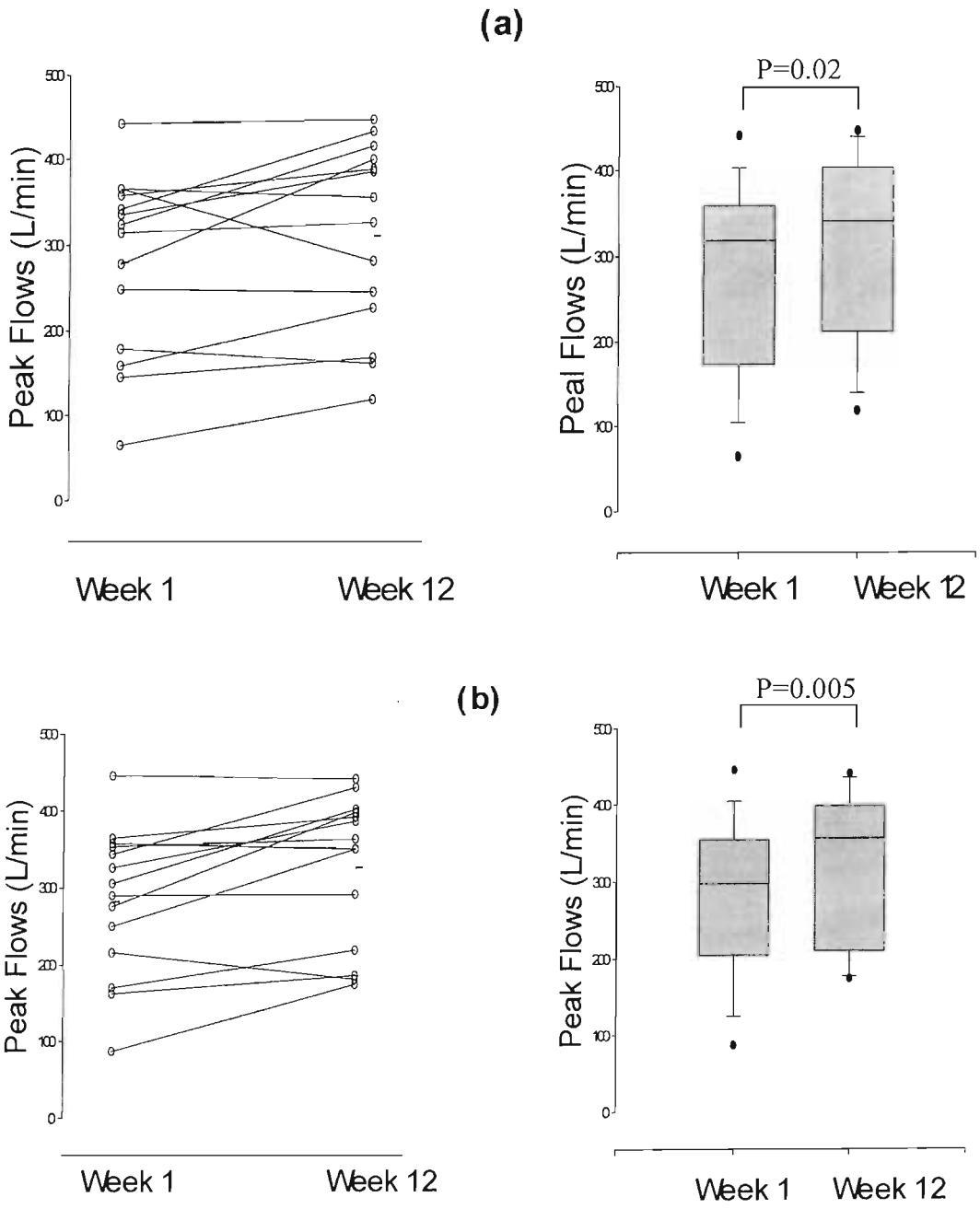


Figure 5-6: Changes in methacholine PC20 before and after 12 weeks of treatment with etanercept from baseline

Data on the Y-axis were log transformed. A p value of >0.05 was considered significant. Panel on left shows the individual subjects and on the right the median (IQR)

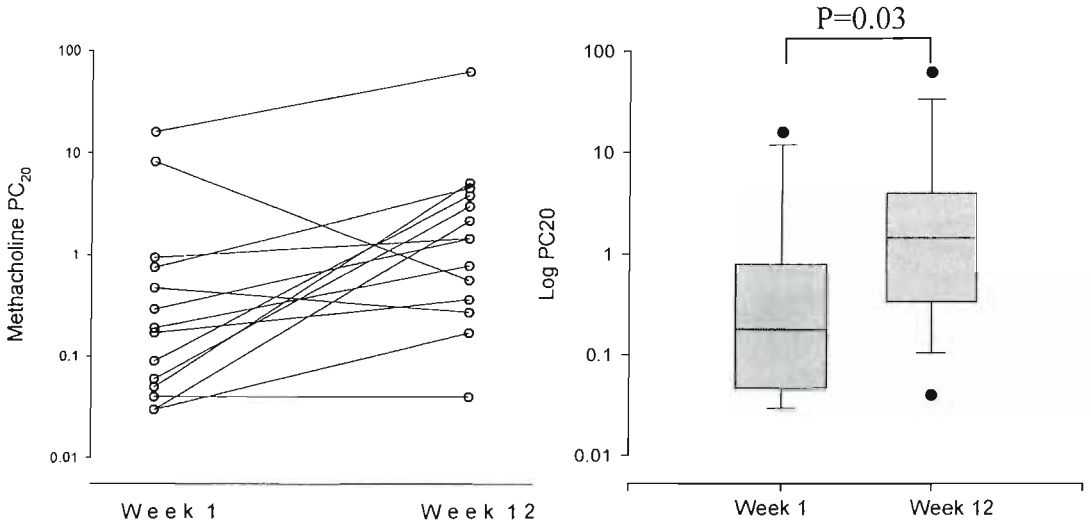
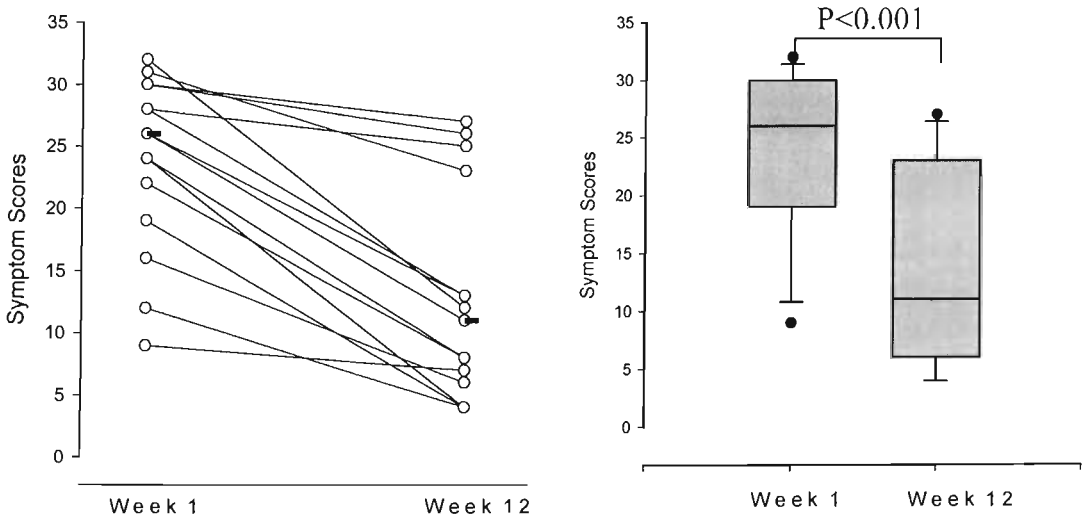


Figure 5-7: Changes in symptom scores before and after 12 weeks of treatment with etanercept from baseline

Panel on left shows individual subjects with the means and on the right represents median (IQR). A p value of < 0.05 was considered significant



5.4.2. Adverse effects

The adverse effects seen were mild and included skin rashes in 4 subjects, injection site reactions in 4 cases. The rashes were maculopapular and urticarial and found at the site of injections. These subsided spontaneously in 4/6 subjects and in 2 subjects oral antihistamines were administered. Injection site reactions included pain and induration at the site of injections. These were managed by rotating the site of administration between the deltoid area, the thighs and the anterior abdominal wall. Three subjects had a weakly positive anti-nuclear antibody at the end of the study. However, there were no other associated features. Nine subjects developed respiratory tract infections during the study and one subject was unable to perform methacholine challenge due to the low baseline FEV_1. Patients developed upper respiratory tract infection with cough and sputum production. This was not associated with fever or changes in the blood biochemistry or full blood count. There were 8 instances of asthma exacerbations but these did not warrant increase in the dose of either the inhaled or oral corticosteroids. The adverse effects encountered are listed in table 5.3.

Table 5-3: Adverse effects observed during the study period

Adverse effect	Number	%
Rash	6	40
Respiratory Tract infections	9	60
Asthma exacerbations	8	53.3
Injection site reactions	4	26.7
Oral Thrush	2	13.3
Joint pains	1	6.7
Nose bleed	1	6.7
Weakly positive ANA	3	20

5.4.3. Induced sputum

Sputum induction was performed using hypertonic saline; however we were able to obtain both pre treatment and post treatment samples from only 11 subjects. The data shown are for 11 subjects

5.4.3.1. Inflammatory cells in sputum

There was no significant change in the inflammatory cells in the expectorated sputum. Data are shown as percentage of cell with mean ± SD (Table 5.4). The mean proportion of eosinophil were 20.2 (4.9%) and following treatment with etanercept they were 16.2 (± 4.1%). The mean proportion of neutrophil were 31.3 (± 8.2 %) at baseline and after treatment with etanercept were 23.5 (± 7.2 %). These results did not reach statistical significance. There was a significant decrease in the absolute neutrophil count before and after 12 weeks of treatment with etanercept. The absolute neutrophil count decreased from 47.9 x 10^4/gm of sputum (2.7-617.2) to 12.3 x 10^4/ gm of sputum (3.1-132.4); p=0.04. There was no significant change in the eosinophil counts in the sputum before and after 12 weeks of treatment with etanercept (Fig 5.8).

