UNIVERSITY OF SOUTHAMPTON

The Leukotriene Pathway in Structural Cells of the Human Airway

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Thesis submitted for degree of PhD

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Respiratory, Cell and Molecular Biology

February 2002

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ABSTRACT

SCHOOL OF MEDICINE
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Doctor of Philosophy

THE LEUKOTRIENE PATHWAY IN STRUCTURAL CELLS OF THE HUMAN AIRWAY

By Anna Julia James

Leukotrienes (LTs) are inflammatory mediators of central importance in the pathogenesis of asthma. Cysteinyl-LTs (cys-LTs) cause bronchoconstriction, oedema, mucus hypersecretion and eosinophil chemotaxis, while LTB_4 is a potent neutrophil chemoattractant and immunomodulator. Cysteinyl-LT_1 receptor ($CysLT_1R$) antagonists have bronchodilator and anti-eosinophilic actions and are effective clinical treatments. LTB_4 receptor (BLT) antagonists reduce neutrophil influx and activation following inhaled allergen challenge.

LTs are thought to be derived predominantly from cells of myeloid origin. Airway structural cell types such as human bronchial epithelial (HBE) cells, human airway smooth muscle (HASM) cells and lung fibroblasts generate pro-inflammatory cytokines and lipid mediators such as prostanoids and 15-lipoxygenase products, but their capacity to generate leukotrienes is unclear. In leukocytes, LT synthesis is initiated from membrane-derived arachidonic acid by 5-lipoxygenase (5-LO) and its activating protein FLAP, followed by conversion of LTA_4 to LTB_4 by LTA_4 hydrolase or to LTC_4 by LTC_4 synthase. We hypothesised that HBE cells, HASM cells and fibroblasts may express 5-LO pathway enzymes and synthesise LTC_4 or LTB_4, either spontaneously or in response to stimulation with inflammatory mediators, and that they may also express $CysLT_1$ and/or BLT.

Using RT-PCR, basal expression of mRNA for 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase was detected in primary HBE cells, HASM cells and bronchial fibroblasts, as well as in the 16-HBE cell line. Immunocytochemistry, Western blotting and FACS analysis demonstrated the presence of 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase protein, and enzyme immunoassays of culture supernatants from each cell-type demonstrated the release of both LTB_4 and LTC_4. The pattern of enzyme expression was similar in the three cell types, all showing greater immunofluorescence for 5-LO, FLAP and LTA_4 hydrolase than for LTC_4 synthase. Levels of enzyme expression in the structural cells were comparable to those in human blood leukocytes. The $CysLT_1$ receptor was present at baseline in HBE cells, HASM cells and fibroblasts, while constitutive expression of BLT was detectable only in HASM cells and fibroblasts.

The effect of treatment with inflammatory mediators on LT pathway proteins in HBE cells, HASM cells and fibroblasts was measured by flow cytometry. Incubation of 16-HBE cells with Ca^{2+} ionophore (A23187, 1μM, 6h) doubled the immunofluorescence of all four LT pathway enzymes ($p<0.05$). Bacterial lipopolysaccharide (2μg/ml), bradykinin (10μM), and a mixture of TNFα, IL-1β, and IFNγ (10ng/ml, 24h) each induced modest increases in enzyme expression (40-90%) in 16-HBE cells.

In HASM cells, incubation for 24 hours with TGFβ (10ng/ml), LTB_4 (10nM) and histamine (10μM) each increased the immunofluorescence of 5-LO pathway enzymes by 50-80%. $CysLT_1R$ expression in these cells was increased 2-fold by TGFβ ($p=0.03$) and 3-fold by a mixture of TNFα, IL-1β and IFNγ ($p=0.05$). $CysLT_1R$ immunofluorescence was not altered by its antagonist MK-571 (1-100nM, 0-72h), suggesting that receptor antagonism does not lead to receptor down-regulation and tachyphylaxis. Treatment of HASM cells with a combination of TNFα, IL-1β and IFNγ (each at 10ng/ml) modestly increased BLT receptor expression.

Myofibroblasts are increased in numbers in asthmatic airways. Treatment of fibroblasts with TGFβ (10ng/ml, 72h) induced transformation of fibroblasts into this contractile phenotype and was accompanied by a 15-fold increase in LTC_4 synthase ($p=0.058$) and a 4-fold increase in $CysLT_1R$ ($p=0.008$), accompanied by a 3-fold down-regulation of LTA_4 hydrolase ($p=0.0014$) and BLT ($p=0.009$).

The anti-inflammatory corticosteroid dexamethasone (1μM, 24h) had no overall effect on the expression of 5-LO pathway enzymes or BLT in HBE cells, HASM cells, or fibroblasts, but intriguingly, in HASM cells, dexamethasone increased $CysLT_1R$ expression by 40% ($p=0.01$).

These results show that HBE cells, HASM cells and fibroblasts constitutively express a complete and active 5-LO pathway for the synthesis of LTB_4 and LTC_4, and that this may be regulated by exposure to inhaled asthma triggers or endogenously released inflammatory cytokines and autacoids. Constitutive expression of $CysLT_1R$ on all three cell-types may be up-regulated in an inflammatory environment leading to increased $CysLT_1$ mediated effects such as collagen or mucus secretion, bronchoconstriction and proliferation. The role of BLT on HASM cells and fibroblasts is not yet clear, although LTB_4 is chemotactic for fibroblasts. LTs released by HBEC, HASM and fibroblasts may lead both to autocrine effects and to leukocyte infiltration, and in combination with other mediators produced by structural cells, contribute to the vicious circle of inflammation and remodelling within the asthmatic airway.

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Acknowledgements

I would like to express my gratitude to the Francis and Augustus Newman Foundation for funding this PhD studentship, Dr Jilly Evans for providing 5-LO, FLAP and LTA_4 hydrolase antibodies, and Prof Frank Austen and Dr John Penrose for providing LTC_4 synthase antibody. I would like to thank Dr S-H Leir for his assistance with epithelial cell culture, Prof Alan Knox and colleagues for providing smooth muscle cells, and Dr Audrey Richter for providing bronchial fibroblasts. I would also like to thank Dr Ian Sayers and Dr John Holloway for their help with RT-PCR, Dr Peter Lackie for his help and advice, and finally I would like to thank Dr Tony Sampson for his enthusiasm, expertise and encouragement.

Abbreviations

5-HETE	12-hydroxyeicosatetraenoic acid
5-HPETE	12-hydroperoxy-eicosatetraenoic acid
5-LO	5-lipoxygenase
A23187	Calcium ionophore
AA	Arachidonic acid
AEC	Aminoethyl carbazole
AIA	Aspirin intolerant asthma
AP-1	Activator Protein 1
APC	Antigen Presenting Cell
ASM	Airway Smooth Muscle
ATA	Aspirin Tolerant Asthma
ATP	Adenosine Triphosphate
β_2-AR	β_2-Adrenergic Receptor
BAL	Bronchoalveolar Lavage
BEC	Bronchial Epithelial Cell
BHR	Bronchial Hyperresponsivness
BK	Bradykinin
BMEM	Bronchial Epithelium Growth Medium
C5a	Complement Factor 5a
cAMP	Cyclic Adenylate Monophosphate
CD-44	Cluster of Differentiation-44
CNS	Central Nervous System
COX-1	Cyclooxygenase-1
$cPLA_2$	Cytosolic Phospholipase A_2
Cys-LT	Cysteinyl-Leukotriene
$CysLT_1R$	Cysteinyl-Leukotriene$_1$ receptor
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethylsulphoxide
EAR	Early Allergic Response
ECP	Eosinophil Cationic Protein
EDN	Eosinophil Derived Neurotoxin
EG2	Eosinophil Marker
EGF	Epidermal Growth Factor
EMEM	Earle's Modified Eagles Medium
EPO	Eosinophil Peroxidase
ET	Endothelin
FACS	Fluorescence Activated Cell Sorting
FcεRI	High affinity IgE receptor
FEV_1	Forced expiratory volume in 1 sec
FCS	Foetal Calf Serum
FGF	Fibroblast Growth Factor
FLAP	5-Lipoxygenase Activating Protein
FMLP	Formyl-methionyl-leucyl-phenylalanine
GAPDH	Glyceraldehyde 3 phosphate dehydrogenase
GC	Glucocorticoid
GCS	Glucocorticoids
GMA	Glycolmethacrylate
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
GRE	Glucocorticoid Response Element
HASM	Human Airway Smooth Muscle
HBEC	Human Bronchial Epithelial Cell
HIS	Histamine
HLA	Human Leukocyte Antigen
HL-60	Promyelocytic cell line
HRP	Horseradish Peroxidase
HRV	Human Respiratory Virus
ICAM	Intercellular Adhesion Molecule

IFN	Interferon
Ig	Immunoglobulin
IGF	Inhibitory Growth Factor
IHC	Immunohistochemistry
FcεRI	Low affinity IgE receptor
FcεRII	High affinity IgE receptor
IGF	Insulin Growth Factor
IL	Interleukin
IP_3	Inositol triphosphate
LAR	Late allergic response
LFA	Lymphocyte Function Associated Antigen
LT	Leukotriene
LTA_4H	Leukotriene A_4 hydrolase
LTC_4S	Leukotriene C_4 synthase
MBP	Major basic protein
MC_T	Tryptase containing mast cell
MC_{TC}	Tryptase and Chymase containing mast cell
MHC	Major histocompatibility complex
MMP	Matrixmetalloproteinase
MFI	Median Fluorescence Intensity
MLCK	Myosin Light Chain Kinase
MPO	Myeloperoxidase
MRP-1	Multidrug Resistance Protein-1
NF-κB	Nuclear Factor κB
NOS	Nitric Oxide Synthase
NSAID	Non-steroidal anti-inflammatory drugs
PAF	Platelet Activating Factor
PBS	Phosphate Buffered Saline
PC_{20}	Provocation concentration causing 20% fall in FEV_1
PCR	Polymerase Chain Reaction
PDE	Phosphodiesterase
PDGF	Platelet Derived Growth Factor
PEF	Peak Expiratory Flow
PG	Prostaglandin
PGI_2	Prostacyclin
PKC	Protein kinase C
$cPLA_2$	Cytosolic Phospholipase A_2
$sPLA_2$	Secreted Phospholipase A_2
PLC	Phospholipase C
PMA	Phorbol-12 myristate 13-acetate
PPARα	Peroxisomal Proliferator Activated Receptor-α
RANTES	Regulated on T-cell activation, normal T-cell expressed and secreted
RAST	Radio-allergosorbent test
RPMI	Rosweli Park Memorial Institue culture medium
RSV	Respiratory Syncitial Virus
RT-PCR	Reverse Transcription PCR
SAB	Streptavidin-biotin
SCF	Stem Cell Factor
$sPLA_2$	Secreted Phospholipase A_2
TGF	Tumour Derived Growth Factor
Th	T helper cell
TIMP	Tissue Inhibitor of Metalloproteinase
TNF	Tumour necrosis factor
TX	Thromboxane
USG	UltroserG (FCS replacement)
VCAM	Vascular Cell Adhesion Molecule
VIP	Vasointestinal Peptide

List of Publications

Expression, regulation, and activity of the leukotriene pathway in human bronchial epithelial cells. AJ James, PM Lackie, I Sayers, JF Penrose, KF Austen, ST Holgate, and AP Sampson. *(In progress)*

Expression of leukotiene pathway in bronchial fibroblasts from asthmatic and normal subjects. AJ James, A Richter, PM Lackie, AP Sampson. 2002 ATS, Atlanta, Georgia 17th-22nd May. (*Abstract*)

Expression of leukotriene pathway enzymes in fibroblasts and myofibroblasts. AJ James, PM Lackie, A Richter, AP Sampson. 2002 ATS, Atlanta, Georgia 17th-22nd May. (*Abstract*)

A Tale of Two CysLTs. AJ James, AP Sampson. Clin Exp Allergy. 2001 Nov;31(11):1660-1664.

Expression and regulation of leukotriene pathway enzymes in human airway smooth muscle cells. AJ James, L Corbett, PM Lackie, AJ Knox, AP Sampson. 2001. Am J Resp Crit Care Med; 163 (5):A513 (*Abstract*)

Cysteinyl-leukotriene receptor-1 expression on human airway smooth muscle cells is not downregulated by the cys-LT_1 receptor antagonist MK-571. AJ James, PM Lackie, AJ Knox, AP Sampson. 2001. Am J Resp Crit Care Med; 163 (5):A513 (*Abstract*)

Evidence for different subgroups of difficult asthma in children. DN Payne, NM Wilson, AJ James, H Hablas, C Agrafioti, A Bush. Thorax.2001;56(5):569-574

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Exhaled nitric oxide in children with very severe asthma: response to two weeks of oral corticosteroids. NM Wilson, AJ James, A Bush. Eur Resp J. 1998;12s28:142 (*Abstract*)

Increased carbon monoxide in exhaled air of normal children with upper respiratory tract infection. CG Uasuf, AJ James, NM Wilson, SA Kharitonov, PJ Barnes. Eur Resp J. 1998;12s28:207 (*Abstract*)

1. Introduction

1.1 Asthma

1.1.1 Definition & diagnosis

Asthma is a complex respiratory syndrome defined as '*a chronic inflammatory disorder of the airways in which many cells play a role, in particular mast cells, eosinophils and T-lymphocytes. In susceptible individuals this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness and cough, particularly in the early morning. These symptoms are usually associated with widespread but variable airflow limitation that is at least partly reversible, either spontaneously or with treatment. The inflammation also causes an associated increase in airway responsiveness to a variety of stimuli (NHLBI/NIH Expert Panel Report, 1997)*'. Several different types of asthma exist provoked by a range of different stimuli such as allergens, respiratory viral infections, occupational agents, exercise, airborne irritants and in some individuals non-steroidal anti-inflammatory drugs (NSAIDs). These stimuli act through different pathways to produce a common end result.

Diagnosis of asthma is based on the pattern of symptoms mentioned above. Diagnostic tests may be used to confirm the clinical criteria such as measurements of bronchial responsiveness to histamine, methacholine and/or exercise challenge as well as lung function tests for example FEV_1 (maximum forced expiratory volume in one second) or PEF (peak expiratory flow) (Lundbäck 1998). The PC_{20}, or provocation concentration of histamine or methacholine that causes a 20% fall in FEV_1, is significantly lower in asthmatics (<8mg/ml) than normals (>8-10mg/ml) and can also correlate with asthma severity (Hargreave 1981 et al., Murray et al. 1981). A common characteristic of asthma is diurnal variations in PEF and diary cards are often kept by patients to monitor changes. Baseline FEV_1 is lower in asthmatics than normals and during an attack FEV_1 measurements can drop to as low as 20% of predicted (McFadden 1998).

1.1.2 Epidemiology

There have been numerous reports that the prevalence of asthma has increased over the last two to three decades with studies on British children showing an increase from approximately 3% in 1982, to 6% in 1985 and to almost 9% in 1988 and 1992 (Hill et al. 1989). In general, studies have shown that asthma is most prevalent in the UK, Australia and New Zealand, but the number of reported cases is increasing steadily everywhere (Lundbäck 1998). Out of 2600 preschool children in the United Kingdom in 1998, 26%

reported current wheezing disorders and 19% of childen had been diagnosed with asthma (Kuehni et al. 2001).

1.1.3 Risk factors

There are several risk factors thought to be important in the development of asthma. The current theory is that an interaction between genetic and environmental factors is important with those persons who develop asthma being both genetically susceptible and exposed to an appropriate environmental stimulus. Atopy is one of the greatest risk factors. This is a state of allergic response to environmental allergens, mediated by IgE, that can be assessed by total serum IgE, allergen specific IgE, or by skin prick testing to common aero-allergens (Weisch et al. 1998). Positive skin prick tests and total IgE have been strongly associated with symptoms of asthma in European, American and Australian studies (Burrows et al. 1989, Kauffmann et al. 1999, Holt et al. 2000). However, although asthma and atopy are closely linked their relationship is complex as many asthmatics are not atopic and vice versa.

A family history of asthma and atopic disease is also recognised as a major risk factor. It has long been known that asthma clusters in families. An individual with a family history of asthma is three to four times more likely to develop the disease than one without family history (Rönmark et al. 1997). Studies carried out on twins have confirmed familial factors. In 2902 Australian twins, the occurrence of asthma in monozygotic twin pairs was significantly higher (30%) than in dizygotic twin pairs (12%) (Duffy et al. 1990).

Exposure to environmental allergens and pollutants is also associated with the development of asthma. Sporik and colleagues showed that out of 93 children who were at high risk of developing asthma on the basis of having a parent with asthma or hay fever, those with higher levels of dust mite in their homes tended to wheeze at a younger age than those with less antigen exposure (Sporik et al. 1990). House dampness and high indoor humidity have also been shown to be risk factors in the development and expression of asthma (Åberg et al. 1996). In adults it has been estimated that occupational chemicals such as dusts, gases and fumes are the cause of approximately 10% of asthma cases (Wiesch et al. 1998). Another possible risk factor associated with the development of asthma includes exposure to tobacco smoke. Studies conducted in Sweden have shown that self smoking is a significant risk for the expression of asthma in adults, and that maternal smoking is a significant risk factor for the development of asthma in children (Lundbäck 1998).

There may be a role for viral infections in the development of asthma. Lower respiratory tract infections in children are common, caused by respiratory syncitial virus, influenza viruses and rhinoviruses. It has been suggested that children with a history of hospitalisation for respiratory infections are more likely to develop asthma as in several studies they have abnormal lung function and increased airway reactivity (Gurwitz et al.1981). However, one of the suggested reasons for the increase in asthma in developed countries is the overall decrease in viral infections experienced during childhood. These infections promote activation of the Th1 immune response that has negative influences on the Th2 lymphocyte profile associated with asthma (Holgate 1997). Several studies have shown that the odds of allergen sensitisation are inversely related to the number of older siblings in the family sensitisation (von Mutius et al. 1994, Strachan et al. 1989). These data raise the possibility that infections early in childhood, which are presumed to be more frequent and occur at an earlier age if there were older siblings at home, might decrease the risk of allergen sensitisation. However, a recent study that set out to examine the importance of the decline in family size in relation to the increasing prevalence of asthma reported that changes in family size do not fully explain the increased occurrence of asthma (Wickens et al. 1999).

1.1.4 Genetics

The observations of familial clustering in asthma from twin and relative studies strongly support a genetic contribution. Inheritance of asthma cannot be described by simple Mendelian genetics as:

- More than one gene may interact to produce the disease
- Different predisposing genes may exist in different individuals
- Interaction with the environment may lead to variable expression and severity.

There are two main approaches used to identify susceptibility genes for a disease. Firstly, specific genes may be evaluated when obvious candidates play a critical function in the onset of a disease. Secondly, genome wide screening may identify genes shared by affected relatives but not normals (Morrison 1998). Using these techniques, it has become possible to identify multiple chromosomal regions linked to asthma, atopy or more specific phenotypes such as bronchial hyperresponsiveness, and within these regions several candidate genes have been identified that are of relevance to the disease.

Chromosome 5q is a region of interest. It has been linked to asthma, BHR and high IgE levels (Marsh et al. 1995, Meyers et al. 1994, Postma et al. 1995, Doull et al. 1996) and

contains a cluster of cytokine genes, including IL-3, 4, 5, 9, 13 and the β chain of IL-12. These cytokines are involved in B-cell switching, proliferation of mast cells and the development and functioning of Th2 cells (Howard et al. 2000, Wilkinson et al. 1996). In addition, the β_2 adrenergic receptor (β_2-AR) and glucocorticoid receptor are contained in this region, receptors for commonly used asthma treatments. The β_2-AR has a high degree of polymorphisms in the population that have a variety of effects such as different affinities for ligands, defective coupling and agonist promoted up or down regulation (Blumenthal 1998). Also, region 5q35 has been shown to contain the LTC_4 synthase gene (Austen et al. 1997).

Chromosome 6 has been linked to eosinophil counts (Daniels et al. 1996) and contains the HLA (human leukocyte antigen) region that is important in antigen presentation. Tumour necrosis factor (TNF) is encoded by a gene within the majorhistocompatibility complex (MHC) region of chromosome 6 and it has been suggested that a polymorphism within this region may be associated with asthma (Morrison 1998). Another region of interest is chromosome 12q that has been linked to asthma in an Afro-Caribbean population (Barnes 1999) and contains the genes encoding nitric oxide synthase (NOS), leukotriene A_4 hydrolase, and IFNγ that inhibits IL-4 activity. IL-4 binding to its receptor is the first signal for production of IgE after allergen exposure and several polymorphisms in its receptor on chromosome 16 have been linked to asthma (Hijazi et al. 2000, Sandford et al. 2000). There are several other chromosomal regions of interest, each with more candidate genes and together with environmental factors these interact in a complex fashion to cause asthma, a multifactorial disorder, in susceptible individuals.

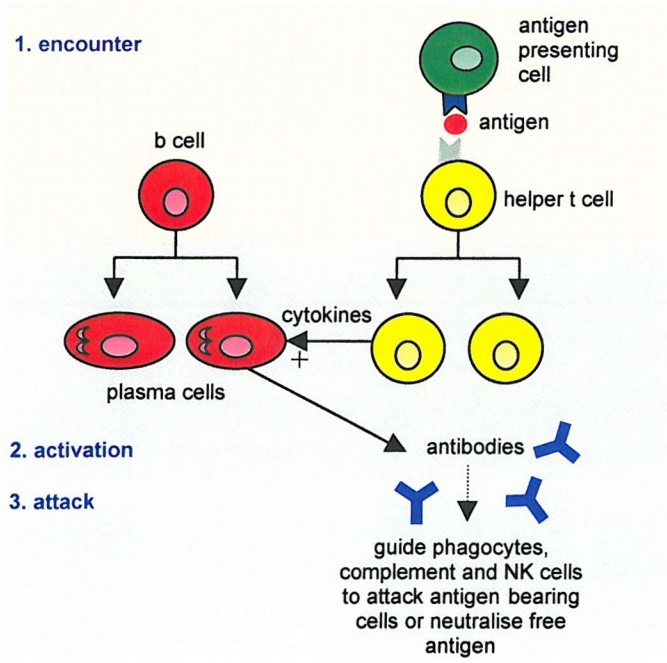


Figure 1. Summary of antigen presentation
(modified from Vander, Sherman & Luciano 1994)

1.1.5 Allergic model of asthma

Atopy is well established as a key factor in the development of asthma and the allergic model of the disease is the easiest to describe (Blumenthal 1998). The first step involves sensitisation of an individual to a particular allergen e.g. Der p1, the major allergen of house dust mite (**figure 1**). The allergen must be taken up by an antigen presenting

cell such as a dendritic cell, macrophage or B cell. Internalisation of the antigen may involve either phagocytosis (macrophages) or binding to immunoglobulin receptors (B cells). In either case the allergen is digested inside the APC into smaller peptides that interact with the major histocompatibility complex (MHC).

There are two types of MHC protein, essential for T cell interaction. Class I MHC proteins are found on the surface of virtually all nucleated cells and are involved in the presentation of endogenous antigens to cytotoxic T cells. In atopic conditions, the peptides interact with the class II human leukocyte antigen (HLA) which is then shuttled to the cell surface and expressed in the plasma membrane. Here the MHC II antigen complex can interact with helper T cells and stimulate cytokine secretion. Along with antigen binding, these cytokines activate B cells. Cytokines are small soluble proteins that by binding to specific receptors may alter the behavior of the cell from which they were secreted or other cells. **Table 1** shows a summary of cytokines of particular relevance to asthma and inflammation.

Table 1. Cytokines important in asthma

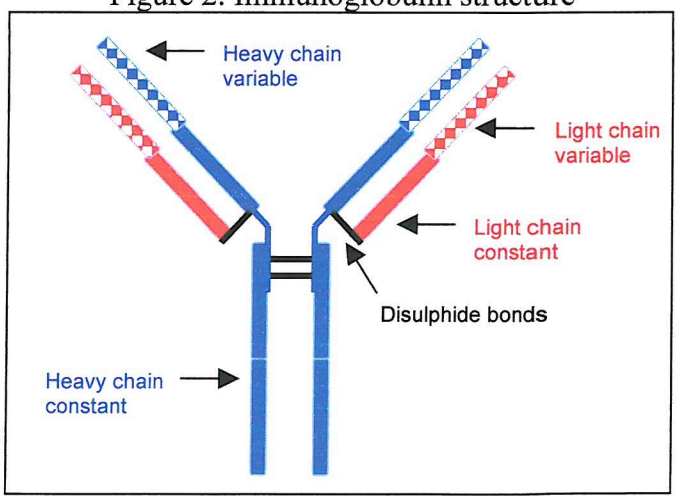
Cytokine	**main source**	**Main target**	**Effect**
IFNγ	Th1 cells, NK cells	Macrophages CD4+ T cells	Activation & differentiation Th2 to Th1 switch
IL-1	monocyte/ macrophages	Th2 cells B cells	Cytokine production Differentiation
IL-3	T lymphocytes	Haematopoietic Stem cells	Proliferation & Differentiation
IL-5	CD4+ and CD8+ lymphocytes	Eosinophils	Chemotaxis Proliferation & activation
GM-CSF	activated T cells and macrophages	Granulocytes, macrophages	Proliferation, activation, ↑ survival
IL-6	monocyte/ macrophages	B cells Monocytes	IgG1 and IgA switch ↑ IL-1 and TNFα secretion
TGFβ	platelets	T cells B cells	↓ growth ↓ IgE production
TNFα	monocyte/ macrophages	Tumour cells almost all cells	↑ apoptosis ↑ cytokine secretion

T helper cells are classified according to the range of cytokines produced. It is now recognised that in asthma, antigen presentation to T helper cells results in differentiation

leading to the Th2 phenotype. Th2 cells specifically produce cytokines such as IL-3, 4, 5, 10, 13 and GM-CSF without significant IFNγ or IL-2. Interaction of these Th2 cells with B cells results in the production of allergen specific IgE. Th1 cells differentiate in the presence of a different range of antigens and are mostly associated with delayed type hypersensitivity responses such as leprosy and tuberculosis. They secrete predominantly IFNγ, IL-2 and TNFβ (Holgate 1993).

This central event of antigen recognition is dependent on antibodies, the antigen-specific family of B-cell products collectively known as immunoglobulins. Immunoglobulins are roughly Y-shaped and share a four polypeptide structure consisting of two identical 50 kDa heavy chains and two identical 25 kDa light chains, **figure 2**. There are two types of light chain, λ and κ without functional difference, but five main heavy chain isotypes each determining the functional activity of an antibody. IgM and IgG provide the bulk of immunity against bacteria and viruses, IgA is important in resistance to multicellular pathogens, the function of IgD is unknown and as mentioned, IgE mediates immediate-type hypersensitivity reactions. The heavy and light chains can be divided into domains on the basis of sequence similarity. The amino terminal of each chain has a variable region (V_L) involved in antigen binding and the remaining domains are constant regions (V_C) that engage the effector functions of the immune system.

Figure 2. Immunoglobulin structure



The allergen-specific IgE antibodies secreted by B cells (or plasma cells once activated) travel all over the body to ineract with their specific antigen and guide the immune reaction. IgE attaches via high affinity IgE receptors ($Ig_εRI$) on e.g. mast cells and basophils and low

affinity IgE receptors ($Ig_{\varepsilon}RII$) on other cells like eosinophils and platelets. This process has the effect of primary sensitisation in an individual who has never come into contact with the allergen before. Upon re-exposure, the allergen will interact with specific IgE bound to the cells, resulting in the production of mediators such as histamine and leukotrienes by mast cells and eosinophils.

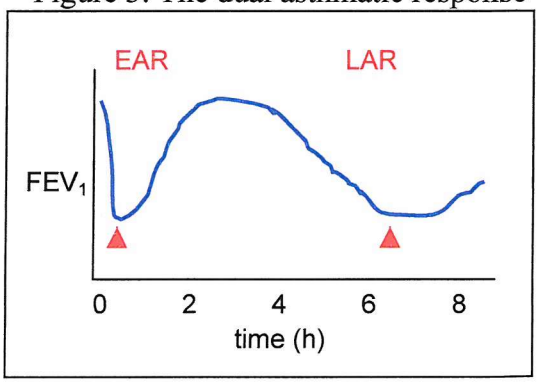
1.1.6 Dual asthmatic response

A commonly used model for studying mechanisms of allergic asthma in humans is the inhaled allergen challenge. The response of the airways to allergen in susceptible patients involves acute bronchospasm, the early airway response (EAR) and in many a later development of airway obstruction, the late airway response (LAR) (**figure 3**). However, this is not true of all asthmatics. It is possible to have isolated early or late responses. Approximately 50% of adult asthmatics will show a dual response upon challenge (Cockcroft 1983, Durham et al. 1990). The early response develops as soon as three minutes after allergen inhalation but subsides within 30-60 minutes. Elevated levels of mast cell mediators such as LTC_4, histamine, tryptase and PGD_2 during the EAR suggests that the degranulation of IgE-stimulated mast cells is responsible for this reaction (Wenzel et al. 1989, Wenzel et al. 1988, Wenzel et al. 1990). These mediators cause rapid airway smooth muscle contraction. A late phase reaction may occur three to eight hours after the early response and is predominantly due to the recruitment and activation of several different leukocytes and further generation of bronchoconstrictor agents (Holgate et al. 1996). The LAR is characterised by bronchial hyperresponsiveness. Bronchial and nasal biopsies from asthmatic patients revealed immunostaining of mast cells for IL-4, IL-5, IL-6 and TNFα (Bradding et al. 1994) and recent evidence has shown that IL-4 is stored pre-formed within mast cell granules (Wilson 2000). The release of such mast cell mediators during the EAR could foster the recruitment of circulating leukocytes and the development of the LAR.

The role of mast cells during the LAR is controversial and another histamine-producing cell type, basophils, are thought be of greater importance to this process. Unlike mast cells, basophils produce histamine and LTC_4 without significant PGD_2 or tryptase, and this profile matches that associated with the late phase reaction in the nasal airway (Naclerio et al. 1985). Basophils have also been identified as the histamine-containing cells in BAL fluid obtained during the LAR (MacDonald 1998). The greatest difference in BAL cells obtained during the EAR and LAR is the dramatic increase in eosinophils that becomes apparent

approximately four hours after challenge and remains for as long as 48 hours (Metzger et al. 1987). This late BAL eosinophilia has been confirmed by many groups and correlates well with elevated levels of BAL fluid IL-5 and GM-CSF (Ohnishi et al. 1993). Along with eosinophils, BAL neutrophils are increased several hours after allergen challenge (Smith et al. 1992, Metzger et al. 1986). Macrophages and monocytes are also associated with the LAR. Macrophages are increased in BAL fluid 48 hours post allergen challenge and are in an activated state as shown by their increased production of IL-6 and TNFα, compared to cells obtained from subjects with an isolated EAR (Metzger et al. 1987, Gosset et al. 1991). Immunocytochemistry of bronchial biopsies obtained 6 hours after allergen challenge has also shown increased numbers of T lymphocytes (Montefort et al. 1994). The later influx of inflammatory cells is thought to involve the upregulation of leukocyte adhesion molecules caused by mediators released during the EAR. Immunostaining for the adhesion molecules ICAM-1 and E-selectin is increased in bronchial biopsies following allergen challenge (Montefort et al. 1994).

Figure 3. The dual asthmatic response



1.2 Cell types involved in asthma

The infiltrating leukocytes associated with asthma originate from stem cells in the bone marrow, polymorphonuclear leukocytes from myeloid progenitor cells and lymphocytes from the common lymphoid progenitor. Following release from bone marrow, they circulate for days or weeks and some are induced to migrate into tissues. When stimulated, they produce a range of mediators that in turn influence other cell types and lead to airway inflammation via a complex network of events.

1.2.1 Lymphocytes

Lymphocytes play a very important role in coordinating the inflammatory response in asthma through the release of specific patterns of cytokines. There are multiple populations and subpopulations of lymphocytes. Briefly, B lymphocytes (B cells) mature in the bone marrow and upon activation by antigen, their main function is to secrete antibodies. There are three main subsets of T cells termed cytotoxic T cells that bind to antigen-bearing cells and kill them, suppressor T cells that inhibit the function of other lymphocytes and lastly helper T cells (Th cells). As described in the context of antigen presentation (p.6), Th cells activate B cells and cytotoxic T cells by the production of cytokines. Different subsets of Th cells produce different combinations of cytokines that in turn will guide a particular type of immune response (Corrigan 1998).

Several observations have confirmed that lymphocytes are key cells in asthma pathogenesis. Increased numbers of activated T cells are seen in the bronchial mucosa and BAL fluid of asthmatics, and their numbers correlate with disease severity and eosinophil counts (Bradley et al. 1991). The cytokines secreted by activated Th2 lymphocytes are relevant to the accumulation and activation of eosinophils in the bronchial mucosa. Significantly elevated percentages of BAL cells express mRNA encoding IL-3, IL-4, IL-5 and GM-CSF (Th2 type cytokines) but not IFNγ (Th1 type) in asthmatics compared to normals and the majority of these are T cells (Robinson et al. 1992). Activated Th2 cells were also detected in the peripheral blood of patients with severe asthma, and numbers were reduced following glucocorticoid therapy to a degree which correlated with clinical improvement (Corrigan et al. 1993).

1.2.2 Neutrophils

Along with eosinophils and basophils, neutrophils comprise the granulocytes so called due to the presence of densely staining granules in the cytoplasm. The main function of neutrophils is phagocytosis of antibody-coated pathogens and activation of bactericidal mechanisms. They also have the capacity to release a number of products of relevance to asthma in particular ones that cause tissue injury such as the proteases elastase, collagenase and gelatinase (O'Byrne 1998). Lipid mediators produced include the prostaglandins, TXA_2, PAF and LTB_4. Although neutrophils produce negligible amounts of LTC_4 themselves they are capable of transcellular biosynthesis of the cys-LTs by providing LTA_4 to structural cell types that possess LTC_4 synthase (Sala et al. 1997). Other neutrophil products include a

factor that activates mast cells and basophils causing degranulation and mediator release (Nadel et al. 1998). Also, when neutrophils are stimulated, a respiratory burst begins resulting in superoxide and hydrogen peroxide release that in turn form more toxic radicals capable of causing tissue damage (Lehrer et al. 1988). The production of neutrophil products occurs in response to fMLP, complement factor C5a, TNFα and GM-CSF. Migration of neutrophils to sites of damage occurs due to chemotactic factors such as C5a, IL-8 and as discussed below, LTB_4 (O'Byrne et al. 1985, Richman-Eisenstat et al. 1993, Berend et al. 1986).

Whether neutrophils play an important role in chronic asthma is controversial but there is increasing evidence that they are particularly involved in severe and acute asthma. Non-allergic triggers of asthma such as nitrogen dioxide and ozone cause an influx of neutrophils into the airways, as does allergen challenge. However, unlike eosinophils, neutrophils do not remain in the airways but disappear rapidly after challenge (Coffey et al. 1996, Teran et al. 1995). Patients who have died of sudden onset asthma have a high number of neutrophils in the submucosa compared to other cell types and nocturnal asthma is also associated with an airway neutrophilia that correlates with severity (Martin et al. 1991, Sur et al. 1993). Patients with severe steroid dependent asthma show blood and sputum neutrohilia compared to those with well controlled mild asthma but it is not known whether this is spontaneous or a consequence of steroid use, which prolongs neutrophil survival by reducing apoptosis (The ENFUMOSA Study Group, Cox et al. 1995).

1.2.3 Eosinophils

Eosinophils, so named due to the intense staining of their granules by the dye eosin have a characteristic bilobed nucleus and a main function of killing antibody coated parasites. Several lines of evidence point to a central role for these cells in asthma, eosinophilia being associated with both hypersensitivity and helminth infections (Kita et al. 1998). Eosinophils generate a wide range of inflammatory mediators. The granules of eosinophils contain four main proteins. Major basic protein (MBP) is localised in the crystalline core and eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase (EPO) are localised in the matrix. These granule proteins are toxic to respiratory epithelium causing desquamation, alterations in ciliary beat frequency and secretion (Gleich 2000). They are also potent helmintho-, cyto- and neurotoxins (Gleich 1986). At nanomolar concentrations they increase vascular permeability and stimulate other cells such as

basophils, mast cells, neutrophils, goblet cells and platelets (Minnicozzi et al. 1995, Gleich 2000).

Several lipid mediators are produced by eosinophils that have cytoplasmic lipid bodies, stores of arachidonic acid (Weller et al. 1994). In contrast to neutrophils they generate relatively large amounts of LTC_4 compared to negligible LTB_4 (Weller et al. 1983). There have been few reports of prostaglandin production although patients with hypereosinophilic syndrome synthesise PGE_1, PGE_2 and TxB_2 (Kita et al. 1998). Compared to neutrophils, comparable quantities of PAF are generated which can cause eosinophil chemotaxis, vasodilation and endothelial adherence (Kay et al. 1992).

Several factors are involved in the recruitment of eosinophils into the airways. Of the eosinophil chemoattractants eotaxin is of particular importance, released by endothelial cells, epithelial cells, smooth muscle cells, fibroblasts and macrophages as well as eosinophils themselves (Lamkioued et al. 1997, Gleich 2000). Others include LTD_4 at concentrations as low as 10^{-10} M, PAF and several T cell products (IL-5, GM-CSF, IL-3) and chemokines (RANTES, MCP3 & 4) (Kita et al. 1998). Eosinophils are primed (partially activated) as a result of migration to the site of inflammation but once there they are fully activated and induced to degranulate by a number of stimuli. Ig receptors, in particular those for secretory IgA and IgG as well as cytokines such as IL-5, GM-CSF and IL-3 cause degranulation and mediator production (Gleich 2000, Kita et al. 1998). Some evidence suggests that eosinophils may express the high affinity IgE receptor (Gounni et al. 1994) as eosinophils isolated from patients with eosinophilia degranulated in response to anti IgE antibody or IgE coated parasites (Gleich 1986). IL-3, IL-5 and GMCSF prolong eosinophil survival by antagonising programmed cell death (Her et al. 1991). The recruitment, activation and survival of eosinophils is strongly inhibited by corticosteroids, an effect which could explain their efficacy (Djukanoviç et al. 1992).

There is much evidence to support a role for eosinophils in asthma. Blood and sputum eosinophilia is associated with asthma and bronchial hyperresponsiveness correlates with blood and BAL eosinophils in both atopic and non-atopic asthma (Kay et al. 1992, Gleich et al. 1998, Wardlaw et al. 1988). Increased eosinophil products (MBP, ECP, EDN) are observed in BAL during allergen induced late phase reactions (Metzger et al. 1987). Eosinophils are prominent cells in histopathological sections obtained from people who have

died from asthma (Dunnill et al. 1960) and high numbers of EG2 positive eosinophils are shown in bronchial biopsies from atopic asthmatics (Azzawi et al. 1990).

In the past decade there has been a lot of evidence to support the description of asthma as a Th2-like disease in which eosinophils are particularly important. However, within this context it is important to mention the recent findings of Leckie et al. concerning the administration of an IL-5 blocking antibody. The effect of this antibody on blood and sputum eosinophil counts, bronchial hyperresponsiveness and the LAR was examined at varying time points up to 16 weeks after intravenous infusion. The most striking result was the >90% reduction in blood and sputum eosinophils confirming the importance of IL-5 in eosinophil formation. However, the blocking antibody had no significant effect on airway hyperresponsiveness or on the late asthmatic reaction to inhaled allergen (Leckie et al. 2000). While a larger and longer clinical study is required, the findings of Leckie and colleagues do highlight the complexity of airway inflammation and the importance of several different cell types. This study particularly questions the role of Th2-cytokine driven eosinophilia in persistent asthma.

1.2.4 Mast cells

Mast cells, with their oval nuclei and numerous granules are major effector cells in allergic disease. They belong to the family of myeloid cells but rather than circulating as mature cells in the blood they circulate as progenitors and complete their differentiation in peripheral tissues, most commonly at sites where they may encounter noxious agents such as the respiratory mucosa, gut and skin. Their main role is to mediate innate immunity to bacteria by the release of granules containing active agents (Schwartz et al. 1998). There are two main types of mast cell in humans, named MC_T and MC_{TC} depending on the main enzyme compositions. MC_T contain tryptase alone, while MC_{TC} contain tryptase, chymase, carboxypeptidase and cathepsin G. MC_T is the predominant type in lung while MC_{TC} is mainly found in the skin (Benyon et al. 1987, Church et al. 1982). Important functions of tryptase include inactivation of fibrinogen and inhibition of fibrogenesis, activation of tissue matrix metalloproteinases (MMP-3), inactivation of neuropeptides, stimulation of fibroblast proliferation and collagen synthesis, eosinophil chemotaxis, and the upregulation of ICAM-1 expression and IL-8 synthesis by BEC (Bingham et al. 2000).

Mast cell derived mediators may be preformed and stored in granules or synthesised *de novo*. Of the granule-associated products histamine is the most studied. In humans, histamine acts at H_1 and H_2 receptors and has effects on smooth muscle, endothelial cells and nerve endings. Histamine is synthesised in the Golgi apparatus by decarboxylation of the precursor amino acid histidine. It is metabolised by histamine N methyltransferase (methylation) and diamine oxidase (oxidation) (Rang & Dale 1991). The importance of histamine in allergic disease is illustrated by the efficacy of H_1 antagonists in modifying diseases such as hayfever (Bousquet et al. 1996) but the failure of antihistamines in asthma therapy suggests that other events are of greater importance in inflammatory processes in the lung (Wood-Baker et al. 1995).