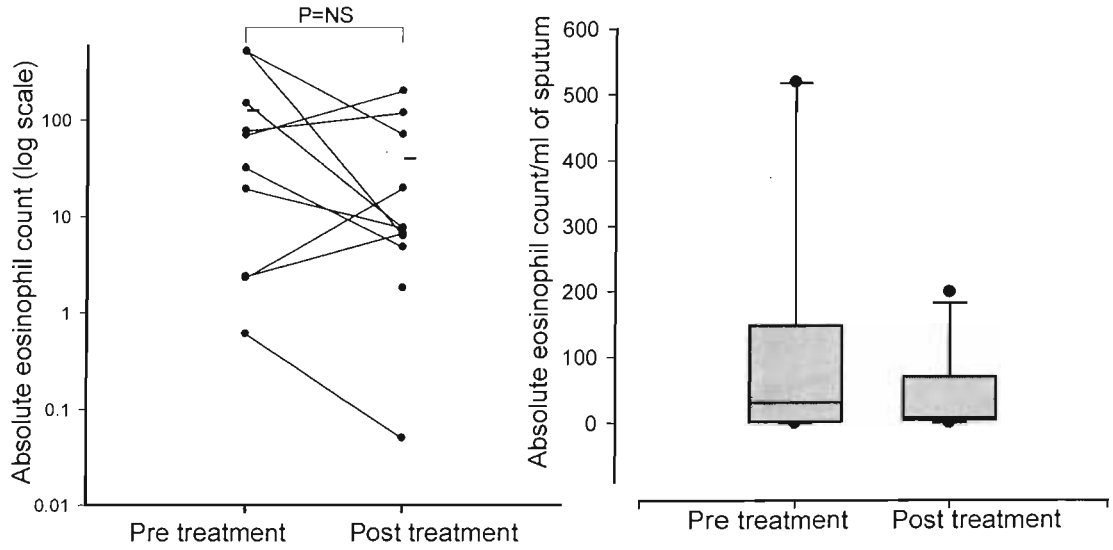
Table 5-4 Inflammatory cells in induced sputum before and after 12 weeks treatment with Etanercept

Inflammatory Cells (n==11)	Pre-treatment (%)	Post-treatment (%)	P value
Eosinophils	20.2 (± 4.9)	16.2 (± 4.1)	NS
Neutrophils	31.3 (± 8.2)	23.5 (± 7.2)	
Macrophages	36.5 (±5.7)	49.5 (± 5.7)	NS
Lymphocytes	2.3 9 (± 0.6)	1.4 (± 0.5)	NS
Epithelial Cells	9.9 (± 6.0)	9.6 (± 5.0)	NS

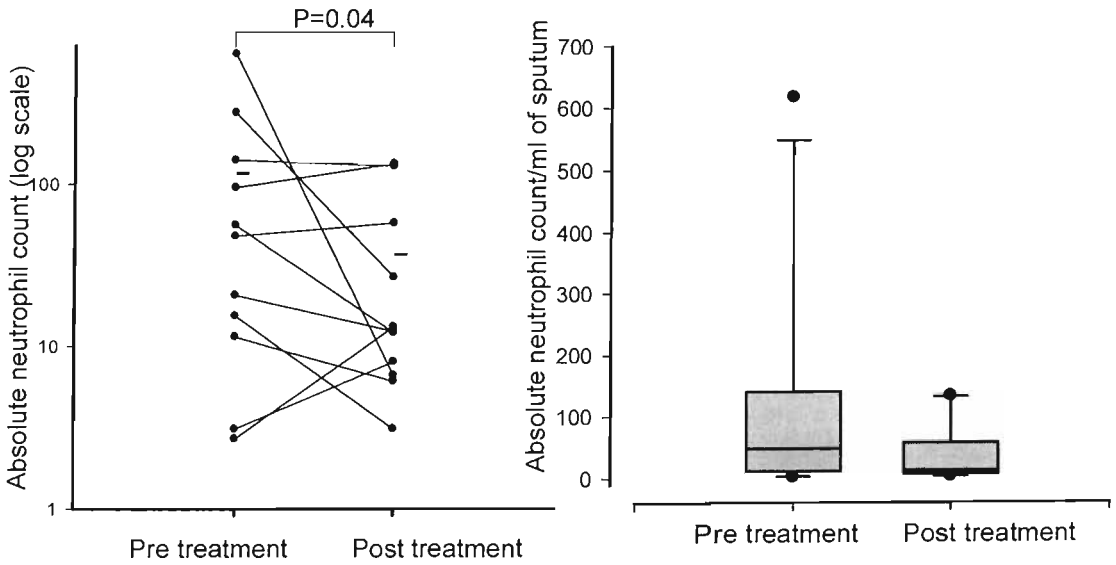
NS- Not significant; Data shown as mean (± SEM)

Figure 5-8 Changes in sputum inflammatory cells before and after 12 weeks of treatment with etanercept

(A) Changes in eosinophils expressed as absolute counts/gm of sputum. Mean is shown as a horizontal bar. Data on the panel on left was log transformed due to the wide variation in cell counts



(B) Changes in neutrophils expressed as absolute counts/gm of sputum. Mean is shown as a horizontal bar. Data on the panel on left was log transformed due to the wide variation in cell counts



5.4.3.2. Cytokine levels in sputum supernatant

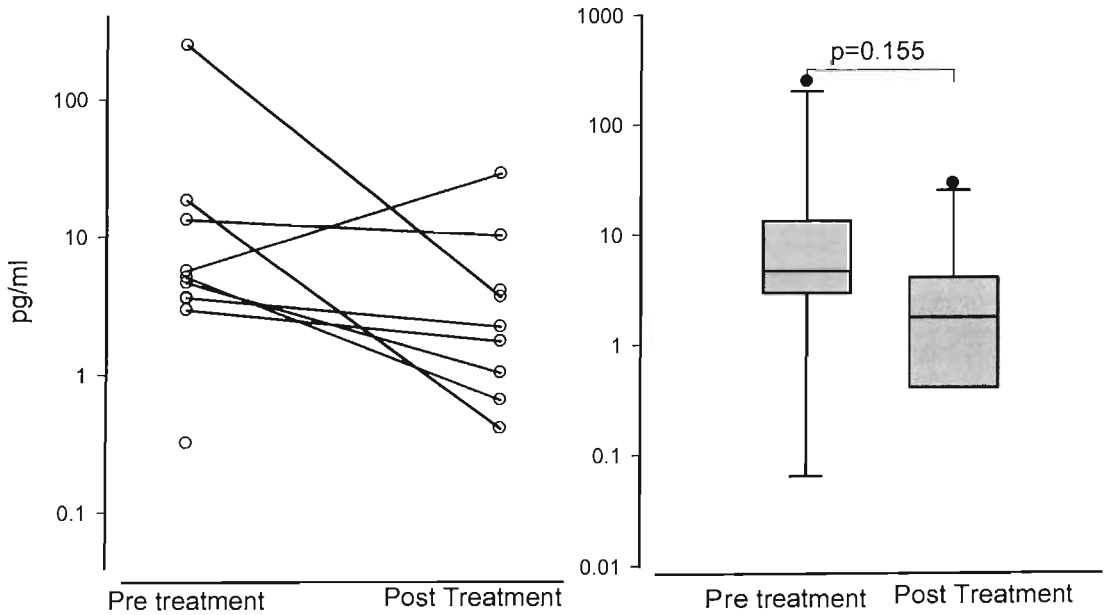
The levels of cytokine protein in the sputum supernatants were determined using commercially available **E**nzyme **L**inked **I**mmuno-**S**orbent **A**ssay (ELISA) kits for the various cytokines in accordance with the manufacturers' instructions. The results are expressed as median (inter quartile ranges).

5.4.3.2.1. Levels of TNF-α in sputum supernatants

The baseline levels of TNF-α was 4.6 (2.9-13.2) pg/ml. After 12 weeks of treatment with etanercept the sputum TNF-α levels were 1.7 (0.4-4.0) pg/ml (Fig 5.9). There was a reduction in the levels of TNF-α but this did not reach statistical significance (p=0.15).

Figure 5-9 Levels of TNF-α in sputum supernatants before and after 12 weeks of treatment with etanercept

A p value 0f <0.05 was considered significant. Panel on the left represents individual subjects plotted on log scale and the box plot represents median (IQR)

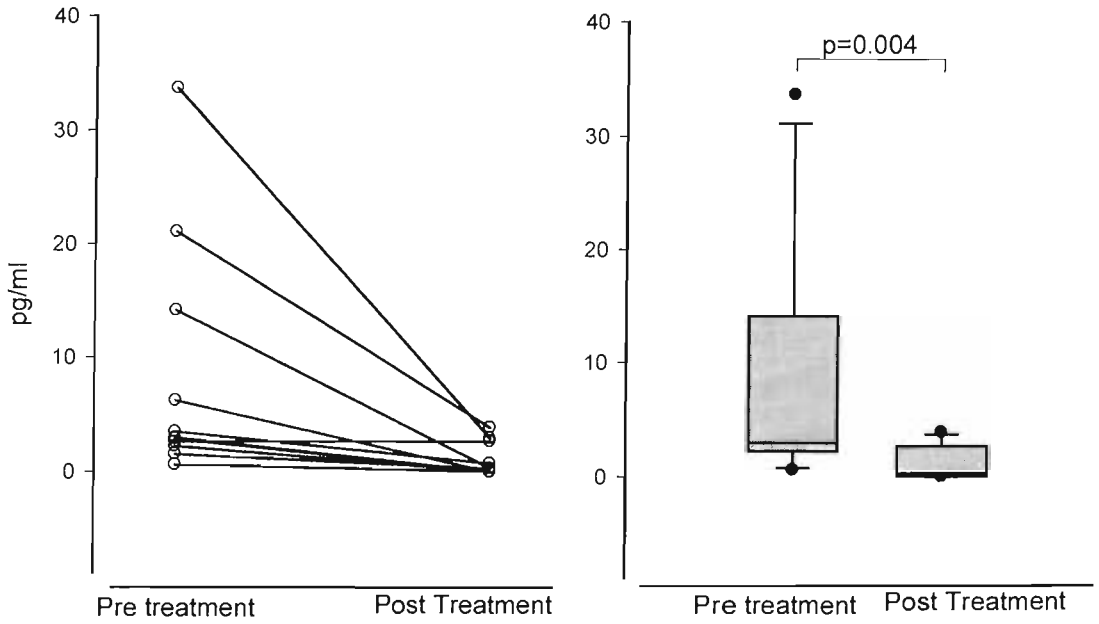


5.4.3.2.2. Levels of IL-8 in sputum supernatants

The baseline IL-8 levels in the sputum supernatants were 3.0 (2.2-14.1) pg/ml. Following treatment with etanercept for 12 weeks the levels reduced to 0.3 (0.0-2.7) pg/ml (Fig 5.10). These results were statistically significant (p=0.004).

Figure 5-10 Levels of IL-8 in sputum supernatants before and after 12 weeks of treatment with etanercept

A p value 0f <0.05 was considered significant. Panel on the left represents individual subjects and the box plot represents median (IQR)



5.4.4. Follow-up

Patients were followed up after 8 weeks following the last dose of the study medication and the FEV_1, FVC and symptom scores reverted to their pre-treatment levels with no significant changes (Table 5.5 and Table 5.6). Patients were not provided a diary card to assess their PEF, therefore PEF were not available for follow up evaluation. Two patients were not available for follow up hence the data reported are for 13 subjects.

Table 5-5: Follow up of patients after 12 weeks of treatment with etanercept

	Pre treatment	Post Treatment	Follow-up
FEV_1 (predicted %)	65.9 (±4.7)	75.4 (±4.7)	66.2 (±5.7)*
FEV_1 (L/min)	1.91 (±0.15)	2.11 (±0.15)	1.95 (±0.2)*
FVC (L)	2.55 (±0.2)	2.88 (±0.2)	2.68 (±0.2)*
FEV_1/FVC (%)	75.5 (±2.3)	75.4 (±3.4)	72.6 (±4.0)*
Morning PEF (predicted %)	54.8 (±5.6)	61.0 (±5.8)	ND
Evening PEF (predicted %)	55.3 (±5.1)	63.9 (±5.1)	ND
Methacholine PC20 (mg/ml)*	0.25 (0.1-0.64)	1.25 (0.53-2.92)	ND
Methacholine AUC†	1.45 (0.98-4.3)	10.6 (3.74-42.61)	ND

ND- Not Done; *the p values were not significant following 8 weeks follow-up

Table 5-6 Lung function and symptom scores of individual subjects before, after and 8 weeks follow up following treatment with etanercept

S. No	FEV_1 (L/min)			FVC (L/min)			FEV_1/FVC (%)			Symptom Scores		
	Week 1	Week 12	Follow-up	Week 1	Week 12	Follow-up	Week 1	Week 12	Follow-up	Week 1	Week 12	Follow-up
1	2.68	2.74	2.74	3.90	4.01	3.65	68.7	68.4	75.1	12	4	15
2	2.33	2.72	2.25	2.64	3.13	2.30	88.4	86.9	97.8	16	6	26
3	1.89	2.64	2.51	3.13	3.73	3.70	60.3	70.9	67.8	24	4	9
4	2.77	2.78	2.85	3.16	3.08	3.30	87.7	90.1	86.4	19	4	15
5	2.01	2.25	1.61	2.94	3.21	2.45	68.5	70.1	65.7	26	13	15
6	2.48	2.73	2.41	3.28	3.48	3.13	75.5	78.4	77.0	26	11	25
7	0.74	1.59	1.41	1.11	2.41	2.19	66.8	66.1	64.4	32	12	26
8	2.12	1.72	1.43	2.79	1.98	2.44	75.8	86.9	58.6	28	25	26
9	1.61	1.79	1.35	2.04	2.02	1.50	78.9	88.6	90.0	30	26	37
10	2.32	2.83	2.67	2.93	3.17	3.07	79.2	89.3	86.9	28	13	24
11	1.43	1.35	0.93	1.99	1.68	1.41	71.6	80.1	66.0	31	23	28
12	1.41	1.83	NA	1.57	2.58	NA	89.9	70.9	NA	30	27	NA
13	1.27	1.35	1.00	1.66	2.51	2.03	76.5	53.8	49.3	24	8	28
14	1.86	2.11	NA	2.31	3.12	NA	80.3	67.5	NA	22	8	NA
15	1.83	1.96	2.19	2.85	3.14	3.71	64.2	63.1	59.0	9	7	7

NA-Not available as patients did not attend follow-up

5.5. Discussion

The therapeutic options for patients with corticosteroid-dependent asthma are very limited. Severe corticosteroid dependent and resistant asthma still represents a great clinical burden accounting for approximately 50% of the health care costs of asthma in view of the multiple admissions for asthma exacerbations and the side effects of long term corticosteroids (299). Thus, 5% of these asthmatic patients account for approximately 50% of total health care costs (300). Therefore, reducing the side-effects of corticosteroids using novel dissociated steroids, soft steroids or with corticosteroid-sparing agents will prove beneficial. Various T cell immunomodulatory agents like cyclosporine, gold, azathioprine and methotrexate have been used with variable success (301-305). Biological agents and cytokine modulators are also being evaluated for the treatment of severe asthma (306). A monoclonal antibody against the FcεRI, the high affinity receptor of IgE has been found to improve asthma control and reduce the need for corticosteroids in patients with severe asthma (305). In view of the economic burden imposed by patients with chronic severe asthma and the large unmet needs in this subset of patients' newer therapeutic options need to be investigated.

This study was a proof of concept study and consequently an open label uncontrolled clinical study. This study was designed to be a hypothesis generating study to evaluate TNF-α blocking strategies in patients with severe asthma. As there were no available data on the role of TNF-α blocking strategies in patients with severe corticosteroid dependent asthma, it was difficult to undertake a placebo-controlled trial. It was also considered not ethical or rational to withdraw or reduce oral or inhaled corticosteroids in patients with such severe disease. In view of these issues, this study followed an open labeled design.

The patients enrolled in this trial were taking regular oral corticosteroids in addition to inhaled corticosteroids. Furthermore, they were on other treatments which included long acting β2 agonists, leukotriene modifying drugs and aminophylline. The need for regular

oral corticosteroids serves as a surrogate marker for severe persistent asthma. In this open labeled study we have shown that regular therapy with etanercept produces remarkable improvements in lung function as seen by improvements in the lung function parameters FEV_1, FVC and PEF.

The improvement in FEV_1 whilst significant was only modest, but the patient population investigated were severe asthmatics and this improvement translates into significant subjective improvement in asthma control. The well established anti-inflammatory therapy for asthma is corticosteroids. In severe asthmatics both oral and inhaled corticosteroids have been studied. In severe asthma, Bosman et al showed that inhaled beclomethasone (1000 mcg) improved FEV_1 from 85 ±18 % to 93 ± 18% while 10 mg of oral prednisolone improved FEV_1 from 86 ±16 % to 91 ±15 % (307). The overall improvement in FEV_1 was 5-8%. Similarly Jenkins et al showed that in patients with chronic severe asthma, inhaled beclomethasone improves FEV_1 from 1.9 L to 2.2 L; an improvement of 0.3 L (308). Limits have not yet been established for corticosteroid therapy to produce changes in FEV_1 .Values of ± 0.15L of FEV_1 or 15L/min of PEF are reasonable estimates (309). Therefore an improvement in the FEV_1 of 0.2L or 9.5% of predicted, though modest is comparable to the standard anti-inflammatory therapies used for the treatment of asthma. Furthermore, the subjects studied had severe airflow obstruction and therefore are more likely to have poor reversibility due to airway wall remodeling. Ulrik and Backer have shown that the subset of non-smokers with moderate to severe asthma have a steeper rate of decline in lung function and poorly reversible or non reversible airflow obstruction (310). They also construed that long term treatment with oral corticosteroids was associated with an increased risk of non reversible airway obstruction. As all the patients recruited for this study had been on oral corticosteroids for variable duration it is likely that there is an element of poor reversibility of airways in the group that was studied.

The improvement in the PEF in this open-labeled trial was also comparable to the efficacy of inhaled corticosteroids in patients with moderate to severe asthma. A recent Cochrane database review showed that in non-oral corticosteroid treated asthmatics with

mild and moderate disease fluticasone resulted in improvements from baseline compared with placebo across all dose ranges in FEV_1 (between 0.13 to 0.45 litres); morning PEF (between 27 and 47 L/min); symptom scores (based on a standardised scale, between 0.5 and 0.85) and reduction in rescue beta-2 agonist use (311). In another study, fluticasone 2mg/day, 1mg/day and budesonide 1.6mg/day was compared in a double blind fashion in patients with severe asthma. The mean morning PEF improved by 24 L/min the fluticasone 2mg group, 21 L/min in the fluticasone 1mg and 13 L/min in the beclomethasone group (312). In the present study mean morning PEF improved by 31 L/min and the mean evening PEF improved by 44 L/min and was statistically significant. This was comparable to the improvements reported with inhaled corticosteroids in patients with moderate to severe asthma.