Newly-formed mediators generated by MC include eicosanoids, such as the bronchoconstrictive LTC_4 and PGD_2 and a range of multifunctional cytokines (Bingham et al. 2000). Bronchial and nasal mucosal biopsies from asthmatic patients revealed immunostaining of mast cells for TNFα, IL-4, IL-5, and IL-6 (Bradding et al. 1994). Interestingly, immunogold electron microscopy recently demonstrated that one of these these cytokines, IL-4 was located within mast cell secretory granules (Wilson 2000). The presence of a store of pre-formed IL-4 has important implications as IL-4 could be rapidly released upon IgE dependent degranulation and contribute to the initiation and maintenance of inflammation.

Classic mast cell activation occurs through FcεRI, the receptor that binds the Fc portion of IgE. When adjacent receptors are crosslinked by multivalent antigen or haptens there is phosphorylation of the FcεRI immunoregulatory tyrosine-activation motifs (ITAMs) that in turn activate other tyrosine kinases (Lyn and Syk) and lead to several pathways of signal transduction (Kinet et al. 1996). Other factors causing mast cell degranulation/mediator production include extracellular matrix proteins e.g. fibronectin and laminin, complement fragments C3a and C5a, eosinophil-derived granule proteins and neuropeptides (el Lati et al. 1995, Bischoff et al. 1992, Church et al. 1991). Primed mast cells will also respond to changes in osmolarity, a mechanism that may contribute to exercise-induced asthma (Eggleston et al. 1990).

Allergen challenge in atopic individuals has shown that mast cells are involved in the early airway response by the measurement of the mast cell mediators LTC_4, histamine, tryptase and PGD_2 in BAL fluid 5 minutes after challenge (Wenzel et al. 1989, Wenzel et al. 1988, Wenzel et al. 1990). Later studies have also suggested that there may be a role for mast cells in the late-phase response. Measurement of the urinary excretion of mast cell markers 9α, 11β–PGF_2 and Ntau-methylhistamine showed that levels were elevated above baseline during both the EAR and LAR of an inhaled allergen challenge in asthmatics (O'Sullivan et al. 1998). Also, a single immunologic stimulus can increase the transcription of mast cell TNFα, IL-4 and IL-5 for up to 72 hours (Okayama et al. 1995), an effect that could explain the cellular influx of the LAR.

1.2.5 Basophils

Basophils are morphologically similar to eosinophils (circulating multilobular granulocytes) but like mast cells they express large amounts of the high affinity IgE receptor FcεRI. Their cytoplasm contains membrane-bound secretory granules, and lipid bodies are also seen. One of the functions of basophils is thought to be in host defence against neoplasms, microorganisms and toxins, but several facts point to an important role in asthmatic airway inflammation. Basophils release both preformed and newly synthesised mediators. Preformed mediators include histamine and the granule protein chondroitin sulphate A. Of the lipid mediators basophils produce mostly cysteinyl lekotrienes and PAF. Cytokines synthesised include IL-4, IL-5, IL-8, IL-13 and MIP-1α (Busse et al. 1998). Like mast cells, basophils produce LTC_4 and histamine, but basophils do not produce tryptase or PGD_2, so the presence of tryptase and PGD_2 in BAL fluid can differentiate between mast cell or basophil activation (MacDonald 1998). A number of different stimuli cause activation and degranulation of basophils. IgE is one of the most studied activators but other stimuli include complement factors C5a and C3a, the bacterial peptide fMLP, PAF and the enzyme PLA_2. Basophils also have surface receptors for several cytokines and chemokines and are activated by mediators such as MCP-1, MCP-3 and RANTES (Grant et al. 1998).

Whereas mast cells are particularly important in the early phase reaction to allergen, basophils are key players in the late phase. After allergen challenge, there is an increase in the number of BAL fluid basophils which is most striking at 24 hours after challenge (Guo et al. 1994). Increased numbers of basophils have been found in the blood and sputum of

asthmatics during clinical exacerbations and enhanced spontaneous release of mediators (histamine and LTC_4) have been noted in basophils from asthmatics compared to normals (Tung et al. 1982). There are also increased numbers of basophils in the airways of subjects who have died from asthma (Koshino et al. 1993). The contribution of basophils to asthma is thought to result from release of inflammatory mediators and increased cytokine production (IL-4, IL-5, IL-13) which can enhance IgE synthesis, Th2 development and eosinophil recruitment.

1.2.6 Monocytes and macrophages

Monocytes and macrophages are cells of the mononuclear phagocyte lineage. A simplistic description of the differentiation of these cells is that bone marrow progenitor cells give rise to blood monocytes from which tissue macrophages or dendritic cells are derived. This terminal differentiation is remarkably tissue-specific and is likely to be controlled by factors released in the local environment. Mononuclear phagocytes can have several functions and factors controlling their differentiation determine whether they phagocytose and kill microorganisms, present antigens to T-cells, release soluble inflammatory mediators or carry out a combination of these events. These functions require the production of a bewildering variety of mediators. Microorganisms are killed by oxygen dependent mechanisms involving the generation of reactive oxygen intermediates such as superoxide (O_2^-) and H_2O_2 and also by generation of the antimicrobial agents lysozyme, cathepsin G, proteinase 3 and elastase. Triggers including IgE-dependent pathways can stimulate the production of leukotrienes, prostaglandins, PAF and lipoxins. Monocytes also produce an array of cytokines of relevance to asthma including GM-CSF, TNFα and IL-6 and many others (Gant et al. 1998). Monocytes and macrophages are also important antigen presenting cells for the activation of allergen-specific Th2 cells. They express MHC class II antigens, adhesion molecules neccesary for T cell binding, co-stimulatory molecules essential for T cell activation and cytokines that promote development of the Th2 phenotype (Clark et al. 1994).

1.3 Airway structural cells

The classical concept of asthma is that it is a Th2 cell orchestrated inflammatory disease. While it is clear that inflammation is central to the pathogenesis of asthma it is increasingly recognised that not just blood lymphocytes and leukocytes are involved in this process. Structural cell types not previously thought to be involved in inflammation have now been shown to release both pro and anti-inflammatory mediators and to play a large part in the

structural changes associated with chronic inflammation. These cell types include airway epithelial cells, smooth muscle cells, fibroblasts and endothelial cells, that in addition to their structural roles, are also able to contribute to airway inflammation and remodelling. The structural changes, or remodelling, that occurs in asthmatic airways includes thickening of the airway wall, increased myocyte mass, myofibroblast hyperplasia, epithelial shedding mucus metaplasia, angiogenesis and subepithelial fibrosis (Elias 2000).

1.3.1 Bronchial Epithelial Cells

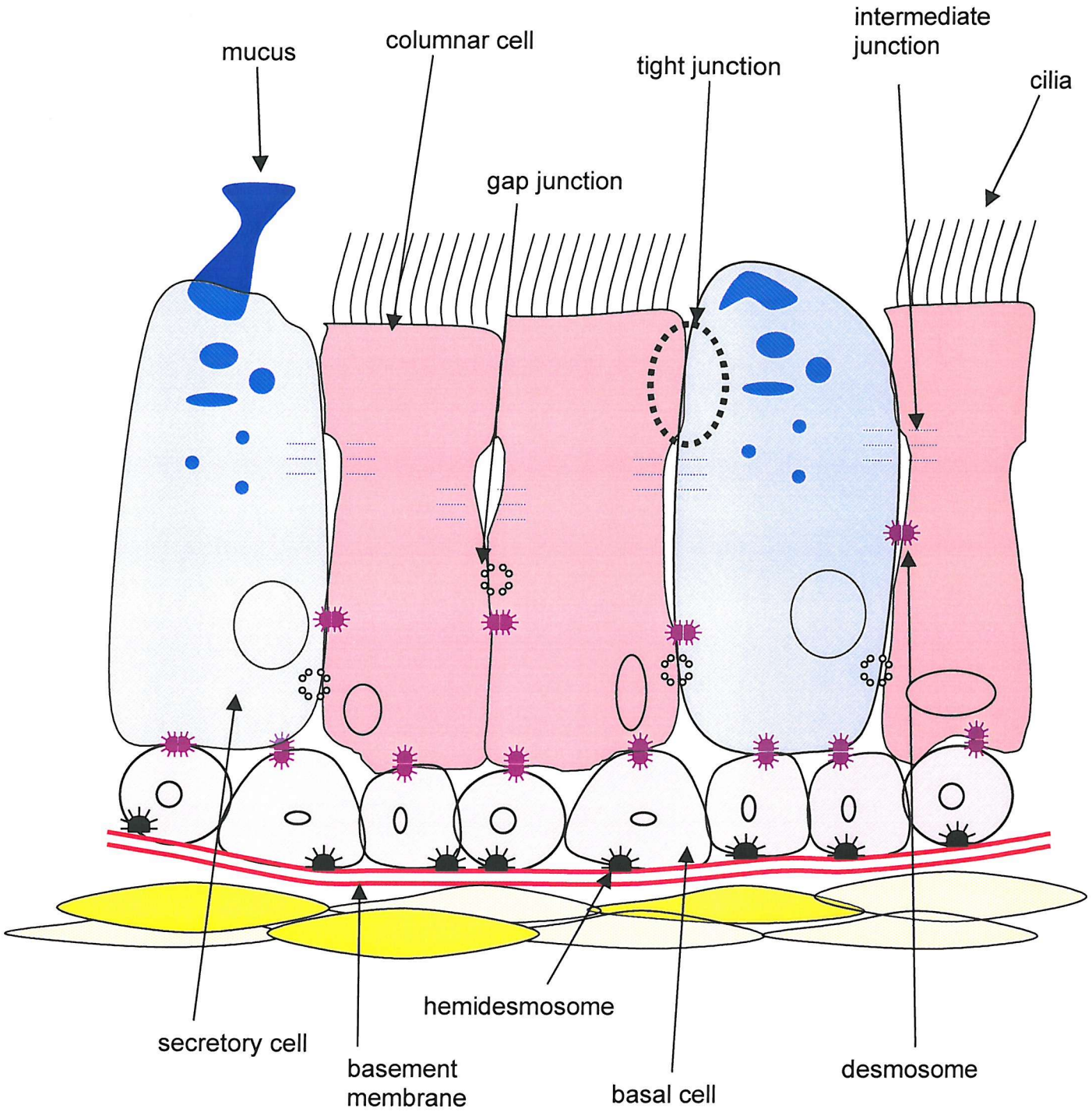
1.3.1.1 Structure

Human bronchial epithelium consists mainly of three cell types, columnar ciliated cells, secretory cells and basal cells (van der Velden et al. 1998). Although for a long time described as pseudo stratified, electron microscopy has now shown that these cells are arranged in a stratified layer with columnar cells adhering to basal cells which adhere to the basement membrane (Roche et al. 1993) (**figure 4**). The structure of the epithelium is maintained through several adhesive mechanisms. Basal cells are firmly attached to the basement membrane by hemidesmosomes, structures made up of integrin proteins, mainly the α6/β4 integrin complex (Sonnenberg et al. 1991). Desmosomes are found between all epithelial cells and comprise several structural filamentous proteins that link adjacent cytoskeletons (Garrod 1993). The passage of molecules between cells is controlled by tight and intermediate junctions. Tight junctions surround each cell at the apical pole and permeability can be modulated by proteases, cytokines, bacterial products, and eosinophil- and neutrophil-derived proteins (Lewis et al. 1995). Intermediate junctions form a ring-like adhesive mechanism around the cell to which adhesion molecules such as E-cadherin have been localised (Schneeberger et al. 1992). Epithelial cells also communicate via gap junctions.

1.3.1.2 Barrier function

Of vital importance to the barrier function of epithelial cells are the strong adhesive mechanisms mentioned, but epithelial cells also provide an active defence system against inhaled noxious agents such as pollutants and particulates. Ciliated cells remove particulate matter by propelling mucus and trapped debris therein upwards and out of the airway (Proud et al. 1998). Ciliary beat frequency can be regulated by several factors such as leukotrienes, bradykinin, histamine and cytokines (Cyrus et al. 1998, van der Velden et al. 1998). Goblet or mucus cells secrete mucus that lines the upper airways and can contribute to the

Figure 4. The structure of bronchial epithelium



detoxification of inhaled substances (Thompson et al. 1995). Products released within this mucus include antibacterial substances (lactoferrin and lysozyme) anti-proteases (metalloprotease inhibitors and the α_1-protease inhibitor) and antioxidants (superoxide dismutase and catalase) (Coonrod 1986, Heffner et al. 1989). Certain mucus cells produce the epithelial transfer component of IgA and components of the complement system that

facilitate efficient phagocytosis of foreign particles by macrophages (Goodman et al. 1981, Rothman et al. 1989). Submucosal glands also exist in the epithelium which are under the control of cholinergic nervous regulation whereas goblet cell secretion is controlled by secretagogues such as proteases, lipid mediators and cytokines (Lundgren et al. 1988). Cell surface peptidases present on epithelial cells can degrade several bronchoconstrictor peptides such as bradykinin and enkephalins, thus protecting the airway smooth muscle (Nadel et al. 1991). Epithelial cells also express a surface aminopeptidase that degrades enkephalins, which in turn modulate the release of acetylcholine from airway nerves (Proud et al. 1998).

1.3.1.3 The epithelium as a target in asthma

Damage to bronchial epithelium is a well known characteristic of asthma. In 1960, Naylor noted the presence of increased numbers of epithelial columnar cell clusters (Creola bodies) in asthmatic sputum (Naylor 1962). Loss of epithelium is also observed in patients who have died from asthma (Dunnill et al. 1960) and more recently the use of bronchoscopes has enabled confirmation of these observations (Laitinen et al. 1987, Montefort et al. 1992). It is mainly the columnar cells that are lost leaving areas of just basal cells although the precise mechanism involved in this shedding is unknown. Despite numerous observations of epithelial shedding in asthma, care should taken when interpreting this characteristic as a recent study suggests that epithelial denudation in bronchial biopsies is merely an artefact of tissue sampling (Ordoñez et al. 2000). This group showed similar epithelial damage in both asthmatic and normal biopsies and found no correlation between the extent of denudation and bronchial hyperresponsiveness

Many agents can cause damage to the bronchial epithelium. The eosinophil is a major cause of epithelial damage in asthma (Frigas et al. 1986). It is recruited to the airways during inflammation and activation causes degranulation with the release of MBP and ECP which are cytotoxic, possibly by promoting target cell pore formation. Effects observed on epithelial cells include ciliostasis, decreased ciliary beat frequency and exfoliation (Montefort et al. 1992). Another eosinophil product EPO is particularly potent in the presence of hydrogen peroxide and halide when highly cytotoxic hypohalous acids are produced (Ayars et al. 1989). Evidence also points to an important role for matrix-metalloproteinases through their capacity to weaken junctional adhesion molecules, in particular MMP-9 whose levels are increased in asthma (Holgate et al. 1998). Other factors

responsible for epithelial damage include pollutants, respiratory viruses, allergens and oxidants (Devalia et al. 1993, Thompson 1998, Wang et al. 1998, Montefort et al. 1992).

Damage to the epithelial layer has numerous effects. The barrier function is lost so noxious agents may reach the underlying smooth muscle more easily, and intra-epithelial sensory nerves involved in neuropeptide release are exposed and stimulated by inhaled agents resulting in reflex bronchoconstriction. A number of protective factors produced by the epithelium such as NO, PGE_2 and antiproteases are lost as well as mucociliary clearance (Folkerts and Nijkamp 1998). In addition there is increased water loss, in particular during exercise which may contribute to the creation of a hypertonic environment that can activate mast cells.

1.3.1.4 Inflammatory function of bronchial epithelium

Bronchial epithelial cells are able to produce a wide range of both pro and anti inflammatory mediators as well as adhesion molecules, summarised in **table 2**. Several lipid mediators are generated. Of the COX products, the bronchoconstrictive $PGF_{2\alpha}$ and relaxant PGE_2 are produced in approximately equal amounts by human bronchial epithelial cells (Holtzman et al. 1988). So far, studies have shown that lipoxygenase products are mostly those of the 12-LO and 15-LO pathways such as 15-HPETE, 15-HETE and 12-HETE, which at relatively high concentrations can cause neutrophil recruitment (Sigal et al. 1991). In human tracheal epithelial cells the 15-LO pathway is particularly predominant (Hunter et al. 1985).

Human bronchial epithelial cells can express an array of cytokines and chemokines such as IL-8, IL-6, IL-5, GMCSF, RANTES and eotaxin, the functions of which include mainly recruitment, activation, and increased survival of eosinophils, neutrophils and lymphocytes. Growth factors synthesised include TGFβ and bFGF that have effects on epithelial growth and differentiation. Other products of bronchial epithelial cells include peptide mediators such as the endothelins which are potent bronchoconstrictors, and NO, a bronchodilator and vasodilator (Black et al. 1989). Both inducible and constitutive isoforms of nitric oxide synthase are present in epithelial cells. NO levels are greatly increased in the exhaled air of asthmatics but the relevance of this remains unknown (Alving et al. 1993, Kharitonov et al. 1994, Folkerts et al. 1998).

Table 2. Pro-inflammatory products of human bronchial epithelial cells

Product	Stimulus
lipid mediators	
PGD_2	
$PGF_{2\alpha}$	Il-1β, TNFα, IFNγ
PGE_2	Il-1β, TNFα, IFNγ, ozone
TXB_2	Il-1β, TNFα, IFNγ, ozone
12/15 HETE	A23187
LTB_4	
Chemokines	
IL-8	viruses, bacterial products, ozone, asbestos, histamine
GROα	TNFα
GROγ	TNFα
MCP-1	TNFα
Eotaxin	TNFα, IFNγ
RANTES	Il-1, TNFα, IFNγ
MIP-2	
Cytokines	
IL-1α/β	asbestos, bradykinin
IL-3	
IL-5	TNFα, IFNγ
IL-6	TNFα, TGFβ, ozone, histamine, viruses
IL-10	
IL-11	TNFα, TGFβ, IL-1, RSV
IL-16	histamine
LIF	
TNFα	NO_2
GM-CSF	Il-1, TNFα, NO_2, histamine, RSV
Growth factors	
PDGF	
IGF	ECP
EGF	
TGFβ	retinoic acid
Adhesion molecules	
ICAM1	HRV, IL-4, IL-5, IL-10, IL-15
LFA-3	
CD44	IFNγ
Others	
PAF	PMA, A23187
NO	histamine
Endothelin	RSV
Fibronectin	
Histamine	influenza virus, Staphylococcus aureus

(van der Velden et al. 1998, Proud et al. 1998, King et al. 1998, Tomee et al. 1998, Bianco et al. 1998, Tristram et al. 1998, Salari et al. 1989, Kumlin et al. 1990, Mitchell et al. 1994, McKinnon et al. 1993, Behera et al. 1998, Salvi et al. 1999)

Bronchial epithelial cells express a range of surface adhesion molecules allowing them to interact with inflammatory blood cells. ICAM-1 and the lymphocyte function associated antigen LFA-3 are expressed on human airway epithelia (Bloemen et al. 1993). ICAM-1 expression is increased by histamine, IL-1β, TNFα, and IFNγ and is also higher on the epithelial cells of asthmatics (Tosi et al. 1993, Vignola et al. 1993). CD44 is found on both asthmatic and normal bronchial epithelium but is increased in asthmatics and at sites of damage, so is thought to be involved in repair processes (Lackie 1997).

There may be a role for epithelial cells in immunoregulation as they express MHC class II antigens such as human leukocyte antigen (HLA-DR), and expression is higher on asthmatic cells (Vignola et al. 1993). Also, epithelial cells can present antigen to T lymphocytes and also stimulate the proliferation of both CD4+ and CD8+ T cells (Mezzetti et al. 1991). The low affinity IgE receptor CD23 has been described in asthmatics but not normals so BEC may be directly activated by IgE dependent mechanisms (Campbell et al. 1994).

1.3.2 Airway Smooth Muscle

1.3.2.1 Structure

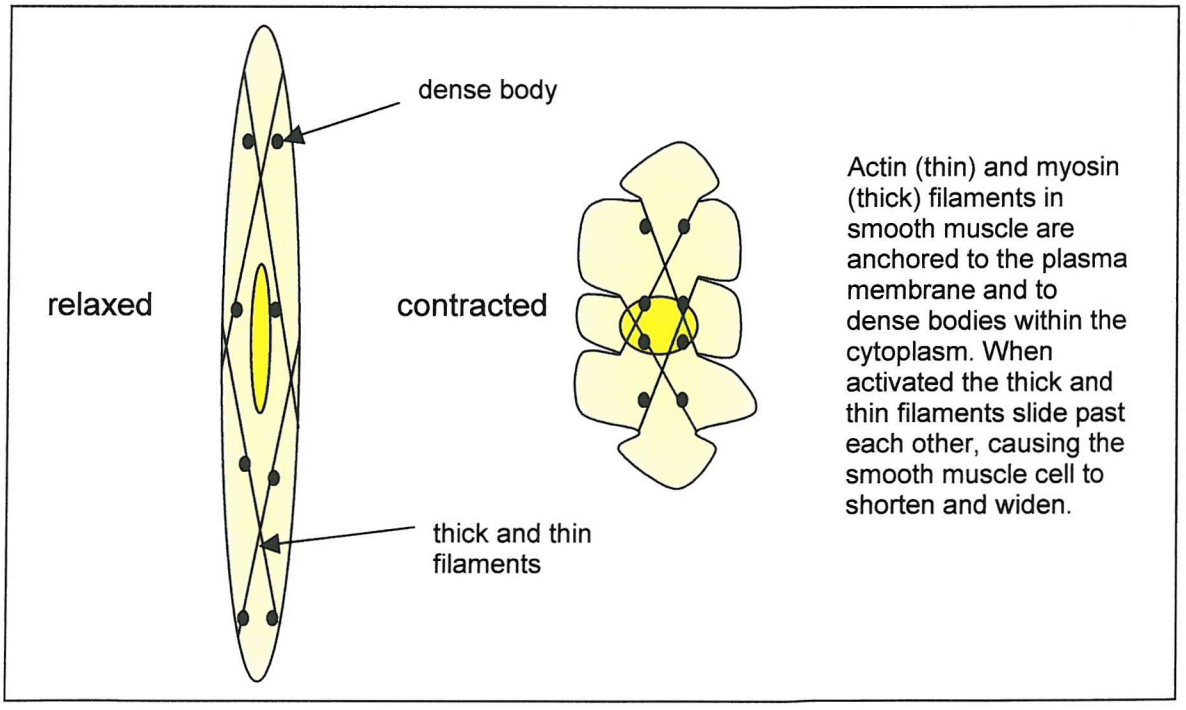
Airway smooth muscle was first described in detail by Reissen in 1822, who used a hand held magnifier and reported that smooth muscle encircles the airway completely below the trachea and to an even greater extent in the peripheral airways. Each individual smooth muscle cell is spindle-shaped, and unlike skeletal muscle, each has a single nucleus and is able to divide. Smooth muscle cells are surrounded by a thin basal lamina and are separated from each other by a gap of 50-80nM. There are also special cell-to-cell contacts, gap junctions, which provide a pathway for metabolite flow between cells. Modulation of gap junction pore opening can be controlled by Ca^{2+}, H^{+} and cAMP which all reduce opening. There are three main functions of smooth muscle cells that are of relevance to asthma: the contractile, proliferative and synthetic functions. Smooth muscle proliferation is discussed within the context of airway remodelling.

1.3.2.2 Contractile function

Two main types of contractile filament are present in the cytoplasm of these cells: thick myosin-containing filaments and thin actin-containing filaments. It is the sliding of adjacent actin and myosin filaments which is thought to generate the contractile force. Changes in the contractile activity of smooth muscle are a consequence of the actions of various chemical

mediators and neurotransmitters that usually bind to a specific receptor, increase intracellular Ca^{2+} and cause contraction. One hypothesis for the initiation of actomyosin activation is that a calcium/calmodulin complex binds to and activates myosin light chain kinase (MLCK). This results in a subsequent phosphorylation of the light chain subunit of myosin and activation of the tension generating, actin-dependent myosin ATPase. Actin filaments are anchored to plasma membrane dense plaques or to cytoplasmic structures known as dense bodies. Contraction causes the cells to shorten and widen as shown in **figure 5**, as tension is transmitted throughout the cell (Gunst et al. 2000). During smooth muscle relaxation the phosphorylated myosin must be dephosphorylated by a phosphatase enzyme which is constitutively present and active. However, when $[Ca^{2+}]_i$ is high, kinase activity predominates. There is also thought to be a role for cAMP in the relaxation process as β-adrenoceptor stimulation increases cAMP levels. A cAMP dependent protein kinase may regulate decreases in $[Ca^{2+}]_i$ or cAMP dependent phosphorylation of MLCK could reduce the sensitivity of the enzyme to Ca^{2+}/calmodulin activation (Barnes 1995).

Figure 5. Smooth muscle contraction



Upon stimulation, the sources of Ca^{2+} include the sarcoplasmic reticulum, internal plasma membrane, mitochondria or through plasma membrane calcium channels. The relaxation process involves a reduction in $[Ca^{2+}]_i$ by sequestration of internal Ca^{2+} involving the organelles above and also translocation across the plasma membrane (Gunst et al. 2000).

The contractile activity of smooth muscle is influenced by neurotransmitters, paracrine agents and other factors such as acidity, O_2 concentration and osmolarity. Airway smooth muscle is innervated by parasympathetic nerves that branch throughout the muscle and release neurotransmitter from swollen vesicle-containing varicosities. Sympathetic neurones are not thought to branch through the muscle. Instead effects are due to circulating catecholamines. α_1 and β_2 adrenoceptors are present on airway smooth muscle, stimulation of which causes contraction and relaxation respectively. The density of β receptors increases from the trachea to the bronchioles. Also, muscarinic M_3 receptors are present, stimulation of which causes contraction (Rang & Dale 1991).

As discussed, a number of inflammatory mediators have effects on airway smooth muscle causing increased or decreased contractile activity depending on the associated changes in $[Ca^{2+}]_i$, second messengers or in some cases indirectly by the production of other mediators. Many of these agents are of particular relevance to asthma, especially the leukotrienes. Cys-LTs are able to contract human airway smooth muscle both in isolated airway strips and rings in vitro as well as in vivo by inhalation (Dahlén et al. 1980, Schmidt et al. 2000). Their potency is at least 1000 times greater than histamine and is a result of their binding to the $CysLT_1$ receptor (Dahlén et al. 1983). The bronchoconstrictor effects of histamine are well known and are mediated by specific receptors (H_1, H_2 and H_3) which respond to stimulation with both calcium influx and prostaglandin synthesis (Joad et al. 1988). Of the prostaglandins, PGD_2, $PGF_{2\alpha}$ and TXA_2 all have contractile activity by their action on TP (the TXA_2 receptor). PGE_2 relaxes canine tracheal smooth muscle, possibly by enhancing intracellular Ca^{2+} sequestration (Tomioka et al. 1982). PAF inhalation causes bronchoconstriction indirectly, possibly by causing histamine or leukotriene release (Smith et al. 1988, Rubin et al. 1987). Several other agents are capable of contractile activity including bradykinin and neurokinins.

1.3.2.3 Biosynthetic function

As well as contributing to airway obstruction, smooth muscle cells are increasingly regarded as a cell type capable of contributing to the chronic inflammation of asthma by their ability to synthesize a wide range of inflammatory mediators. See **table 3** for a list of the cytokines, chemokines and other agents produced. Also, adhesion molecules for blood leukocytes are expressed constitutively on HASM cells e.g. CD44 and ICAM-1. Upon stimulation with

Table 3. Inflammatory mediators produced by airway smooth muscle cells

Product	Stimulus	Cell type
Cytokines		
IL-6	asthmatic serum, IL-1β, IFNγ, TNFα	human
IL-1β	RSV	human, rabbit
IL-5	basal + ↑ by asthmatic serum	human, rabbit
GM-CSF	basal + ↑ by asthmatic serum	human, rabbit
IL-2	basal + ↑ by asthmatic serum	human, rabbit
IL-12	basal + ↑ by asthmatic serum	human, rabbit
IFNγ	basal + ↑ by asthmatic serum	human, rabbit
SCF		human
Chemokines		
MCP-1, 2, 3		human
RANTES	IL-1β	human
IL-8	asthmatic serum, IL-1β, IFNγ, TNFα	human
Eotaxin		human
Growth factors		
bFGF	chlamydia pneumoniae	human
EGF		human
IGF-2		rabbit
IGFBP-2		rabbit
PDGF-BB	IL-1β	guinea pig
Adhesion molecules		
ICAM-1	TNFα	human
VCAM-1	TNFα	human
Lipid mediators		
PGF2α	IL-1β, IFNγ	human
TxB_2	IL-1β, IFNγ	human
PGE_2	AA, BK, IL-1β, IFNγ	human, cow
Others		
NO	LPS	pig

(John et al. 1997, Panettieri et al. 1995, Belvisi et al. 1997, Delamere et al. 1994, Rodel et al. 2000, Pype et al. 1999, Kassel et al. 1999, Hakonarson et al. 1999)

TNFα, the expression of ICAM-1 and VCAM-1 may be increased, along with an increase in lymphocyte adhesion to the smooth muscle cells (Panettieri et al. 1995).

1.3.2.4 Phenotype plasticity

It has been proposed that airway smooth muscle cells are able to switch between functional phenotypes. Cells in primary culture which proliferate slowly show greater amounts of actin and myosin contractile proteins and lower amounts of cytoskeletal proteins (contractile phenotype). However, rapidly proliferating cells like those grown in the presence of foetal calf serum show lower amounts of actin and myosin, more cytoskeletal protein and also increased Golgi apparatus and rough endoplasmic reticulum (synthetic phenotype) (Johnson et al. 1997). Although not proven, the synthetic, proliferative phenotype could be the one involved in airway remodelling and obstruction (Hirst 1996).

1.3.3 Fibroblasts

Fibroblasts are a connective tissue cell of mesenchymal origin that constitute the major cell type of the lung interstitium. They are extremely versatile and display a remarkable capacity to differentiate into other members of the connective tissue family such as cartilage, bone, adipocyte and smooth muscle cells (Buckley et al. 2001). The fibroblast is a spindle shaped, elongated cell with a large oval nucleus containing one or more nucleoli. Cytoplasmic microfilaments and intermediate filaments are not prominent in normal fibroblasts but another type of fibroblast, the "contractile interstitial cell" or myofibroblast has been described which exhibits features of both fibroblasts and smooth muscle cells. Myofibroblasts have been observed to increase in a variety of pathological conditions such as pulmonary fibrosis. The main functions of lung fibroblasts include maintenance of the extracellular matrix and tissue repair, and recent studies have shown that fibroblasts also participate in the orchestration of inflammation.

1.3.3.1 Connective tissue formation and repair

The primary role of the fibroblast is in deposition of the extracellular matrix and fibroblasts are therefore responsible for the secretion of proteins such as collagen which maintain the integrity of the alveolar compartment. Types I and III collagen are the major secretory products of lung fibroblasts and make up 65% of the extracellular matrix. Other matrix constituents such as fibronectin are also produced. In addition to collagen secretion,

fibroblasts are a major source of the matrix metalloproteinases MMP-2 (collagenase) and MMP-9 (gelatinase) enabling them to break down connective tissue (Knight 2001).

The repair mechanisms that follow lung injury are akin to normal wound healing in which tissue injury is followed by inflammation and repair by scar formation. The healing process requires migration, proliferation and apoptosis of fibroblasts. After injury, fibroblasts at the wound margin begin to proliferate and migrate into the denuded area where they lay down a collagen rich matrix. During this process many fibroblasts transform into myofibroblasts that generate strong contractile forces in order to draw the wound margins towards each other (Aoshiba et al. 1999). These normal fibroblast functions of matrix deposition and repair play a role in the structural changes that take place in the asthmatic lung and are discussed further within the context of airway remodelling.

1.3.3.2 Fibroblasts and inflammation

While fibroblasts remain largely quiescent under normal conditions, they are activated during inflammation to produce an array of inflammatory mediators. These cells release several classes of compound that can have profound effects on the surrounding environment (**table 4**). Cytokines expressed by fibroblasts include IL-1, which may activate T-cells in the bronchial wall, GM-CSF which may activate eosinophils and IL-6 which has pleiotropic effects (Roche 1991). Fibroblasts have also been shown to secrete chemotactic factors for neutrophils, eosinophils, T lymphocytes, monocytes and macrophages in response to inflammatory insults such as smoke extract, bradykinin and lipopolysaccharides, and also in response to bleomycin and methotrexate which may cause fibrosis and pneumonitis (Sato et al. 1999, Koyama et al. 2000, Koyama et al. 2000b, Takamizawa et al. 1999 and Sato et al. 2001). This chemotactic activity was shown to consist of GM-CSF, G-CSF, MCP-1, TGFβ, IL-8 and LTB_4. A lipid mediator released in large quantities by lung fibroblasts is PGE_2, which is thought to inhibit the proliferation of surrounding fibroblasts (McAnulty et al. 1997).

Of particular relevance to chronic inflammation are the interactions that take place between fibroblasts and cells of myeloid origin. Co-culture of mast cells and fibroblasts can modulate the proteoglycan content of mast cell granules (Levi-Schaffer et al. 1986), induce mast cell cytokine synthesis (Razin et al. 1991) and also increase stimulus-induced release of mast cell histamine, LTB_4, LTC_4 and PGD_2 (Levi-Schaffer et al. 1987). Co-culture of human IL-5

Table 4. Inflammatory mediators produced by pulmonary fibroblasts

Mediator	Stimulus
cyto/chemokines	
IL-1	
GM-CSF	smoke, BK, bleomycin, methotrexate
IL-6	
G-CSF	LPS, BK, bleomycin
TGFβ	BK, bleomycin
IL-8	smoke, LPS, BK
MCP-1	smoke, BK, bleomycin
eotaxin	IL-4, TNFα
RANTES	TNFα
IL-11	IL-17
GROα	IL-17
SCF	
lipid mediators	
LTB_4	smoke, BK
PGE_2	IL-1
prostacyclin	PDGF
15-HETE	IL-1
TxA_2	mellitin, A23187
TxB_2	
adhesion molecules	
ICAM-1	IL-1β, TNFα, IFNγ and IL-4
VCAM-1	IL-1β, TNFα, IFNγ and IL-4
Integrins β1 and β3	TGFβ
matrix proteins	
collagens I & III	IL-1β, TGFβ
MMP-2	IL-6
MMP-9	IL-6
fibronectin	
tenascin	

(Goerig et al.1988, Knight 2001, Sato et al. 1999, Koyama et al. 2000, Koyama et al. 2000b, Takamizawa et al. 1999, Sato et al. 2001, Roche 1991, McAnulty et al. 1997, Feinmark et al. 1982, Spoelstra et al. 2000, Teran et al. 1999, Molet et al. 2001, Zhang et al. 1996)

stimulated eosinophils with the 3T3 fibroblast cell line significantly increased the viability of the eosinophils and accelerated their conversion into the hypodense phenotype (Rothenburg et al. 1989). Incubation with the 3T3 fibroblasts also enhanced helminthic cytotoxicity (Rothenburg et al. 1989). Thus fibroblasts may regulate the number and function of infiltrating inflammatory cells. Fibroblasts themselves are also activated by haematopoietic cells through CD40-CD40 ligand interactions. Engagement of CD40 on fibroblasts leads to NFκB activation and the synthesis of IL-6, IL-8, COX-2 products and the polysaccharide hyaluronan (Smith et al. 1997).

Expression of adhesion molecules on resident cells in the lung plays an important role in leukocyte infiltration and pulmonary fibroblasts express a number of these molecules. ICAM-1 and VCAM-1 are present on airway fibroblasts and their levels are increased following exposure to IL-1β, TNFα, IFNγ and IL-4 but reduced after incubation with budesonide and formoterol (Spoelstra et al. 2000). Other surface receptors include the integrins, a large family of receptors for components of the extracellular matrix. Ligation of these receptors is essential for cell survival, differentiation and for the activation of a wide variety of genes. β1 and β3 integrins are constitutively expressed by pulmonary fibroblasts and may be upregulated by growth factors in an inflammatory environment (Knight 2001).

1.3.4.Endothelial Cells

The smooth, single-celled layer lining the inner surface of blood vessels is the endothelium. These structural cells serve as a physical barrier but also have several other biological functions, many of which are of particularly important in the control of inflammation such as haemostasis, vascular tone, permeability and leukocyte transendothelial migration.

1.3.4.1 Haemostasis

Inflamed airways show evidence of vascular damage by the presence of fibrin and red blood cells in the alveolar space, which in turn can further amplify lung injury. Fibrin is a leukocyte chemoattractant and dying red blood cells release iron which is a key intermediate in free radical-generating reactions that produce cell injury and matrix degradation (Varani 1998). Also, the endothelium by virtue of its role in limiting haemorrhage, serves to further amplify the proinflammatory consequences of vascular damage. The activation of the clotting and fibrinolytic cascades releases agents such as factor XII, tissue factor, urokinase-type plasminogen activator and plasminogen activator inhibitor-1 which can all participate in inflammatory reactions (Hekman et al. 1987).

1.3.4.2 Vascular tone

Endothelial cells secrete substances that act on adjacent vascular smooth muscle to affect its tone. Under normal circumstances, vascular tone is maintained through complex regulatory pathways involving peptide and lipid mediators that control vessel constriction and relaxation. These include the vasodilators prostacyclin, NO and the vasoconstrictors endothelin-1, PDGF, thromboxanes and PAF (Varani 1998). Endothelial cells have also been shown to produce LTB_4, LTC_4, LTD_4 and LTE_4, not alone but only when cultured with

polymorphonuclear leukocytes which provide the substrate LTA_4 (Claesson et al. 1988). Impaired vascular tone has the potential to affect leukocyte accumulation in the lung and may therefore contribute to pulmonary damage.

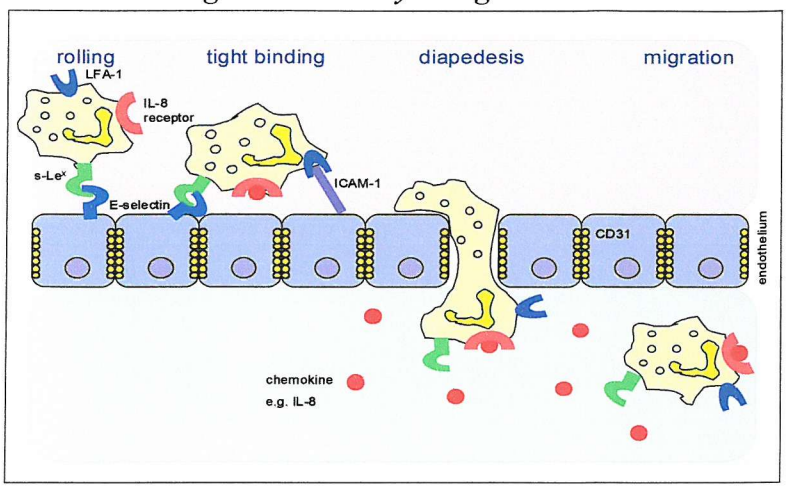
1.3.4.3 Permeability

Impairment of the endothelial permeability barrier is a well known characteristic of inflammation. The permeability barrier may be compromised by agents that interfere with cytoskeletal elements to cause endothelial cell shape change and retraction. Retraction leads to the opening of junctions that allows the efflux of soluble plasma components and oedema. Mediators that contribute to increased vascular permeability in the asthmatic airway include histamine, bradykinin, LTC_4, LTD_4, TxA_2, PAF and neurokinins A and B (Goldie et al. 1995).

1.3.4.4 Leukocyte Migration

The mobilisation of leukocytes out of blood vessels and across endothelial cells to the site of inflammation is thought to occur in four steps: rolling, tight binding, diapedesis and migration, **figure 6** (Imhof et al. 1997). Rolling is mediated by adhesive molecules called selectins. Endothelial cells express P-selectin rapidly after exposure for example to LTB_4, or E-selectin after a few hours upon exposure to bacterial LPS. Selectins interact with the carbohydrate epitopes of leukocyte surface glycoproteins and cause reversible adherence to the endothelium, "rolling". For the strong interaction of the second step there must be interactions between specific leukocyte integrins such as LFA-1 (CD11a:CD18) with endothelial molecules such as ICAM-1, and this results in firm, arrested binding. ICAM-1 has been shown to be significantly upregulated on the endothelial cells of atopic asthmatics 6 hours after endobronchial allergen challenge (Montefort et al. 1994). Crossing the endothelial wall, or diapedesis involves LFA-1 (lymphocyte function-associated antigen-1) as well as interaction with the immunoglobulin like molecule CD31, expressed on leukocytes and endothelial intercellular junctions. These interactions allow the leukocyte to squeeze between endothelial cells. Movement through the basement membrane occurs with the aid of proteolytic enzymes and the final step of movement through tissues to the site of inflammation occurs under the influence of chemoattractant molecules.