It was interesting to note that the FVC also improved following administration of etanercept. Asthma is traditionally characterized by a reduced FEV_1 and an essentially normal FVC. However patients with chronic severe asthma share some features of COPD, in that there is an element of air trapping akin to COPD (98, 121). Improvement in the FVC therefore implies improvement in the air trapping. As both the FEV_1 and FVC improved the ratio which is characteristically reduced in obstructive airway disease there was no statistically significant change in the ratio.

Etanercept resulted in significant improvement in asthma control. Asthma control was evaluated using a validated questionnaire developed by Juniper and colleagues (298). This questionnaire includes seven items. The first five involves subjective evaluation by the patient of their symptoms. The sixth item is the use of rescue medications and the last item is the FEV_1 at the time of hospital visits. This questionnaire therefore evaluates asthma control over the past one week using both subjective and objective measures of evaluation. Although there was no difference in the use of the metered dose inhaler as the rescue medication, all but one patient completely and voluntarily withdrew from their use of nebulised salbutamol. At the end of the study two patients requested continuation of etanercept therapy on compassionate grounds and in the remainder, the beneficial effect was maintained for 2-4 weeks before their asthma gradually returned to the pre-treatment

state. This suggests that Etanercept had a beneficial effect on asthma in this group of subjects but the effects are not long lasting as seen in patients treated with anti TNF-α for rheumatoid arthritis (313). All but one subject had an improved asthma control and a feeling of well-being within 4 weeks of starting treatment with etanercept.

The most striking feature of this study was the improvement in airway hyperresponsiveness. Airway hyperresponsiveness is a characteristic feature of asthma and any improvement in this element is believed to be associated with improved asthma control. When measuring BHR in asthma using repeated methacholine challenge, it is accepted that the error in PC_{20} determination is within one doubling dilution. Following experimental allergen exposure or natural allergen exposure, changes of 1-2 doubling dilutions in PC_{20} are considered clinically significant (314). Thus, although we had no data on which to base a power calculation prior to commencement of the study, our findings of an improvement of 2.5 doubling dilutions in PC_{20} is well outside the natural variation and consistent with a significant clinical improvement, in addition to any beneficial effect that may have already been achieved using inhaled and oral corticosteroids.

Studies have shown that TNF-α regulates the function of airway smooth muscle by modulating the secretion of cytokines and chemokines and the level of expression of adhesion molecules (193). TNF-α is believed to affect airway hyperresponsiveness both by reducing the influx of inflammatory cells into the airway smooth muscle and by a direct effect on the calcium homeostasis in the smooth muscle. TNF-α is well known to directly influence the modulation of BHR and this has been shown in various animal and human experiments (191, 272). In tracheal preparations of Dunkin-Hartley pigs recombinant TNF-α increased maximal isotonic contraction to methacholine which was completely inhibited by co-incubation in the organ bath with a dimeric recombinant TNFR p80 construct (315). In keeping with these observations, this is the first clinical study to demonstrate the abrogation of BHR by blocking the effects of TNF-α in patients with severe asthma. Though this study was open-labeled this significant improvement in the airway responsiveness is unlikely to be a placebo phenomenon. In addition the return

of the lung function to pre-treatment levels during the 8 week follow up suggests an operational role for TNF-α blocking strategies in patients with chronic severe asthma.

We attempted to study the effects of etanercept on indices of inflammation using induced sputum. Despite the severity of their asthma, we were able to obtain paired sputum samples from 11 of the 15 subjects involved in the study. However, although reductions in eosinophil and neutrophil numbers were observed, only the neutrophil numbers reached statistical significance. This observation that inhaled TNF-α increases BHR and leukocyte influx into human airways (191, 272) suggests that further studies involving tissue biopsies before and after therapeutic intervention may better address the effects of etanercept on endothelial cell activation and inflammatory cell recruitment.

In patients with asthma there is a strong correlation between the levels of IL-8 and the percentage neutrophils and the levels of MPO points to a role of IL-8 in the recruitment and activation of neutrophils in the airway lumen (316). Chronic severe asthma is characterized by neutrophilic airway infiltration and neutrophil influx occurs during allergen challenge (317). Neutrophils are recruited into the airways in asthma by a number of mediators and chemokines, the most important of which is interleukin IL-8 (80). TNF-α activates the transcription factor nuclear factor-κB (NF-κB), which in turn activates the IL-8 gene in epithelial cells and macrophages. In the present study we have shown that IL-8 levels were significantly decreased following treatment with etanercept. While, the TNF-α levels were reduced this did not reach statistical significance. The finding that sputum TNF-α level was not significantly decreased after treatment with etanercept may be due to TNF-α not being secreted adequately into the sputum. In a recent study it was found that the assay of TNF-α is very variable in the sputum supernatants (318). The baseline IL-8 levels were high in 4/11 subjects however these subjects did not show any specific differences in their lung function, airway responsiveness or changes in other parameters following treatment with etanercept. As all the ELISA were done in duplicates it is unlikely to be a chance finding. The reduction in the levels of IL-8 in the sputum supernatants suggests the possibility of etanercept affecting the cytokine *milieu* and as a consequence on the improvement in asthma

control. The reduction in neutrophils associated with a decrease in IL-8 levels in the sputum following treatment with etanercept points to a possible role for TNF-α in modulating IL-8 and hence a possible role for TNF-α blocking strategies in the management of patients with severe asthma. Further studies are required to evaluate the exact role of TNF-α blocking strategy on the inflammatory parameters in asthma and to understand the mechanistic role of TNF-α blocking strategy.

Most of the actions of TNF-α in asthma occur in combination with other cytokines as part of a complex interacting cytokine network. In chronic severe asthma, neutrophils become increasingly prominent in addition to eosinophils (72). In rheumatoid arthritis blocking the effects of TNF-α reduces the production of downstream cytokines IL-6, IL-8 and adhesion molecules ICAM-1 and VCAM-1 (319). Since T lymphocytes, macrophages, eosinophils and neutrophils use the same spectrum of adhesion molecules it is likely that TNF-α blockade in asthma reduces cellular trafficking into the airways. TNF-α regulates IL-8 release from a range of cells including epithelial cells, fibroblasts, smooth muscle and endothelial cells which mediate neutrophil chemotaxis and activation. Thus, by blocking the effects of TNF-α, etanercept is able to down regulate IL-8 release thereby reducing neutrophil trafficking.

The development of novel therapeutic strategies for asthma is chiefly focused on identifying new targets that could help to inhibit or prevent asthmatic airway inflammation. Several cytokines and their receptors are considered as possible candidates for such strategy. Although multiple cytokines have been implicated in the pathogenesis of allergic disease some cytokines play a more critical role in the inflammatory process. Studies with anti IL-5 was effective in decreasing the eosinophils in the blood and BAL, however this treatment had no effect on airway hyperresponsiveness (49). IL-4 and IL-13 blocking strategies have shown clinical efficacy in patients with mild to moderate asthma. Nebulised soluble IL-4 receptor has shown effectiveness in preventing the deterioration in asthma after reduction of inhaled corticosteroids (320, 321) however two large phase III trials did not show significant improvements in asthma and hence this molecule has been dropped from further development.

In the present study a soluble receptor for TNF-α was effective in improving asthma symptoms, rescue medication use, and most importantly, airway responsiveness in a sub group of asthmatics with chronic and severe disease. TNF-α is expressed in asthmatic airways and plays a key role in amplifying the airway inflammation through activation of NF-κB, AP-1 and other transcriptional factors. Current therapies for asthma are effective in the majority of patients but there is a need to develop new treatments that deal with more severe asthma which is currently not well controlled by high doses of inhaled corticosteroids. Blocking the effects of TNF-α could therefore be a logical approach to asthma therapy particularly in patients with severe disease.

The most common adverse effects encountered in this study were injection site reactions which presented as a localized maculopapular and urticarial rash. Some patients had itching. There was no ulceration or blistering. To overcome this problem the patients had their injection sites rotated between their arms, thighs and the anterior abdominal wall. The incidence of rashes was reduced after rotating the sites of injection. Two subjects reported generalized rashes which have been described in the past with etanercept. These rashes settled with oral antihistamine treatment and did not warrant withdrawal of the subjects from the study or admission to the hospital, suggesting that these were either mild or moderate. The respiratory tract infections experienced by the 4 subjects would not be unusual in such patients with severe disease. It is difficult to suggest a causal association between the respiratory tract infections and the study medication as this was an open labeled study. This was associated with worsening of asthma control which was managed by increasing the dose of rescue medications. None of the subjects needed an increase in either their oral or inhaled corticosteroids suggesting a possible beneficial effect of etanercept in these subjects. Etanercept as other TNF-α modifying therapies are well-known to predispose patients to upper and lower respiratory tract infections. The incidence of upper respiratory tract infections was 29% for etanercept in placebo controlled trials (322). In addition the incidence of tuberculosis has been reportedly higher in patients on long term TNF-α blocking treatment. In the present study, we did not see any patients develop symptoms and signs suggestive of tuberculosis. Three

subjects had a weakly positive anti-nuclear antibody (ANA) following treatment with etanercept. This was not associated with any clinical features suggestive of SLE. Earlier reports have suggested that etanercept is associated with development of auto-antibodies. Of the patients evaluated for ANA the percentage of patients who developed new positive ANA (≥ 1:40) was higher in patients treated with etanercept (11%) than in the placebo treated group (5%). However no patients developed clinical signs of lupus like syndrome or other new auto-immune diseases (323). Hence this form of therapy could be a relative contraindication for patients with SLE. Follow-up of 10/15 patients by contacting their General Practitioners by telephone two years following the study did not reveal any increased incidence of malignancies.

5.6. Conclusions

This is the first study to evaluate the effects of TNF-α blockade in patients with severe asthma. This study provides evidence for the role of TNF-α in asthma and opens up the possibility of blocking TNF-α as a therapeutic option. While it could be argued that changes could result from a placebo effect, this is most unlikely on account of the clear beneficial effects of treatment on baseline lung function. Of particular note is the dramatic 5-10 fold reduction in BHR that occurred after treatment with etanercept. This study has not established whether anti-TNF-α therapy is efficacious in milder forms of asthma, however, in those with severe disease, the improvement in the lung function, asthma symptoms and rescue medication use were significant. Since severe, asthma makes up a substantial portion of the health costs for this disease, we believe these observations may be of clinical significance in identifying TNF-α as a new therapeutic target. The success of this treatment provides evidence for an active role for TNF-α in the pathophysiology of chronic severe asthma. The effects of anti-TNF therapy in severe asthma now require confirmation in larger placebo controlled studies.

6. Discussion and future studies

6.1. Summary of Results

Asthma treatment has not changed significantly over the last 10 years. Since the introduction of leukotriene receptor antagonists' back in 1995, the only other new treatment introduced for asthma is anti IgE (Omalizumab). But, the addition of omalizumab herald a new era in asthma therapy as this is the first biological molecule introduced for asthma treatment. With the increasing incidence of asthma and the recognized unmet needs for the patients with severe disease, it is essential to identify new targets and therapeutic strategies. The application of modern molecular and cellular techniques combined with the ability to obtain clinical samples by fibreoptic bronchoscopy from the airways of patients with asthma has proved to be a useful tool in this pursuit. This has facilitated the study of inflammatory cells and cytokines mediating airway inflammation and the effects of novel therapeutic molecules for asthma. The studies presented in this thesis have examined the effects of allergen exposure on airway inflammation and in particular TNF-α in the airways, the effects of blocking the effects of TNF-α in an *in vitro* culture system and the clinical effects of blocking the effects of TNF-α in patients with severe asthma.

Asthma, irrespective of severity is a chronic airway inflammatory disease associated with airway hyperresponsiveness, airflow limitation, and as a consequence, respiratory symptoms. Initiation and regulation of airway inflammation is influenced by various factors including, the environment, the cell type, inflammatory mediators and the genetic makeup (susceptibility) of the individual. Allergen challenge has been used to study airway inflammation and the roles of various cytokines in asthma when there is an allergic basis to the disease.

The use of the repeated low dose allergen challenge model to assess the airway inflammation in patients with mild asthma has been evaluated in earlier studies (142, 250). While one study showed changes in airway inflammation without significant

changes in asthma control (243) other studies have shown worsening of BHR following low dose allergen challenge (142) and worsening asthma symptoms with changes in lung function (143). The response pattern may, for example, depend on patients' characteristics, the level of allergen exposure, the duration of exposure and the type of allergen; however this experimental model has more resemblance to the situation of natural allergen exposure. In this thesis, I have shown that repeated low dose allergen exposure daily three days a week for four weeks in patients with mild atopic asthma is a safe procedure and can produce sub-clinical changes in the airway inflammation without producing significant changes in the lung function or airway responsiveness. The first chapter of this thesis studies the role of low dose allergen challenge and its effects on TNF-α and adhesion molecules in subjects with mild allergic asthma.

The studies with repeated low dose allergen exposure in subjects with mild allergic asthma have demonstrated that:

a) Repeated low dose allergen challenge with house dust mite is a safe procedure and in my hands was not associated with significant changes in airway hyperresponsiveness, asthma control, lung functions and rescue medication use.

b) Repeated low dose allergen challenge resulted in up-regulation of TNF-α in bronchial biopsies associated with a parallel increase in the number of mast cells in the submucosa, thereby suggesting the possible source of TNF-α as the mast cell.

c) The up-regulation of TNF-α in the bronchial biopsies was associated with an increase in the expression of adhesion molecules VCAM-1 and ICAM-1 emphasising the importance of adhesion molecules which are involved in the recruitment of inflammatory cells into the airways.

Repeated low dose allergen exposure model is a useful model to assess airway inflammation in asthma but it involves administration of allergen to patients with asthma. For that reason this study would be difficult to perform in patients with moderate or

severe asthma. Furthermore repeated bronchoscopies and stopping the inhaled corticosteroids and long acting β2 agonists prior to bronchoscopy in patients with moderately severe asthma can be associated with deterioration of asthma. We therefore used an explant culture system which has been validated in earlier experiments in our department (34-36) to study the effects of house dust mite on the airway tissues. The usefulness of this model have been shown from various studies from our department.

The use of immunohistochemistry has helped to define the resident inflammatory cells in the bronchial mucosa of mild and moderately severe asthmatics. The application of inflammatory bio-markers by this technique has helped identify the presence of $CD3^+$, $CD4^+$ and $CD8^+$ T lymphocyte population, neutrophils, eosinophils, mast cells and macrophages in the airway mucosa. This technique was also employed to identify the expression of TNF-α and adhesion molecules in the bronchial biopsies. Molecular techniques including ELISA employed to the explant supernatant and the sputum supernatant has helped us to determine the cytokines in the airways and the effects TNF-α blockade on these cytokines. The explant culture method provides a means of evaluating newer molecules for the treatment of asthma before undertaking clinical trials on human volunteers.

The studies with explant culture methods on the bronchial biopsies of moderately severe asthmatics demonstrated that:

a) CDP 870, the TNF-α blocking PEGylated Fab fragment masked the assay of TNF-α in the supernatants by 33%. Hence a correction was used for the assay of TNF-α in the explant supernatants.

b) As found in the low dose allergen challenge model *in vivo*, stimulation of bronchial explant cultures *in vitro* from moderately severe asthmatics with house dust mite allergen extract resulted in increased expression of TNF-α in the bronchial biopsies. However, in this model it was also possible to show that the levels of TNF-α were significantly reduced both in the biopsies and in the supernatant following exposure to CDP 870. Furthermore, in the presence of CDP

870 the levels of adhesion molecule ICAM-1 were reduced below the baseline levels.

c) Analysis of cytokines from the explant supernatants showed significant reduction in the levels of TNF-α and IL-8 in the presence of CDP 870. However there was no change in the levels of either IL-1β and IL-5 levels.

The gold standard to evaluate any new therapeutic intervention in medicine is through clinical trials. In this thesis I have assessed the role of TNF-α blockade with etanercept- a soluble TNF-α receptor in patients with chronic severe asthma. Given that this was a pilot study and the dearth of information available on the role of TNF-α blockage in asthma it was felt appropriate to conduct an open-labelled study to look at the role of this intervention in patients with severe asthma.

The clinical trial with etanercept in patients with chronic severe asthma showed that:

a) Administration of 25 mg of etanercept twice a week for 12 weeks in patients with severe asthma resulted in a significant improvement in FEV_1 and interestingly in FVC. This was associated with a concomitant improvement in both the morning and evening PEF. As both the FEV_1 and FVC improved, the FEV_1 /FVC ratio did not show significant improvement.

b) Etanercept also improved asthma control as assessed by Juniper's asthma control questionnaire. All but one patient were able to withdraw completely from the use of their nebulised β2 agonist. There was a significant improvement in airway responsiveness following treatment with etanercept. The airway responsiveness as assessed by methacholine challenge showed a 2.5 doubling dose increase.

c) The improvement in lung function and asthma control lasted for the duration of treatment and the patients' lung function had returned to their baseline levels by 8 weeks of follow-up after completing the trial.

d) Sputum IL-8 levels were significantly reduced following treatment with etanercept. This was associated with a reduction in the neutrophils in the sputum of the treated patients.

e) Etanercept was safe and well tolerated. The adverse effects encountered were rashes, injection site reactions and upper respiratory tract infection. These have been reported with the use of etanercept in patients with rheumatoid arthritis.

Mild asthma is characterised by eosinophilic inflammation but it is difficult to say whether all the clinical features of asthma is eosinophil mediated. Recent studies with allergen challenge of mice with total ablation of the eosinophil lineage was associated with increases in airway hyperresponsiveness and mucus secretion similar to those observed in wild-type mice, but the eosinophil-deficient mice were significantly protected from peribronchiolar collagen deposition and increases in airway smooth muscle suggesting that eosinophils contribute substantially to airway remodelling but are not obligatory for allergen-induced lung dysfunction (324). On a similar note, administration of anti IL-5 antibodies in patients with asthma resulted in reduction of both blood and airway eosinophils and the deposition of tissue matrix proteins with no changes in the asthma control (49, 50). These studies suggest that eosinophils have a role in asthma pathogenesis but do not explain all the clinical manifestations of asthma despite some asthmatics not exhibiting airway eosinophilia (325). While mild asthmatics are well controlled with inhaled steroids it is the steroid unresponsive severe asthmatics that pose a major problem.