Figure 6. Leukocyte migration



1.3.5 Airway remodelling

Although asthma was traditionally regarded as an entirely reversible disorder it is now recognised that this may not be an accurate picture. Although some asthmatics do 'grow out' of their symptoms many experience an accelerated rate of respiratory function deterioration with age. Some patients develop an irreversible airway obstruction and most asthmatics have persistent airway hyperresponsiveness. To explain these observations much attention has been directed at the structural changes, or remodelling, that may occur in asthmatic airways. These changes include thickening of the airway wall, increased myocyte mass, myofibroblast hyperplasia, mucus metaplasia, angiogenesis and subepithelial fibrosis (Elias 2000).

Many of these changes are thought to be associated with epithelial damage and restitution. Under normal circumstances damaged epithelium is able to repair itself rapidly. An *in vivo* model has shown that post-injury, epithelial cells at the edge of the damaged area de-differentiate, flatten and rapidly migrate to provide a covering layer over the denuded basement membrane (Erjefält et al. 1995). Following denudation, endothelial gaps are formed, plasma exudation is initiated and a network of fibrin and other plasma proteins is formed on the basement membrane. Thus the extracellular milieu is filled with growth factors, cytokines, proteases and anti-proteases all of which can affect the tissue responses discussed below.

The response of the epithelium to proteolytic attack is to enter a repair phenotype as shown by CD44 overexpression. Damaged epithelial cells also overexpress EGF and EGF receptors

(Puddicombe et al. 2000). EGF is an important regulator of epithelial repair as it influences epithelial proliferation, differentation and survival. While EGFR upregulation is important in repairing damaged epithelium, impairment of this receptor may promote abnormal healing and remodelling, possibly through TGFβ release (Knight 2001).

1.3.5.1 Subepithelial fibrosis

Early postmortem studies described what was believed to be thickening of the asthmatic basement membrane and electron microscopy has revealed that this thickening is the result of a fibrotic response in the lamina reticularis, a phenomenon present even in very mild asthma cases (Elias 2000). This response is due to enhanced accumulation of the repair-like proteins fibronectin, tenascin and types I, III and V collagens (Roche et al. 1989). The epithelial basement membrane proteins collagen IV and laminin are absent. These findings indicate that the layer is composed of mesenchymal-derived structural proteins, pointing to the involvement of subepithelial fibroblasts, in particular myofibroblasts. Myofibroblasts have increased synthetic organelles, contractile apparatus and are associated with wound healing and fibrosing processes. Myofibroblastic cells are found beneath the bronchial epithelium of asthmatic and normal subjects but were higher in the asthmatics and correlated with the depth of the collagen layer (Brewster et al. 1990).

These structural changes are thought to arise from interactions between epithelial cells and fibroblasts. Epithelial repair is a normal process following inflammatory damage and repairing epithelial cells release a variety of factors that influence fibroblast migration, matrix production and remodelling. In epithelium-fibroblast co-culture systems, chemical or physical damage to the BEC resulted in increased collagen gene expression in the underlying fibroblasts due to the combined effects of bFGF, PDGF, IGF-1, TGFβ and endothelin-1 (Zhang et al. 1999). In a similar system, stimulation of epithelial cells with ozone (which causes lung fibrosis), caused enhanced gene expression of I and III procollagen as well as TGFβ1 in the human lung fibroblasts (Lang et al. 1998).

Fibroblast matrix deposition is regulated at several levels. Control of the synthesis/secretion and breakdown of matrix proteins as well as the rate of cell division and apoptosis can all affect this process. Multiple growth factors such as EGF and PDGF directly influence matrix deposition (Mutsaers et al. 1997). TGFβ is one of the best characterised growth-factors and

controls multiple processes in wound repair. TGFβ increases collagen, fibronectin and tenascin production by fibroblasts and epithelial cells. TGFβ can also inhibit the synthesis and activity of enzymes that degrade the extracellular matrix while increasing the activity of inhibitors of these enzymes such as TIMP-1 (tissue inhibitor of matrix metalloproteinases) (Mutsaers et al. 1997). Interestingly, LTC_4 has been shown to enhance collagenase mRNA expression in human lung fibroblasts (Medina et al. 1994) whereas LTD_4 significantly increases collagen production by human dermal fibroblasts (Abe et al. 2000).

1.3.5.2 (Myo)fibroblast hyperplasia

Several factors have been shown to affect fibroblast proliferation and apoptosis. The classical mast cell mediators histamine, heparin and tryptase are connective tissue mitogens, and cytokines capable of introducing fibroblast proliferation include IL-1, EGF, FGF, PDGF, TGFβ, TNFα, IL-5, IL-13, IL-4, IGF-1, GM-CSF (Kovacs 1991, Redington 2000, Elias 2000, Kähler et al. 1996). Cysteinyl-leukotrienes may have proliferative effects on fibroblasts. While LTD_4 alone had no effect on dermal fibroblast proliferation (Abe et al. 2000) it was able to stimulate fibroblast proliferation if the endogenous synthesis of prostaglandins was blocked (Baud et al. 1987). PGE_2 is an inhibitor of fibroblast proliferation (Kähler et al. 1996). In the normal process of wound healing, apoptosis is also important as after scar development (myo)fibroblasts are removed, their role in the repair processs being complete. Recent evidence suggests a role for lipoxygenease products in the induction of apoptosis. 12-LO overexpression and 12-HPETE stimulation of Chinese hamster ovary fibroblasts both lead to increased levels of apoptosis (Gu et al. 2001), and in cultured human lung fibroblasts, inhibitors of the 5-LO pathway are able to protect against apoptosis induced by thiol depletion (Aoshiba et al. 1999).

1.3.5.3 Increased myocyte muscle mass

In 1992, Huber and Koessler observed that the thickness of the airway smooth muscle layer of patients who had died of asthma was markedly increased. The hyperresponsiveness of asthmatic airways is thought to partly involve this increase in muscle layer thickness (Hirst 2000). Although increased airway wall thickness has little effect on airway resistance in the absence of smooth muscle shortening, in the thickened airway it amplifies the effect of normal amounts of smooth muscle shortening leading to a much greater narrowing of the airway and to airflow obstruction, thus hyperresponsiveness (Lambert et al. 1993).

The thickening of the airway smooth muscle layer may be the result of several processes including increased cell survival (increased smooth muscle mass due to decreased cell removal), increased myocyte extracellular matrix deposition or hyperplasia (increase in cell number) and/or hypertrophy (increase in cell size) (Ebina et al. 1993, Thomson et al. 1998). Most studies have concentrated on mitogenesis as a cause and indeed several factors have been shown to increase smooth muscle proliferation. Growth factors with a stimulatory effect on human smooth muscle mitogenesis include EGF, bFGF, PDGF, TNFα and TGFβ. Certain inflammatory mediators such as cys-LTs, histamine and endothelin (ET-1), and extracellular matrix proteins such as laminin and heparin also have a role in stimulating the proliferation of cultured airway smooth muscle cells. Inhibition of smooth muscle proliferation has also been observed by treatment with PGE_2, implying the existence of complex regulatory pathways for smooth muscle cell proliferation (Hirst 2000).

1.3.5.4 Vascular Changes

Changes to the vasculature such as increased vascularity, vasodilation and microvascular leakage have been described in patients who died from fatal asthma (Carrol et al. 1997). In normal healthy individuals up to 10% of a bronchial biopsy section may be occupied with blood vessels but in sections from mild asthmatic airways up to 17% is vascular (Wilson 2000). Severe, steroid-dependent asthmatics also have increased numbers of submucosal vessels, and interestingly these also show greater ICAM-1 expression (Vrugt et al. 2000). These findings imply that there is increased proliferation of vessels in asthmatic airways and accordingly endothelial cells from asthmatics show evidence of active replication (Wilson 2000). The mechanism of angiogenesis involves signals from endothelial cells that increase vascular smooth muscle proliferation. *In vitro*, factors that can induce angiogenesis include tryptase, histamine, heparin, TNFα, TGFβ, bFGF, VEGF and IL-8, many of which are elevated in asthma (Wilson 2000). Cys-LTs may also have a role in angiogenesis as studies using animal cells have shown that LTC_4 promotes a dose-dependent increase in the growth of aortic endothelial cells, and LTC_4 and LTD_4 both increase the proliferation of cultured vascular smooth muscle cells (Palmberg et al. 1987, Porreca et al. 1995, Modat et al. 1987).

1.3.5.5 Mucus metaplasia

A remodelling response associated with changes to the epithelial layer is that of mucus metaplasia. An increase in the number of mucus secreting cells, mucus hypersecretion and airway obstruction due to mucus plugging are well-documented features of chronic and fatal

asthma (Aikawa et al. 1992). An increase in the number of goblet cells may be controlled by factors that regulate epithelial proliferation such as EGF and LTD_4 (Leikauf et al. 1990). Alternatively goblet cell metaplasia may occur that involves differentiation of pre-existing epithelial cells into goblet cells. This process can occur very rapidly and has been shown to occur in response to neutrophil elastase, neutrophil lysates and cigarette smoke (Rogers et al. 1986, Lundgren et al. 1988). Goblet cells in human airways do not have direct neuronal innervation and environmental stimuli (smoke, SO_2) and inflammatory mediators (LTC_4, LTD_4, PGs, histamine, ECP, IL-6) all stimulate goblet cell secretion (Fahy 1998). Of the cytokines, IL-13 may be of particular relevance to mucus metaplasia as transgenic mice overexpressing IL-13 showed greatly enhanced mucus production (Elias 2000).

1.4 Treatment of asthma

As exposure to allergens causes a worsening of asthma in susceptible patients, allergen avoidance measures may be introduced upon identification of provoking factors. Allergen avoidance has been shown to effectively reduce asthma symptoms for patients living at high altitude and after cessation of occupational exposure (Varga 1998). However, it should be noted that measures taken to reduce allergen exposure in the home environment are not always reflected in a convincing improvement in the clinical condition of allergic asthmatics (Cloosterman et al. 1999). In addition to avoidance measures, which may not always be appropriate or feasible, there are several medications currently used to treat asthma, both as rescue medication and as prophylactic therapy.

1.4.1 β_2 agonists

β_2 adrenergic receptors (β_2-AR) are G-protein coupled receptors and agonists improve airway function largely by a direct relaxant effect on airway smooth muscle. Agonist binding causes an increase in intracellular cAMP via the Gs/adenylyl cyclase effector pathway and relaxation of airway smooth muscle. The most commonly used β_2 agonists today are salbutamol (abuterol), terbutaline and salmeterol of which salbutamol is short acting (0-4 hours) and salmeterol, due to its lipophilicity and repeated stimulation of the β_2AR is long acting (up to 24 hours) (Johnson 1993). The main route of administration of these drugs is aerosol inhalation because of the rapid onset of action and relative lack of side effects. The principal adverse effects of β_2 agonists are tremor caused by stimulation of

skeletal muscle β_2 ARs, an increased heart rate, more common with less selective β_1/β_2 agonists (e.g. fenoterol) and hypokalaemia.

A major disadvantage of these agents is downregulation of β_2 receptor expression with continued use, resulting in a decreased sensitivity to the drug. Several mechanisms are responsible for this down regulation including sequestration (internalisation of receptors away from cell surface), degradation and downregulation by alterations at transcriptional and translational levels (Barnes 1999). Several polymorphisms of the β_2 AR gene exist, and further examination has suggested that these may have modulating effects on asthma severity and response to therapy due to the differing degrees of downregulation that occur with the various genotypes (Reihsaus et al. 1993).

In addition to their actions on smooth muscle, β_2 agonists also have effects on other cell types. On post capillary venular endothelial cells they inhibit plasma exudation by preventing separation of endothelial cells (Baluk et al. 1994). The adhesion of neutrophils and eosinophils to endothelial cells is also reduced by β_2 agonists and epithelial cell ciliary beat frequency may be reduced (Bowden et al. 1994, Devalia et al. 1992). On mast cells some β_2 agonists reduce histamine release (Assem et al. 1969). There are also reports of reduced mediator release by eosinophils, neutrophils, T cells and epithelial cells, but whether these *in vitro* studies are relevant to *in vivo* β-agonist use is uncertain as relatively high concentrations are required for these effects (Barnes 1999).

1.4.2 Glucocorticosteroids

Glucocorticoid (GC) hormones of the adrenal cortex and their synthetic analogues e.g. dexamethasone, prednisolone (oral) and beclomethasone dipropionate, budesonide (topical) are the most effective class of drugs used to treat inflammation. The action of these drugs requires binding to a specific steroid receptor within the cell after diffusion across the plasma membrane. Binding to the GC receptor causes a conformational change to take place involving phosphorylation and dimerisation of the receptor which then translocates to the nucleus and binds to a specific GRE (glucocorticoid response element) in the chromatin. GRE binding then modulates gene transcription either directly by stimulating/inhibiting a gene promoter or independently of GRE, by interfering with other transcription factors, inducing transcription factor inhibitors or destabilising target gene mRNA (Adcock 2000).

Glucocorticosteroids (GCS) reduce mucosal oedema and inflammation due to modulation of eosinophils, mast cells and lymphocytes. Inhaled beclomethasone treatment of asthmatics during a six week trial caused significant increases in lung function, reduced BHR and reduced β_2 agonist use. Bronchial biopsies obtained post treatment showed significantly fewer epithelial and mucosal mast cells and eosinophils (Djukanoviç et al. 1992). These effects are likely to be due to:

1. Inhibition of the production of growth factors and cytokines involved in inflammatory cell proliferation, chemotaxis and survival (Schleimer et al. 1985)
2. The induction of cytokines that downregulate Th2 cells (Sousa et al. 1995)
3. Inhibition of the release of arachidonic acid and thus reduction of eicosanoid production via induction of lipocortin-1 transcription (Adcock et al. 1982)
4. Reduction of endothelial cell permeability (Schleimer 1998)

Interestingly, glucocorticoids also increase the transcription of the β_2 AR gene in rat and human lung and may prevent its desensitisation (Mak et al. 1995a, Mak et al. 1995b), a beneficial effect in asthma treatment.

As mentioned above, it is well known that lipocortin-1 induced by GCS inhibits PLA_2. In theory this effect should reduce the synthesis of all arachidonate-derived mediators, although experiments have found that this is not true of leukotriene production. Several studies have shown a lack of effect of GCS on LT production by humans *in vivo*. In normal subjects neither oral prednisolone nor inhaled budesonide (60mg/day and 1600µg/day respectively) taken for a week had any effect on urinary levels of LTE_4 (Sebaldt et al. 1990, Manso et al. 1992). A similar lack of effect on urinary LTE_4 was observed in asthmatic subjects treated with fluticasone propionate (1000µg/day) for two weeks (O'Shaughnessy et al. 1993). There are reports of reduced LT production by inflammatory cells *ex vivo* following steroid therapy, but the results vary depending on cell type. For example, alveolar macrophages obtained from GCS-treated subjects showed a reduced capacity for LTB_4 synthesis, although steroid treatment had no effect on leukotriene production in blood leukocytes (De Caterina et al. 1993). As will be discussed further later, GCS therapy has surprising effects on the expression of leukotriene pathway enzymes. Treatment with dexamethasone has been found to increase 5-LO and/or FLAP expression in human blood monocytes, neutrophils and eosinophils (Cowburn et al. 1999, Pouliot et al. 1994, Riddick et

al. 1997). The overall lack of effect of glucocorticoids on leukotriene production provides support for the combined use of GCS and anti-leukotrienes in asthma therapy.

The main disadvantage of corticosteroid therapy is the side effects although these are mainly a problem with orally administered drugs and not with inhaled treatments. These include suppression of growth in children, CNS effects such as euphoria or depression, deposition of fat in the face and abdomen, thinning of the skin and impaired wound healing (Schleimer 1998). Although inhaled steroids show fewer systemic effects, high doses of inhaled beclomethasone taken for a long period of time may cause osteoporosis in adults (>2000 μg/day) and growth suppression in children (400μg/day) (Woodcock 1998, MacKenzie 1998). Such effects can be largely eliminated by the use of inhaled fluticasone propionate, which undergoes complete first-pass metabolism in the liver after absorption of the orally-ingested fraction (Barnes et al. 1998). Local effects such as oral thrush can also be a problem with inhaled corticosteroids although this can be reduced by rinsing the mouth with water after inhalation, and by the use of spacer devices. A rare subset of asthmatics are steroid resistant due to incomplete absorption of the drug, increased GC metabolism or a decreased ability of the glucocorticoid receptor to bind to GRE (Adcock 2000).

1.4.3 Phosphodiesterase (PDE) inhibitors

Cyclic AMP (cAMP) is generated from ATP by adenylate cyclases that are activated by G-protein-coupled receptors. cAMP counteracts a variety of inflammatory cell functions involved in the development of asthma such as bronchoconstriction, oedema and smooth muscle proliferation. Cyclic nucleotide hydrolysing phodphodiesterases are a family of enzymes that break down cAMP and cGMP, so it is postulated that PDE inhibitors have anti-asthmatic effects by increasing levels of cAMP (Tenor and Schudt 1999).

There are several PDE families, each with further subtypes, and each has different substrate, activator or inhibitor sensitivities. Theophylline, a non-selective PDE inhibitor, is an orally administered methylated xanthine drug, related to the naturally occurring xanthine caffeine, that has been used to treat asthma for over 50 years due to its bronchodilator and anti-inflammatory effects. Members of the theophylline family include aminophylline, a soluble form of theophylline and choline theophyllinate, which is longer acting. While theophylline inhibits several pathogenic effects such as cytokine synthesis and release, inflammatory cell activation, microvascular leakage and bronchoconstriction (Barnes et al. 1994), there has

been a decline in theophylline use due to its adverse effects. At therapeutic serum concentrations of 10-20mg/L side effects include nausea, vomiting, headaches and insomnia but concentrations above 20mg/L are potentially fatal causing cardiac arrhythmias and seizures (Markham & Faulds 1998).

It has been shown that there is cell specific expression of the different PDE families. As PDE3 and PDE4 are found in most inflammatory cells, as well as in airway and vascular smooth muscle, it was suggested that specific inhibitors of these enzymes might provide benefit as efficient anti-asthma drugs. However, the first PDE4 inhibitor, rolipram failed due to its presence in neuronal cells which led to CNS side effects, nausea and vomiting at therapeutic plasma concentrations. The new concept is that PDEs are present in diverse functional conformations, for example, in association with other proteins or membranes. Two conformational states of PDE4 have been distinguished by their different affinities for rolipram. An enzyme with high affinity for rolipram predominates in neuronal cells and one with low affinity in inflammatory cells. Compound SB207499 (Ariflo®) associates more with the low affinity PDE4 conformation and is undergoing clinical investigation as an anti-asthmatic drug. Also, there are two different types of PDE3 encoded by different genes. PDE3A is found in the myocardium and vasculature whereas PDE3B is found in lymphocytes. It has been shown that PDE3 inhibition may amplify the effects of PDE4 inhibitors so a dual selective PDE3B and PDE4 (rolipram low affinity) may be an attractive concept for the future development of PDE inhibitors (Tenor and Schudt, 1999).

1.4.4 Cromones

The cromone family comprises sodium cromoglycate (cromolyn) and nedocromil sodium, two structurally unrelated compounds with similar actions, used to treat inflammation in the airways. The mechanism of action of these compounds was thought to be by 'stabilising' cell membranes and preventing degranulation and mediator release, but this has not been shown at therapeutic concentrations. It has been suggested that they block a specific chloride channel and therefore prevent the hyperpolarisation necessary to maintain a sustained calcium influx, essential for degranulation (Norris et al. 1998). Cromolyn sodium and nedocromil sodium reduce mediator release from mast cells, macrophages, eosinophils (Eady 1986). Taken by inhalation these drugs are safe and well tolerated but they do not significantly reduce airway inflammation compared to placebo in adults (Devalia et al.

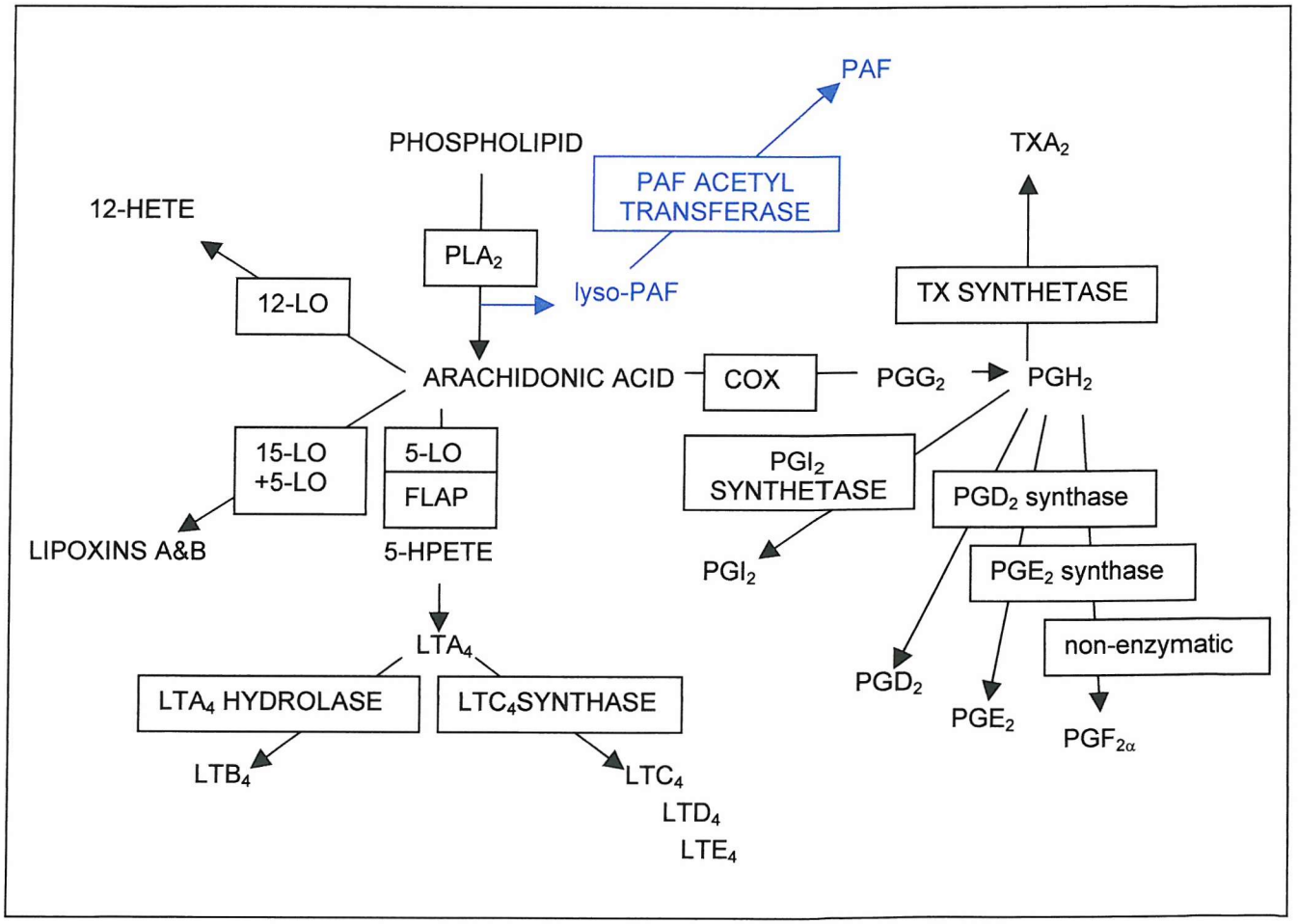
1996). In children however, nedocromil sodium does show a significant prophylactic effect against asthma (Armenio et al. 1993), possibly due to a mild anti-eosinophilic effect.
The newest anti-asthma drugs, the leukotriene modifiers, are discussed in the next section.

1.5 Lipid Mediators

1.5.1 Arachidonate

Eicosanoids are lipid mediators derived from oxidative metabolism of arachidonic acid, a major structural component of membrane phospholipids, and are generated *de novo* in response to various stimuli. The term eicosanoid originates from the greek *eicosa*, indicating 20 carbon atoms and *enoic* meaning an acid with double bonds. Arachidonic acid is a 20 carbon unsaturated fatty acid with four double bonds, usually found esterified in phospholipids at the sn-2 position. Along with LTs, the eicosanoids include prostaglandins, thromboxanes, lipoxins and several products of the 12-LO and 15-LO pathways, **figure 7**.

Figure 7. Eicosanoid pathways



1.5.2 Prostaglandins and Thromboxanes

Prostaglandins are synthesised from arachidonic acid by cyclooxygenase (COX) isoenzymes. COX is bound to the endoplasmic reticulum and catalyses the formation of the cyclic endoperoxides PGG_2 and PGH_2. There are two isoforms of COX. COX-1 is thought to be constitutive while COX-2 is encoded by an immediate early gene and is induced by agents such as cytokines, growth factors and other allergic mediators (Goetzl et al. 1995). COX-1 is associated with the production of prostanoids with housekeeping functions, including cytoprotective actions in the gastric mucosa, haemostasis and the regulation of renal blood flow. COX–2 products are associated with inflammation, pain and pyrexia. Depending on the specific downstream enzymes present, different products of the prostanoid pathway are produced by different cell types. Specific synthase/synthetase enzymes exist for the generation of TXA_2, PGI_2, PGD_2 and PGE_2. For example, in platelets the COX pathway leads mainly to the synthesis of TXA_2, which acts at TP receptors to cause vasoconstriction, platelet aggregation and in guinea pigs, bronchoconstriction. Prostacyclin, PGI_2 is produced by vascular endothelial cells and is involved in the suppression of platelet adherence and vasodilation by acting at IP receptors. PGD_2, PGE_2 and $PGF_{2\alpha}$ are produced by leukocytes as well as structural cell types and are involved in several processes, including inflammation, by their actions on DP, EP and FP receptors respectively. Actions of relevance to asthma include increased vascular permeability and bronchodilation by PGE_2, bronchoconstriction (at TP receptors) and vasodilation by PGD_2 and bronchoconstriction and microvessel constriction by $PGF_{2\alpha}$ (An et al. 1998). The role of PGD_2 in asthma has also been examined by the generation of mice deficient in the DP receptor. Following ovalbumin sensitisation and subsequent challenge, these animals had greatly reduced levels of Th2-type cytokines, less lymphocyte and eosinophil accumulation in the airways and they failed to develop airway hyperreactivity (Matsuoka et al. 2000). These findings indicate an important role for PGD_2 in the initiation of asthmatic responses.

1.5.3 Platelet Activating Factor (PAF)

Another compound, not an eicosanoid but often described in the context of lipid mediators is platelet activating factor (PAF), so named after its discovery as a proaggregatory mediator of anaphylaxis in the rabbit. PAF is a modified phospholipid mediator of hypersensitivity and inflammation that shares with eicosanoids the derivation of its precursor from cellular membranes by a phospholipase activation dependent mechanism. PAF causes some aggregation of platelets in humans but also has effects on other cell types including

vasodilation, leukocyte chemotaxis and smooth muscle bronchoconstriction (Braquet et al. 1987). Interestingly, some of the actions of PAF such as bronchoconstriction appear to be leukotriene dependent as they are inhibited by cys-LT receptor antagonists and are accompanied by increases in urinary LTE_4. However, in allergen challenge models, PAF antagonists were extremely disappointing (Spina et al. 1989).

1.5.4 Lipoxygenase pathways (other than 5-LO)

There are three major lipoxygenase enzymes in mammalial cells, 5-LO, 12-LO and 15-LO, the number indicating the C atom of arachidonic acid at which one oxygen molecule is introduced. Unstable hydroperoxyeicosatetraenoic acid (HPETE) products are formed by these enzymes and converted into the more stable 5, 12 and 15-HETEs that have been shown to possess biological activity. The 12-LO pathway is found mainly in platelets and leukocytes and leads to 12-HETE formation (Sun et al. 1980). 12-HETE induces neutrophil degranulation and augments the IgE-mediated release of mast cell products (Holtzman 1992). It also has chemotactic properties, enhances tumour cell adhesion to the subendothelial matrix (Honn et al. 1989) and may stimulate migration of aortic smooth muscle cells (Nakao et al. 1984). The biological activities of 15-LO are of particular interest as they represent a major pathway for arachidonic acid metabolism in human lung tissue and levels of 15-HETE are increased in lung tissue from asthmatic subjects (Hamberg et al. 1980). 15-LO products are the most abundant archidonate products of airway epithelial cells and eosinophils (Holtzman 1992). 15-HETE stimulates LTC_4 production from mastocytoma cells, mucus production from cultured airways and under certain circumstances may be mitogenic (Goetzl et al. 1983, Marom et al. 1983 and Holzman et al. 1992). However, both 12-HETE and 15-HETE effects occur predominantly at micromolar rather than nanomolar concentrations, which gives the impression that they have little biological activity. Complex interactions between the 5-, 12- and 15-lipoxygenase enzymes and their products may give rise to the production of another class of mediator, the liopxins.

1.5.5 Lipoxins

Lipoxins, an acronym for lipoxygenase interaction products, are trihydroxytetraenes typically formed by transcellular metabolism initiated by the sequential actions of 15- and 5- or 5- and 12-LOs on arachidonic acid. LXA_4 and LXB_4 are the principal species formed in mammals. Under certain conditions, aspirin-induced acetylation of COX-2 inhibits prostanoid production and promotes the formation of 15-HETE which is then converted by

5-LO to either 15-epi-LXA_4 or 15-epi-LXB_4. These aspirin triggered lipoxins may produce beneficial effects as they can down-regulate the action of certain granulocytes (McMahon et al. 2001). Many of the proinflammatory actions of leukotrienes are inhibited by lipoxins such as leukocyte chemotaxis, adhesion and cytokine production. Their actions are not restricted to leukocytes. In fibroblasts they inhibit MMP synthesis and stimulate TIMP expression (Sodin-Semrl et al. 2000). A high affinity G-protein coupled LXA_4 receptor has been described in several myeloid and non-myeloid cells including neutrophils, eosinophils, endothelial, epithelial and fibroblast cells (McMahon et al. 2001). Interestingly, it has recently been shown that aspirin- triggered lipoxins may bind to the $CysLT_1$ receptor which could explain their modulation of cys-LT mediated inflammatory processes (Gronert et al. 2001).

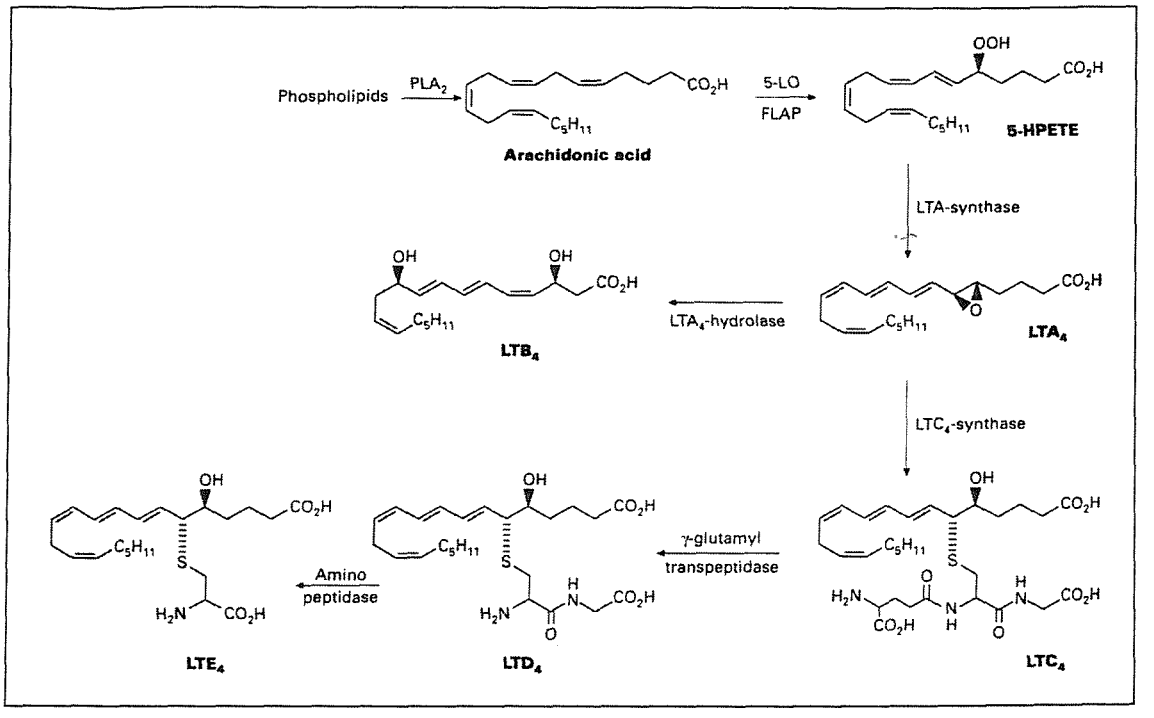
1.5.6 Leukotriene synthesis

There are two main classes of compound that make up the leukotriene (LT) family distinguished on the basis of their structures. Leukotrienes C_4, D_4 and E_4 each incorporate a cysteine residue and are thus termed the cysteinyl LTs (cys-LTs) whereas LTB_4 is a dihydroxy acid (**figure 8**). The first step in LT synthesis is the liberation of arachidonic acid from cell membranes by phospholipase A_2. This arachidonic acid is presented to 5-LO by FLAP and then converted into the unstable intermediate 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and further converted into the epoxide, LTA_4 by 5-LO. LTA_4 is then either hydrolysed to form LTB_4 by LTA_4 hydrolase or processed by LTC_4 synthase, a glutathione S-transferase, to form LTC_4. The LTC_4 molecule is actively transported out of cells by the multidrug resistance associated protein (MRP-1) and then cleaved extracellularly by liver-derived γ-glutamyl transpeptidase to LTD_4, which is further cleaved by a leukocyte derived dipeptidase to LTE_4. LTC_4, LTD_4 and LTE_4 are known as the cysteinyl leukotrienes due to the presence of a cysteinyl group and these molecules have different biological effects to LTB_4. The effects of the cysLTs are mediated via the cysLT receptors (section 1.5.10) and LTB_4 via binding to the BLT receptor (section 1.5.11).

1.5.6.1 Phospholipase A_2

Phospholipases are enzymes that release free fatty acids from cellular phospholipids by hydrolysis. The PLA_2 family is a large and diverse group of proteins that cleave phospholipids at the sn-2 position, and can be divided into two main subfamilies. Firstly, there are extracellular PLA_2s known as type II or secretory PLA_2s ($sPLA_2$). These enzymes

Figure 8. Structures of the leukotrienes



From 'SRS-A to LEUKOTRIENES, The Dawning of a New Treatment'. Edited by S-E Dahlén & ST Holgate.

are released from cells, have a relatively low molecular mass of 14 kDa, hydrolyse a variety of phospholipids and require high Ca^{2+} concentrations (mM) for catalytic activity (Dennis 1994). Several different types of $sPLA_2$ have been categorised and there are currently five distinct $sPLA_2$ groups, referred to as groups IB, IIA, IIC, V and X (Lambeau et al. 1999). One of the most studied is the group IB digestive $sPLA_2$ released by the pancreas (Verheij et al. 1981). The group IIA enzyme is also called inflammatory-type $sPLA_2$ as it is found in high concentrations in inflamed tissues. The most potent inducers of group IIA $sPLA_2$ expression include pro-inflammatory cytokines such as IL-1α, IL-1β and TNFα, and endotoxins such as lipopolysaccharide. On the other hand, glucocorticoids, anti-inflammatory cytokines and certain growth factors are potent suppressors of the induction of group IIA $sPLA_2$ enzymes (Lambeau et al. 1999). Intriguingly, reports indicate that in addition to their catalytic activity, secreted PLA_2s also have receptor-mediated effects. At present, two main types of $sPLA_2$ receptors have been identified in mammals although the physiological properties of these receptors are not yet clearly understood (Lambeau et al. 1999). It has been suggested that activation of $sPLA_2$ receptors is involved in the stimulation of cell proliferation, smooth muscle contraction, chemokinesis and fertilisation (Kishino et al. 1992, Sommers et al. 1992, Kanemasa et al. 1992, Fry et al. 1992). While there is a role for $sPLA_2$ enzymes in eicosanoid synthesis (Fonteh et al. 1994), a second phospholipase

subfamily, the cytosolic PLA_2 ($cPLA_2$) is the one thought to be important in leukotriene synthesis for the reasons outlined below.

Cytosolic PLA_2 is located within cells and has several defining characteristics. Purification of the enzyme from various cells has revealed a relatively high molecular mass of $\approx$ 85 KDa (Kramer et al. 1991, Gronich et al. 1990). $cPLA_2$ also shows a strong preference for arachidonate-containing substrates (Clark et al. 1991) and responds to increases in intracellular Ca^{2+} concentrations with translocation from the cytosolic to the particulate fraction and greatly enhanced activity (Channon et al. 1990). In support of a key role in the leukotriene pathway, $cPLA_2$ has been shown to co-localise with 5-LO and FLAP at the nuclear membrane upon stimulation in both neutrophils and macrophages (Peters-Golden et al. 1993, Pouliot et al. 1996).

The 749 amino acid $cPLA_2$ shows no sequence homology with the known forms of $sPLA_2$. As well as the PKC-like domain responsible for the Ca^{2+} dependent binding of $cPLA_2$ to membranes or phospholipid substrate, $cPLA_2$ also contains numerous phosphorylation sites for both serine/threonine and tyrosine protein kinases (Sharp et al. 1991). The characteristics of $cPLA_2$ are ones that would be expected of an intracellular enzyme involved in *de novo* mediator generation as numerous stimuli such as growth factors, mitogens, vasoactive peptides, crosslinking of Fc receptors, cytokines and interferons have all been shown to mobilise arachidonic acid from phospholipids by $cPLA_2$ activation.

1.5.6.2 5-lipoxygenase

5-lipoxygenase is a member of a family of lipoxygenases that includes 12 and 15-lipoxygenase. It catalyses the first two steps in the biosynthesis of leukotrienes, firstly oxygenation of arachidonic acid to produce 5-HPETE and secondly the dehydration of 5-HPETE to produce the unstable epoxide LTA_4 (Samuelsson et al. 1991). As well as arachidonic acid, 5-LO is also able to oxygenate other substrates, for example, in the presence of excess 15-HETE 5-LO can form 5,12-diHETE (Petrich et al. 1996). 5-LO also has a role in lipoxin formation by converting 15-HPETE or 15-HETE into LXA_4 and LXB_4.

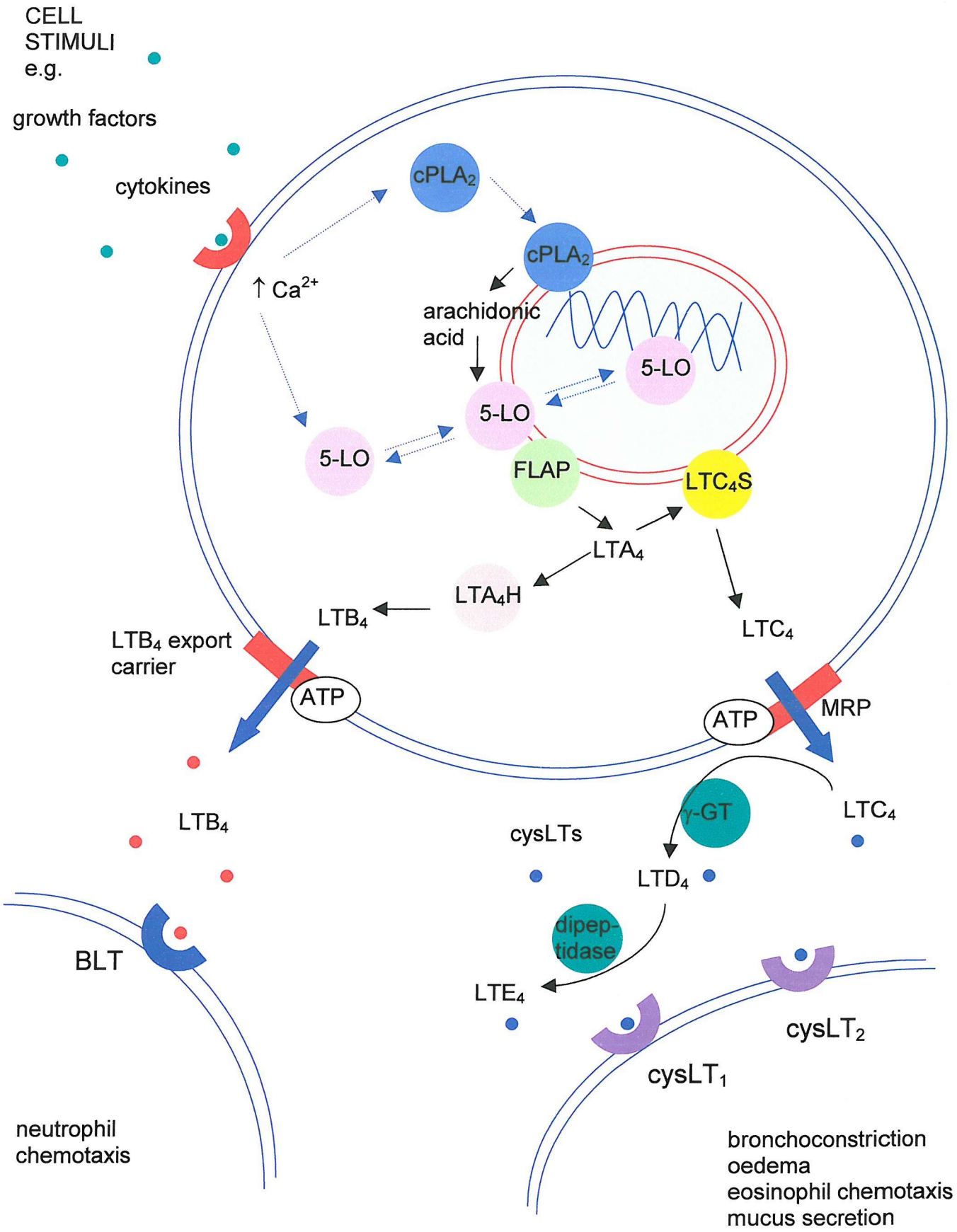
Human 5-LO has been purified, characterised and cloned and is shown to be a 78kDa, 673 amino acid protein thought to be expressed mainly in cells of myeloid origin (Matsumoto et al. 1988, Funk et al. 1988). The 5-LO enzyme requires several components for activity.