It is unclear why severe asthmatic patients respond poorly to inhaled and oral corticosteroids. While it could be argued that severe asthma is a more complex form of mild asthma it is also possible that this is a different form of disease. A distinct pathophysiology which might account for this difference is the predominance of neutrophilic inflammation (295). In severe asthma, epithelial damage has the potential to contribute to neutrophilic inflammation through enhanced production of IL-8 via EGFR-dependent mechanisms (76). The clinical efficacy of glucocorticoids can be attributed to

several actions on eosinophils, including reduction of circulating number; inhibition of the recruitment of eosinophils to sites of inflammation after allergen provocation in *in vitro* and *in vivo* animal models and reduction in the number of eosinophils and their secretory products in the blood, BAL fluid, nasal fluid, and the airway mucosa (326, 327). It is believed that apoptosis of airway tissue eosinophils is a major component of the pharmacologic profile of steroids used for the treatment of asthma (328). While corticosteroids promote eosinophil apoptosis, glucocorticoid treatment inhibits neutrophil cell death *in vitro* (329). In view of the insensitivity of neutrophils to the anti-inflammatory actions of corticosteroids, there is a need for novel pharmacological strategies to control the harmful pro-inflammatory activities of these cells.

It is likely that there is a spectrum of corticosteroid responsiveness with the rare resistance at one end and a relative resistance seen in patients who require high doses of inhaled and oral corticosteroids (330). The reduction in corticosteroid responsiveness has been ascribed to a reduction in the number of glucocorticoid receptors, altered affinity of the ligand to the receptor and the increased expression of inflammatory transcription factors like AP-1 that compete with DNA binding (331, 332). Furthermore, studies have revealed that there is reduced suppression of IL-4 and IL-5 mRNA in BAL cells obtained from steroid resistant patients after one week of treatment with prednisolone when compared to steroid sensitive asthmatics (333). Hence, the inflammatory cell profile and the cytokine profile are different in the mild and severe group of asthmatics.

In this thesis, I have shown that TNF-α is involved in the modulation of airway inflammation. I have shown that with low dose allergen exposure TNF-α can be modulated and mast cells are likely the key cell type associated with this change. This correlated with a similar result from the explant study where TNF-α was elevated following *in vitro* culture of bronchial biopsies of moderately severe asthmatics with *Der p*. Furthermore, the expression of adhesion molecules was similarly up regulated both in the *in vivo* model and in the *ex vivo* explant model.

The results of the low dose allergen exposure studies although showed evidence of up regulation of TNF-α in patients with mild allergic asthma, the lack of standardization of this model coupled with the very small sample size should be interpreted with caution and is a major limitation of this thesis. Furthermore, the explant model has had its limitations in not being to repeat measure of ELISA due to the small amount of available supernatant and the inability to perform all the markers in all the samples. The explant model though appears a viable model has its limitations in view of the inability to perform repeated measures and difficulty in performing intra-class correlations due to the small numbers. Blocking the effects of TNF-α with a monoclonal antibody resulted in a decrease of TNF-α, adhesion molecules and IL-8 in patients with moderately severe asthma. Within the confines of this study and despite its limitations we could say blocking the effects of TNF-α seems an effective option in patients with severe corticosteroid dependent asthma

Airway inflammation in asthma is associated with tissue injury and IL-1β, IL-6 and TNF-α were detected in BAL from patients with symptomatic asthma (278). The pro-inflammatory effects of IL-1β, IL-6 and TNF-α are antagonised by IL-10, interleukin-1 receptor antagonist (IL-1Ra) and soluble tumour necrosis factor receptors I and II (sTNFR) which have anti-inflammatory properties and contribute to limit the inflammatory response in controlled asthma and in asthmatic subjects following allergen exposure (278, 335). Tille-Leblond and colleagues evaluated the levels of pro-inflammatory mediators (IL-1, IL-6 and TNF-α) and anti-inflammatory mediators (IL-10, IL-1Ra and sTNFR I & II) in broncho-alveolar lavage samples from patients with status asthmaticus. The levels of IL-10, IL-1Ra and sTNFR I & II were higher in patients with status asthmaticus than in patients with controlled asthma. Despite the presence of higher levels of these anti-inflammatory cytokines in BAL the net inflammatory activity was found to be pro-inflammatory and this was mainly due to the levels of IL-1β and TNF-α (132). This pro-inflammatory activity mediated by IL-1 and TNF-α could play key role in status asthmaticus. It is possible that these cytokines also play important roles in severe asthma. The distribution of these pro and anti-inflammatory cytokines in the airways of patients with asthma shares a similarity with the distribution of cytokines in the synovial

fluid of patients with rheumatoid arthritis where blocking the effects of TNF-α is being used as a therapeutic option.

IL-1 and TNF-α shares many biological activities including stimulation of adhesion molecule expression, secretion of chemokines by epithelial and endothelial cells and recruitment and activation of leukocytes in tissues (336, 337). TNF-α promotes neutrophil recruitment concomitantly in the lung with the appearance of BHR in normal subjects (191). It is therefore possible that the presence of Th2 cytokines like IL-4 and IL-5 may relate to the general component of the disease like eosinophilic inflammation and the disease process in general but the possibility of specific cytokines not necessarily those belonging to the Th2 family may be associated with the more severe disease. This is evidenced by a recent study from our department which showed that TNF-α levels in the BAL co-related with the severity of asthma. The severe asthmatics had significantly higher median (range) concentrations of TNF-α in BALF than either the healthy controls or those with mild asthma, the latter two groups not differing significantly. Relative TNF-α mRNA levels were also significantly higher in biopsies from the severe asthmatic subjects compared to mild asthmatic subjects (294).

In addition to its pro-inflammatory properties, TNF-α also plays a role in the induction and potentiation of BHR. Although BHR probably is mediated by the inflammatory mediators and the inflammatory cell infiltration into the airways, studies have shown that the airway smooth muscle by itself has the ability to modify its contractile susceptibility in the presence of cytokines (338, 339). TNF-α regulates the function of ASM not only by inducing a contractile phenotype by enhancing calcium signalling through different pathways but also by modulating the secretion of other cytokines and the level of expression of adhesion molecules (193). In our explant work we were able to demonstrate that in the presence of CDP 870 the expression of adhesion molecules in the bronchial biopsies of moderately severe asthmatics were significantly lower.

In our proof of concept study we demonstrated improvements in bronchial hyperresponsiveness and all the parameters of asthma symptoms and lung function

following 12 weeks of treatment with etanercept. We would ideally like to have conducted the explant study and the clinical trial with the same molecule but we had the opportunity to evaluate different molecules to block the effects of TNF-α. It would be interesting to note whether there are any differences between these molecules in the treatment of asthma as studies from Crohn's disease has shown dissimilar responses.

6.2. Future Directions

This thesis has provided an insight into the role of TNF-α in mild, moderate and severe asthma. The explant model was used to assess airway inflammation and response to CDP 870 in patients with severe asthma. Studies with explant culture system have shown interesting insights into the pathogenic mechanisms of asthma (34-36). The explant model provided a stepping stone to evaluate TNF-α blocking strategy in patients with chronic severe asthma.

We had the opportunity to use a soluble receptor against TNF-α (etanercept) in the proof of concept study and a monoclonal antibody against TNF-α (CDP 870) in the explant studies. CDP 870 is still in very early stages of clinical trials in rheumatoid arthritis. Studies in patients with Crohn's disease have shown that the efficacy of infliximab and etanercept are different (197, 340). While infliximab was found to clinically effective in the treatment of Crohn's disease and etanercept had no effect. Although various reasons were attributed to this difference (341, 342) it would be interesting to see whether this disparity is present in patients with asthma treated with infliximab, adalimumab and etanercept.

This clinical trial was an open labeled study and it is well recognized that asthma studies have a placebo effect. Therefore, a double blind placebo controlled clinical trial would be the next step in evaluating the role of etanercept in patients with severe asthma. In a small crossover trial by Berry and colleagues treatment of patients with refractory asthma with etanercept lead to a significant improvement in lung function, airway hyperresponsiveness and asthma quality of life at 10 weeks (318). It is the patients on the severe end of the spectrum who would benefit most from expensive biological therapies

in view of the number of hospital visits, hospital admissions and man hours lost, but it would be of interest to look at the effects of TNF-α blocking strategies in patient with mild and moderate asthma. If there are differences in the effectiveness of this therapy between the different groups of severity this could provide us some insight into the pathogenic mechanisms of asthma in relation to its severity.

The efficacy of TNF-α in patients' with asthma could serve to assess the mechanistic basis of the role of TNF-α in asthma. As allergen induces the up regulation of TNF-α, the role of environment in propagating airway inflammation needs to be addressed. We know that TNF-α polymorphism exists in patients with asthma (343, 344). The TNF-α and lymphotoxin-alpha (LTA) genes belong to the TNF gene super family located within the human major histocompatibility complex on chromosome 6p in a region repeatedly linked to asthma. Various TNF-α polymorphisms have been noted in asthma. However the results have been variable. The polymorphism in the promoter region of the TNF-α gene at nucleotide –308, relative to the transcription start site, may be important in determining the host TNF-α response. Studies looking at extensive linkage disequilibrium present on chromosome 6 have found that extended haplotypes account for association of TNF-α single nucleotide polymorphisms (SNPs). The extended haplotype LTαNco*1/TNF-308*2/HLA-DRB1*02 was associated with a significant association with asthma with an odds ratio of 6.68 and an even more significance with BHR with an odds ratio of 21.9 (345, 346). Hence, genotyping patients with chronic severe asthma can be used as a possible way to decide on which group of patients would benefit from this therapy. It is likely that the beneficial response to TNF-α blocking strategy would not be seen in all subjects as was seen in patients with rheumatoid arthritis (347) but a way to segregate this small subset of asthmatics could provide a useful tool to direct this form of therapy. Several new biologics are being tested in clinical trials that promise to soon enhance the therapeutic armamentarium to fight RA. The biological therapies being investigated include blockade of T cell co-stimulation by a CTLA4Ig fusion protein (abatacept), blockade of interleukin (IL)-6 signaling with an antibody to the IL-6 receptor, neutralizing IL-15 by a monoclonal antibody, and targeting B cells with an anti-CD20 antibody (rituximab). While there are notable similarities between chronic severe

asthma and rheumatoid arthritis it would be interesting to see the effects of these new molecules on asthma symptoms and asthma control.