Unlike other lipoxygenases it is very strongly activated by Ca^{2+} at approximately 4-10μM (Yamamoto 1992, Rådmark 2000). The 5-LO amino acid sequence does not contain an obvious Ca^{2+} binding domain although it does show some weak sequence homology with a group of calcium dependent membrane binding proteins such as calmodulin and lipocortin (Dixon et al. 1988). The mechanism by which Ca^{2+} activates 5-LO is likely by causing the enzyme to attach itself to phospholipid membranes. Under basal conditions 5-LO is found in soluble form in the nucleus and cytosol but when the cell is activated or exposed to Ca^{2+} the enzyme translocates to the perinuclear membrane and becomes active (Peters-Golden et al. 1993) (**figure 9**). While this is true of many cell types it has been shown that in certain cells, particularly those involved in airway inflammation such as alveolar macrophages and infiltrating granulocytes, there is a soluble nuclear pool of 5-LO. In human blood eosinophils it is thought that this nuclear pool of 5-LO represents a novel mode of enzyme regulation as there is a decrease in LTC_4 generation associated with the adherence dependent nuclear import of 5-LO (Brock et al. 1999). However, alveolar macrophages that have intranuclear 5-LO, produce more LTB_4 than their progenitors, blood monocytes which have cytosolic 5-LO (Bigby et al. 1987).

Recently 5-LO was shown to interact with the cytoskeletal protein coactosin-like protein (CLP), a possible anchor that may retain 5-LO in the cytosol of resting cells and/or silence its activity by steric hindrance (Provost et al. 1999) but as yet the mechanisms of translocation remain speculative. A proposed mechanism involves the Src homology 3 (SH3) binding motif found on 5-LO. This allows 5-LO to bind to the SH3 domain of growth factor bound receptor protein 2 (Grb2). Inhibition of Grb2 binding interfered with the translocation of 5-LO from cytosol to nucleus in neutrophils thus suggesting a role for SH3 in nuclear import (Lepley et al. 1994).

The action of 5-LO is ATP and iron dependent (Yamamoto 1992). 5-LO activation by ATP is dependent on the presence of Ca^{2+} and typical concentrations required for maximal stimulation are 1mM (Ford-Hutchinson et al. 1994). Active 5-LO contains 1 mol iron/mol protein (Percival 1991). This iron at the active site of the enzyme is necessary for the two redox cycles that result in LTA_4 formation. Another important aspect of 5-LO regulation is its oxidative inactivation by its own catalytic end product LTA_4, a process known as suicide inactivation (Lepley et al. 1994). With prolonged stimulation (15 min, Ca ionophore) this inactivation is irreversible so to sustain 5-LO output there must be *de novo* production of the

Figure 9. Cellular location of the LT pathway



enzyme. However, with transient stimulation (0.5-5 min) 5-LO retains enzymatic activity (Brock et al. 1998).

The promoter region of the 5-LO gene is lacking the typical TATA and CCAAT boxes expected of inducible genes and instead it exhibits features common to the promoter regions of housekeeping genes (Funk et al. 1989). Housekeeping genes usually display wide tissue distribution, perform essential metabolic functions and show little regulation. However, there must be mechanisms for restriction of 5-LO expression as it is expressed mainly in cells of myeloid lineage and not many other cell types. Also differentiation of several leukocyte cell lines using DMSO can cause an increase in 5-LO mRNA levels (Jakobsson et al. 1992). The 5-LO gene may be regulated by cis-acting nucleotide sequences as it contains binding sites for many known transcription factors including Sp1, Sp3, Egr-1, Egr-2, NFκB, GATA, Myb and AP family members (Silverman et al. 1998). These have both positive and negative regulatory influences on transcription (Hoshiko et al. 1990).

Several lines of evidence suggest transcriptional regulation of the 5-LO gene. Models used to show myeloid cell maturation often involve differentiation of leukaemic cell lines such as HL-60. In several leukocyte cell lines, increased 5-LO mRNA levels are observed following differentiation and activation in response to DMSO, PMA, TGFβ, GM-CSF, IL-3, low density lipoprotein (LDL), Ca ionophore and vitamin D3 (Stankova et al. 1995, Steinhilber et al. 1993a, Steinhilber et al. 1993b, Jakobsson et al. 1992, Piechele et al. 1993). Upregulation of 5-LO expression has been observed in the absence of differentiation caused by GM-CSF stimulation in PMNLs. After a short exposure there is a 3-fold increase in 5-LO caused by post-transcriptional mechanisms but after longer exposure mRNA levels increase as well (Pouliot et al. 1994, Stankova et al. 1995). Surprisingly, it has been shown that stimulation with dexamethasone is able to upregulate 5-LO mRNA and protein in human mast cells and peripheral blood monocytes (Colamorea et al. 1999, Riddick et al. 1997). Athough no obvious glucocorticoid response element has been identified in the 5-LO promoter, this effect may be modulated by GCS via indirect effects on the binding of transcription factors such as NFκB, and other cell signalling processes including post-transcriptional events. The physiological relevance of this dexamethasone-induced upregulation remains unknown.

1.6.6.3 5-lipoxygenase activating protein (FLAP)

An essential co-factor for the first step in leukotriene biosynthesis is FLAP, the 5-lipoxygenase activating protein. It was first discovered when the mechanism of action of the leukotriene synthesis inhibitor MK-886 was studied. This drug not only blocked leukotriene production *in vivo* and *in vitro* (Gillard et al. 1989) but also prevented the translocation of 5-lipoxygenase to the nucleus upon stimulation (Rouzer et al. 1990). As MK-886 had no effect on purified 5-LO or any of the other LT pathway enzymes (Gillard et al. 1989), the search for a target protein began. An 18kDa protein was detected in leukocyte membranes using a photoaffinity probe and purified using MK-886 affinity columns (Miller et al. 1990).

There is considerable evidence to show that FLAP is essential for leukotriene synthesis. When human osteosarcoma cells were transfected with either 5-LO or FLAP alone and stimulated with Ca ionophore, no arachidonic acid metabolites could be detected but if both proteins were transfected into these cells then stimulation resulted in leukotriene synthesis, which could be inhibited by MK-886 (Dixon et al. 1990). There is also a strong correlation between the affinity of compounds for FLAP and their ability to inhibit LT synthesis (Charleson et al. 1992).

The initial mechanism by which FLAP was thought to facilitate leukotriene synthesis was by acting as a docking protein to hold 5-LO at the nuclear membrane where it could then interact with arachidonic acid (Rouzer et al. 1990). However, in certain cell types such as rat alveolar macrophages, 5-LO may be associated with membrane fractions even at rest and in this case MK-886 is unable to affect translocation, although it can still inhibit leukotriene synthesis (Coffey et al. 1992). Therefore the current hypothesis is that FLAP presents free arachidonic acid to 5-LO in order to stimulate efficient utilisation (Abramovitz et al. 1993, Mancini et al. 1993).

Cloning of the human FLAP gene has revealed a gene spanning 31 kilobases and consisting of five small exons and four large introns localised to human chromosome 13. Examination of the promoter region shows a possible TATA box and potential AP-2 and glucocorticoid receptor binding sites suggesting that FLAP is a more inducible and regulated gene than 5-LO (Kennedy et al. 1991). The proposed model for the structure of FLAP consists of three transmembrane domains, connected by two hydrophilic loops, with the N and C termini on opposite sides of the membrane (Miller et al. 1990) and with no consensus sites for

phosphorylation or glycosylation (Dixon et al. 1990). FLAP is thought to be expressed largely in cells of myeloid origin and its subcellular localisation appears to be at the nuclear envelope and endoplasmic reticulum in all cell types studied, both in stimulated and unstimulated cells (Pouliot et al. 1996, Woods et al. 1993).

Several studies support the finding that FLAP appears to be highly regulated. Glucocorticoid receptor binding sites can function as both negative and positive regulators of transcription and their presence in the FLAP promoter suggests regulation by anti-inflammatory steroids. Surprisingly, experiments show a paradoxical increase in FLAP mRNA and/or protein levels after incubation with dexamethasone in differentiated THP-1 cells, a monocyte cell line, as well as in human blood monocytes, eosinophils and neutrophils (Goppelt-Struebe et al. 1997, Pouliot et al. 1994, Cowburn et al. 1999, Riddick et al. 1997). Induction of cell differentiation can also regulate FLAP expression. Vitamin D3 causes maturation of human blood monocytes into macrophages and has a strong effect on increasing FLAP expression (Coffey et al. 1993). DMSO induced differentiation of HL-60, a promyelocytic cell line, towards granulocytes also induces both 5-LO and FLAP expression (Bennett et al. 1993, Reid et al. 1990).

Certain cytokines have been shown to be responsible for an upregulation of FLAP expression. For example, GM-CSF, a cytokine that aids the proliferation and differentiation of myeloid progenitors, can increase levels of both FLAP mRNA and protein in human neutrophils (Pouliot et al. 1994). IL-5, a cytokine that selectively activates eosinophils and prolongs their survival can increase the proportion of normal blood eosinophils immunostaining for FLAP as well as increasing FLAP protein levels, translocating 5-LO to the nucleus and priming the cells for enhanced A23187 stimulated LTC_4 synthesis (Cowburn et al. 1999).

1.5.6.4 Leukotriene A_4 hydrolase

Leukotriene A_4 hydrolase is the enzyme responsible for the stereoselective hydrolysis of LTA_4 resulting in LTB_4 formation. It is a bifunctional enzyme, with the ability to hydrolyse epoxides as well as having aminopeptidase activity. LTA_4 hydrolase is active as a monomer and requires no cofactors, although it incorporates a zinc ion that is essential for catalytic activity (Medina et al. 1991). Site directed mutagenesis has determined the three zinc binding ligands as His-295, His-299 and Glu-318, a feature shared with other zinc-

metalloproteinases (Toh et al. 1990, Medina et al. 1991). Mutations at Glu-297 have been able to distinguish the two enzyme activities as certain amino acid substitutions inhibited peptidase activity and others the hydrolase activity (Medina et al. 1992).

Chloride ions have been shown to stimulate LTA_4 hydrolase, but only with respect to its peptidase activity (Wetterholm et al. 1992). This is a factor that may restrict the proteolytic function of the enzyme to the extracellular space where the Cl^- concentration is higher. Another characteristic of the LTA_4 hydrolase enzyme is that it shows a gradual inactivation caused by its substrate known as suicide inactivation. This was first observed when LTB_4 production stopped within 30 seconds of adding LTA_4 to a batch of human leukocyte LTA_4 hydrolase (McGee et al. 1985, Evans et al. 1985). However, this effect could also partly be put down to the short half-life of LTA_4 in solution. The mechanism of suicide inactivation has been investigated and showed that LTA_4 by virtue of its chemical reactivity attacks the Tyr 378 residue at the active site of the enzyme.

Cloning of the human LTA_4 hydrolase gene from a placental λ phage genomic library has revealed a relatively large gene (>35 kb) containing 19 exons and localised to chromosome 12q22 (Mancini et al. 1995). The enzyme cloned from lung cDNA libraries has a calculated molecular mass of 69kDa (Funk et al. 1987), but there may be different isoforms of the enzyme as the erythrocyte derived enzyme is smaller (54 kDa) (Rådmark et al. 1984). In addition, the human airway epithelial cell LTA_4 hydrolase activity does not show substrate inactivation like other LTA_4 hydrolases (Bigby et al. 1989).

Sequencing of the 5' flanking region of the gene has shown several potential transcription factor sequences including AP-2 and two xenobiotic-response elements, but no TATA box (Mancini 1995). AP-2 is a phorbol ester response element and PMA is able to double LTB_4 production in human neutrophils stimulated by fMLP (McColl et al. 1987). Recently LTA_4 hydrolase expression was shown to be increased by stimulation of human polymorphonuclear leukocytes with IL-4 and IL-13 (Zaitsu et al. 2000).

Unlike 5-LO and FLAP there is not an obvious association of LTA_4 hydrolase with cell membrane fractions either at baseline or after stimulation with ionophore in human leukocytes (Rådmark et al. 1990) and it is generally assumed that the enzyme resides in the cytosol. However, it has been reported that in liver cells there is an association of LTA_4

hydrolase with cell membranes (Gut et al. 1987). LTA_4 hydrolase has been detected in virtually all tissues so far examined, from several different species including humans, and in a wide range of cultured cells. It is particularly abundant in intestine, spleen, lung and kidney, whereas in the blood, neutrophils, monocytes, lymphocytes and erythrocytes have large amounts while eosinophils, basophils and platelets are virtually devoid (Haeggstrom 2000). Surprisingly, many of the cell types expressing LTA_4 hydrolase do not express 5-LO. As the other functions of LTA_4 hydrolase remain unknown, transcellular biosynthesis of LTB_4 (the substrate LTA_4 being provided by another cell type) may be physiologically important. Transcellular biosynthesis has been demonstrated between neutrophils and endothelial cells or airway epithelium (Brady et al. 1992). An effect of the widespread distribution of LTA_4 hydrolase could be to increase LTB_4 production at sites of inflammation following recruitment of leukocytes to the area, leading to further leukocyte migration (Rådmark et al. 1990).

1.5.6.5 Leukotriene C_4 synthase

Leukotriene C_4 synthase is responsible for the formation of leukotriene C_4, the parent compound of the cysteinyl leukotrienes. It is a member of the glutathione-S-transferase family of enzymes but rather than being involved in the detoxification of cells like the other members, it appears to exclusively conjugate leukotriene A_4 with reduced glutathione (Yoshimoto et al. 1985). The mechanism of LTC_4S action has been shown by site directed mutagenesis. One LTC_4S residue (Arg-51) opens the epoxide ring of LTA_4 and another (Tyr-93) provides the thiolate anion of reduced glutathione (Lam 1997).

Experiments using purified LTC_4 synthase revealed that enzyme activity is dependent on divalent cations (Mg^{2+}>Ca^{2+}) and phospholipids (Nicholson et al. 1992). The enzyme exhibits inactivation by its substrate glutathione at concentrations above 5mM, but at lower concentrations this reduced glutathione stabilises the enzyme (Nicholson et al. 1993). As determined by SDS-PAGE, LTC_4 synthase is an 18kDa protein that is active as a homodimer. Dimerisation provides the correct protein folding to enable catalytic function (Lam 1997).

Surprisingly, cloning of LTC_4 synthase revealed that the 150 amino acid protein shows no sequence homology with other GSH transferases but is most similar to FLAP (Welsch et al. 1994), and is even inhibited by the FLAP inhibitor MK-886 at very high concentrations

(Lam et al. 1994). The sequence has two potential phosphorylation sites as well as a glycosylation site (Welsch et al. 1994). In accordance with the presence of a phosphorylation site on the enzyme, it has been shown that PMA can suppress LTC_4 synthase activity via protein kinase C in both human granulocytes and in an eosinophilic substrain of HL-60 cells (Ali et al. 1994, Sjölinder et al. 1995). This action was prevented by the specific PKC inhibitor staurosporine and is thought to represent a way in which PKC activation can shift the production of eicosanoids from cysLTs to prostanoids.

Genomic cloning of the leukotriene C_4 synthase gene has shown that like FLAP it has five exons and four introns, although the LTC_4 synthase gene is considerably smaller (Penrose et al. 1996). The 5' flanking region of the gene has sequences consistent with SP-1, AP-1 and AP-2 binding sites. The presence of these sites is consistent with the finding that LTC_4S activity is induced in human erythroleukaemia cells after treatment with PMA (Söderström et al. 1992). The regulation of LTC_4S expression in eosinophils thus appears to be a maturation related phenomenon. During IL-3 and IL-5-induced eosinophil development from human umbilical cord progenitor cells, there is no expression at 7 days in the mixture of early granulocytes but at 14 days after development into eosinophils and basophils, LTC_4S is present (Boyce et al. 1996), and this is subsequent to the sequential induction of PLA_2, 5-LO and FLAP.

Chromosomal localisation has mapped the LTC_4 synthase gene to chromosome 5q35, a region close to a cluster of genes for cytokines and receptors thought to be of central importance to asthma, as mentioned above (Austen et al. 1997). Another characteristic of the LTC_4 synthase gene that is of particular relevance to asthma, is the presence of a polymorphism in its promoter region at nucleotide -444 (Sanak et al. 1997). There is a profound overexpression of LTC_4 synthase in the bronchial biopsies of aspirin sensitive asthmatics compared to controls and aspirin tolerant asthmatics (Sampson et al. 1997), and aspirin sensitive patients also have a higher frequency of occurrence of the less common C_{-444} allele (Sanak et al. 1997). Thus it appears that this polymorphism may be directed towards regulation of LTC_4 synthase expression and could predispose to aspirin induced asthma. However, unlike this Polish study, a study in the United States did not find any significant differences in the occurrence of C_{-444} between groups of normals, asthmatics and aspirin sensitive asthmatics (Van Sambeek et al. 2000).

Like FLAP, LTC_4 synthase is an integral membrane protein as suggested by the presence of mostly hydrophobic residues in its amino acid sequence and it also appears to be largely perinuclear as shown by immunostaining of alveolar macrophages (Nicholson et al. 1993, Penrose et al. 1995). A limited number of cell types have been shown to express LTC_4 synthase, most of which are of myeloid origin including eosinophils, mast cells, monocyte/macrophages and leukaemic cell lines (Lam et al. 2000), but not neutrophils, which generate exclusively LTB_4 (Haeggström 2000). In addition, certain structural cell types such as endothelial cells and vascular smooth muscle, that lack the proximal enzymes of the LT pathway, are able to synthesise LTC_4 by a transcellular mechanism involving the LTC_4S-related microsomal glutathione transferase type II enzyme (Feinmark et al. 1986, 1987).

1.5.6.6 Multidrug Resistance Protein (MRP)

The export of LTC_4 from cells displays characteristics such as saturability, time-, temperature- and energy-dependence and competition between LTC_4 and LTC_5, all of which point to the presence of a distinct cellular export system for LTC_4 (Lam et al. 1989, Sjölinder et al. 1999). This export system has been shown to be the multidrug resistance protein (MRP) which transports glutathione, glucuronate and sulphate conjugates (Jedlitschky et al. 1996). The expression of MRP correlates closely with LTC_4 transport (Jedlitschky et al. 1994). MRP was discovered by its overexpression in the multidrug resistant cell line H69AR (Cole 1992). MRP deficient mice appear to have a reduced inflammatory response to arachidonic acid injection in the ear, due to reduced LTC_4 export (Wijnholds et al. 1997).

The mechanism of LTC_4 transport was discovered by studies looking at the mechanisms by which tumour cells resist cytotoxic drugs. One mechanism is by excretion of these agents by members of the ATP-binding cassette (ABC) family of proteins. There are two ABC proteins that can render cells drug resistant, P-glycoprotein (Pgp) and the multidrug resistance-associated protein (MRP) that transports glutathione conjugates. To date five different MRP homologues exist termed MRP-1, MRP-2 etc. MRP-1 is expressed in most human tissues, as is MRP-5, but MRP-2 and MRP-3 are expressed mostly in the liver with little expression elsewhere (Kool et al. 1997). Within cells, MRP is located primarily in the plasma membrane (MRP-2 mainly apical plasma membrane) and endoplasmic reticulum (Keppler et al. 1996).

Characterisation of the LTC_4 transporter in mouse mastocytoma cells has shown that it is an integral membrane glycoprotein with a mass of approximately 190kDa and its most potent competitive inhibitor is MK-571, a LTD_4 antagonist with a Ki value of 0.8μM (Leier et al. 1993). Recent evidence suggests that NSAIDs may also block the action of MRP, as they can increase cytotoxic drug toxicity by preventing the cellular export of the cytotoxic drugs doxorubicin and vincristine (Roller et al. 1999). MRP has three membrane spanning domains and two nucleotide binding domains (Gao et al. 1998) and has several potential glycosylation sites and targets for kinases (Loe et al. 1996). MRP-1 has been mapped to chromosome 16 (Cole 1992) and the MRP-2 gene to chromosome 10 (Taniguchi et al. 1996). The MRP promoter is similar to that of a house keeping gene but the 5' flanking region has binding sites for proteins such as SP-1 (Zhu et al. 1994). Functional involvement of MRP-1 in the export of LTC_4 has been shown in platelets and mast cells (Sjölinder et al. 1999, Bartosz et al. 1998).

1.5.6.7 LTB_4 export

There is little information available regarding the export of LTB_4 from cells, but it is not exported by the LTC_4 transporter MRP. The transport of LTB_4 out of leukocytes has been shown to be a carrier mediated process with saturation-, time- and temperature-dependence (Lam et al. 1990).

1.5.6.8 Leukotriene catabolism

The degradation of LTB_4 can occur by two main pathways. One is by ω-oxidation (the addition of an OH group) in a two step reaction resulting in the formation of 20-OH-LTB_4 followed by 20-COOH-LTB_4. The enzyme required for this reaction is a specific cytochrome P-450 that is highly expressed in neutrophils (Kikuta et al. 1998). β-oxidation is known to be a predominant pathway of LTB_4 metabolism in the hepatocyte after the initial ω-oxidation of the methyl terminus and alcohol dehydrogenase-mediated formation of an ω-carboxylmoity (Shirley et al. 1992). Alternatively, in kidney, lung and liver, oxidation of the 12-hydroxl group of LTB_4 may occur (Kumlin et al. 1990, Kasimir et al. 1991). The enzyme responsible is LTB_4 12-hydroxydehydrogenase that yields the product 12-keto-LTB_4 and can also metabolise prostaglandins (Yokomizo et al. 2000). LTB_4 is relatively stable in blood showing some slow degradation by cellular dependent ω-oxidation (Zakrzewski et al. 1988).

LTC_4 and LTD_4 are converted into LTE_4, which has a longer biological half-life, before inactivation. Incubation of LTC_4 with whole blood *in vitro* results in the rapid formation of LTD_4 and LTE_4 (Zakrzewski et al. 1989). *In vivo* arterial injection of radioactive LTC_4 in monkeys shows rapid clearance from the blood, after 15 sec 40% of the radioactivity was converted into LTD_4 and LTE_4 (Tagari et al. 1989). Initially, LTE_4 is thought to be metabolised by ω-oxidation to 20-COOH-LTE_4 in the liver, a process again thought to involve cytochrome P-450 (Orning 1987, Keppler 1992). Succesive β-oxidation reactions of the 20-carboxy-LTE_4 then result in the formation of 18-, 16-, and 14-carboxy-LTE_4 metabolites (Sala et al. 1990). Alternatively, LTE_4 may undergo acetylation to N-acetyl-LTE_4 catalysed by N-acetyl transferase, which is found in the liver, kidney and lung. N-acetyl-LTE_4 retains some biological activity but is then further metabolised by ω-oxidation and β-oxidation to various inactive carboxylated N-acetyl products. N-acetylation is the main route of catabolism in rodents (Hagmann et al. 1986), but not in humans (Maltby et al. 1990). LTE_4 levels can be measured in urine to provide information about total cys-LT entry into circulation as the quantity of LTE_4 excreted into the urine is approximately 5% of the LTC_4 injected into the vascular system (Maltby et al. 1992). Similarly, levels of inhaled LTD_4 also correlate with the amounts of LTE_4 excreted in the urine (Verhagen et al. 1987).

1.5.7 Cys-LT actions

In 1940 Kellaway and Trethewie demonstrated that when antigen-sensitised guinea pig lungs are stimulated, they release a substance which contracts smooth muscle (Kellaway et al. 1940). This substance was initially called slow reacting substance (SRS), the biological activity of which was later shown to be due to a group of compounds called the cysteinyl-LTs, LTC_4, LTD_4 and LTE_4.

Of particular interest to asthma are the bronchoconstrictive effects of the cys-LTs. Dahlén and colleagues carried out the first *in vitro* experiments using human tissue and showed that LTC_4 and LTD_4 caused contraction of isolated bronchial preparations (Dahlén et al. 1980). LTC_4 and LTD_4 were approximately equipotent and at least 1000 times more potent than histamine (Dahlén et al. 1980). Holroyde in 1981 was the first to report on the *in vivo* effect of inhaled LTC_4 and LTD_4 in two normal volunteers. LTC_4 and LTD_4 were found to be equally effective in reducing expiratory flow in two subjects and the cys-LT antagonists FPL55712 and FPL59257 partially inhibited this bronchoconstriction (Holroyde et al. 1981).

Several more detailed studies followed using greater numbers of subjects and different markers of airway obstruction, and all confirmed these preliminary findings. It was also found that in normal subjects, inhaled LTD_4 is more effective at causing contraction of peripheral airways than the central airways as greater concentrations of LTD_4 were required to cause a 15% reduction in FEV_1 (more sensitive to changes in the state of the large airways) than required to cause a 30% reduction in $V_{30}P$ (thought to be more sensitive to state of peripheral airways) (Bisgaard et al. 1983). Inhaled LTD_4 is more than 100 times more potent than LTE_4 at causing airway obstruction in normal subjects although the effect of LTE_4 is longer lasting (Davidson et al. 1987).

In vivo studies have shown that inhaled cys-LTs are also potent bronchoconstrictors in asthmatic subjects. Griffin and colleagues were the first to show that LTD_4 and LTC_4 inhalation caused airway obstruction in asthmatic subjects (Griffin et al. 1983) and later studies suggested that the response to cys-LT inhalation may be exaggerated in asthmatics compared to normals (Adelroth et al. 1986), as is the case with other mediators such as histamine. There are reports that the aerodynamic site of response to LTC_4 inhalation is more widespread in asthmatics than in normals, involving both the central and peripheral airways (Pichurko et al. 1989). It may be that there is a difference in the distribution of $CysLT_1$ receptors in normals and asthmatics although this has not yet been examined.

Cys-LTs also contribute to the process of airway tissue oedema and recruitment of leukocytes to the site of inflammation. Vascular permeability can be examined in animals by the extravasation of markers like Evans Blue dye or radioactive/fluorescent macromolecules. Using such techniques, LTC_4 and LTD_4 have been found to increase the formation of endothelial gaps in several studies (Chanarin et al. 1994). Airway eosinophilia is a prominent feature of asthma and inhaled LTE_4 has been shown to cause a dose dependent increase in the number of eosinophils in the lamina propria of asthmatic subjects (Laitinen et al. 1993). Several mechanisms have been suggested to explain this eosinophilia. Assessment of cell migration has shown that LTD_4 is a potent and selective chemoattractant for eosinophils at concentrations as low as $10^{-10}M$ (Spada et al. 1994). Cys-LTs also increase rolling of leukocytes on endothelial cells by increasing P-selectin expression on both cell types (Pedersen et al. 1997, Kanwar et al. 1995). In addition, it has been shown recently that cys-LTs promote eosinophil survival by reducing apoptosis (Lee et al. 2000).

In asthma there is excess mucus secretion which obstructs the airways and may lead to plugging. A technique involving the culture of human airways in the presence of radiolabelled amino sugars that become incorporated into mucus glycoproteins has shown that cys-LTs are potent secretagogues (Marom et al. 1982). Cys-LTs also affect respiratory epithelial cell ciliary motility. Some studies suggest a decrease in motility that is abolished by LTRAs (Bisgaard et al. 1987, Granbo et al. 1996) and others an increase in ciliary beat frequency that is also abolished by LTRAs (Cyrus et al. 1998, Tamaoki et al. 1991).

Evidence also suggests that cys-LTs play a role in airways remodelling as they are potent mitogens for airway epithelial cells (Leikauf et al. 1990) and cys-LTs increase collagen production and collagenase activity in human dermal and lung fibroblasts (Abe et al. 2000, Medina et al. 1994). Cysteinyl-leukotrienes appear to have a role in cell-restructuring events such as migration and pattern formation, by complex interactions with growth factor signalling pathways. EGF regulates organisation of the actin microfilament system in several cell types and activation of the EGF receptor leads to $cPLA_2$ activation and subsequent arachidonic acid release (Clark et al. 1991). Metabolism of the released AA leads to an EGF-induced production of both prostaglandins and leukotrienes (Peppelenbosch et al. 1992). EGF induced Ca^{2+} influx results from intermediary LT production (Peppelenbosch et al. 1992) and the mitogenic response of syrian hamster embryo fibroblasts to EGF can be blocked by inhibition of LT synthesis (Glasgow et al. 1992). EGF induced actin reorganisation in a variety of structural cell lines appears to depend on LTs acting as second messengers (Peppelenbosch et al. 1993). Several mitogenic responses are also thought to be a result of interaction between cys-LTs and growth factor pathways. In cultured rabbit airway smooth muscle cells IGF, but not LTD_4 was mitogenic but when added in combination these two agents exerted a significant synergistic effect (Cohen et al. 1995). It was shown that LTD_4 enabled more free IGF to interact with its receptors by reducing levels of an IGF binding protein (IGFBP) (Cohen et al. 1995). LTD_4 also significantly augments EGF induced HASM proliferation (Panettieri et al. 1998).

1.5.8 LTB_4 actions

LTB_4 was not identified as a component of SRS-A but instead by its potent chemotactic activity for neutrophils, being equipotent with complement factor C5a and fMLP (Ford-Hutchinson et al. 1980). LTB_4 can also cause directional migration of eosinophils at 10^{-8}M (Spada et al. 1994). Inhalation of a single dose of LTB_4 has no effect on bronchial

hyperresponsiveness or lung function although it causes a rapid blood neutropaenia (5 min) followed by a later neutrophilia (30 min) (Sampson et al. 1997). Although neutrophils are implicated in nocturnal and sudden onset asthma (Fahy et al. 1995, Martin et al. 1991), treatment with the BLT antagonist LY293111 had no bronchodilator effect in allergen challenged patients but significantly reduced BAL fluid neutrophils and IL-8 compared to placebo (Evans et al. 1996). However, in a dog model LTB_4 was found to increase airway reactivity to acetylcholine (O'Byrne et al. 1985). Guinea pig lung parenchyma shows contractile activity in response to LTB_4 but this effect is thought to be due to the release of other mediators, for example histamine, cys-LTs or TxA_2 (Dahlén et al. 1983, Piper et al. 1982). Other LTB_4 effects on neutrophils include increasing neutrophil-endothelial cell interactions and stimulation of neutrophil activation leading to degranulation and mediator release (Hoover et al. 1984).

There is thought to be an immunomodulatory role for LTB_4 as it can induce the production of IL-5 by T-lymphocytes and therefore promote the effects of IL-4 on IgE production by B lymphocytes (Yamaoka et al. 1994). LTB_4 may also affect the expression of low-affinity receptors for IgE on B-lymphocyte cell lines (Odlander et al. 1988). There are reports that LTB_4 may be immunosuppressive through the induction of suppressor T-cells (Payan et al. 1984) or immunostimulatory as the LTB_4 antagonist ONO-4057 inhibits T-cell proliferation and cytokine production (Morita et al. 1999). Other LTB_4 effects include NF-κB activation, c-fos and c-jun gene transcription and AP-1 DNA binding in monocytes (Aoki et al.1998, Brach et al. 1992, Stankova et al. 1992). LTB_4 may also be involved in inflammatory pain due to a lowering of the nociceptive threshold (Levine et al. 1984).

1.5.9 Knockout mice

Insight into the functions of leukotrienes has also been achieved using the technique of targeted gene disruption in mice. These studies confirm that the major roles of the leukotrienes are in inflammatory processes. Mice lacking in $cPLA_2$ show reduced allergic responses but also reproductive and developmental defects most likely due to their impaired prostaglandin production (Uozumi et al. 1997). Disruption of 5-LO and FLAP does not give rise to any obvious defects in mice. 5-LO deficient mice have blunted inflammatory responses to inflammatory stimuli such as arachidonic acid-induced ear inflammation and ovalbumin-induced airway inflammation. However, these mice are also more susceptible to death from parasitic infection (Funk 1996). Similarly, FLAP-deficient mice also show

reduced inflammatory responses. The severity of collagen-induced arthritis is reduced in these animals (Griffiths et al. 1997). As 5-LO and FLAP disruption will affect both LTB_4 and cys-LT synthesis, LTA_4 hydrolase deficient mice have been compared with 5-LO knockout mice in order to isolate LTB_4 functions. Mice lacking LTA_4 hydrolase develop normally, their oedema formation was lower than in wild type animals although not as reduced as in 5-LO knockouts and these mice are also resistant to the systemic shock induced by PAF (Byrum et al. 1999). LTC_4 synthase deletion also has no effect on the development of mice but the response to induction of cutaneous anaphylaxis in the ear was significantly diminished in these animals (Kanaoka et al. 2001).

1.5.10 CysLT receptors

The presence of cys-LT receptors was suggested even before the structures of the LTs were identified, as the compound FPL-55712 appeared to be an antagonist of SRS-A (Augstein et al. 1973). Experiments using the available LT antagonists have outlined two main classes of cysteinyl receptor (Spector 1997). These have been named $CysLT_1$ and $CysLT_2$ of which $CysLT_1$ mediated effects are blocked by several antagonists such as MK-571 and MK-476 whereas $CysLT_2$ mediated effects are blocked by the dual antagonist, BAY u9773 (Dahlén 2000).

The existence of two different cys-LT receptors was suggested by the finding that LTD_4-induced contractions of guinea pig parenchymal preparations were blocked by FPL55712 but those induced by LTC_4 were not (Drazen et al. 1980). Similarly, it was found that the LTC_4 induced constriction of guinea pig airways could not be antagonised by FPl55712 when the metabolic conversion of LTC_4 into LTD_4 was inhibited (Fleisch et al. 1982). However, when this type of experiment was repeated using human bronchi, FPL55712 antagonised LTC_4 and LTD_4 effects equally, suggesting that in humans LTC_4 and LTD_4 act at the same receptor (Buckner et al. 1986). Evidence suggesting the presence of $CysLT_2R$ in human lung derives mainly from experiments on vascular tissue. Labat et al. showed that a number of antagonists that block cys-LT-induced contractions of bronchi fail to inhibit contractile responses in venous preparations (Labat et al. 1992). They also found differences in the agonist sensitivity of these responses with LTE_4 being a comparatively weak agonist. Also, these pulmonary vessel contractions were antagonised by the experimental compound BAYu9773, at present the only $CysLT_2$ antagonist, but a dual $CysLT_1/CysLT_2$ antagonist (Gardiner et al. 1990). In contrast to vasoconstriction at higher concentrations (10^{-6}-10^{-5}M),

cys-LTs at lower concentrations (10^{-8}-10^{-7}M) relax many arterial preparations from dogs, guinea-pigs, and humans (Secrest et al. 1985, Sakuma et al. 1987, Ortiz et al. 1995). In pulmonary vessels, this mechanism has been proposed to involve $CysLT_2$ receptors on the vascular endothelium that produce indirect relaxation via the synthesis of nitric oxide and prostanoids.

As well as there being different subtypes of receptor, the tissue distribution of the receptor varies and species differences in the receptors also occur. In guinea pigs, FPL55712 is more potent in ileum than in lung preparations (Fleisch et al. 1982) and the potency of ICI-198615, a selective $cysLT_1$ antagonist is significantly different in guinea pig and rat lung preparations (Snyder et al. 1986).

The human $CysLT_1$ and $CysLT_2$ receptors were both recently characterised at the molecular level (Lynch et al. 1999, Takasaki et al. 2000). Both are classical G-protein-coupled serpentine receptors with seven putative transmembrane regions and their functional activation involves calcium mobilisation. The $CysLT_2$ receptor polypeptide shows 31% amino acid identity to Cys-LT_1. The molecular studies largely clarified and extended the previous pharmacological observations. At the $CysLT_1$ receptor, LTD_4 is approximately 10-fold more potent as an agonist of calcium mobilisation than LTC_4 or LTE_4. At $CysLT_2$ receptors, LTC_4 and LTD_4 are roughly equipotent with each other, but both have lower affinity for $CysLT_2$ than LTD_4 has for the $CysLT_1$ receptor. The specificity of the 'lukast' drugs for $CysLT_1$, and the dual antagonism of $CysLT_1$ and $CysLT_2$ by BAYu9773 was also confirmed by the molecular studies. The $CysLT_1R$ gene has been mapped to the X chromosome (Lynch et al. 1999) whereas $CysLT_2$ is localised to chromosome 13q14 (Takasaki et al. 2000).

There are striking differences in tissue distribution between $CysLT_1$ and $CysLT_2$. Expression of $CysLT_1$ mRNA is mainly in the spleen, peripheral blood leukocytes and airway smooth muscle (Lynch et al. 1999), while large amounts of $CysLT_2$ mRNA are found in the heart (Purkinje fibres), placenta, spleen and leukocytes, but not in the lung (Takasaki et al. 2000). A recent study assessing the roles of $CysLT_1$ and $CysLT_2$ in the heart confirmed minimal $CysLT_1$ expression with $CysLT_2$ being expressed at high levels in both the atrium and ventricle, more specifically on myocytes, fibroblasts and vascular smooth muscle but not on endothelial cells (Kamohara et al. 2001).

1.5.11 Leukotriene B_4 receptors

The specific binding of LTB_4 to an unknown receptor on human polymorphonuclear leukocytes (Kreisle et al. 1983) was later found to be to the BLT receptor, the first of the leukotriene receptors to be cloned (Owman et al. 1997). Its gene encodes a 352 amino acid protein with seven transmembrane spanning domains (Owman et al. 1996). BLT is a G-protein coupled receptor (Yokomizo et al. 1997). Its structure shows two potential glycosylation sites and several PKC phosphorylation sites that are well conserved in different species (Yokomizo et al. 2000). In chinese hamster ovary (CHO) cells expressing the receptor, ligand binding causes an increase in intracellular Ca^{2+}, IP_3 accumulation and inhibition of adenyl cyclase, effects that are only partially blocked by pertussis toxin, implying that BLT couples to both PT sensitive and insensitive G-proteins (Yokomizo et al. 1997). Northern blotting experiments of various human tissues have shown that the expression of BLT is highest in leukocytes followed by the thymus and spleen with little expression elsewhere (Yokomizo et al. 1997). CHO cells expressing BLT show prominent chemotaxis towards LTB_4 as well as LTB_4 induced chemokinesis, two of the main pharmacological effects of LTB_4 (Yokomizo et al. 1997). The human BLT gene is located on chromosome 14q (Owman et al. 1996). It is a small gene (≈5kb) with 3 exons (Yokomizo et al. 2000) but as yet the promoter region has not been analysed and possible regulatory mechanisms are unknown.

The classic BLT receptor described above has recently been re-named BLT1 due to the discovery of a second LTB_4 receptor, designated BLT2. BLT1 and BLT2 show 45.2% similarity in their amino acid sequences but their main differences are that LTB_4 binds to BLT2 with a lower affinity than BLT1, and whereas BLT1 is expressed predominantly in leukocytes, BLT2 is ubiquitously expressed (Yokomizo et al. 2000). The functions of LTB_4 that are mediated by BLT2 have not yet been fully described and future research may reveal more roles for this leukotriene.

Peroxisome proliferator-activated receptors (PPARs) are transcription factors that regulate gene expression of enzymes associated with lipid homeostasis (Lemberger et al. 1996). As well as binding to BLT, LTB_4 can bind and activate one of these receptors, PPARα, ultimately increasing the expression of enzymes that degrade lipid mediators including LTB_4 itself (Devchand et al. 1996). Thus, LTB_4 is involved in a feedback mechnism that can control the duration of an inflammatory response.

Intriguingly, in addition to its chemoattractant properties the BLT receptor has another recently discovered function. It is a type of HIV-1 coreceptor that mediates the entry of primary HIV-1 into CD4 positive cells (Owman et al. 1998). After HIV binds to CD4 there is a conformational change in its glycoprotein envelope allowing the virus to bind to a G-protein linked receptor, one of which is BLT. This in turn facilitates fusion of the envelope with the cell membrane and thus viral entry (Owman et al. 2000). The implications of this effect are unclear.

1.5.12 Leukotriene modifiers

There are two main types of antileukotriene drugs, those that block LT synthesis and those that block LT receptors. Some examples of each type of drug are shown in **table 5**. Leukotriene synthesis inhibitors usually block either FLAP or 5-LO and the most well known of these is zileuton, a 5-LO inhibitor licensed for use in the USA (Chanarin et al. 1994). There are several cys-LT receptor antagonists used to treat asthma, those licensed for use are montelukast, zafirlukast and pranlukast (Barnes 2000). The BLT receptor antagonist LY293111 is not used to treat asthma due to its lack of clinical benefit, although it does reduce neutrophil influx upon allergen challenge after one week of treatment (Evans et al. 1995).

Initial experimental studies usually involve inhalation challenges with allergen and other stimuli and test the ability of drugs to block the bronchoconstrictor reactions, as well as BHR as measured by methacholine or histamine. Both zileuton and the cys-LT antagonists effectively reduce both the EAR and LAR after allergen, by 84% and 60% respectively, showing that cys-LTs are vital in both the acute bronchoconstriction caused by mast cell degranulation and the late reaction characterised by granulocyte infiltration (O'Byrne 1997). As well as allergen challenges, other agents such as cold air, exercise, aspirin and the cysLTs themselves can be used to provoke bronchoconstriction. Virtually all leukotriene modifiers tested, of both types, will shift the dose response curve of a LTD_4 challenge to the right and show protection against allergen, aspirin, cold air and exercise induced bronchoconstriction (Spector 1997).

In clinical asthma, treatment with zafirlukast (20mg twice a day), montelukast (10mg once a day) or zileuton (600mg 4x/day) reduces several common asthma symptoms such as night time awakening, morning and daytime asthma scores and β_2 agonist use, and also improve

lung function tests (Spector 1997). In some cases there is also a reduced need for corticosteroid therapy. Interestingly, studies in stable asthmatics have shown that cys-LT antagonist inhalation causes an immediate bronchodilation, an effect that is not seen in normals and which is additive to inhaled β_2 agonist induced relaxation (Holgate et al. 1996, Barnes 2000). This suggests that cys-LTs contribute to the residual impaired lung function of asthmatics and that these drugs are not solely smooth muscle relaxants (Barnes 2000).

Table 5. Leukotriene modifying agents

Mechanism of action	Drug	Reference
SYNTHESIS INHIBITORS		
5-LO inhibitors	Zileuton (A-64077)	Cohn 1994
	ABT-761	Bell 1997
	Z-D2138	Kusner 1994
FLAP inhibitors	Quiflapon (MK-0591)	Diamant 1993
	MK-886	Friedman 1993
	BAYx1005	Hatzelmann 1994
RECEPTOR ANTAGONISTS		
CysLT antagonists	Zafirlukast (ICI-204,219)	Aharony 1998
	Pobilukast (SK&F 104353-Q)	Aharony 1998
	Tomelukast (LY171883)	Cloud 1989
	Verlukast (MK-679)	Dahlén 1993
	Pranlukast (ONO-1078)	Barnes 1995
	Montelukast (MK-0476)	Aharony 1998
BLT antagonist	LY293111	Evans 1995

Although many of the early leukotriene modifying drugs were disappointing due to poor efficacy and gastrointestinal and liver side effects, those in use today are very safe and well tolerated. Reported minor side effects include dyspepsia (zileuton) and headaches (zafirlukast and zileuton) although neither is significant compared to placebo (Barnes 2000). Zileuton has an approximately 2% incidence of liver function abnormalities as shown by mild elevated liver enzyme levels, so monitoring of liver function is required with this drug.

There is evidence that anti-leukotriene treatment is particularly beneficial to a subpopulation of asthmatics in whom the overproduction of leukotrienes is an important characteristic. Hasday et al. showed that treatment with zileuton only reduced antigen-induced indices of inflammation in a subset of allergic asthmatics who overproduced cys-LTs upon ragweed challenge (Hasday et al. 2000). Another type of asthma in which leukotriene overproduction is a key characteristic is that of aspirin sensitive asthma.

Approximately 10% of adults with asthma will experience bronchospasm after ingestion of aspirin or other non-steroidal anti-inflammory drugs (NSAIDs) (Szczeklik et al. 2000). This distinct clinical syndrome is characterised by chronic nasal congestion, often with nasal polyps, an eosinophilic inflammation in the upper and lower airways and acute asthma attacks following NSAID ingestion (Szczeklik et al. 2000). A key characteristic of these individuals is a chronic overproduction of cys-LTs indicated by an approximate 3- to 7-fold increase in basal urinary LTE_4, further exacerbated by aspirin ingestion (Knapp et al. 1992, Israel et al. 1993). The theory proposed to explain AIA involves the shared pharmacologic action of NSAIDs including aspirin, that is inhibition of the COX enzyme. Blockade of the prostaglandin pathway may remove the braking effect of PGE_2 on 5-LO (Kuehl et al. 1984) resulting in augmented cys-LT production as well as making more free arachidonic acid available to the 5-LO pathway.

Possible explanations as to why aspirin sensitivity does not occur in all asthmatics include a difference in the susceptibility of COX enzymes to aspirin/NSAIDs or a difference in the 5-LO pathway. The expression of both COX-1 and COX-2 is similar in bronchial biopsies from AIA and aspirin tolerant asthma (Cowburn et al. 1998, Sousa et al. 1997). The expression of LTC_4 synthase (but not other LT pathway enzymes) is 5 times higher in AIAs than aspirin tolerant asthmatics and 19 times higher than controls (Cowburn et al. 1998). Recently an A to C transversion polymorphism was discovered in the LTC_4 synthase gene promoter 444 nucleotides upstream from the transcription start site, C_{444} (Sanak et al. 1997). This C_{444} allele is 60% more common in AIAs than aspirin tolerant asthmatics and in these individuals there is upregulated LTC_4 synthase mRNA expression in eosinophils (Sanak et al. 1997, Sanak et al. 1998). The AIA associated allele has an additional transcription activator-binding motif and so may cause increased inducibility of LTC_4 synthse (Szczeklik et al. 2000).

1.6 Hypothesis and Aims

The critical role of cys-LTs in the pathogenesis of asthma is well recognised. The cys-LTs cause bronchoconstriction, oedema, mucus hypersecretion and eosinophil chemotaxis, while LTB_4 is a potent neutrophil chemoattractant (Hay et al. 1995). LT levels in bronchoalveolar lavage fluid and urine from asthmatic subjects are elevated compared to normals (Arm et al. 1993) and LT synthesis inhibitors and cysLT receptor antagonists are effective asthma treatments (Drazen et al. 1999). LTs are thought to be generated by cells of myeloid origin,

including mast cells, eosinophils, neutrophils, basophils, and monocytes. Relatively few studies have investigated airway structural cell types as a potential source of LTs, despite the fact that these cells are increasingly recognised to have an active pro-inflammatory role in asthma. Bronchial epithelial cells, airway smooth muscle cells and fibroblasts generate a range of cytokines, chemokines and growth factors and lipid mediators. However, while they are established as a source of arachidonic acid metabolites such as the prostanoids and thromboxanes (van der Velden et al. 1998, Johnson et al. 1997), their capacity to generate LTs via the 5-LO pathway has not been thoroughly investigated.

Literature searches have not provided any evidence that HASM possess a 5-LO pathway although vascular smooth muscle has been shown to generate cys-LTs when provided with LTA_4 by another cell type (Feinmark et al. 1987). While there is evidence of leukotriene production by fibroblasts from tissues other than the lung, reports of LT production by airway fibroblasts are few. For example, systemic sclerosis causes overexpression of 5-LO in dermal fibroblasts (Kowal-Bielecka et al. 2001) and LTB_4 production by skin fbroblasts has also been detected (Calderwood et al. 1989). Airway fibroblasts have been shown to produce leukocyte chemotactic activity in response to stimuli such as smoke extract, bradykinin, lipopolysaccharides and the drugs bleomycin and methotrexate. One of the components of this activity has been suggested to be LTB_4 by the use of a LTB_4 receptor antagonist, lipoxygenase inhibitors and radioimmunoassay (Sato et al. 1999, Koyama et al. 2000, Koyama et al. 2000b, Takamizawa et al. 1999 and Sato et al. 2001). A foetal lung fibroblast cell line was shown to release cys-LTs in response to thiol depletion (Aoshiba et al. 1999).

Reports of LT production by BEC are more numerous than in smooth muscle and fibroblasts but findings are varied. The first report of leukotriene production in epithelial cells was by Holtzman and colleagues in 1983, who showed that dog tracheal epithelial cells generate primarily LTB_4 and very small amounts of the cys-LTs, but only after incubation with arachidonic acid (Holtzman et al. 1983). More recently, there have been reports that LTB_4 is produced by bovine bronchial epithelial cells and that smoke, endotoxin, acetylcholine and substance P can all increase this production (Koyama et al. 1998 & 1991). In humans, some reports conclude that BEC do not have a 5-lipoxygenase pathway or generate LTs (Hunter et al. 1985, Tristram et al. 1998). Others suggest that the only way in which human tracheal epithelial cells can generate a leukotriene product is by transcellular synthesis i.e. LTA_4 is

provided by inflammatory cells and then converted into LTB_4 by an epithelial LTA_4 hydrolase (Bigby et al. 1989). This group found no evidence of cys-LT formation. McKinnon et al detected both LTB_4 and cys-LTs in supernatants from BEAS-S6 cells by HPLC, but only after exposure to ozone (McKinnon et al. 1993). Behera at al were also able to detect both cysLT and LTB_4 production by enzyme immunoassay in two HBE cell lines after respiratory syncytial virus infection, but this group could not detect 5-LO mRNA at baseline (Behera et al. 1998).

There are no published studies in primary epithelial cells, fibroblasts or smooth muscle cells from human lung or in HBE cell lines that combine immunoassays of released LTs with investigations of the expression and regulation of all four LT pathway enzymes. *I hypothesised firstly that bronchial epithelial cells, fibroblasts and airway smooth muscle cells may express 5-LO pathway enzymes and generate LT either spontaneously or after exposure to pro-inflammatory stimuli, and hence may contribute to asthma pathophysiology.*

In addition to their effects described on other cell types, cys-LTs may have direct effects on BEC, HASM and fibroblasts cells such as increased proliferation, bronchoconstriction, and mucus and collagen secretion (Hay et al. 1995). These effects are likely to be mediated by the $CysLT_1$ receptor that has recently been cloned and characterised (Lynch et al. 1999). As yet, there are no reports of expression of $CysLT_1R$ on HBEC, or of its regulation in HASM and fibroblasts. The LTB_4 receptor BLT1 is predominantly expressed on neutrophils but it is not clear whether this receptor is found on structural cell types in the airway. LTB_4 has been shown to mediate fibroblast chemotaxis *in vitro* (Mensing et al. 1984) and the expression of BLT on fibroblasts and HASM could mediate such effects. *Secondly, I hypothesise that $CysLT_1$ and BLT are present on BEC, fibroblasts and HASM and that there may be regulation of their expression by inflammatory stimuli or receptor antagonists.*

Therefore, my aims were:

1. To examine using RT-PCR, immunocytochemistry, SDS-PAGE/Western blotting, flow cytometry, and enzyme immunoassays the baseline expression of the 5-LO pathway enzymes in HBEC, fibroblasts and HASM.

2. To examine LT biosynthetic enzyme expression after incubation with pro-inflammatory stimuli such as calcium ionophore (A23187), autacoids (bradykinin, histamine), bacterial

lipopolysaccharide (LPS), and cytokines and growth factors known either to activate BEC, fibroblasts and HASM or to modulate the 5-LO pathway in myeloid leukocytes.

3. To investigate the expression of $CysLT_1$ and BLT in human bronchial epithelial cells, airway smooth muscle cells and bronchial fibroblasts, at baseline and after treatment with inflammatory stimuli.

2. Methods

2.1 Materials. Table 6

RPMI, EMEM, DMEM and Ham's F12 media Leibovitz's L-15 and LHC-9 media HBSS and PBS L-glutamine and Antibiotic/antimycotic solution Trizol Foetal calf serum, Human serum & UltroserG Trypsin/EDTA EGF	Gibco, Life Technologies Ltd, Paisley, UK
Calcium ionophore A23187 Hydrogen peroxide Bovine serum albumin Chloroform, Isopropanol, Paraformaldehyde, Saponin Ethidium bromide Cytokeratin-18 antibody Actin, Myosin, Vimentin and Desmin antibodies Trypan blue, Cell dissociation medium Dexamethasone PCR reagents (Mg^{2+}, jumpstart enzyme) Glycine, Dextran, Agarose, Tris buffer,TEMED, Tween	Sigma, Poole, Dorset, UK
LTB_4 and LTD_4 authentic standards BLT antibody MK571	Cayman Chemical, Ann Arbor, USA
Biotinylated swine anti rabbit antibody Biotinylated rabbit anti mouse antibody Biotinylated rabbit anti goat antibody Steptavidin-biotin-horseradish-peroxidase kit Rabbit and goat serum Rabbit and Mouse immunoglobulins	DAKO Ltd, High Wycombe, Bucks, UK
Il-1β, IL-3, IL-5, IL-6, TNFα, TGFβ, IFNγ & GM-CSF	R&D systems, Minneapolis, USA
Omniscript reverse transcription kit	Qiagen, Crawley, UK
Cell culture plasticware	Falcon Primaria, Beckton Dickenson, UK
Goat anti rabbit/phycoerythrin antibody Rabbit anti mouse/FITC antibody Rabbit anti goat/FITC antibody β_2-AR antibody	Southern Biotechnologies, Cambridge, UK
Biotrak $LTC_4/D_4/E_4$ + LTB_4 enzyme immunoassay kits SuperPlus ECL substrate	Amersham International, Little Chalfont, UK
Acrylamide bis solution Sodium dodecyl sulphate (SDS) Molecular weight markers Ammonium persulphate	Bio-Rad, Hemel Hempstead, UK
5-LO, FLAP, LTA_4H and $CysLT_1R$ antibodies	gift from Dr J Evans (Merck&Co, West Point, PA, USA)
LTC_4 synthase antibody	gift from Prof KF Austen & Dr JF Penrose (Harvard, Boston, USA)

Experiments were performed in human bronchial epithelial cells (BEC), specifically in 16-HBE and H292 cell lines and in primary BEC from resected lung, and in human airway smooth muscle and human airway fibroblasts.

2.2 Culture of 16-HBE and H292 BEC lines

Two human bronchial epithelial cell lines were cultured. H292, a tumour derived cell line was obtained from the American Type Culture Collection (Rockville, MD, USA) and 16-HBE, a simian virus (SV40)-immortalised cell line was provided by Dr Gruenert at the University of California (Cozens et al. 1994). Both cell lines were grown in a humidified incubator at 37°C with 5% CO_2. 16-HBE cells were cultured in Eagle's Minimal Essential Medium (EMEM) supplemented with 10% foetal calf serum (FCS), l-glutamine (2mM), penicillin-streptomycin (100U/ml and 100μg/ml) and amphotericin B (0.25μg/ml) and H292 cells in RPMI with 10% FCS and penicillin-streptomycin (100U/ml and 100μg/ml) and amphotericin B (0.25μg/ml). To passage cells for experiments, confluent cells in 75cm^2 culture flasks were washed twice with Ca^{2+}/Mg^{2+} free HBSS and incubated at 37°C for 15 minutes with 2ml 1% trypsin/EDTA. The contents of the flask were added to 10ml culture medium containing 10% FCS and centrifuged (1000rpm) at 4°C for 10 minutes. The cell pellet was resuspended in 10ml culture medium with FCS. Cell viability was assessed by exclusion of Trypan Blue (0.05%). For immunocytochemistry, approximately 30,000 cells were seeded on to sterile 13mm glass coverslips. The cells were then cultured for a further 2-4 days until confluent by light microscopy. For flow cytometry cells were cultured in 6 well plates until confluent and detached using trypsin/EDTA. For experiments involving $CysLT_1R$, cells were non-enzymatically detached using Cell Dissociation Medium to prevent damage to cell surface receptors.

2.3 Culture of primary BEC from human lung explants

Primary BEC were provided by Dr S-H Leir (RCMB, Southampton University School of Medicine). Bronchial tissue was obtained from patients undergoing surgical resection for lung tumours. Morphologically normal areas of bronchial tissue were collected by a pathologist and immediately transferred to Leibovitz's L-15 medium and kept at 4°C for up to 24h. Using a dissection microscope under sterile conditions, the epithelial layer was removed from cartilage and submucosa and cut into 1-2mm^2 sections. These were placed upside down in 24 well plates in LHC-9 medium supplemented with 2% USG, 5ng/ml EGF,

2mM L-glutamine, penicillin-streptomycin (100U/ml and 100μg/ml) and amphotericin B (0.25μg/ml). Culture medium was replaced every 3-4 days and cells were used in experiments when they covered at least 50% of the well. Cell morphology was carefully monitored using inverted phase contrast microscopy and any wells with visible fibroblast contamination were discarded.

2.4 Culture of primary HBE cells obtained by bronchial brushing

Bronchial brushed primary BEC were generously provided by Dr James Lordan (RCMB, Southampton University School of Medicine). Bronchial brushings were obtained by bronchoscopy using a fibreoptic bronchoscope in accordance with standard published guidelines [National Heart and Lung Institute, 1992]. Epithelial cells were collected using a standard sterile single-sheathed nylon cytology brush that was passed by direct vision via the bronchoscope channel into the lower airways, and six consecutive brushings were sampled from a 3-4cm^2 area of the lower airway epithelium. Cells were harvested into 5ml of sterile PBS after each brushing. After completion of the procedure, 5ml RPMI with 10% FCS were added and the sample was centrifuged at 150g for 5 minutes. Cell cultures were established by seeding freshly-brushed cells into culture dishes containing 5ml serum-free, hormonally-supplemented bronchial epithelium growth medium (BEGM; Clonetics San Diego, CA) supplemented with penicillin-streptomycin (100U/ml and 100μg/ml) and amphotericin B (0.25μg/ml). When confluent, cells were passaged using trypsin and were used for experimentation at passage 2 to 3. Viability was assessed by exclusion of Trypan Blue, and was consistently >90%.

2.5 Characterisation of HBE cells

Primary HBEC and 16-HBE and H292 cell lines were all characterised by morphology and immunocytochemistry. Under the light microscope, cells have the classic polygonal 'cobblestone-like' morphology characteristic of epithelial cells (Devalia et al. 1991) and consistently stain positively (>95%) for the epithelium-specific, structural protein cytokeratin-18, either by FACS or immunostaining of cells grown on coverslips (**figure 10**).

2.6 Culture of primary HASM Cells

Primary cultures of the HASM cells were provided by Dr AJ Knox, Dr L Pang and L Corbett (Respiratory Medicine Unit, City Hospital, Nottingham, UK) and sent to

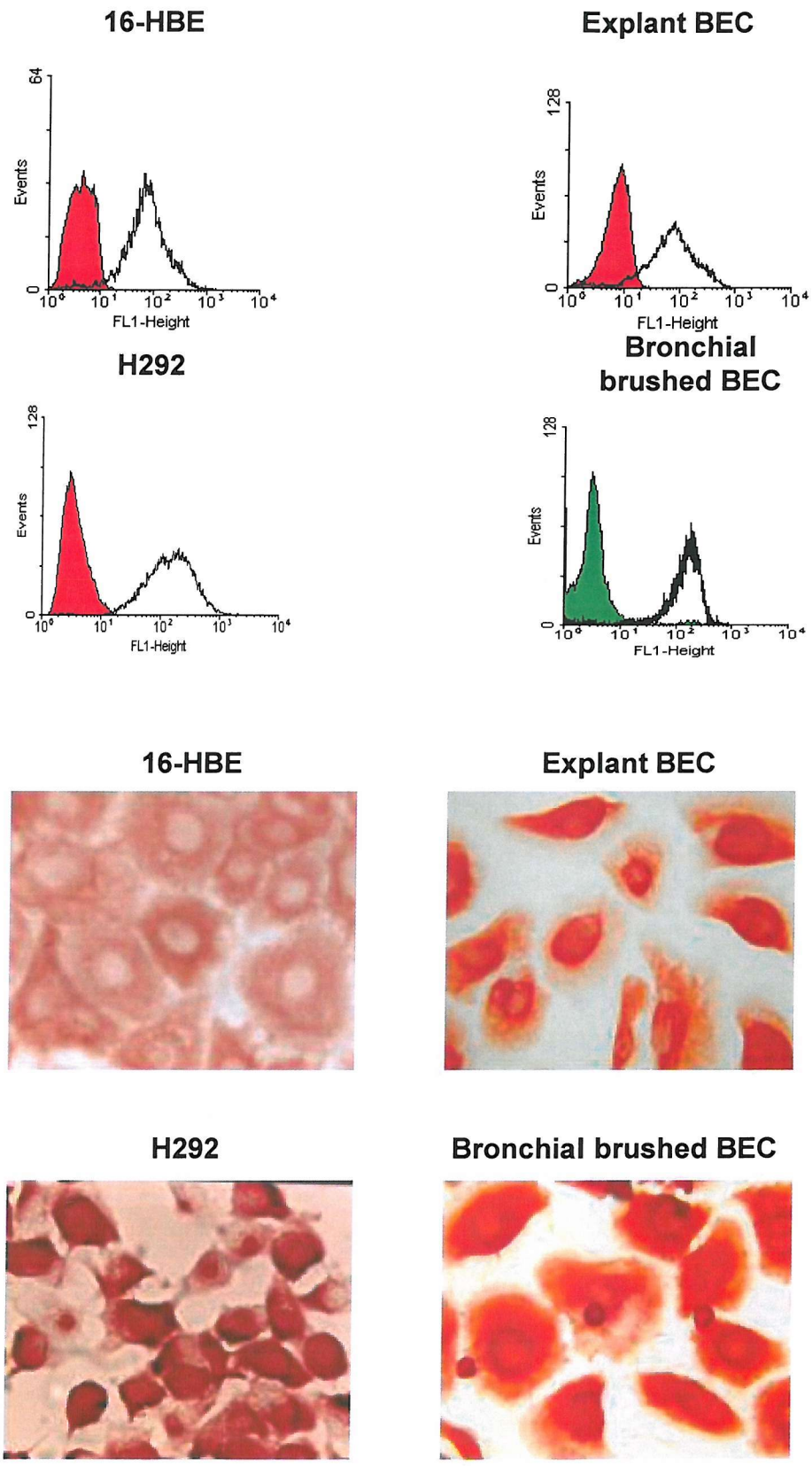


Figure 10. FACS histograms and photomicrographs showing cytokeratin-18 staining in 16-HBE, H292, bronchial brushed primary BEC and explant primary BEC. Epithelial cells were fixed, permeabilised and stained for cytokeratin. Upper panel: filled FACS graphs show isotype control and open graphs show staining with cytokeratin antibody. Lower panel: Photomicrographs (40x) showing cytokeratin immunostaining. Positively stained cells appear red.

Southampton frozen in foetal calf serum with 10% DMSO. The HASM cultures were originally prepared from explants of airway smooth muscle obtained from post-mortem individuals within 12 hours of death (Pang et al. 1997). The patients had no history of lung disease and morphologoically normal airways. Smooth muscle tissue was transported in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS, penicillin-streptomycin (100U/ml and 100μg/ml), amphotericin B (0.25μg/ml) and L-glutamine (2mM). Tissue was washed several times in the same medium before removal of the trachealis muscle from the epithelium and connective tissue under sterile conditions. 2x2 mm^2 explants were then excised and placed in Petri dishes. The explants were submerged in the culture medium and kept in a humidified, 5% CO_2, 37°C incubator. HASM cells were allowed to grow out from the tissue and become confluent (Pang et al. 1997).

2.7 Culture of primary bronchial fibroblasts

Bronchial fibroblast cultures were provided by Dr Audrey Richter (RCMB, Southampton University School of Medicine). Fibroblasts were grown as explants from bronchial biopsy tissue obtained from 5 asthmatic and 5 normal subjects. Before biopsy, the lung function and airway responsiveness of each subject were tested. Subject details are shown in **table 7**. Six submucosal biopsies from each subject were placed in a Petri dish with 10% FCS/DMEM containing 50U/ml penicillin, 50μg/ml streptomycin and 2mM L-glutamine, and cut into pieces using sterile scalpel blades. The tissues were incubated in a humidified incubator at 37°C, 5% CO_2 for approximately one week. During this time fibroblasts migrated from the tissue and proliferated on the base of the culture dish. The fibroblast cultures were fed every 2-3 days, passaged weekly and used for experiments between passages 2 to 8.

Table 7. Subject details of fibroblast donors

Patient no.	Male/Female	Asthmatic status	FEV_1 (% predicted)	PC_{20} (histamine mg/ml)
GB9	M	Mild	79	0.71
GB14	F	Mild	83	5.69
GB15	F	Mild	75	1.49
GC8	M	Moderate	78	1.17
EX35	F	Mild	67	8
GA8	M	Normal	100	>32
GA9	M	Normal	100	>32
GA10	M	Normal	93	>32
GA12	F	Normal	97	>32
GA13	M	Normal	89	>32

2.8 Characterisation of HASM and fibroblasts

Like HBE cells, HASM cells and fibroblasts were characterised both morphologically and by immunocytochemical staining. Under the light microscope, HASM cells were spindle-shaped and when confluent showed the characteristic hill-and-valley appearance described by Campbell and Campbell (1993). Fibroblasts are also spindle-shaped, elongated cells with a large oval nucleus (Absher et al. 1989). Due to their similar shape, experiments were carried out to compare the basal expression of contractile (α-actin and myosin) and cytoplasmic (vimentin and desmin) proteins in these two cell types by FACS analysis (**figure 11**). Significantly greater amounts of all four proteins were present in the smooth muscle cells, demonstrating that the fibroblasts and HASM cells used in this project were sufficiently different to allow their description as two different cell types. However, care should be taken when interpreting data obtained from these cell types as their defining characteristics are not clear cut, and depending on culture conditions their phenotypes can be altered.

2.9 Experimental design

Prior to experimentation, cells were grown to confluence to mimic *in vivo* conditions by forming a more stable, less proliferative population. Cells were then starved of FCS for 24 hours to arrest growth and to reduce the risk of background stimulation by growth factors or other agents found in serum. However, the effect of 10% FCS was examined and shown not to have an effect on LT biosynthetic enzyme expression, as measured by FACS analysis in H292 cells **(figure 12)**. Stimulation of cells was then carried out in the presence of 2% USG, a synthetic FCS replacement. USG was used to standardise experiments as the composition of FCS is not uniform and batches may vary. In the case of fibroblasts, tests were carried out in an experiment medium recommended for these cells consisting of 1:1 Ham's F12 medium and DMEM with 1% BSA and 1x ITS (Insulin Transferrin Sodium Selenite) supplement. This medium was applied 24 hours prior to incubation with stimuli.

2.10 Dextran sedimentation of mixed leukocytes

Mixed blood leukocytes were used as positive control cells for the expression of 5-LO pathway enzymes in SDS-PAGE/Western blotting experiments. Normal venous blood (50ml) was collected in EDTA Vacutainer tubes (Becton-Dickinson), and added to 10ml of PBS containing 5% dextran (MW 70kD) and 2% FCS, and after sedimentation for 60 min at

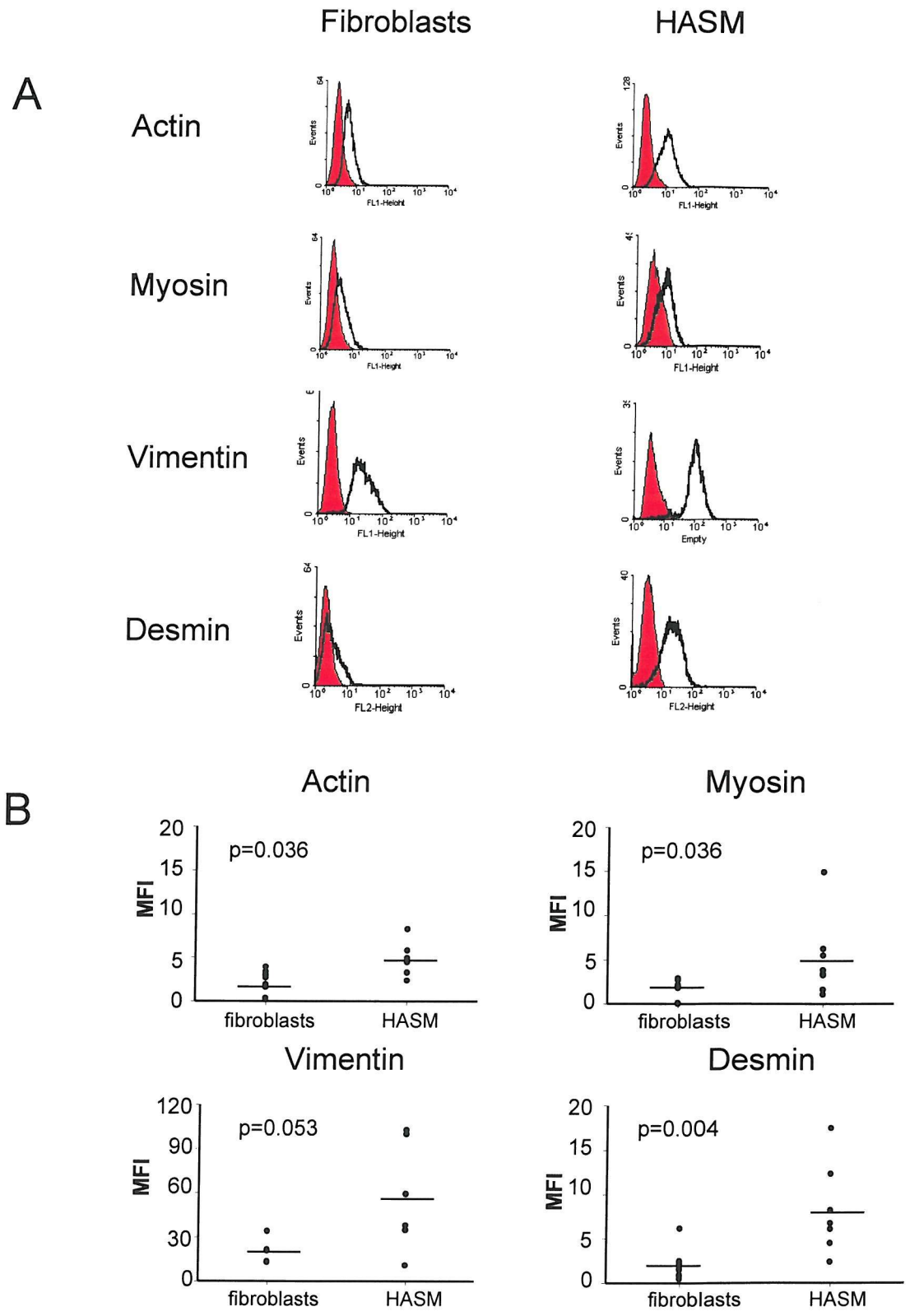


Figure 11. Expression of contractile and cytoskeletal proteins in human airway smooth muscle cells and fibroblasts as determined by FACS analysis. (A) Representative FACS histograms showing baseline expression of actin, myosin, vimentin and desmin in fibroblasts and smooth muscle. Filled curves show isotype control and open curves show fluorescence intensity of specific antibody staining. (B) Comparison of baseline median fluorescence intensity of contractile and cytoskeletal protein expression in fibroblasts and smooth muscle cells. Results were compared using the Mann Whitney U-test (n=6-9).

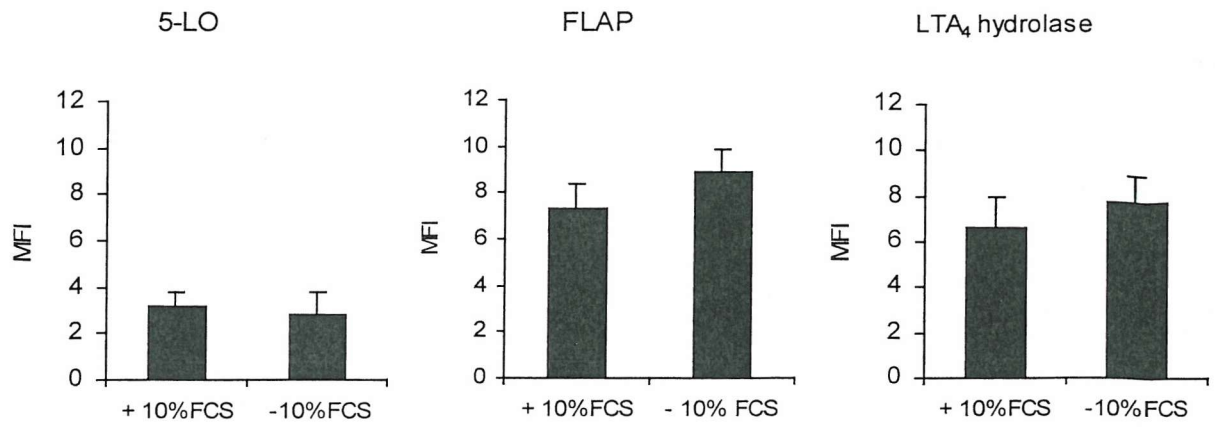


Figure 12. Flow cytometry showing the effect of 10% FCS on LT enzyme expression in H292 cells. H292 cells were incubated either with or without 10% FCS for 24 hours and the expression of 5-LO, FLAP and LTA_4 hydrolase was examined by FACS analysis. Results show mean±SEM and are not significantly different by Student's paired t-test ($p>0.05$, $n=4$).

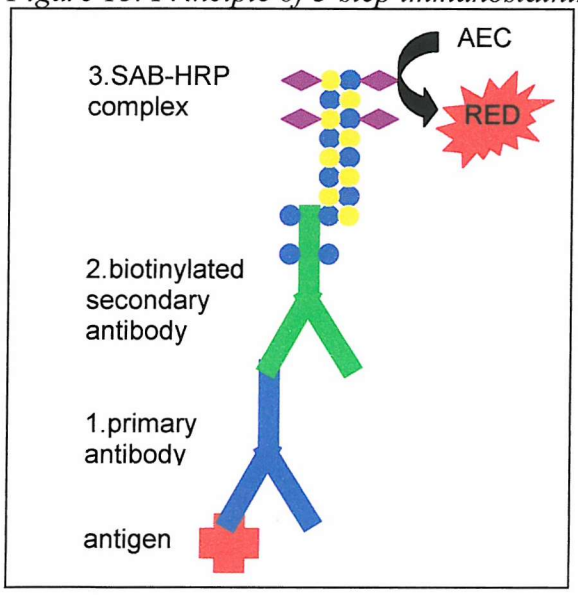
room temperature, the upper leukocyte-rich layer was separated from the lower erythrocyte layer and added to an equal volume of 2%FCS/PBS before centrifugation at 300g for 10min at 20°C. The resulting pellet was subjected to a hypotonic red cell lysis by resuspension in 5 ml distilled water for 45 sec followed by flooding with a large excess of 2% FCS/PBS to restore isotonicity. Cells were centrifuged again at 300g for 10min at 20°C and resuspended in 2% FCS/PBS for counting. This procedure produced mixed leukocytes composed of approximately 60% neutrophils, 1% basophils and eosinophils, 20% monocytes and 20% lymphocytes, and with a viability of >95%. Certain Western blotting experiments also used eosinophils as a positive control for LTC_4 synthase expression. The technique of eosinophil isolation was as previously described by Cowburn et al. (Cowburn et al. 1999). Briefly, eosinophils were purified from blood by Ficoll-Paque (1.077g/ml) discontinuous density gradient centrifugation and hypotonic lysis; contaminating neutrophils, T-cells, and monocytes were removed by negative immunomagnetic selection with anti-CD16, anti-CD3, and anti-CD14 magnetic beads respectively (MACS system, Miltenyi Biotech, Bisley, Surrey, UK), and contaminating platelets were removed where necessary by a low-speed centrifugation (150g). This produced eosinophils at >98% purity and >95% viability.

2.11 Immunocytochemistry

LT pathway enzymes were visualised in HBEC, HASM cells and fibroblasts by the technique of immunostaining using rabbit antisera containing polyclonal antibodies to the human enzymes (**figure 13**). To fix the cells prior to staining, coverslips were washed twice in PBS, submerged in ice-cold methanol for 10 minutes and then washed twice again in PBS. Immunostaining of cells on coverslips was analagous to the technique of staining

GMA embedded biopsies (Britten et al. 1993). To block endogenous peroxidases, coverslips were lowered onto 100µl drops of 0.3% H_2O_2 in 0.1% sodium azide on flat parafilm for 30 min at room temperature. Coverslips were then washed three times by lowering onto 100µl drops of Tris-buffered saline (TBS) for five min. To reduce non-specific binding, cells were incubated with blocking medium (Dulbecco's modified Eagle's medium, 10% FCS and 1% bovine serum albumin) for 30 min. Primary antibodies were applied for one hour at appropriate concentrations (5-LO 1:1000, FLAP 1:1000, LTA_4 hydrolase 1:1000, LTC_4 synthase 1:500). After washing, biotinylated secondary swine-anti-rabbit antibody (1:600) was applied for one hour followed by streptavidin-biotin-horseradish-peroxidase (SAB-HRP) complex (1:200) for one hour. Finally, immunostaining was visualised using the chromagen AEC that is converted by the HRP to a red product. This three step staining protocol allows amplification of the antigen signal as the large SAB-HRP complex has several sites for AEC conversion. Cell nuclei were counterstained using Meyer's haematoxylin and coverslips were mounted onto slides using Moviol gel.

Figure 13. Principle of 3-step immunostaining



Optimal antibody dilutions were found by previous titration. Several increasing dilutions of primary antibody were applied to cells and staining was performed as above. The appropriate dilution showed cell associated red staining as opposed to no visible staining or to all-over coloration, that would indicate non-specific binding. **Figure 14** shows a typical titration of LTA_4 hydrolase in 16-HBE cells. After titration, the appropriate dilution of each antibody was used in all subsequent experiments.

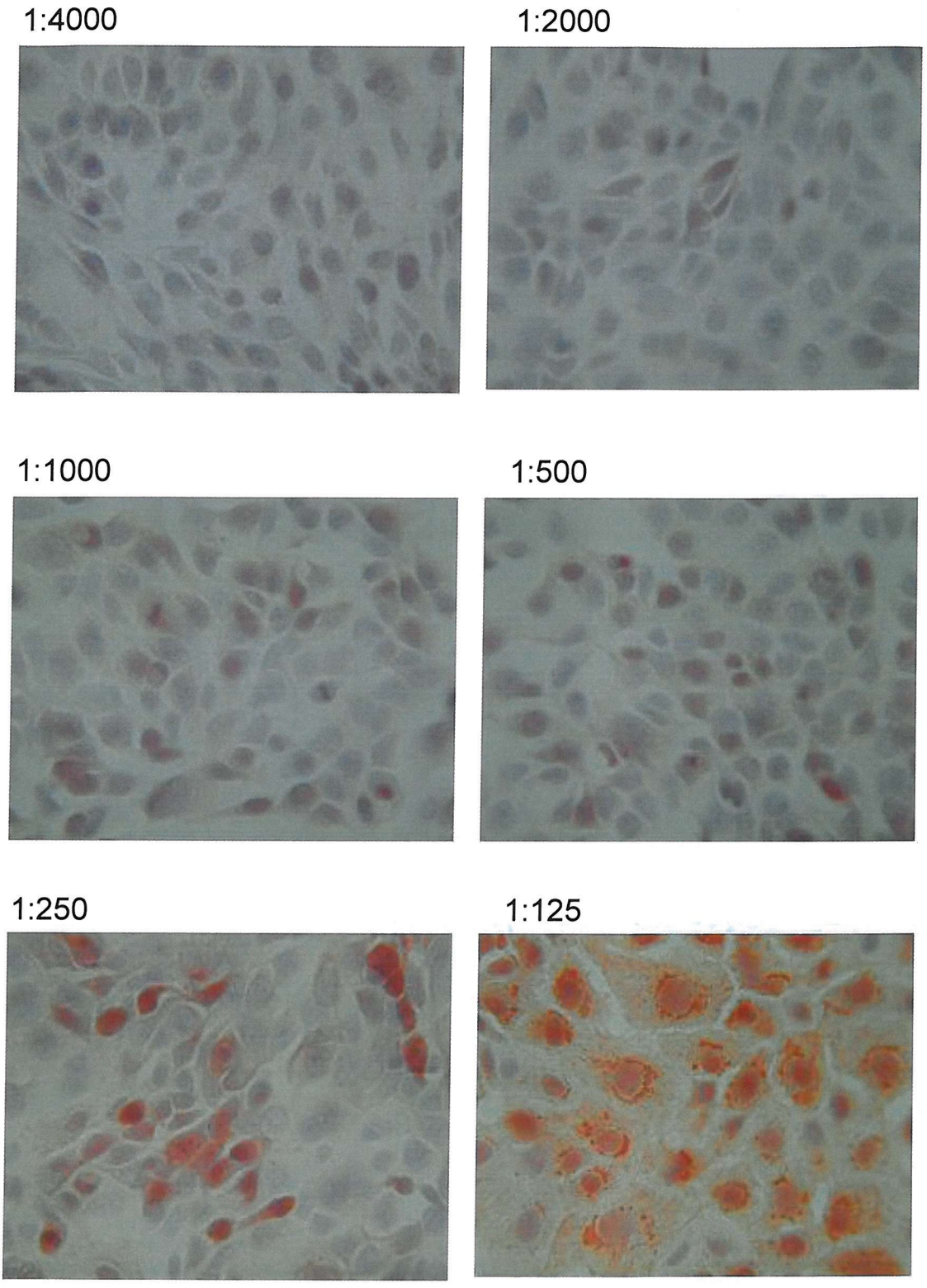


Figure 14. Photomicrographs (40x) showing a representative LTA_4 hydrolase titration in 16-HBE cells. 16-HBE cells were stained using antibody concentrations from 1:125 to 1:4000 with optimal staining shown at a concentration between 1:500 and 1:1000. Positive AEC staining is red and nuclei are stained blue with Meyer's haematoxylin.