In summary this thesis has revealed a significant role for TNF-α in asthma. This thesis provides evidence, that TNF-α production is increased in mild and moderately severe asthma in response to allergen. At a functional level, blocking the effects of TNF-α in severe corticosteroid-dependent asthma its significance has been established by showing clear improvements in clinical and physiological measures of asthma following 12 weeks treatment with the soluble TNF-α receptor-IgG1Fc fusion protein, etanercept. Etanercept had a dramatic effect in improving asthma symptoms, baseline lung function and bronchial hyperresponsiveness. Since severe, corticosteroid refractory asthma makes up a substantial portion of the health costs for this disease, these novel observations are of major clinical significance in identifying TNF-α as an key target with significant implications on the future therapy of asthma. Large double blind placebo controlled studies are required to evaluate the efficacy of this novel therapy in patients with severe corticosteroid dependent severe asthma.

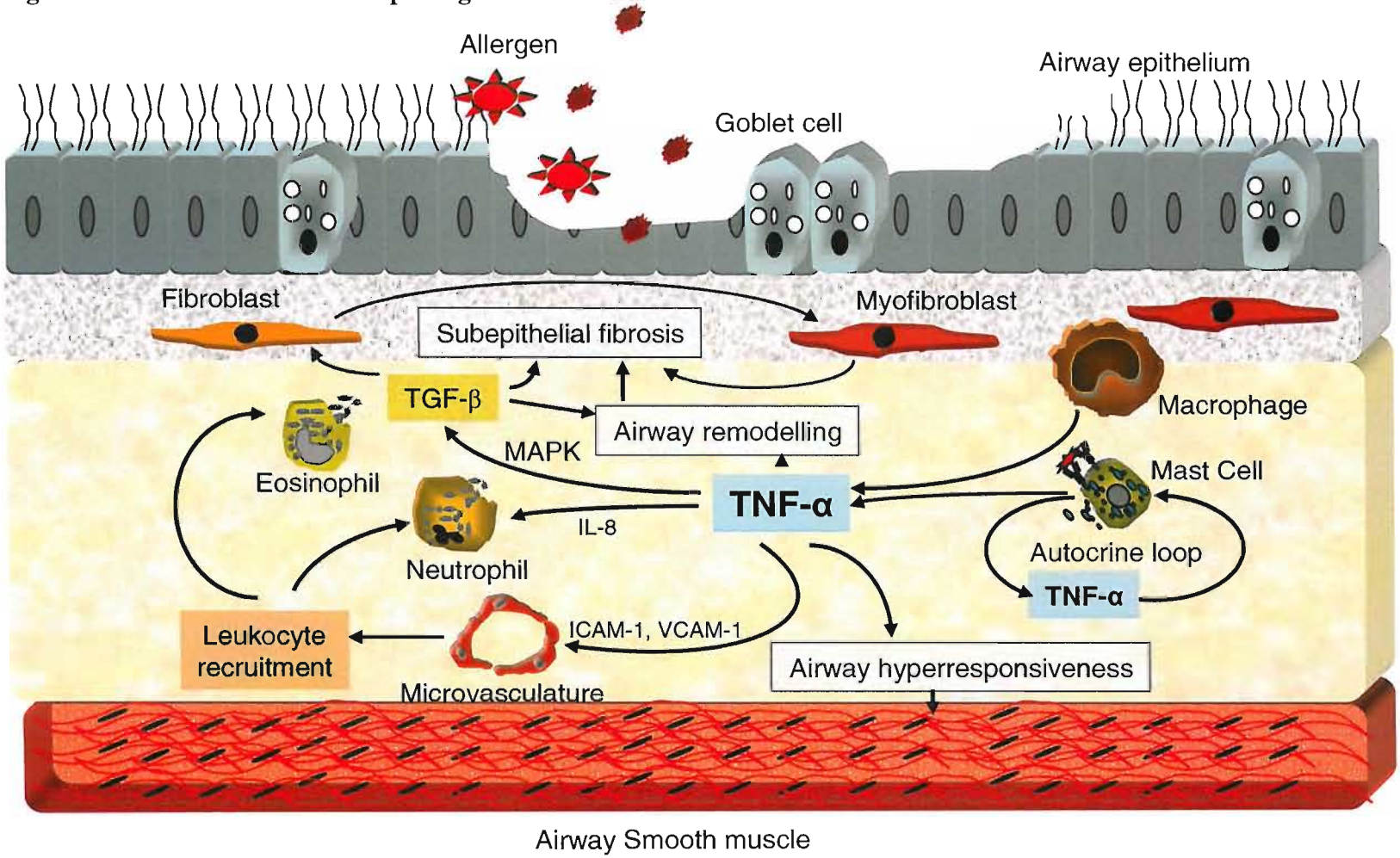
6.3. Possible mechanistic role of TNF-α in asthma

As in the pathogenesis of RA where TNF-α plays a key role, there is strong evidence to suggest an important role for TNF-α in asthma (Fig 1). TNF-α is released from mast cells and macrophages following exposure to allergen through IgE dependent mechanisms. We have shown this in our first study where low dose allergen challenge lead to an increased expression of TNF-α in the airways. There are evidence to suggest that TNF-α once released by mast cells can sustain their production through an autocrine loop mechanism where in TNF-α induces further production of TNF-α there by leading to a constant inflammatory *milieu* (157). Once released into airways TNF-α acts by inducing a pro-inflammatory response through release of other pro inflammatory cytokines including IL-1, IL-2, IL-4, IL-6, IL-8, IL-10 and IL-12 and by up-regulation of adhesion molecules ICAM-1 and VCAM-1 (348). These adhesion molecules facilitate the migration of eosinophils and neutrophils into the airways. This leads to airway inflammation and airway remodelling, a characteristic feature of asthma. Following low dose allergen

challenge we have shown that there is an up regulation of adhesion molecules ICAM-1 and VCAM-I in the bronchial biopsies and this was a consistent finding seen in the explant cultures from moderately severe asthmatics. IL-8 is a key chemotactic factor for neutrophils and TNF-α seems to modulate the regulation of IL-8. In the explant cultures we have shown that blocking the effects of TNF-α by CDP 870 lead to a reduction in IL-8 levels. Furthermore, blocking the effects of TNF-α in patients with chronic severe asthma lead to a decrease in the IL-8 levels in the sputum, which was associated with a reduction in the sputum neutrophils. As severe asthma is associated with a predominance of neutrophils in the bronchial biopsies and in sputum it is likely that inhibition of IL-8 levels could play a significant role in asthma control in patients treated with etanercept.

TNF-α is implicated in activation and proliferation of myofibroblasts thereby contributing to sub-epithelial fibrosis and tissue remodelling. TNF-α could also play a significant role in airway remodelling by modulating epithelial EGFR signalling and mucus secretion. TNF-α acts on fibroblasts through its actions on TGF-β generated mainly through MAP kinase to affect airway remodelling (349). Perhaps the most significant effects of TNF-α in asthma is the potentiation and induction of BHR. TNF-α regulates the function of airway smooth muscles by its effects on other cytokines and adhesion molecules. But TNF-α is also capable of inducing a hypercontractile phenotype by enhancing calcium signalling through various pathways including an indirect emptying of intracellular calcium stores through the generation of second messengers and by directly activating inositol phosphate turnover (193, 195). We have shown in our clinical study that treatments with etanercept for 12 weeks improve methacholine response by 2.5 doubling dilutions which are consistent with the effects of TNF-α on airway responsiveness. This thesis has provided both experimental and clinical evidence that TNF-α plays a major role in the pathogenesis of asthma thereby emphasising the importance of TNF-α as a therapeutic target in patients with chronic severe asthma.

Figure 6-1: Role of TNF-a in the pathogenesis of asthma



TGF-β-Transforming growth factor β, MAPK- MAP Kinase, ICAM- Intercellular adhesion molecule, VCAM- Vascular cell adhesion molecule, TNF-α- Tumour necrosis factor alpha

7. References

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APPENDIX I

Materials

Bronchoscopy

Bronchoscope (Olympus FB-20D, Tokyo, Japan)

Biopsy Forceps (Olympus, Tokyo, Japan)

Bronchial brush (Olympus, Tokyo, Japan)

Medications obtained from hospital pharmacy

Lignocaine spray (10%)

Lignocaine solution (1%)

Midazolam (6 mg IV)

Atropine (0.6 mg)

Salbutamol nebules (5 mg)

Ipratropium bromide (0.5 mg)

Methacholine

APPENDIX II

ELISA PROTOCOLS

IL-5 ELISA

Reconstitution and Dilution of Hu IL-5 Standard

- The standard was reconstituted to 7500 pg/ml with Standard Diluent Buffer. Swirl or mix gently and sit for 10 minutes to ensure complete reconstitution. The standard was used within 1 hour of reconstitution.
- 100 ml of the reconstituted standard was added to a tube containing
- 0.900 ml Standard Diluent Buffer to constitute 750 pg/ml of IL-5.
- 0.300 ml of Standard Diluent Buffer to each of 6 tubes labelled 375, 187.5, 93.7, 46.8, 23.4 and 11.7 pg/ml IL-5.
- Serial dilutions of the standard were made as advised in the dilution table.

Dilution of Streptavidin-HRP

- The Streptavidin-HRP 100x concentrate was in 50% glycerol. 10 µl of this was diluted with 1 ml of Streptavidin-HRP Diluent for each 8-well strip used in the assay.