2.12 Flow cytometry (FACS)

Fluorescence activated cell sorting (FACS) enables the quantification of cellular proteins. Cells are stained in a similar way to that described for cells on coverslips, but are suspended in solution and labelled with a fluorescent secondary antibody. For intracellular staining, cells were fixed in 4% paraformaldehyde in PBS for at least 30 minutes and then washed once in PBS before treatment with glycine buffer (0.1M in PBS) for 10 minutes to reduce possible antibody/paraformaldehyde interactions. All steps were carried out on ice. Cells were then washed twice with PBS and permeabilised in saponin-buffer for 30 minutes (PBS, 0.5% saponin & 0.5% BSA). All subsequent steps were performed in saponin buffer as the permeabilisation is reversible. Cells were adjusted to a concentration of approximately one million per ml buffer. 100µl of this suspension was placed in each FACS tube and incubated with appropriate amounts of the primary antibody for 30 minutes. **Table 8** shows antibody dilutions.

Table 8. Antibody dilutions for FACS staining

5-LO	1:40
FLAP	1:40
LTA_4hydrolase	1:40
LTC_4 synthase	1:20
$CysLT_1R$	1:100
BLT	1:40
β_2-AR	1:100
Actin	1:40
Myosin	1:40
Vimentin	1:40
Desmin	1:40
Cytokeratin	1:100

Both primary and secondary antibodies were diluted in saponin buffer plus 10% human serum to reduce non-specific binding. The cells were washed three times and incubated with the fluorescence-labelled secondary antibody for 30 minutes (1:200). Before analysis, cells were washed a further three times and suspended in 250µl saponin buffer before being passed through the flow cytometer (FACscan, Becton Dickinson) and analysed using Cellquest software (Becton Dickinson). Extracellular staining of live cells requires no fixation or permeabilisation. Cells were washed in FACS buffer (1% BSA, 0.1% sodium azide) and staining steps were carried out as for intracellular staining but in FACS buffer with 10% human serum.

The correct antibody dilutions for FACS were established by titration. A graph showing the fluorescence intensity of several increasing antibody dilutions should show three phases corresponding to insufficient antibody, correct antibody and excess antibody concentration. **Figure 15** shows a typical antibody titration. FACS analysis also requires the use of relevant isotype controls for each primary antibody to confirm specificity of the antibody of interest and to enable subtraction of background staining. The protein concentrations of both the isotype control and the primary antibody need to be known in order to be matched. These are either provided by the manufacturer or measured by protein assay.

2.13 Protein Assay

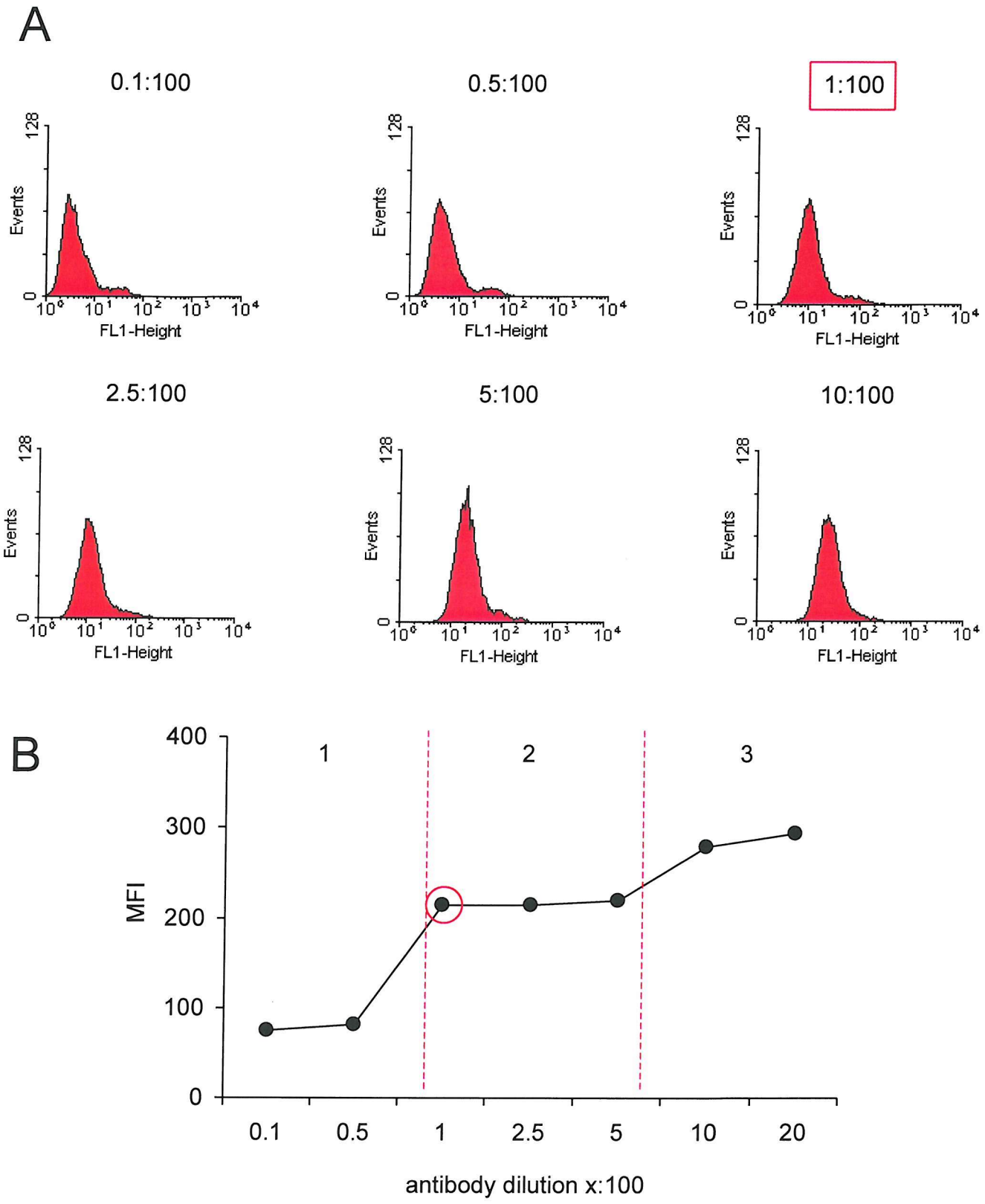
To use equivalent concentrations of antibodies and isotype controls for FACS analysis, samples of each were analysed using the Bio-Rad DC Protein Assay, following the manufacturer's protocol. Briefly, alkaline copper tartrate solution and Folin reagent was added to samples and rabbit immunoglobulin standards (0-1.5mg/ml) in a 96-well plate. Depending on protein concentration, a characteristic blue colour develops, the absorbance of which can be read at 750nM by a spectrophotometer. Antibody and isotype control concentrations are shown in **table 9,** as well as the dilution factor required to compare control staining with antibody staining.

Table 9. Protein concentrations of antibodies used

antibody	concentration	isotype control	concentration	isotype dilution factor
5-LO	~50mg/ml	rabbit serum	~100mg/ml	1:2
FLAP	~50mg/ml	rabbit serum	"	1:2
LTA_4 hydrolase	~50mg/ml	rabbit serum	"	1:2
BLT	~10mg/ml	rabbit serum	"	1:10
LTC_4 synthase	~1mg/ml	rabbit Ig	~20mg/ml	1:20
β_2-AR	0.5mg/ml	rabbit Ig	"	1:40
$CysLT_1$	~30mg/ml	goat serum	~100mg/ml	1:3
Actin	0.2mg/ml	mouse IgG_1	~20mg/ml	1:100
Myosin	"	mouse IgG_1	"	"
Desmin	"	mouse IgG_1	"	"
Vimentin	"	mouse IgG_1	"	"
Cytokeratin	"	mouse IgG_1	"	"

2.14 SDS PAGE/Western blotting

SDS-PAGE/Western blotting allows the quantification by immunostaining of small amounts of protein separated by electrophoresis on a polyacrylamide gel. The technique thus uses the specificity of a primary antibody combined with determination of molecular weight to



gure 15. CysLT$_1$R FACS titration in 16-HBE cells. A) FACS histograms showing CysLT$_1$R staining in -HBE at concentrations ranging from 0.5-10µl in 100µl. Cells were grown to confluence and detached ing non-enzymatic cell dissociation medium. B) Graph showing median fluorence intensity at the different tibody concentrations. Titration curves have three parts corresponding to (1) staining is too weak, (2) tibody concentration is correct and staining is specific and (3) antibody concentration is too high and there non-specific staining. The concentration of CysLT$_1$R antibody used (1:100) is circled.

identify and quantify the target protein. Immunoblotting for leukotriene pathway enzymes was performed essentially as described (Cowburn et al. 1999). Approximately 10^6 cells were suspended in 250µl protease inhibitor solution and sonicated on ice for three 10 second pulses at 16µm. SDS reducing buffer (0.06M Tris, 10% SDS, 10% β-mercaptoethanol, 50% glycerol, 0.05% bromophenol blue) was then added to the lysed cell suspension at a 1:3 dilution and heated at 80°C for 5 minutes. 5-LO and LTA_4 hydrolase required 10% polyacrylamide gels and FLAP and LTC_4 synthase, being smaller molecules, required 12% polyacrylamide gels. The lanes were loaded with 20-25µl of sample or 5µl molecular weight markers and the gels were run at 0.04 amps for approximately 45 minutes in BIO-RAD mini-Protean II equipment. Proteins were semi-dry transferred to nitrocellulose membrane (20 volts, 20 min) and the nitrocellulose membrane was then stored in PBS/5% dry milk powder overnight to reduce non-specific binding. For Western blotting, the antibodies were made up in PBS, 0.05% Tween non-ionic detergent and 1% BSA. Primary antibodies were applied for 90 minutes at a concentration of 1:1000 and the membrane was washed for 3 x 5 minutes in 0.05% Tween. The secondary biotinylated swine-anti-rabbit antibody was applied for 90 minutes at a concentration of 1:2000 followed by three washes. SAB-HRP complex was then applied for 1 hour at a concentration of 1:2000. Following three washes, the protein bands were visualised using the chemiluminescent substrate SuperPlus (Amersham, UK).

2.15 Primary Antibodies

The source of the primary antibodies used for immunocytochemistry, flow cytometry and Western blotting is as shown in section 2.1, Materials. The 5-lipoxygenase antisera (LO-32) is a rabbit polyclonal raised against native purified human leukocyte 5-LO which has been reported to recognise both rat and human leukocyte 5-lipoxygenases (Woods et al. 1993). FLAP rabbit antiserum (H4) was raised against the thyroglobulin conjugate of amino acid residues 41-52 of FLAP. This region is completely conserved between rat and human FLAP and the antiserum recognises FLAP in the membrane fraction of leukocytes from both of these species (Mancini et al. 1992). LTA_4 hydrolase antiserum was raised in rabbits against purified human neutrophil derived LTA_4 hydrolase and was found to recognise this enzyme in human leukocytes in bronchial biopsies, as shown by Cowburn et al. (1998). LTC_4 synthase antibody was raised against a peptide in the carboxy terminus of LTC_4 synthase and is highly specific for human LTC_4 synthase. This affinity purified rabbit polyclonal

antibody shows no cross-reactivity to FLAP, mGSTII or mouse LTC_4S (Penrose et al. 1995). Recombinant $CysLT_1$ receptor was expressed in *Escherichia coli* and the purified antigen was used to raise a goat anti-$CysLT_1$ receptor-specific antisera which has been found to recognise $CysLT_1R$ in human lung and and peripheral blood leukocytes (Figueroa 2001). The BLT1 antibody is commercially available (Cayman Chemicals) and was raised against a peptide from the C-terminus of BLT1, cloned from HL-60 human leukaemia cells. This BLT-1 antibody reacts with both human and mouse BLT-1 and is affinity-purified rabbit IgG.

2.16 RNA extraction and cDNA synthesis for RT-PCR

The polymerase chain reaction is a sensitive and rapid technique for detecting the presence of a specific DNA or cDNA. The first step is the synthesis of cDNA from mRNA that has been isolated from cells. Total mRNA was isolated from cells grown in 10 cm^2 Petri dishes using the Trizol purification method as recommended by the manufacturer (Gibco, UK). Briefly, 1ml Trizol was applied to cells and after homogenisation and addition of glycogen, samples were extracted with chloroform. After centrifugation at 13,000 rpm for 15 min at 4°C, the upper aqueous phase was collected and added to an equivalent volume of isopropanol. RNA was precipitated overnight at -20°C. The RNA pellet was recovered by centrifugation (13,000 rpm, 4°C, 15 min) and washed twice in 80% ethanol with DEPC-treated water. The pellet was allowed to air dry before resuspension in 20µl DEPC water. For first-strand cDNA synthesis, 2µg RNA was incubated for one hour at 37°C in a total volume of 20µl with DEPC treated water, 2µl 10x RT buffer, 2µl dNTP mix, 2µl oligo-dT primer (10µM), 1µl RNase inhibitor (10units/µl) and 1µl Omniscript reverse transcriptase according to the Qiagen Omniscript Reverse Transcriptase kit protocol.

2.17 Reverse transcription PCR

To amplify the specific genes of interest, RT-PCR was carried out using the following primer sequences, designed using Oligo Primer Analysis Software version 5 (National Biosciences, Inc):

5-LO	upper	5'-TAC ATC GAG TTC CCC TGC TAC-3'
	lower	5'-GTT CTT TAC GCT GGT GTT GCT-3'
FLAP	upper	5'-GAT GCG TAC CCC ACT TTC CTC-3'

	lower	5'-GAA TAT GCC AGC AAC GGA CAT-3'
LTA_4H	upper	5'-GCC CGA GAT AGT GGA TAC CTG-3'
	lower	5'-TTT TTG CTC AAA GCG ATA GGA-3'
LTC_4S	upper	5'-CTG TGC GGC CTG GTC TAC CGT 3'
	lower	5'-GGG AGG AAG TGG GCG AGC AG-3'
$CysLT_1$	upper	5'-CCG CTG CCT TTT TAG TCA GTT TC-3'
	lower	5'-GGG TAC ATA AGT CAC GCT GGA CA-3'
GAPDH	upper	5'-GCT TGT CAT CAA TGG AAA TCC-3'
	lower	5'-AGG GGA TGC TGT TCT GGA GAG-3'

PCR was performed with 2μl of reverse transcription products in a total volume of 25μl containing DEPC-treated water, 10x PCR buffer (2.5μl), minimum Mg 25mM (2.5μl), dNTP 10mM (0.5μl), jumpstart 2.5 U/μl (0.25μl) and 1μl of each upper and lower primer per tube (1.5μl GAPDH primers). Using a Peltier Thermal Cycler (PTC-225, MJ Research), thirty PCR cycles were carried out at 96°C for 5 min, 94°C for 30 sec, 58°C (for 5-LO, FLAP, and LTA_4 hydrolase), 57°C (for GAPDH, the positive control), 64°C (for LTC_4 synthase) or 64°C (for $CysLT_1R$) for 30 sec, followed by 72°C for 7 min. The different primers required different annealing temperatures to optimise binding to the DNA strand. Reaction products were separated on a 2% agarose gel and bands were visualised using ethidium bromide (0.1%). The gel was scanned using a Fluorimager 595 (Molecular Dynamics, Little Chalfont, UK) and Phoretix advanced software (Nonlinear Dynamics Ltd, Newcastle upon Tyne, UK). As a negative control, PCR was carried out in the absence of cDNA for each set of primers.

2.18 DNA Sequencing

To prove that the PCR product generated had the expected sequence, automated DNA sequencing was also performed. This technique involves the application of four different chain-terminating nucleoside triphosphates that will generate a ladder of DNA fragments when incubated with DNA polymerase and either an upper or lower primer. Analysis of the DNA fragments produced determines the nucleotide sequence of the original DNA strand. This technique firstly involves removal of unincorporated dNTPs and primers from the double stranded PCR product. dNTPs were dephosphorylated with shrimp alkaline phosphatase (SAP) while excess primers were destroyed by exonuclease I (ExoI) (AP

Biotech) in a reaction mixture containing 5µl PCR product, 1µl SAP, 0.1µl Exol in a total volume of 7µl with SAP dilution buffer (0.9µl). For the sequencing reaction, 4µl BigDye Terminator Ready Reaction Mix, 4µl sequencing buffer, 3.5µl DNA template, 3.5µl of one relevant primer (either upper or lower) and 5.3µl H_2O were added to a PCR tube and placed in a thermal cycler. Samples were heated to 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 minutes for a total of 35 cycles. Samples were then precipitated using ethanol before electrophoresis on the ABI Prism 373 with BigDye Filter Wheel to find out the DNA sequence of the original PCR product.

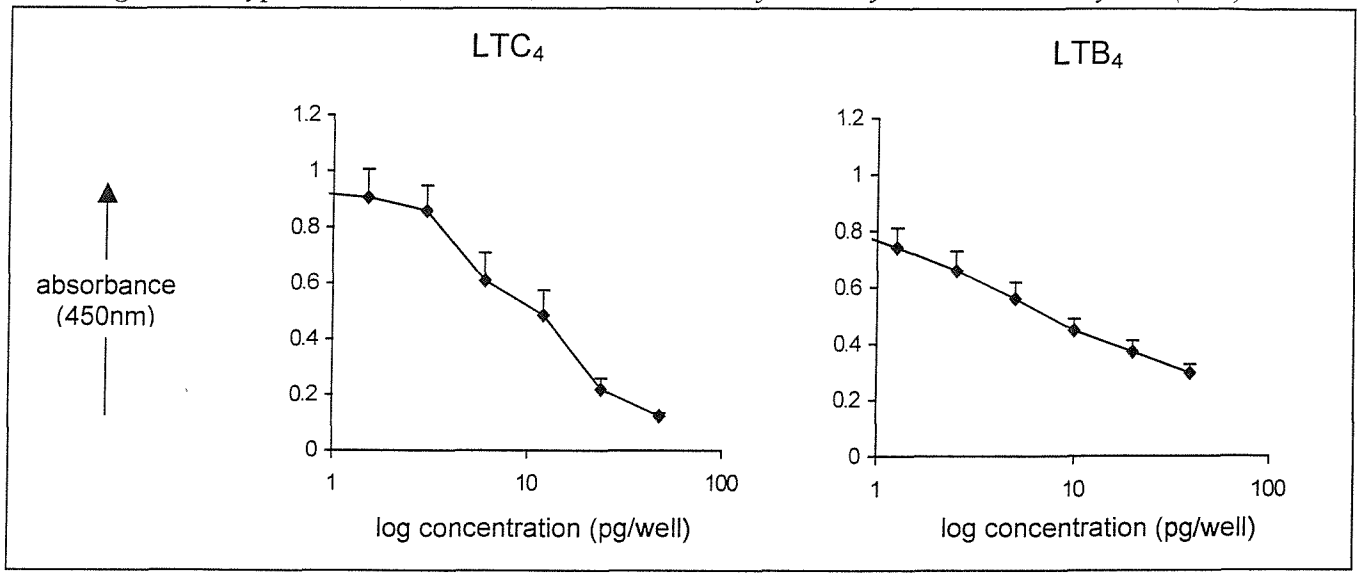
2.19 Enzyme immunoassay (EIA)

Cell culture supernatants were stored at -20°C prior to assay of LT concentrations using Biotrak $LTC_4/D_4/E_4$ and LTB_4 EIA kits in accordance with the manufacturer's instructions. The assays are based on competition between unlabelled leukotriene and a fixed amount of peroxidase-labelled leukotriene for binding to a specific polyclonal antibody. The cys-LT EIA was calibrated with standard LTC_4 from 0.75-48 pg/well (**figure 16**), and used a polyclonal antiserum that cross-reacts with LTC_4 (100%), LTD_4 (100%), LTE_4 (70%) and their 11-trans isomers, but negligibly with LTB_4 (0.3%), prostaglandin $PGF_{2\alpha}$, PGD_2, PGE_2, 6-keto-$PGF_{1\alpha}$, TXB_2 or glutathione (<0.006%). In the cys-LT EIA kit, within-assay variability for duplicate samples was approximately 5% for 12 pg/well LTC_4 and 9% for 1.6pg/well LTC_4. Between-assay variability was approximately 6% for 10.5pg/well and 18% for 1pg/well LTC_4. The LTB_4 EIA was calibrated with standard LTB_4 from 0.30-40 pg/well (**figure 16**) and uses a polyclonal antiserum that cross-reacts with LTB_4 (100%), 20-OH-LTB_4 (2%) and 6-trans-LTB_4 (25.5%), but negligibly with LTC_4, LTD_4, and 12-LO and 15-LO products. For the LTB_4 EIA kit, within-assay variability was approximately 7% for 29pg/well LTB_4 and 9% for 1pg/well LTB_4. Between-assay variability was approximately 6% for 10pg/well and 12% for 0.5pg/well LTB_4. LT levels are quantified as pg/well and expressed as pg per million cells.

To achieve results as accurately as possible from enzyme-immunoasssay kits foetal calf serum was not present in the supernatants being tested. Initial experiments showed that RPMI medium containing 10% FCS had background levels of LTB_4 immunoreactivity. Instead, during experiments when culture medium was collected, foetal calf serum replacements such as UltroserG were used which reduced FCS interference by

approximately 10 fold. Also, to correct for the possibility of non-specific interactions, all enzyme-immunoassay standard curves were made up in the same medium as that in which the cells had been incubated. Experiments were designed such that levels of both LTC_4 and LTB_4 consistently fell within the middle of the standard curves. For measurement of cys-LTs this involved a 10-fold concentration of samples by centrifugation and evaporation to dryness *in vacuo* and resuspension in PBS. It should be recognised that all EIA data in this thesis represent LT-like immunoreactivity, as LTB_4 and the cys-LTs were not identified by other techniques such as mass spectroscopy.

Figure 16. Typical LTC_4 and LTB_4 standard curves from enzyme-immunoassay kits (n=5).



2.20 Methylene Blue Proliferation Assay

To measure levels of cell proliferation in response to stimulation with different agents, a simple type of proliferation assay was performed using methylene blue dye, as previously described (Richter et al. 2001). Initially, cells were placed in 24 well plates at a density of $2.5x10^4$ in 0.5ml culture medium with 10% FCS and allowed to adhere overnight. At t=0, the culture medium was removed and replaced with medium containing the mediators being tested but without FCS (to reduce background proliferation). After incubation for the relevant length of time, the medium was aspirated and cells were fixed using 4% formaldehyde in saline for 30 minutes. After removal of fixative, cells were stained using a filtered solution of 1% methylene blue in borate buffer (disodium tetraborate 0.01M, pH 8.5) for 30 minutes. The plate was thoroughly washed with borate buffer and the methylene blue was eluted with 1:1 solution of ethanol and 0.1M HCl. After shaking the plates were read at 630nm using a spectrophotometer. The higher the absorbance at 630nm, the more cells were

present in the well. In order to estimate how many cells are present from the absorbance levels, standard curves were made for each cell type by placing known numbers of cells in the 24 well plate, allowing them to adhere (but not proliferate) and then staining them in the way described above. Such standard curves are shown in **figure 17**.

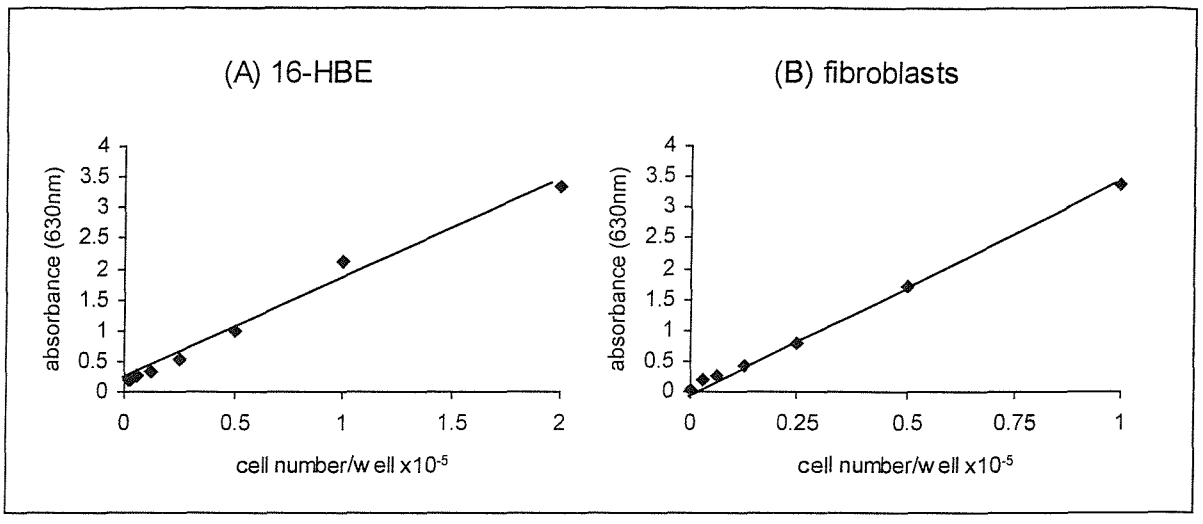


Figure 17. Methylene blue proliferation assay standard curves for 16-HBE and fibroblasts.

2.21 Statistical analyses

As all datasets in this thesis are n<30, Normality cannot be assumed and comparisons between groups were performed by non-parametric analyses, principally Wilcoxon's signed rank test for paired data and the Mann-Whitney U-test for unpaired data. p<=0.05 was accepted as significant. Where the data distribution was not obviously skewed, additional Student's t-tests were performed to explore significant differences emerging from non-parametric analyses. The Figures show individual data points with median bars, but in some Figures with multiple histogram bars, mean and SEM values may be shown for clarity. All analyses were carried out using the Minitab statistical software package (Minitab Inc, State Co., PA, USA).

FACS data may be expressed as percent positive cells i.e. the percentage of cells with a fluorescence intensity greater than the isotype control, or as median fluorescence intensity (MFI). In this study MFI is used for comparisons of data as it is a more sensitive way of detecting changes in the level of protein expression.

3. Results

The leukotriene pathway in human bronchial epithelial cells

To test the hypothesis that human bronchial epithelial cells may express 5-LO pathway enzymes and produce leukotrienes, a number of experiments were carried out using the HBE cell lines 16-HBE and H292, with critical experiments also extended to primary cells. The expression of 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase was examined both at baseline and after stimulation with Ca ionophore, histamine, bradykinin, bacterial LPS, pro-inflammatory cytokines and anti-asthma drugs, using techniques including flow cytometry, immunocytochemistry, Western blotting and RT-PCR. As well as enzyme expression, LTC_4 and LTB_4 production was examined by enzyme immunoassay. Finally, a possible role for leukotrienes in the proliferation of 16-HBE cells was examined.

3.1 Baseline expression of LT pathway proteins in 16-HBE and H292 cell lines

At baseline, mRNA for all four 5-LO pathway enzymes was detected by RT-PCR in 16-HBE cell lysates, with amplicons being generated that were consistent with 5-LO (124 bp), FLAP (210 bp), LTA_4 hydrolase (291 bp), and LTC_4 synthase (155 bp) (**Figure 18a**). Negative control experiments carried out in the absence of cDNA showed no bands, and GAPDH positive controls were consistent between experiments. As a further positive control, LTC_4 synthase mRNA was also investigated by RT-PCR in the KU812F basophilic cell line; LTC_4 synthase mRNA bands in 16-HBE cells and KU812F cells were comparable in density. As a further negative control, LTC_4 synthase mRNA was shown to be absent in HeLa cells (**Figure 18a**). DNA sequencing of the LTC_4 synthase mRNA amplicon was also carried out and confirmed that the sequence of basepairs generated corresponded with that predicted for LTC_4 synthase.

SDS-PAGE/Western blotting of lysates from 16-HBE cells and mixed leukocytes showed immunopositive bands for 5-LO (~80 kDa), FLAP (~18 kDa), LTA_4 hydrolase (~70kDa) and LTC_4 synthase (~19 kDa) proteins (**Figure 18b**). Each lane represents equivalent numbers of cells, indicating that the 16-HBE cells expressed quantitatively more 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase per cell than the mixed leukocytes. In identical experiments in which equal amounts of cellular protein were added to each lane, the density of the bands for all four proteins in 16-HBE cells was relatively lower, but remained comparable to those in mixed leukocytes (**Figure 18c**). The difference from figure 18B may reflect the larger size of 16-HBE cells compared to mixed leukocytes. The bands for 5-LO and FLAP co-migrated with purified recombinant human 5-LO (Denis et al. 1991) and insect Sf9 cell membranes expressing high levels of human FLAP respectively (Mancini et

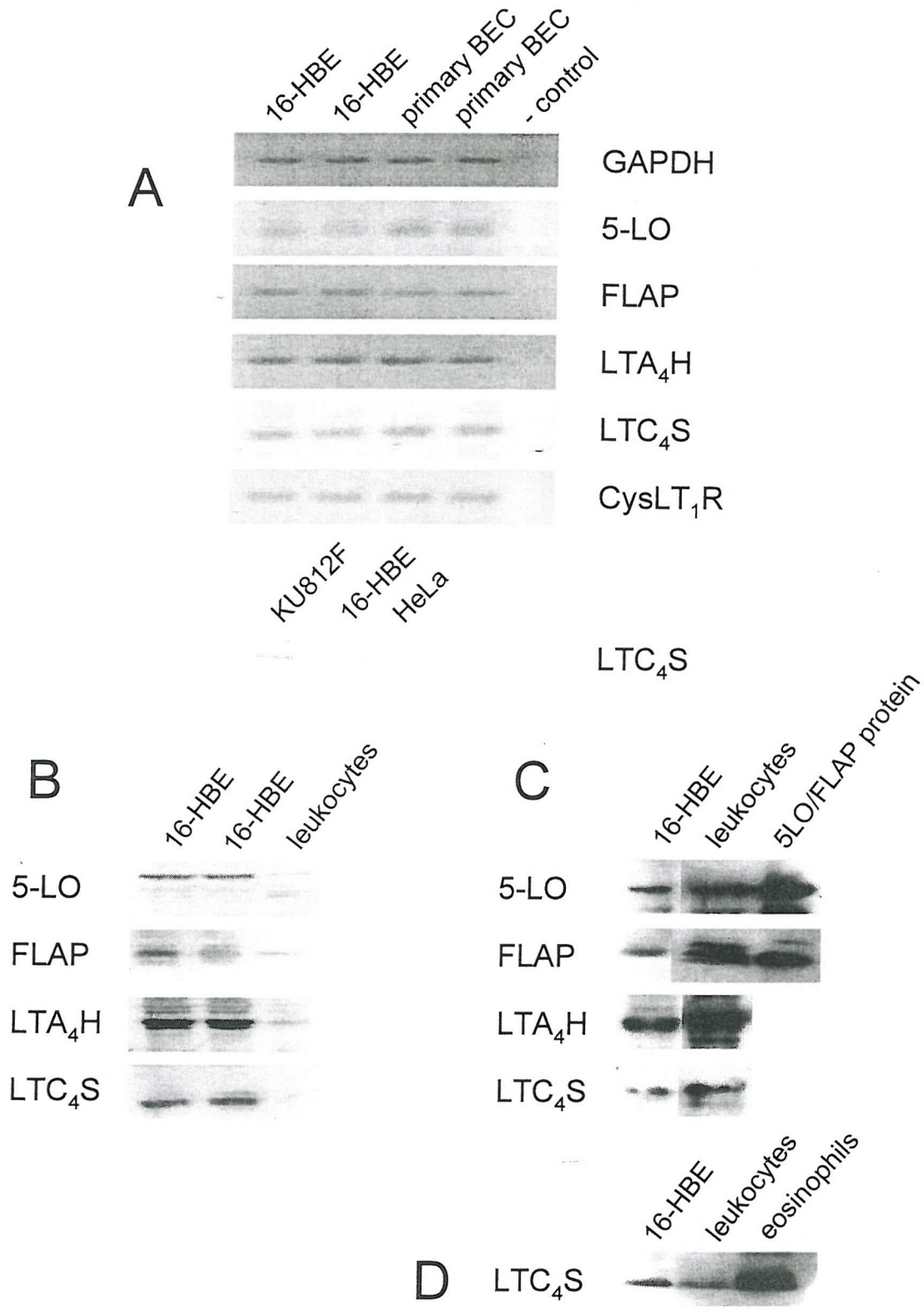


Figure 18. Expression of mRNA and protein for 5-LO pathway enzymes in HBE cells. (A) RT-PCR analysis of 5-LO, FLAP, LTA_4 hydrolase, LTC_4 synthase and $CysLT_1R$ mRNA in 16-HBE cells and primary BEC. Total RNA was isolated and RT-PCR was performed using gene specific primers. GAPDH was the internal positive control and as a negative control, PCR was carried out in the absence of RT products. LTC_4S mRNA was also present in the basophilic KU812 cell line (+ control) but not in HeLa cells (- control). (B&C) SDS-PAGE/Western blotting experiments show immunoreactive bands for 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase at approximately 80, 18, 70 and 18 kDa respectively. Lysates containing equal cell numbers (B) or equal amounts of cell protein (C) were resolved at 0.04A for 45 min on 10% polyacrylamide gels for 5-LO and LTA_4 hydrolase and 12% gels for FLAP and LTC_4 synthase. Mixed leukocyte lysates were used as positive controls for 5-LO pathway enzyme expression and (D) eosinophils were used as a further positive control for LTC_4S expression.

al. 1993). To provide an additional positive control for LTC_4 synthase, 16-HBE cells were compared to immunomagnetically-purified normal human blood eosinophils (>98% pure) (**Figure 18d**). When corrected for equal amounts of cellular protein, 16-HBE cells expressed less LTC_4 synthase compared to eosinophils, but comparable amounts to mixed leukocytes.

FACS analysis of leukotriene pathway enzyme expression in 16-HBE and H292 cells showed constitutive expression of 5-LO, FLAP and LTA_4 hydrolase in both cell lines. LTC_4 synthase immunofluorescence was low in 16-HBE cells and undetectable in H292 cells (**Table 10, Figure 19**). In H292 cells, FLAP and LTA_4 hydrolase immunofluorescence was approximately three times greater than that of 5-LO ($p=0.03$ and $p=0.01$ respectively, $n=5$). Baseline 5-LO expression was also strikingly low in H292 cells when compared to 16-HBE cells ($p=0.05$, $n=5$). Levels of 5-LO, FLAP and LTA_4 hydrolase immunofluorescence were all significantly greater than LTC_4 synthase in 16-HBE cells ($p=0.03$, $p=0.03$ and $p=0.03$ respectively).

Table 10. Baseline expression of LT pathway enzymes in HBE cell lines

	5-LO	FLAP	LTA_4 hydrolase	LTC_4 synthase
16-HBE	9.2±2.2	9.5±1.3	10.4±1.4	0.6±0.2
H292	2.9±0.9	8.9±1.1	7.8±1.2	ND

units = MFI, mean±SEM, ND=not detectable

In addition to 5-LO pathway enzymes, the spontaneous expression of the LTB_4 receptor (BLT) and the cys-LT receptor, $CysLT_1$ was examined. RT-PCR results showed that both 16-HBE and primary BEC express $CysLT_1$ mRNA (**Figure 18a**). Immunocytochemistry and FACS analysis confirmed constitutive $CysLT_1R$ expression in 16-HBE and primary BEC (**Figure 20**). However, expression of the LTB_4 receptor was not detectable by flow cytometry in 16-HBE cells. FACS titrations were carried out using two BLT antibodies, one polyclonal and one monoclonal, but neither showed any positive staining above that of the relevant isotype controls at any of the dilutions tested (**Figure 21**). As a positive control, FACS analysis showed abundant BLT expression in neutrophils (MFI 10.35), (**Figure 21**).

3.2 The effect of calcium ionophore A23187 on 5-LO pathway enzyme expression and leukotriene production in HBE cells

The regulation of 5-LO pathway enzymes in 16-HBE cells was investigated initially by immunocytochemistry in cells treated with or without calcium ionophore A23187. Cells

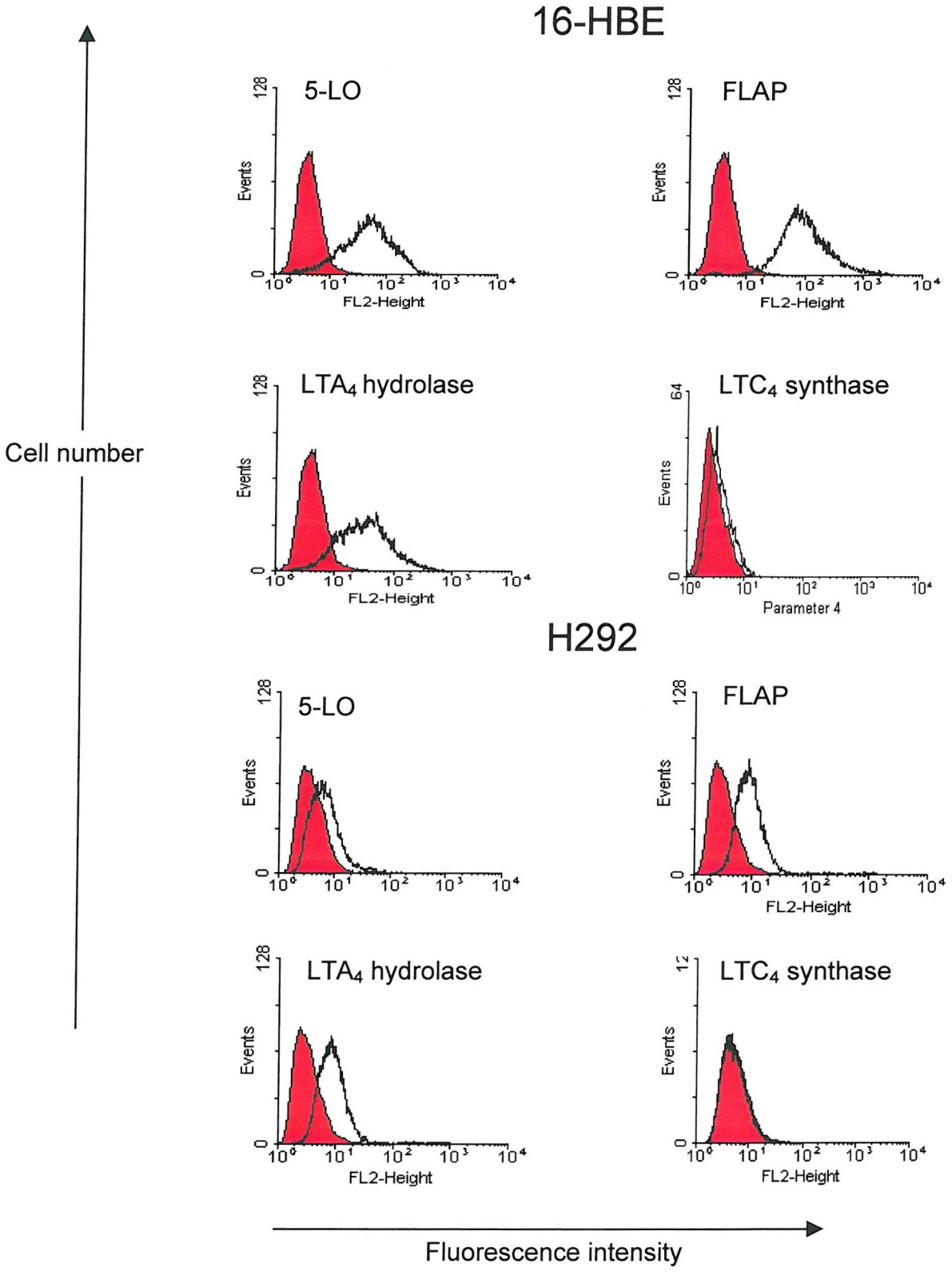


Figure 19. FACS histograms showing baseline LT pathway enzyme expression in 16-HBE and H292. 16-HBE and H292 cells were fixed, permeabilised and stained for 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase. Filled graphs show fluorescence intensity of relevant isotype control and open graphs show specific antibody staining. Positive 5-LO, FLAP and LTA_4 hydrolase staining was observed in both 16-HBE and H292. LTC_4synthase staining was low in 16-HBE and not different to isotype control in H292 cells.

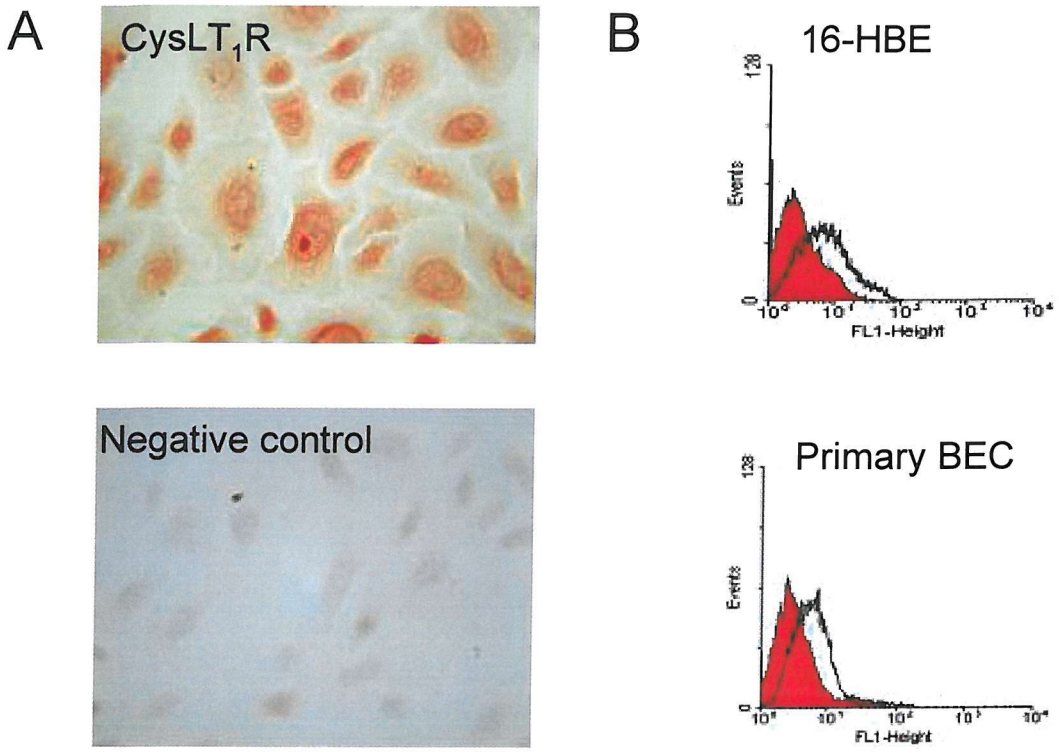


Figure 20. Representative photomicrographs and FACS histagrams showing $CysLT_1$ staining in 16-HBE and primary bronchial epithelial cells. (A) 40X photomicrographs showing $CysLT_1R$ and negative control in primary HBE cells. Positive AEC staining is red against Meyer's haematoxylin nuclear counterstain. (B) FACS plots. Live 16-HBE and primary HBE cells were stained for $CysLT_1R$. Filled curves show staining with isotype control and open curves show $CysLT_1$ staining.

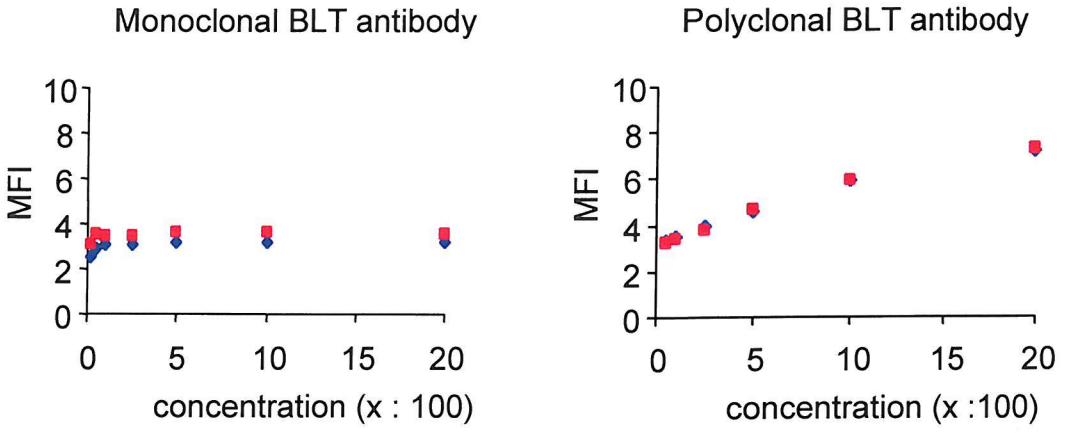


Figure 21. FACS titrations of BLT antibodies in 16-HBE cells. 16-HBE cells were fixed, permeabilised and stained using two different BLT antibodies at dilutions from 1:400 to 1:20. No positive BLT staining (■) was measured above that of the relevant isotype controls (♦), at any of the concentrations tested.

were cultured up to 12 hours in the presence or absence of A23187 (2μM), or for 6 hours with 0-2 μM A23187, then immunostained for the LT pathway enzymes (**Figure 22**). The percentages of cells immunopositive for 5-LO, FLAP and LTC_4 synthase (but not LTA_4 hydrolase) increased significantly in both a time- and dose-dependent manner in response to A23187. For statistical analysis, ANOVA was used to compare all A23187 stimulated time points with all corresponding unstimulated time points in the time course (5-LO $p=0.001$, FLAP $p=0.006$, LTC_4 synthase $p=0.06$). Although ANOVA did not show a general time dependent increase in the number of cells expressing LTA_4 hydrolase, it appears that at the 12 hour time point alone, A23187 had a stimulatory effect on this enzyme ($p=0.07$). After 12 hours, there were more ionophore stimulated cells expressing LTA_4 hydrolase (31.4±6.5) than unstimulated cells (14.2±2.6). For the dose response curve, all A23187 stimulated values were compared to control by ANOVA (5-LO $p<0.01$, FLAP $p<0.01$ and LTC_4 synthase $p=0.01$, $n=5$). The vehicle control (DMSO) had no significant effect on LT pathway enzyme expression in 16-HBE cells. Representative photomicrographs illustrating immunocytochemical expression of 5-LO and FLAP in 16-HBE cells cultured with or without A23187 (1μM) for 6 hours are shown in **Figure 23**.

The effect of calcium ionophore A23187 in combination with PMA, a phorbol ester that mimics the endogenous protein kinase C activator, diacylglycerol (DAG) was also examined by immunocytochemistry in 16-HBE cells (**Figure 24**). 16-HBE cells were incubated with A23187 (1μM) and PMA (50nM) on coverslips for 6 hours and immunostained for LT pathway enzymes as above. The percentage of cells staining positively for 5-LO (3.1±1.2), FLAP (2.6±0.9) and LTA_4 hydrolase (10.4±2.7), was significantly increased by stimulation to 14.3±4.6, 8.4±2.0 and 19.2±3.0 ($p=0.05$, $p=0.04$ and $p=0.02$ respectively). Levels of LTC_4 synthase were unchanged (0.9±0.5 vs 0.7±0.5, $p=0.8$).

The effect of A23187 on enzyme expression was confirmed by FACS analysis in 16-HBE and H292 cells (**Figures 25&26**). Consistent with the immunocytochemistry results, 5-LO immunofluorescence was significantly increased by stimulation of 16-HBE cells with A23187 (1μM) for 6 hr from 15.1±4.7 to 24.0±7.6 ($p=0.05$), and FLAP was increased from 10.8±1.9 to 18.5±4.4 ($p=0.04$, $n=6$). In contrast to the immunocytochemistry data, A23187 also significantly increased LTA_4 hydrolase immnuofluorescence from 9.7±1.8 to 17.3±4.3

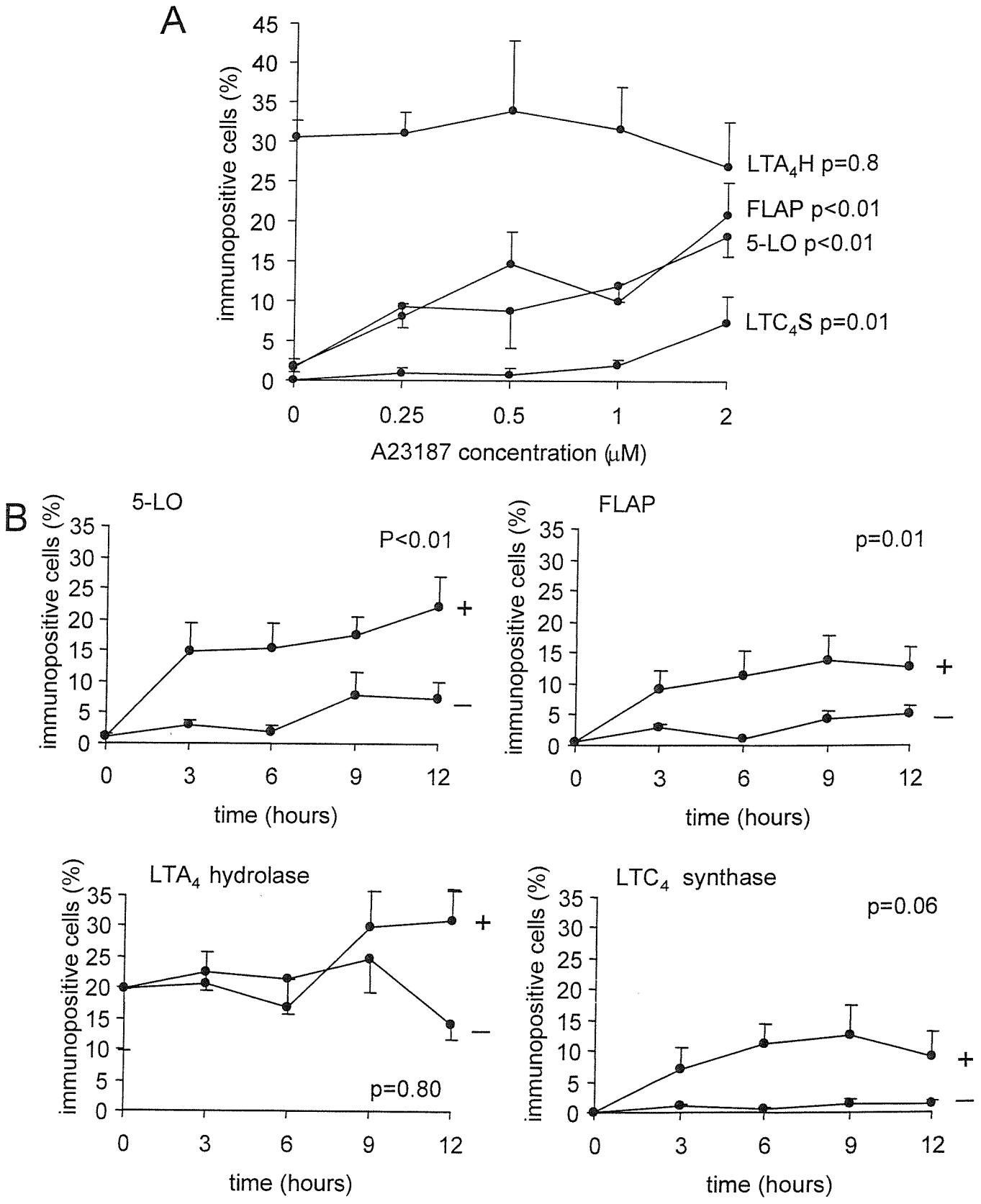


Figure 22. Effect of Ca ionophore A23187 on 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase immunostaining in 16-HBE cells. 16-HBE cells were incubated for A) 6 hours in the presence or absence of A23187 (0-2μM) or B) for 0-12 hours with (+) or without (-) 2μM A23187, and immunostained for 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase. Positive cells were counted by light microscopy by a blinded observer. Values are mean +/- SEM (n=5). A23187 stimulation significantly increased the proportion of cells immunostaining for 5-LO, FLAP and LTC_4 synthase in a dose- and time-dependent manner (ANOVA), but had no effect on the high basal levels of LTA_4 hydrolase expression.

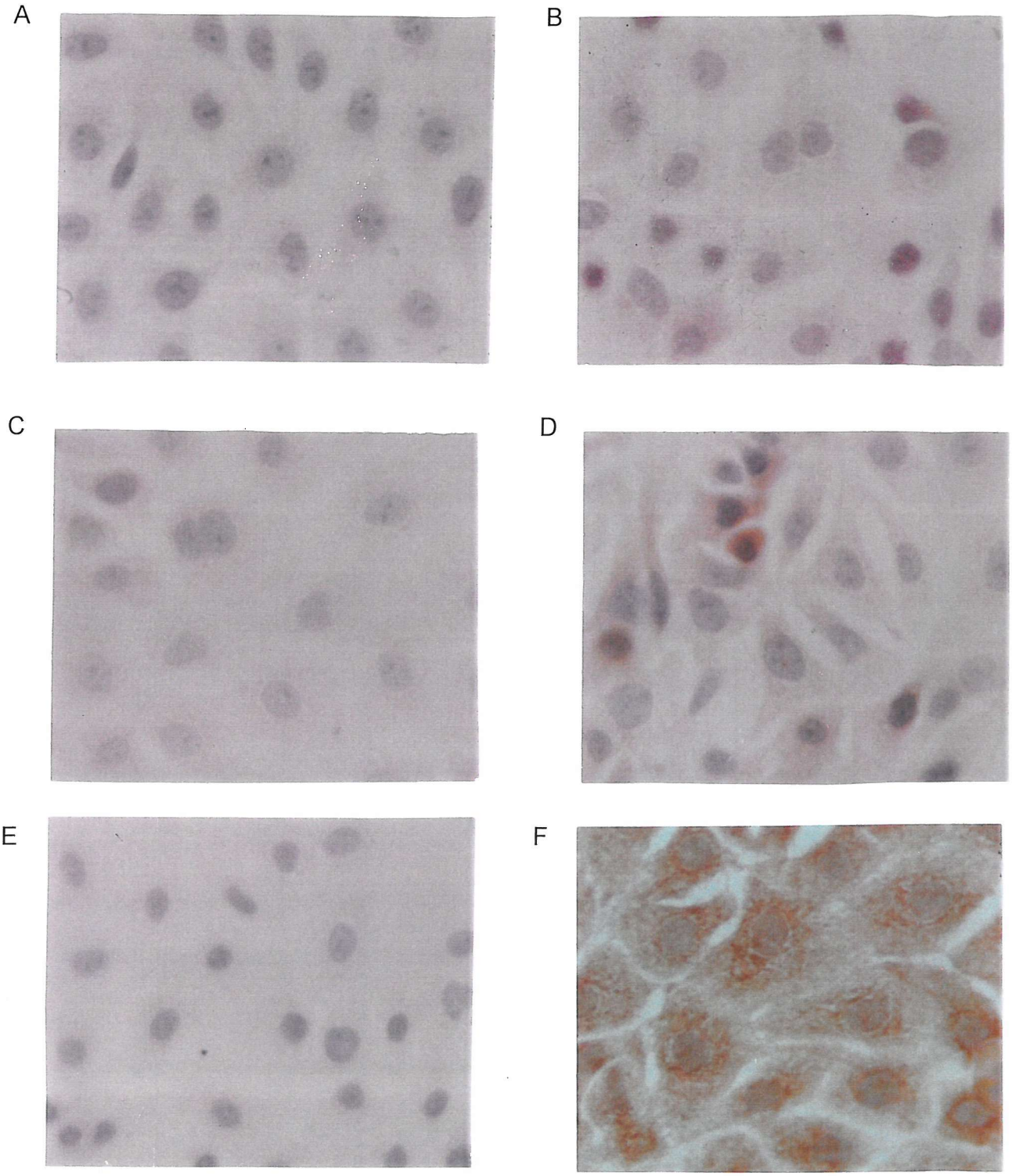


Figure 23. Effect of Ca ionophore A23187 on 5-LO and FLAP immunoreactivity in 16-HBE cells. Representative photomicrographs (40x) show 5-LO and FLAP immunostaining in 16-HBE cells grown on coverslips, in the presence or absence of A23187 (1μM, 6h). Staining is shown for A) 5-LO control, B) 5-LO+A23187, C) FLAP control, D) FLAP+A23187, E) negative control (irrelevant primary antibody) and F) cytokeratin-18 positive control. Positive AEC staining is red and cells were counterstained with Meyer's haematoxylin.

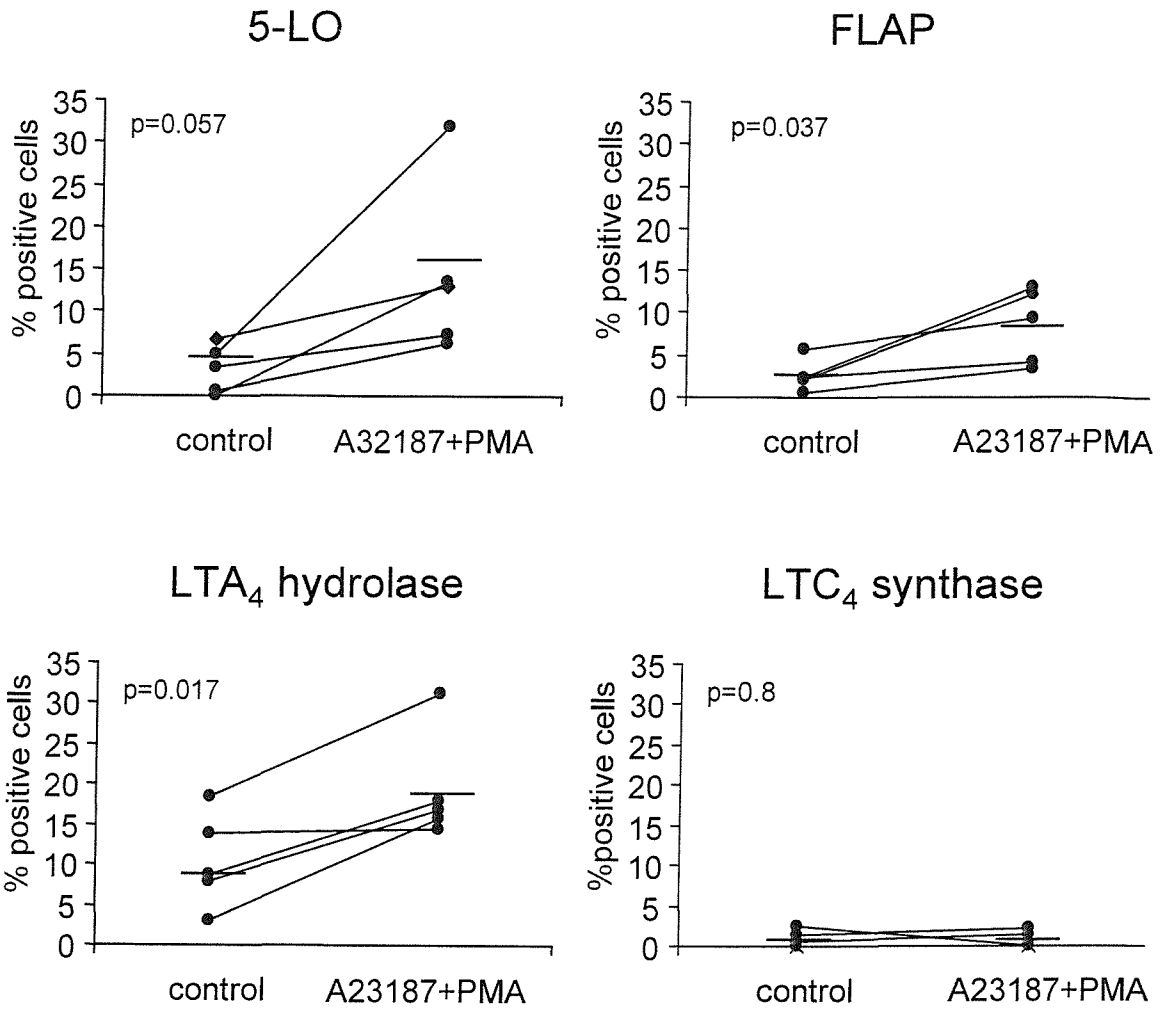


Figure 24. Immunocytochemical analysis of the effect of PMA and A23187 on 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase expression in 16-HBE cells. The effects of PMA (50nM) and Ca ionophore A23187 (1μM) on LT pathway enzyme expression was examined in 16-HBE cells incubated for 6h. Cells were immnuostained for 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase and counted by light microscopy by a blinded observer. Results are shown as individual data points with median bars. The proportion of cells immunostaining positive for 5-LO, FLAP and LTA_4 hydrolase was increased by PMA+A23187 but no effect was seen on LTC_4 synthase expression (Wilcoxon's signed rank test, n=5).

1.3±0.3 (p=0.04, n=4) (**figure 25**). Similar FACS data were obtained in H292 cells (**Figure 26**). Treatment with A23187 (1μM) for 6 hr significantly increased 5-LO immunofluorescence from 3.4±1.3 to 9.7±3.6 (p=0.05), FLAP from 8.1±2.1 to 16.7±4.69 (p=0.04), and LTA_4 hydrolase from 8.1±1.9 to 17.3±4.9 (p=0.04, n=5). However, LTC_4 synthase expression was not detectable by FACS in H292 cells at baseline or after A23187 stimulation. Control and stimulated MFI levels of 5-LO expression were lower in H292 cells than in 16-HBE cells (control H292 MFI 3.4±1.3, 16-HBE 15.1± 4.7, p=0.03, stimulated H292 MFI 9.6± 3.6, 16-HBE 24.0± 7.6, p=0.06), while FLAP and LTA_4 hydrolase immnuofluorescence were broadly comparable between the two cell types.

The capacity of 16-HBE cells to produce LTC_4 and LTB_4 when incubated with or without A23187 (1μM) for 6 hr was examined by EIA of cell supernatants. LT-like immunoreactivity was detectable after 6 hr in 16-HBE cells. Total cys-LT immunoreactivity rose from 27.1±1.9 in unstimulated cells to 55.3±13.4 pg/10^6 in cells treated with A23187 (p=0.05, n=10), and LTB_4 immunoreactivity rose from 119±12.8 to 148±17.5 pg/10^6 cells (p=0.02, n=5) (**Figure 27**).

3.3 FACS analysis of the effects of bradykinin, histamine, and bacterial LPS on 5-LO pathway enzyme expression in 16-HBE cells

5-LO pathway enzyme expression was assessed by FACS in 16-HBE cells incubated for 24 hours with the autacoids bradykinin (0-10μM) and histamine (0-100 μM), or with the bacterial toxin LPS (0-2 μg/ml), three common physiological stimuli of BEC.

Compared to control values, 5-LO expression assessed by FACS analysis was not significantly changed by any concentration of LPS up to 2μg/ml. FLAP expression (MFI) was significantly increased by 1μg/ml LPS from 15.8±4.0 to 30.0±7.0 (p=0.036, n=6) and LTA_4 hydrolase was significantly increased by 2μg/ml LPS from 12.4±3.4 to 18.7±4.9 (p=0.036, n=6) (**Figure 28**). LTC_4 synthase expression remained low, both at control levels and following stimulation with LPS.

Compared to control values, the MFI of 5-LO was significantly increased by 0.1μM and 10μM bradykinin (control 11.4±2.9 vs 0.1μM BK 15.8±2.4, p=0.046 and control 11.4±2.9 vs 10 μM BK 16.5±3.2, p=0.003, respectively, n=6) (**figure 29**). Bradykinin had no effect

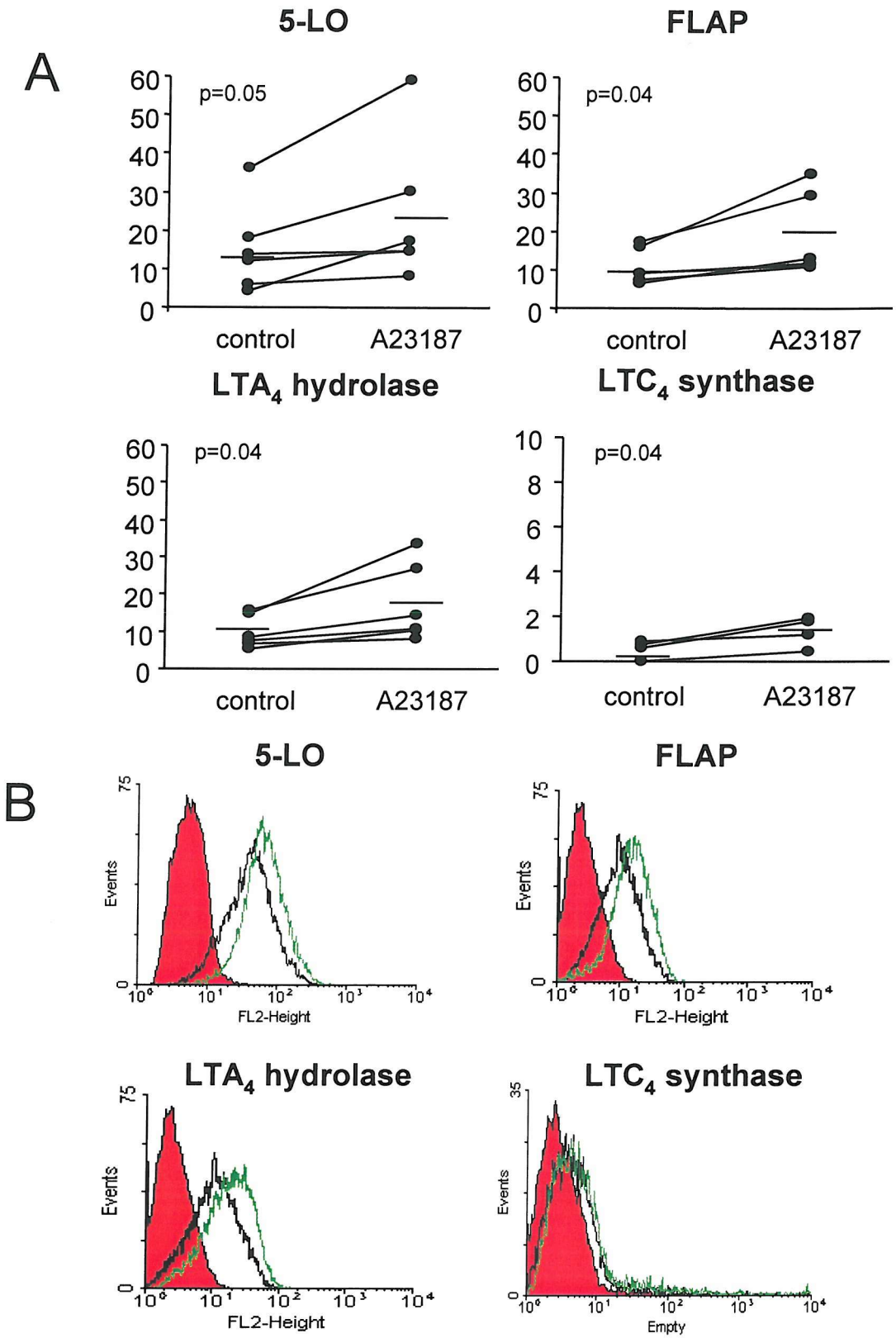


Figure 25. FACS analyses showing the effect of Ca ionophore A23187 on 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase expression in 16-HBE cells. Cells were cultured for 6 hours in the presence or absence of A23187 (1μM), fixed, permeabilised and stained for 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase as described in methods. Panel (A) shows that the median fluorescence intensities of 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase were significantly increased by A23187 stimulation (p=0.05, p=0.04, p=0.04 and p=0.04 respectively, n=6). Panel (B) shows representative FACS histograms (red graph=isotype control, black=control, green=+A23187). Results are presented as individual data points with median bars and analysed using Wilcoxon's signed rank test.

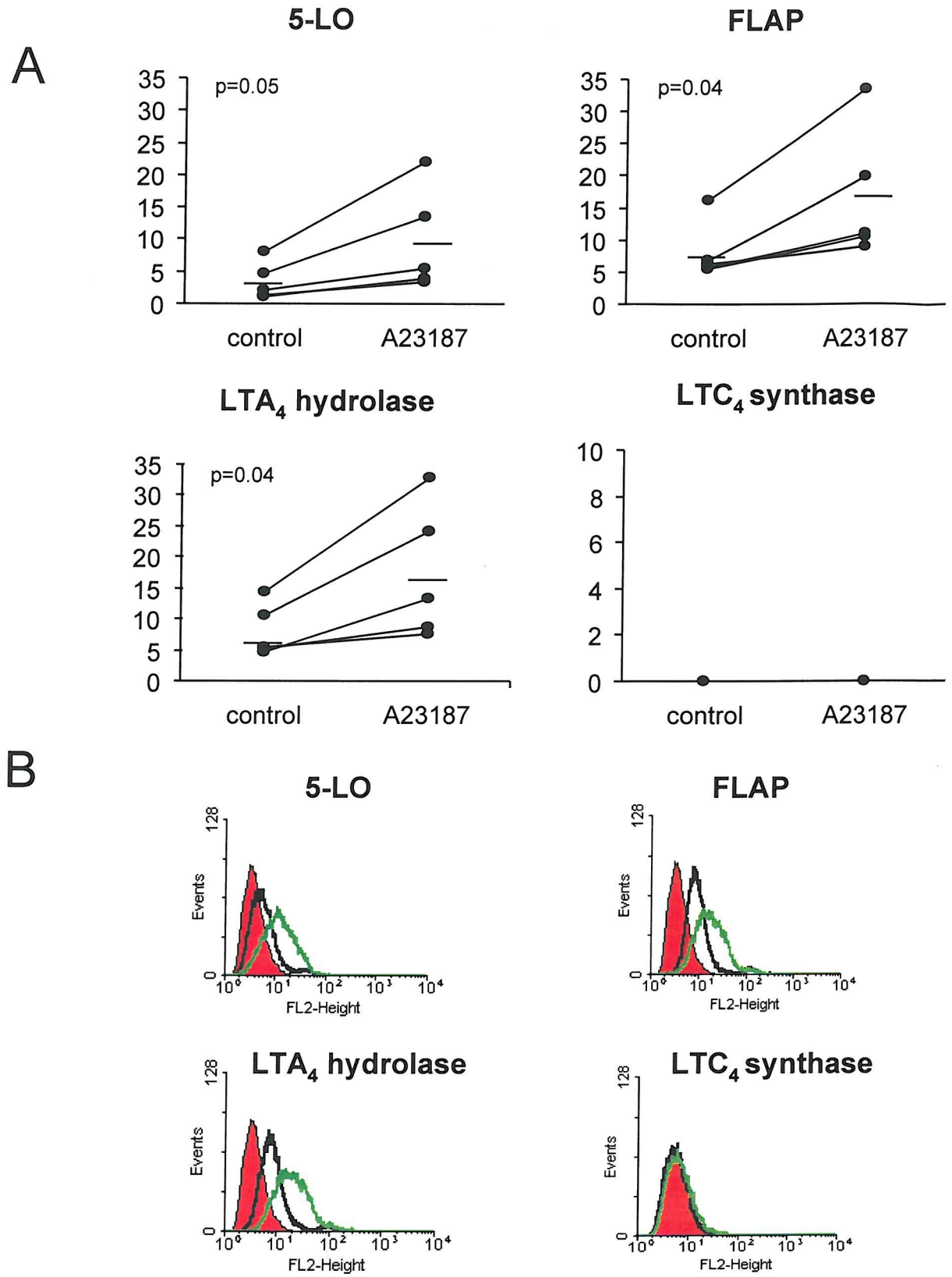


Figure 26. FACS analyses showing the effect of Ca ionophore A23187 on 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase expression in H292 cells. Cells were cultured for 6 hours in the presence or absence of A23187 (1μM), fixed, permeabilised and stained for 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase as described in methods. Panel (A) shows that the median fluorescence intensities of 5-LO, FLAP and LTA_4 hydrolase were significantly increased by A23187 stimulation (p=0.05, p=0.04, and p=0.04 respectively, n=6). Panel (B) shows representative FACS histograms (red graph=isotype control, black=control, green=+A23187). Results are presented as individual data points with median bars and were analysed using Wilcoxon's signed rank test. LTC_4 synthase immunofluorescence was not detectable in H292 cells.

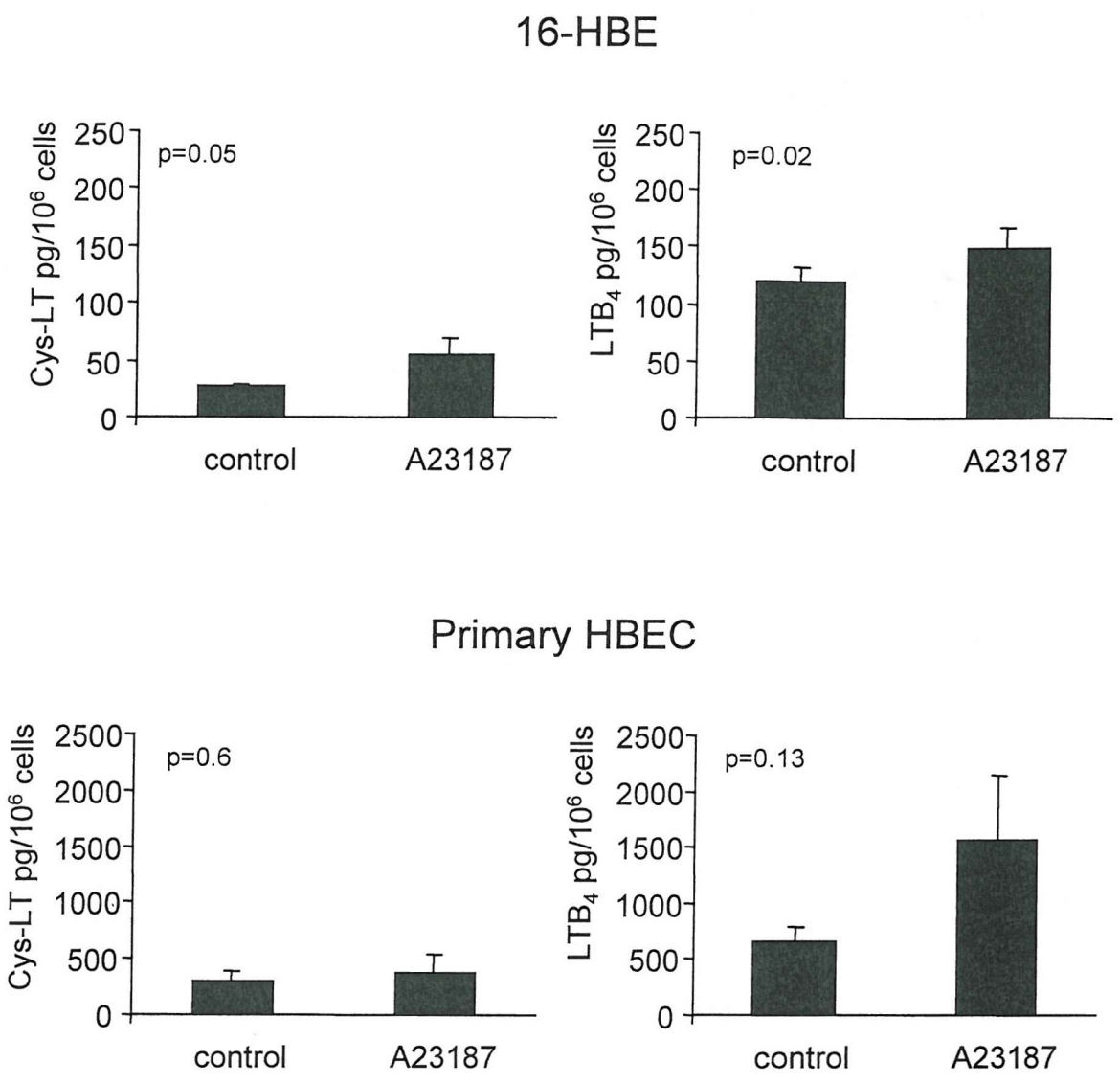


Figure 27. Effect of Ca ionophore A23187 on cys-LT and LTB_4 release in 16-HBE and primary BEC. Total cys-LTs (LTC_4,LTD_4 and LTE_4) and LTB_4 were measured by enzyme immunoassay of 16-HBE and primary HBEC cell supernatants incubated for 6 hours in the absence or presence of Ca ionophore (1μM) for 6 hours. Leukotriene production (mean+SEM) is expressed as pg/million cells. Unstimulated 16-HBE and primary HBE cells produced detectable amounts of both cys-LTs and LTB_4. Incubation with Ca ionophore significantly increased cys-LT and LTB_4 production in 16-HBE cells (p=0.05, n=10 and p=0.02, n=5 respectively, paired t-test) but did not significantly change either cys-LT or LTB_4 production in primary BEC (n=7). Production of LTC_4 and LTB_4 is approximately 10 times greater in primary HBEC than in 16-HBE (LTC_4 p=0.006 and LTB_4 p=0.005), as indicated by the different y-axis ranges.

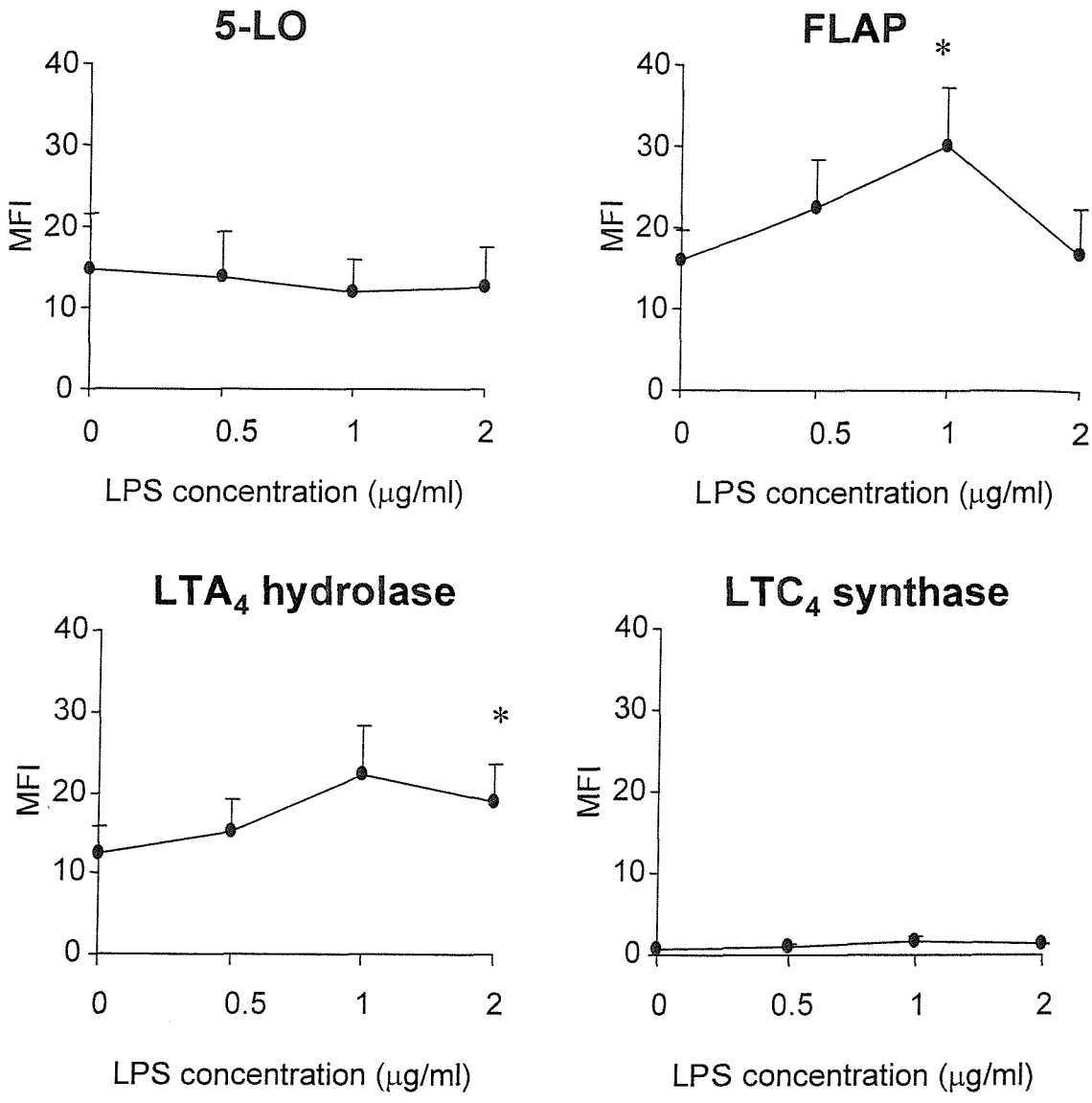


Figure 28. FACS analyses showing the effect of bacterial lipopolysachharide (LPS) on 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase in 16-HBE cells. 16-HBE cells were incubated for 24 hours with LPS (0-2μg/ml), fixed, permeabilised and stained for 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase as described in Methods. Results (mean+SEM) are expressed as median fluorescence intensity and were compared using Student's paired t-test (n=7). Compared to control, LPS had no significant effect on the expression of 5-LO and LTC_4 synthase but significantly increased the expression of FLAP and LTA_4 hydrolase at 1 and 2 μg/ml respectively (*$p<0.05$).

on FLAP expression compared to control, but LTA_4 hydrolase expression was significantly increased by 10μM bradykinin (7.8±2.7 vs 12.3±2.8, p=0.05, n=6). In contrast to bradykinin, concentrations of histamine up to 100μM had no effect on the expression of 5-LO, FLAP or LTA_4 hydrolase in 16-HBE cells compared to control cells (**figure 30**). LTC_4 synthase expression remained low in 16-HBE cells, both at baseline and following stimulation with bradykinin and histamine.