Dilution of Wash Buffer

- The 25 time concentrate of wash buffer was allowed to reach room temperature and mix to ensure that any precipitated salts have redissolved. 1 volume of the 25x wash buffer concentrate with 24 volumes of deionized water (e.g., 50 ml may be diluted up to 1.25 litres, 100 ml may be diluted up to 2.5 litres).

Assay Method

- 100 µl of the Standard Diluent Buffer was added to zero wells. Well(s) reserved for chromogen blank were left empty.
- 100 µl of standards, samples or controls to the appropriate were added to microtiter wells and the plates were covered with plate cover and incubated for 2 hours at room temperature.
- After thoroughly washing, 100 µl of biotinylated anti-IL-5 (Biotin Conjugate) solution was pippetted into each well except the chromogen blank(s).
- The plates were covered with plate cover and incubated for 30 minutes at room temperature.

- The solutions was thoroughly aspirated from wells and discarded. The wells were washed 4 times.
- 100 µl of streptavidin-HRP Working Solution was added to each well except the chromogen blank(s).
- The plates were covered with the plate cover and incubated for 30 minutes at room temperature.
- After thorough washing 100 µL of Stabilized chromogen was added to each well and the liquid in the wells will begin to turn blue. This was incubated for 30 minutes at room temperature and in the dark.
- 100 µl of stop solution was added to each well and the solution in the wells changed from blue to yellow.
- The absorbance of each well was read at 450 nm within 2 hours after adding the stop solution.
- The absorbance of the standards was plotted against the standard concentration and the Human IL-5 concentrations for unknown samples and controls were read from the standard curve.

IL-1β ELISA

Reconstitution and Dilution of Hu IL-1β Standard

- The standard was reconstituted to 2500 pg/ml with standard diluent buffer and allowed to sit for 10 minutes to ensure complete reconstitution. The standards were used within 1 hour of reconstitution.
- 100 µl of the reconstituted standard was added to a tube containing 900 µl of standard diluent buffer. This was labelled as 250 pg/mL Hu IL-1β.
- 250 µl of standard diluent buffer was added to each of 6 tubes labelled 125, 62.5, 31.2, 15.6, 7.8 and 3.9 pg/mL Hu IL-1β.
- Serial dilutions of the standard were made as described in the dilution table.

Dilution of Streptavidin-HRP

- The Streptavidin-HRP 100x concentrate was in 50% glycerol. 10 µl of this was diluted with 1 ml of Streptavidin-HRP Diluent for each 8-well strip used in the assay.

Dilution of Wash Buffer

- The 25 time concentrate of wash buffer was allowed to reach room temperature and mix to ensure that any precipitated salts have redissolved. 1 volume of the 25x wash buffer concentrate with 24 volumes of deionized water (e.g., 50 ml may be diluted up to 1.25 litres, 100 ml may be diluted up to 2.5 litres).

Assay Method

- 50 µl of the standard diluent buffer were added to zero wells. Well(s) reserved for chromogen blank should be left empty.
- 50 µL of standards, samples or controls were added to the appropriate microtiter wells.
- 100 µL of biotinylated anti-IL-1β (Biotin Conjugate) solution was pippetted into each well except the chromogen blank(s).
- The plates were covered with plate cover and incubated for 2 hours at room temperature.
- After thoroughly washing the plate 100 µl of streptavidin-HRP working solution was added to each well except the chromogen blank.
- The plates were covered with plate cover and incubated for 2 hours at room temperature.

- After washing the wells 100 µl of stabilized chromogen was added to each well. The liquid in the wells began to turn blue. This was incubated for 25 minutes at room temperature and in the dark.
- 100 µL of stop solution was added to each well and the solution in the wells changed from blue to yellow.
- The absorbance of each well was read at 450 nm having blanked the plate reader against a chromogen blank within 2 hours after adding the stop solution.
- The absorbance of the standards was plotted against the standard concentration and the Human IL-1β concentrations for unknown samples and controls were read from the standard curve.

IL-8 ELISA

Reconstitution and Dilution of Hu IL-8 Standard

- The standards were reconstituted to 10.0 ng/ml with standard diluent buffer and allowed to sit for 10 minutes to ensure complete reconstitution. The standards were used within 1 hour of reconstitution.
- 100 µl of the reconstituted standard was added to the tube containing 900 µl of standard diluent buffer. This was labelled as 1000 pg/ml Hu IL-8.
- 300 µl of standard diluent buffer was added to each of 6 tubes labelled 500, 250, 125, 62.5, 31.2, and 15.6 pg/ml Hu IL-8.
- Serial dilutions of the standard were made as described in the manufacturers dilution table.

Storage and Final Dilution of Streptavidin-HRP

- 10 µl of the100 times concentrated solution was diluted with 1 ml of Streptavidin-HRP diluent for each 8-well strip used in the assay. This was labelled as Streptavidin-HRP working solution.

Dilution of Wash Buffer

- The 25 time concentrate of wash buffer was allowed to reach room temperature and mix to ensure that any precipitated salts have redissolved. 1 volume of the 25x wash buffer concentrate with 24 volumes of deionized water (e.g., 50 ml may be diluted up to 1.25 litres, 100 ml may be diluted up to 2.5 litres).

Assay Method

- The number of 8-well strips needed for the assay was determined and were inserted into the frame(s) for current use.
- 50 µl of the standard diluent buffer was added to zero wells and the well(s) reserved for chromogen blank were left empty.
- 50 µl of standards, samples or controls were added to the appropriate microtiter wells.
- 50 µl of biotinylated anti-IL-8 (Biotin Conjugate) was added into each well except the chromogen blank(s).
- The plates were covered with a plate cover and incubated for 1 hour and 30 minutes at room temperature.

- After thoroughly washing the plates, 100 µl of Streptavidin-HRP working solution to each well except the chromogen blank(s).
- The plates were covered with a plate cover and incubated for 30 minutes at room temperature.
- The plates were washed thoroughly and 100 µl of stabilized chromogen was added to each well. The liquid in the wells would turn blue. This was incubated for 30 minutes at room temperature and in the dark.
- The assay was stopped by adding 100 µl of stop solution to each well. The solution in the wells should change to yellow.
- The absorbance of each well at was read at 450 nm having blanked the plate reader against a chromogen blank composed of 100 µl each of stabilized chromogen and stop solution. The plates were read within 2 hours after adding the stop solution.
- The absorbance of the standards was plotted against the standard concentration and the Human IL-8 concentrations for unknown samples and controls were read from the standard curve.

APPENDIX III

Junipers Asthma Control Questionnaire

Please answer question 1-6.

Circle the number of the response that best describes how you have been during the past week

On average, during the past one week, how often were you **woken by your asthma** during the night?

0 Never
1 Hardly ever
2 A few minutes
3 Several times
4 Many times
5 A great many times
6 Unable to sleep because of asthma

On average, during the past one week, how **bad were your asthma symptoms when you woke up** in the morning?

0 No symptoms
1 Very mild symptoms
2 Mild symptoms
3 Moderate symptoms
4 Quiet severe symptoms
5 Severe symptoms
6 Very severe symptoms

In general, during the past one week, how **limited were your activities** because of your asthma?

0 Not limited at all
1 Very slightly limited
2 Slightly limited
3 Moderately limited
4 Very limited
5 Extremely limited
6 Totally limited

In general, during the past one week, how much **shortness of breath** did you experience because of asthma?

0 None
1 A very little
2 A little
3 A moderate amount
4 Quiet a lot
5 A great deal
6 A very great deal

In general, during the past one-week, how much of the time did you **wheeze**?

0 Not at all
1 Hardly any of the time
2 A little of the time
3 A moderate amount of the time
4 A lot of the time
5 Most of the time
6 All the time

On average, during the past one week, how many **puffs of short acting bronchodilator** (eg. Ventolin) have you used each day?

0 None
1 1-2 puffs most days
2 3-4 puffs most days
3 5-8 puffs most days
4 9-12 puffs most days
5 13-16 puffs most days
6 More than 16 puffs most days

To be completed by the clinic staff

FEV1 pre-bronchodilator:.....
FEV1 predicted:.....
FEV1 % predicted:.....
(Record actual values on the dotted lines and score the FEV1 % predicted in the next column)

0 >95% predicted
1 95-90%
2 89-80%
3 79-70%
4 69-60%
5 59-50%
6 <50% predicted

APPENDIX IV

DIARY CARD

OVERNIGHT ASTHMA

(Symptoms during the night)

0 = No symptoms. Slept through the night
1 = Woke once for a short while due to asthma
2 = Woke more than once due to asthma, but slept in-between
3 = Awake most, but not all night due to asthma
4 = Did not sleep at all. Awake all night due to asthma

MORNING ASTHMA

(Symptoms of cough/wheeze/breathlessness/chest tightness when you get up in the morning)

0 = No symptoms at all
1 = Some symptoms but hardly noticeable
2 = Symptoms noticeable and a little uncomfortable
3 = Symptoms definitely noticeable and uncomfortable
4 = Symptoms very bad and extremely uncomfortable

DAYTIME ASTHMA

(Symptoms during the day)

0 = No symptoms at all during day
1 = Some symptoms but barely noticeable
2 = Symptoms noticeable and interfered to some extent with usual daily activity
3 = Symptoms definitely noticeable and interfered a lot with usual daily activity
4 = Symptoms bad. Unable to carry out usual daily activity or go to work.

Please contact the study doctor at the telephone number provided if your peak flow measurements drop below _____ L/min On 2 consecutive days or below _____ L/min at any time.

DIARY CARD

DATE	OVERNIGHT ASTHMA	MORNING ASTHMA	MORNING PEFR	DAYTIME ASTHMA	EVENING PEFR	VENTOLIN USE (No of Puffs)