3.4 FACS analysis of the effect of inflammatory cytokines and dexamethasone on 5-LO pathway enzyme expression in 16-HBE cells

5-LO pathway enzyme expression was assessed by FACS in 16-HBE cells stimulated with a selection of pro-inflammatory cytokines, some of which have been reported to have an effect on LT enzyme expression in leukocytes (Riddick et al. 1999, Pouliot et al. 1994, Cowburn et al. 1999). Cells were incubated with 20ng/ml of IL-3, IL-5, IL-6, TNFα, GM-CSF, or TGFβ, or a combination of all six cytokines (**Figures 31a & 31b**). Compared to control cells cultured for 24 hr, the median fluorescence intensities of 5-LO, FLAP and LTA_4 hydrolase were not significantly increased by any individual cytokine or the cytokine mixture. Expression of 5-LO was significantly, but modestly, down-regulated by IL-6 from 19.0±3.2 to 14.7±3.0 (p=0.013, n=6). FLAP immunofluorescence was modestly reduced by the cytokine mixture from 10.7±2.5 to 8.8±2.3 (p=0.035, n=6). LTA_4 hydrolase immunofluorescence was reduced by TGFβ from 13.3±3.1 to 8.6±1.6 (p=0.049, n=6) and by the cytokine mix from 14.8±3.4 to 11.0±2.7 (p=0.05, n=6). Dexamethasone (1μM) had no effect on the expression of 5-LO, FLAP or LTA_4 hydrolase. Basal, cytokine- and dexamethasone-stimulated levels of LTC_4 synthase expression were low and no changes were detectable by FACS.

Following experiments showing that IL-3, IL-5, IL-6, TNFα, GM-CSF and TGFβ had little effect on LT pathway enzyme immunofluorescence, 16-HBE and H292 cells were incubated with an alternative combination of cytokines with several known positive effects on HBE cell activation. Cells were incubated for 24 hours with a mixture of IL-1β (20ng/ml), TNFα (200U/ml) and IFNγ (200U/ml) and 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase expression was examined by FACS analysis. In 16-HBE cells (**Figure 32**), the cytokine mix significantly increased FLAP from 14.2±3.5 to 23.3±5.1 (p=0.04, n=7) but had no effect on

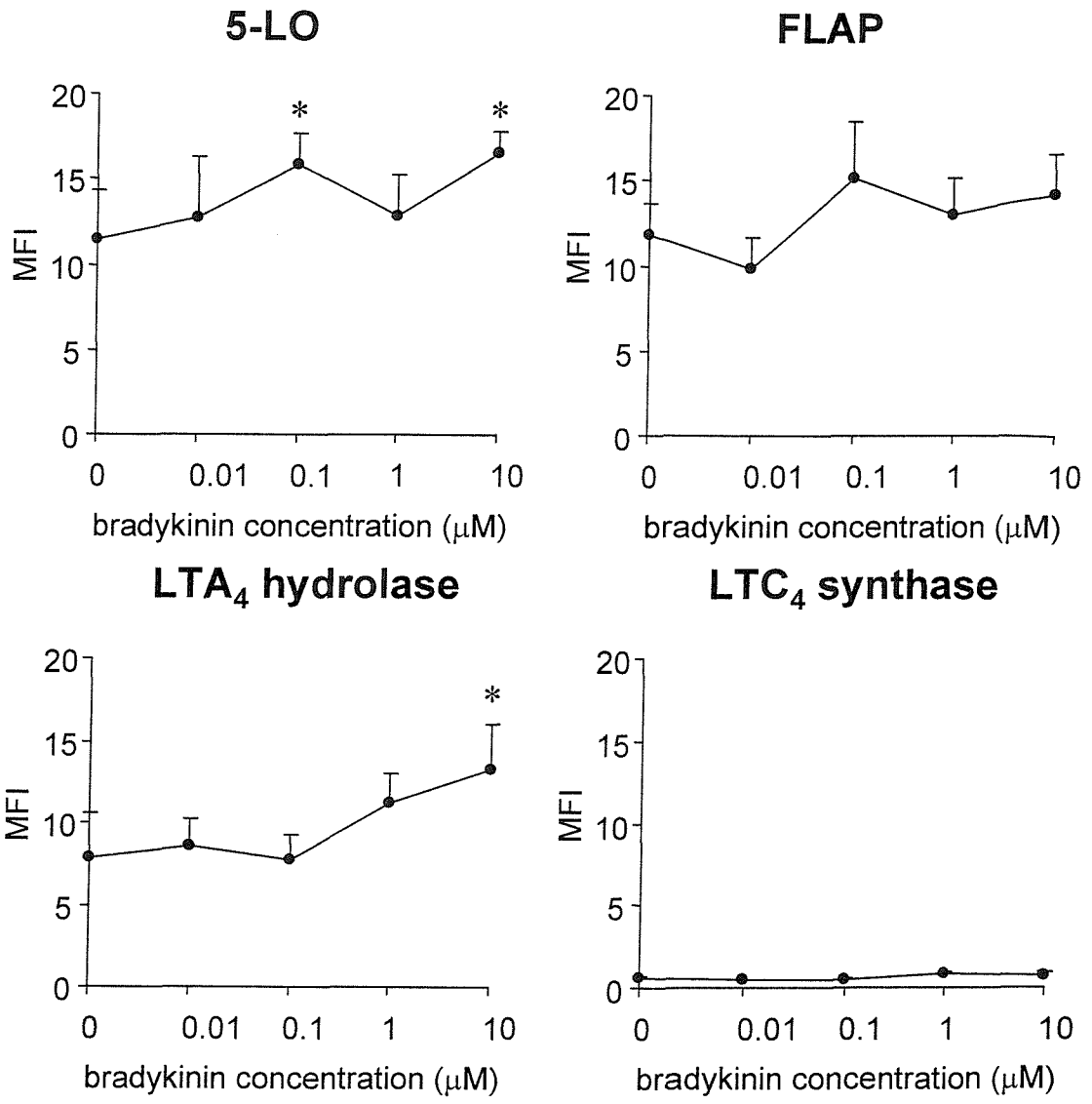


Figure 29. FACS analyses showing the effect of bradykinin on 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase in 16-HBE cells. 16-HBE cells were incubated for 24 hours with bradykinin (0-10μM), fixed, permeabilised and stained for 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase as described in Methods. Results (mean+SEM) are expressed as median fluorescence intensity and were compared using Student's paired t-test (n=7). Compared to control, bradykinin significantly upregulated 5-LO and LTA_4 hydrolase at 10μM but had no effect on FLAP or LTC_4 synthase expression (*$p<0.05$).

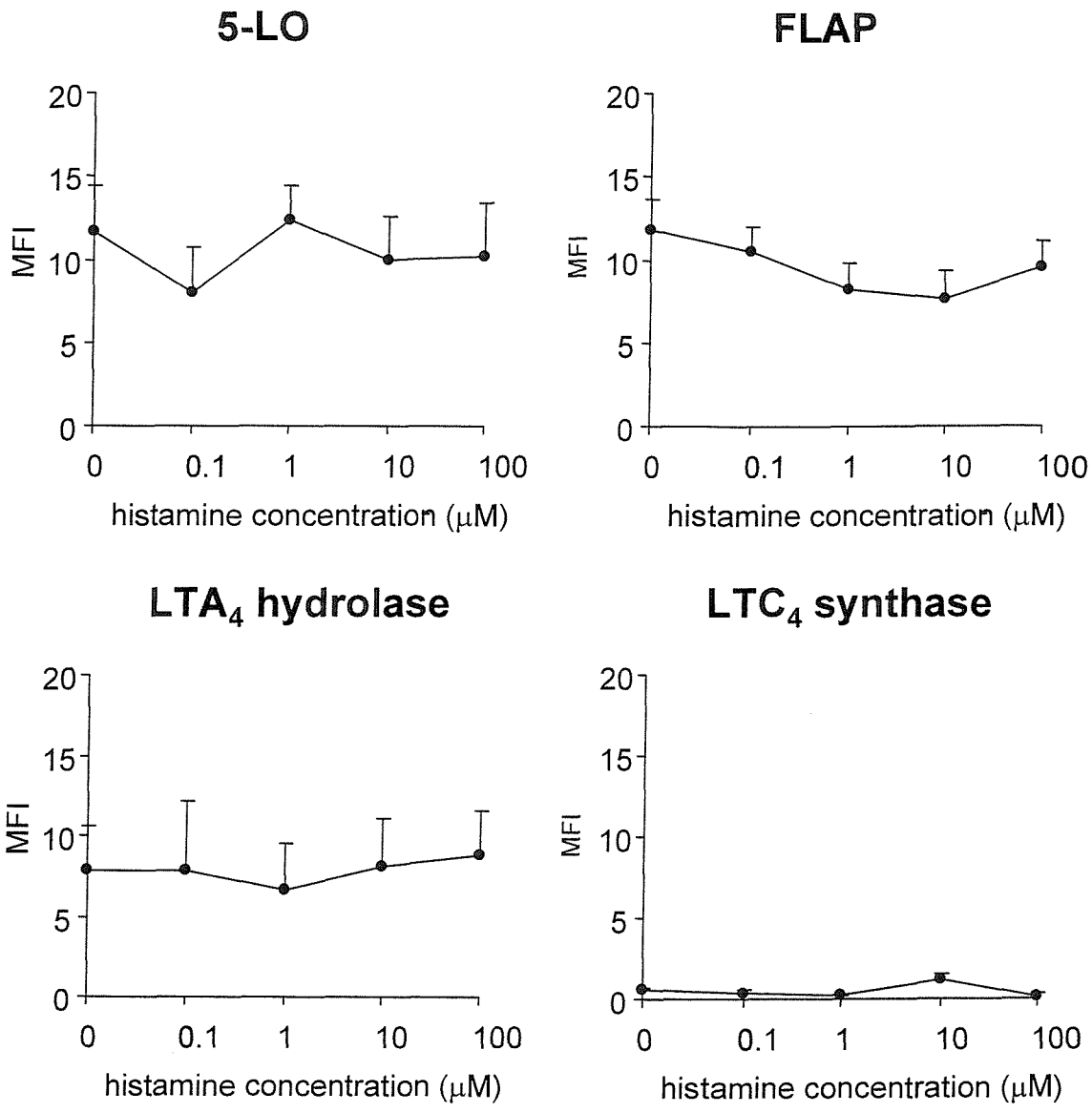


Figure 30. FACS analyses showing the effect of histamine on 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase in 16-HBE cells. 16-HBE cells were incubated for 24 hours with histamine (0-100μM), fixed, permeabilised and stained for 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase as described in Methods. Results (mean+SEM) are expressed as median fluorescence intensity and were compared using Student's paired t-test (n=7). Compared to control histamine had no significant effect on the immunofluorescence of 5-LO, FLAP, LTA_4 hydrolase or LTC_4 synthase in 16-HBE.

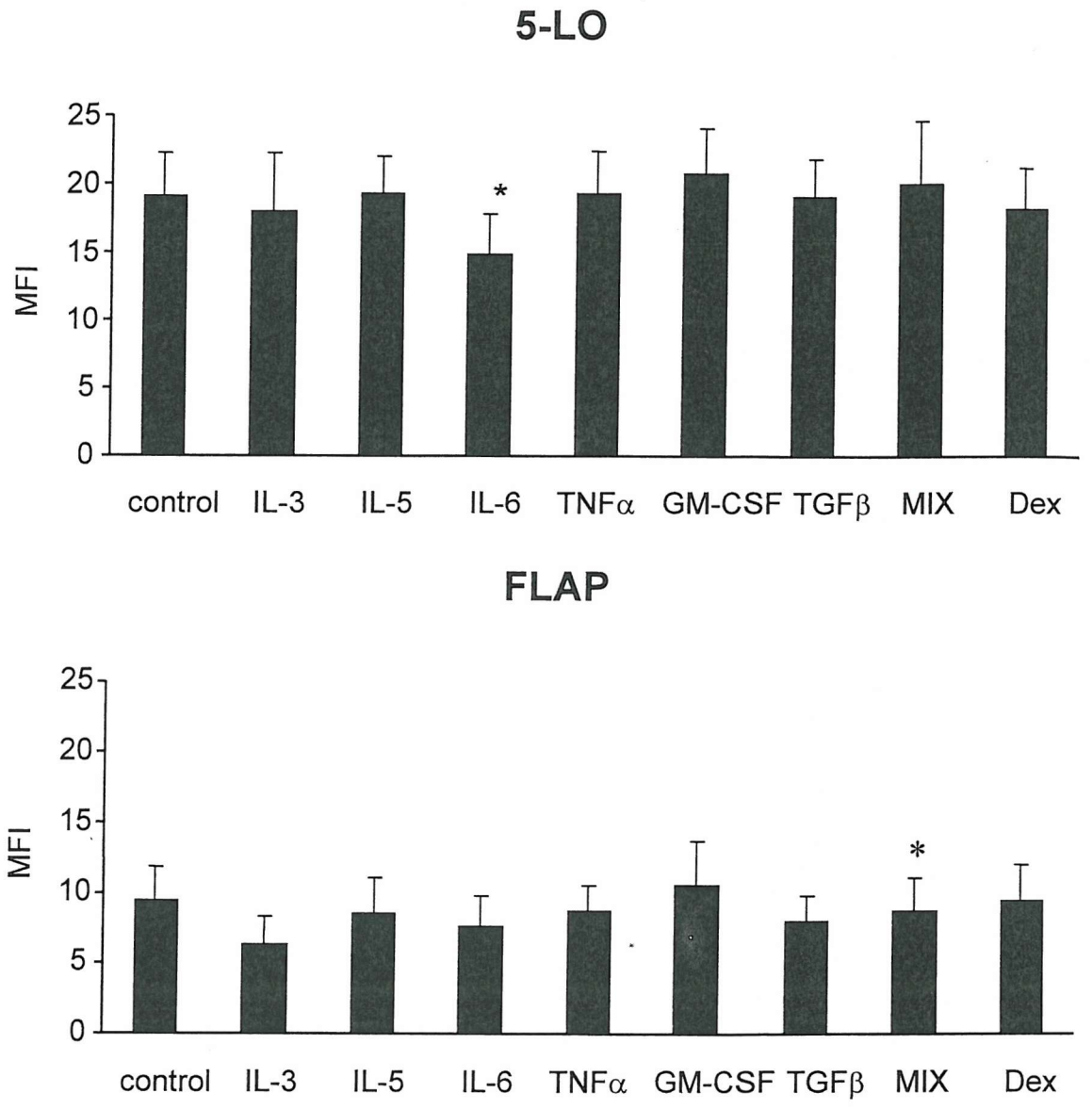


Figure 31a. Effect of pro-inflammatory cytokines and dexamethasone on 5-LO and FLAP expression in 16-HBE cells as measured by FACS analysis. Leukotriene pathway enzyme expression was examined in 16-HBE cells incubated for 24 hours with 20ng/ml of IL-3, IL-5, IL-6, TNFα, GM-CSF, TGFβ or a combination of all cytokines (MIX) or 1μM dexamethasone (Dex). Results show mean+SEM MFI and were compared using Student's paired t-test (*p=<0.05, n=6). Compared to controls, 5-LO expression was significantly reduced by IL-6 (p=0.01) and FLAP expression was significantly reduced by the cytokine mix (p=0.04).

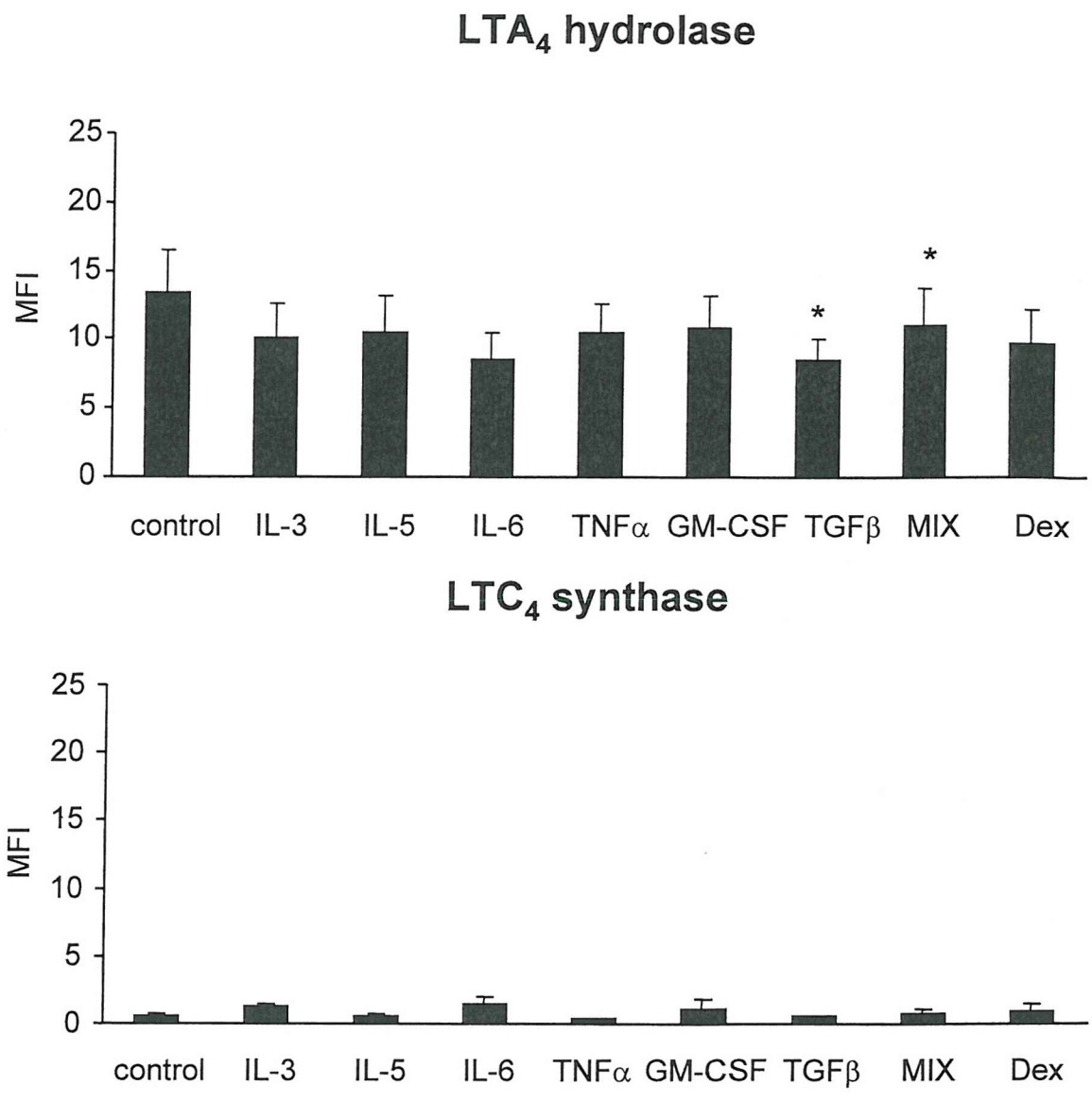


Figure 31b. Effect of pro-inflammatory cytokines and dexamethasone on LTA_4 hydrolase and LTC_4 synthase expression in 16-HBE cells as measured by FACS analysis. Leukotriene pathway enzyme expression was examined in 16-HBE cells incubated for 24 hours with 20ng/ml of IL-3, IL-5, IL-6, TNFα, GM-CSF, TGFβ or a combination of all cytokines (MIX) or 1μM dexamethasone (Dex). Results show mean+SEM MFI and were compared using Student's paired t-test (*p=<0.05, n=6). LTA_4 hydrolase expression was significantly reduced by TGFβ and the cytokine mix (p=0.05 and p=0.05 respectively) but levels of LTC_4 synthase were not changed.

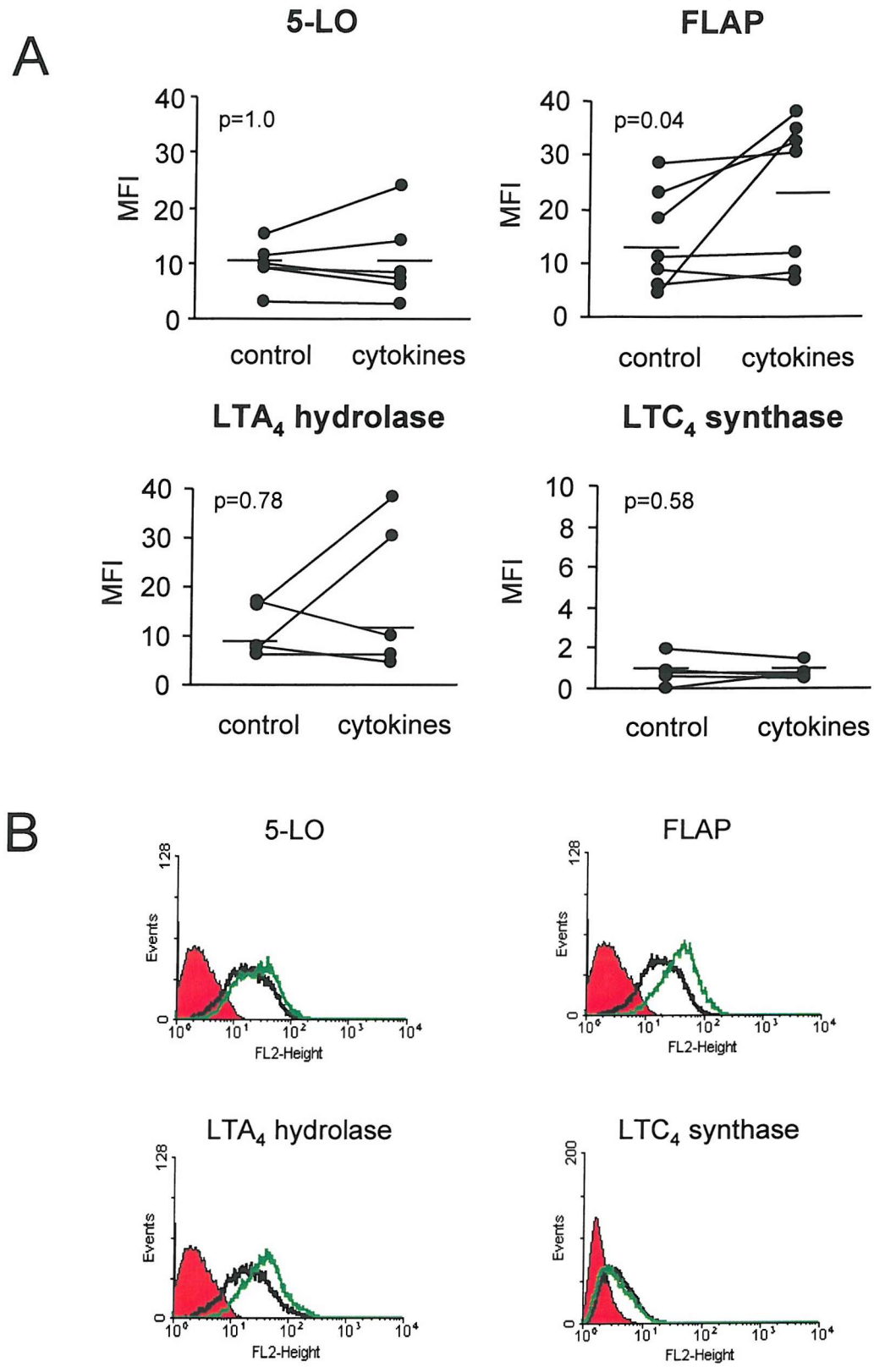


Figure 32. FACS analysis of LT pathway enzyme expression in 16-HBE cells after stimulation with IL-1β, TNFα and IFNγ. The expression of 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase was examined by FACS analysis in 16-HBE cells, after incubation with a combination of IL-1β (20ng/ml), TNFα (200U/ml) and IFNγ (200U/ml) for 24 hours. Results are shown as individual data points with median bars and were compared using Wilcoxon's signed rank test (panel A). Panel B shows representative FACS plots (red=isotype control, black=-cytokines, green=+cytokines). The cytokine mix increased the expression of FLAP in 16-HBE (p=0.04) but had no effect on 5-LO, LTA_4 hydrolase or LTC_4 synthase.

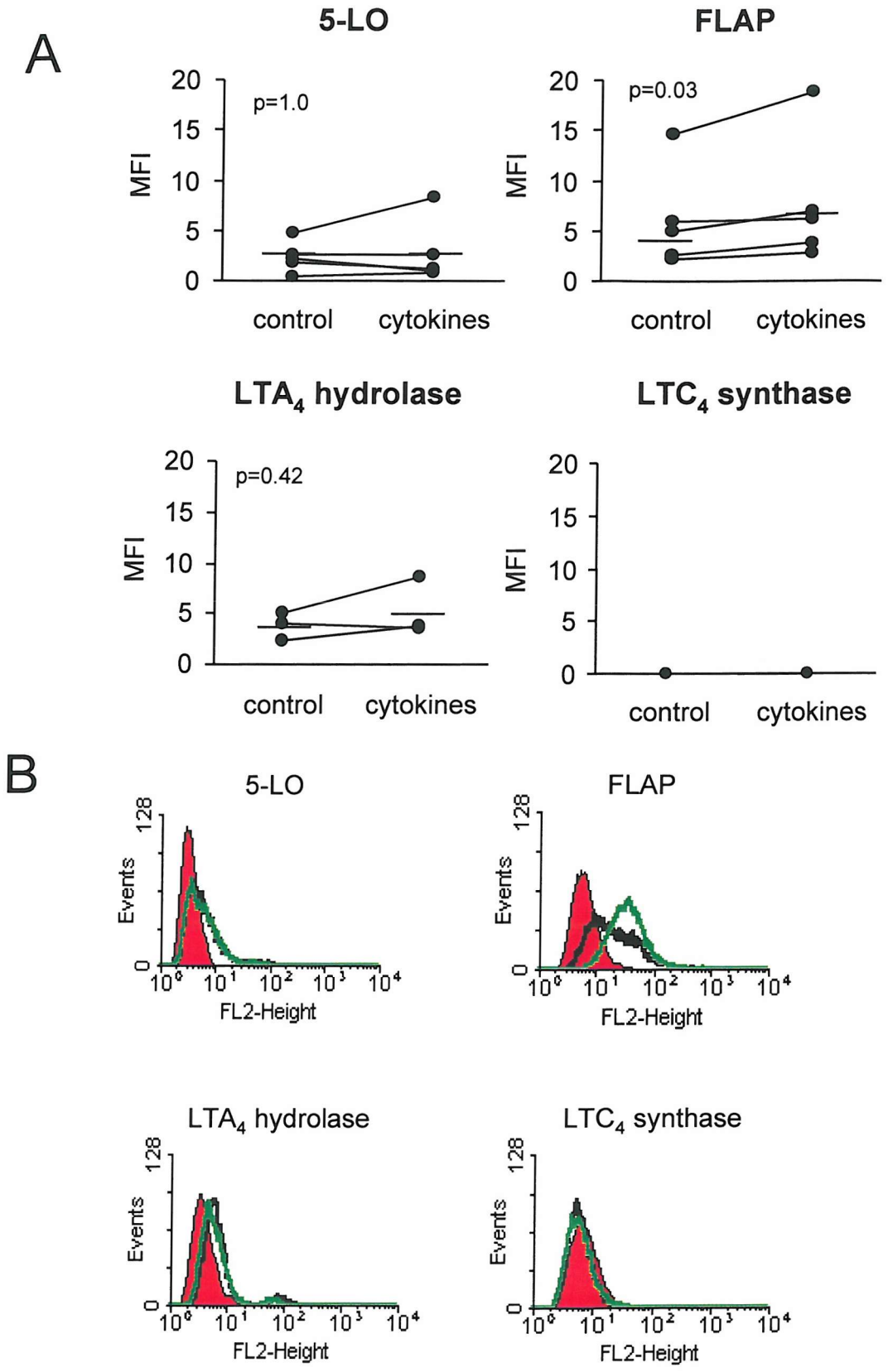


Figure 33. FACS analysis of LT pathway enzyme expression in H292 cells after stimulation with IL-1β, TNFα and IFNγ. The expression of 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase was examined by FACS analysis in H292 cells, after incubation with a combination of IL-1β (20ng/ml), TNFα (200U/ml) and IFNγ (200U/ml) for 24 hours. Results are shown as individual data points with median bars and were compared using Wilcoxon's signed rank test (panel A). Panel B shows representative FACS plots (red=isotype control, black=-cytokines, green=+cytokines). The cytokine mix increased the expression of FLAP in H292 (p=0.03) but had no effect on 5-LO, LTA_4 hydrolase or LTC_4 synthase.

5-LO, LTA_4 hydrolase or LTC_4 synthase. Similarly, in H292 cells (**Figure 33**), IL-1β, TNFα and IFNγ had no effect on 5-LO and LTA_4 hydrolase, but FLAP was significantly increased from 6.1±2.3 to 8.0±2.8 (p=0.03, n=5). LTC_4 synthase immunofluorescence was not detectable by FACS, in control or cytokine-stimulated H292 cells.

3.5 FACS analysis of the effects of dexamethasone and MK-571 on 5-LO pathway enzyme expression in 16-HBE cells.

Following the lack of effect of dexamethasone on the baseline expression of 5-LO pathway enzymes, we also investigated whether this corticosteroid had any effect on the upregulation of LT pathway enzymes. Of all the stimuli tested, Ca ionophore was the most effective in increasing the immunofluorescence of 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase, so the effect of dexamethasone (1μM) was examined in combination with A23187 (1μM). We also hypothesised that $CysLT_1$ receptor antagonism might modulate the 5-LO pathway, so we examined the effect of MK-571 (10nM), a $CysLT_1$ antagonist, on the expression of the LT pathway enzymes, both at baseline and following upregulation with A23187. Results are shown in **Figures 34a and 34b**.

As expected, stimulation with Ca ionophore increased the immunofluorescence of 5-LO (from 5.2±1.3 to 12.8±2.3, p=0.009), FLAP (from 5.8±1.2 to 10.4±1.2, p=0.06), LTA_4 hydrolase (from 5.5±0.7 to 12.0±1.8, p=0.005) and LTC_4 synthase (from 0.3±0.3 to 1.3±0.5, p=0.026). As shown in the previous section, dexamethasone alone had no effect on the expression of 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase. However, dexamethasone was able to reduce the expression of 5-LO after A23187 upregulation from 12.8±2.3 to 9.9±2.4 (p=0.04). Dexamethasone did not significantly modify levels of ionophore stimulated FLAP, LTA_4 hydrolase or LTC_4 synthase.

MK-571 was also without effect on the baseline expression of the LT pathway enzymes. Incubation with MK-571 modestly reduced the expression of ionophore stimulated 5-LO from 12.8±2.3 to 7.5±0.9 (p=0.069), although this effect did not reach statistical significance. MK-571 did not have any significant effects on ionophore stimulated levels of FLAP, LTA_4 hydrolase or LTC_4 synthase.

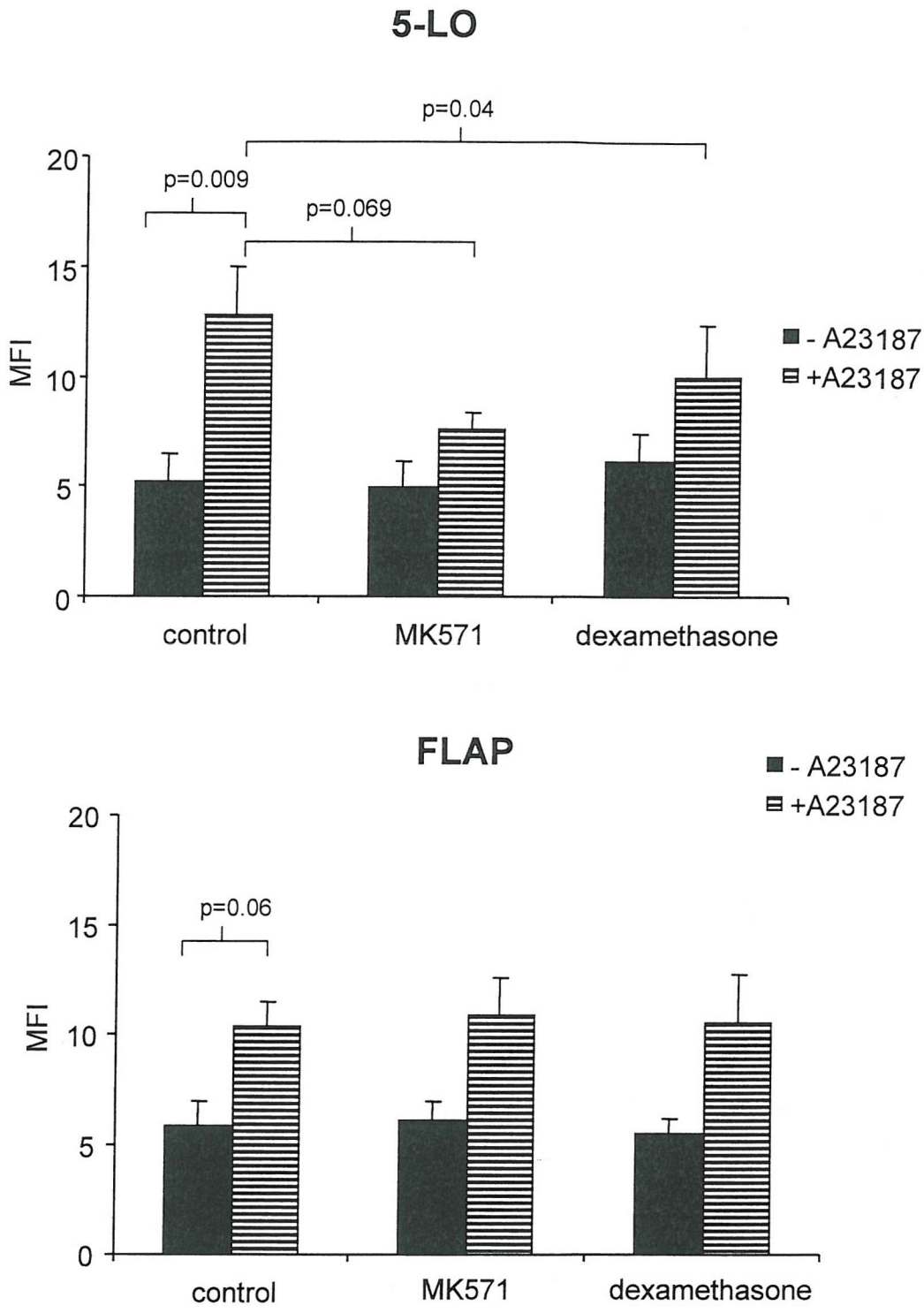


Figure 34a. Effect of MK571 and dexamethasone on 5-LO and FLAP immuno-fluorescence in 16-HBE cells incubated with or without A23187. 16-HBE were cultured for 24 hours in either serum free medium, 1μM dexamethasone or 10nM MK571 for 24 hours. The effects of dexamethasone and MK571 were also examined in cells stimulated with A23187 (1μM) for 24 hours. Cells were fixed, permeabilised and stained for 5-LO and FLAP. Results are shown as mean+SEM and were compared using Student's paired t-test (n=6).

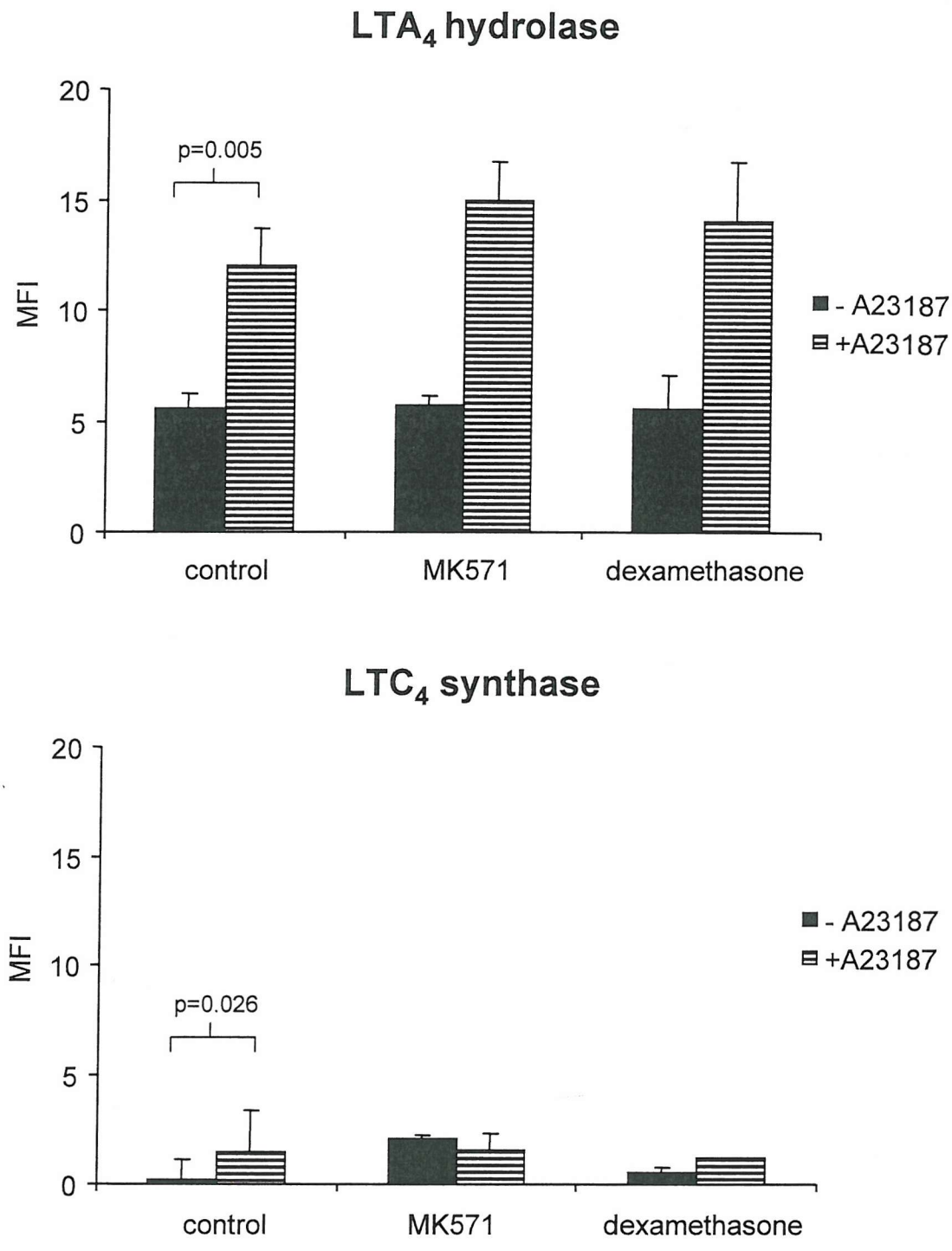


Figure 34b. Effect of MK571 and dexamethasone on LTA_4 hydrolase and LTC_4 synthase immunofluorescence in 16-HBE cells incubated with or without A23187. 16-HBE were cultured for 24 hours in either serum free medium, 1μM dexamethasone or 10nM MK571 for 24 hours. The effects of dexamethasone and MK571 were also examined in cells stimulated with A23187 (1μM) for 24 hours. Cells were fixed, permeabilised and stained for LTA_4H and LTC_4S. Results are shown as mean+SEM and were compared using Student's paired t-test (n=6).

3.6 Expression of 5-LO pathway enzymes and LT production in primary HBE cells.

To investigate whether LT pathway enzymes are expressed and regulated *in vivo*,expression of 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase was examined by FACS analysis at baseline and after stimulation for 6hr with Ca ionophore A23187 (1μM) in primary HBE cells obtained from normal lung tissue (as described in Methods). These cells were grown as explants from tissue obtained from nine subjects. FACS analysis demonstrated distinct baseline expression of 5-LO, FLAP and LTA_4 hydrolase in HBE cells from all nine subjects (MFI 4.0±1.6, 21.7±8.3 and 15.3±13.0 respectively) (**Figure 35**). In primary BEC, baseline LTC_4 synthase immunofluorescence (MFI 0.88±0.69) was significantly lower than that of 5-LO and FLAP ($p=0.05$ and $p=0.02$ respectively) and baseline levels of FLAP were approximately 5 times greater than 5-LO ($p=0.04$). Stimulation of primary HBE cells with 1μM A23187 for 6 hours increased FLAP immunofluorescence from 21.7±8.3 to 65.0±39.6 although this change did not reach statistical significance ($p=0.076$, $n=9$). No significant changes in expression of 5-LO, LTA_4 hydrolase or LTC_4 synthase were observed. However, LTC_4 synthase expression was increased in three out of the nine samples (control 1.6±1.9, stimulated 8.4±1.5, $p=0.05$), but not expressed or upregulated in the remainder.

Basal and 1μM A23187-stimulated production of LTC_4 by primary HBEC was examined by EIA of cell supernatants after 6 hr. Spontaneous immunoreactivity for LTC_4 (308±80 pg/10^6 cells, $n=7$) and LTB_4 (653±140 pg/10^6 cells, $n=7$) was detected (**figure 27b, p102**), and non-significant increases were observed after incubation with A23187 (LTC_4 381±154 pg/10^6 cells, $p=0.6$ and LTB_4 1568±576 pg/10^6 cells, $p=0.13$). Baseline immunoreactivity for LTC_4 and LTB_4 was approximately 10 times greater in primary HBE cells than in similarly treated 16-HBE cells (LTC_4 $p=0.006$ and LTB_4 $p=0.005$).

Baseline expression of LT pathway enzymes was also examined by FACS analysis in primary HBE cells obtained by bronchial brushing from 3 normal and 3 asthmatic subjects (**Figure 36**). FACS analysis confirmed baseline 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase expression in cells from both normal and asthmatic subjects. There were no marked differences between the subject groups in any enzyme, although the power of the study to detect such differences ($n=3$ each group) was limited.

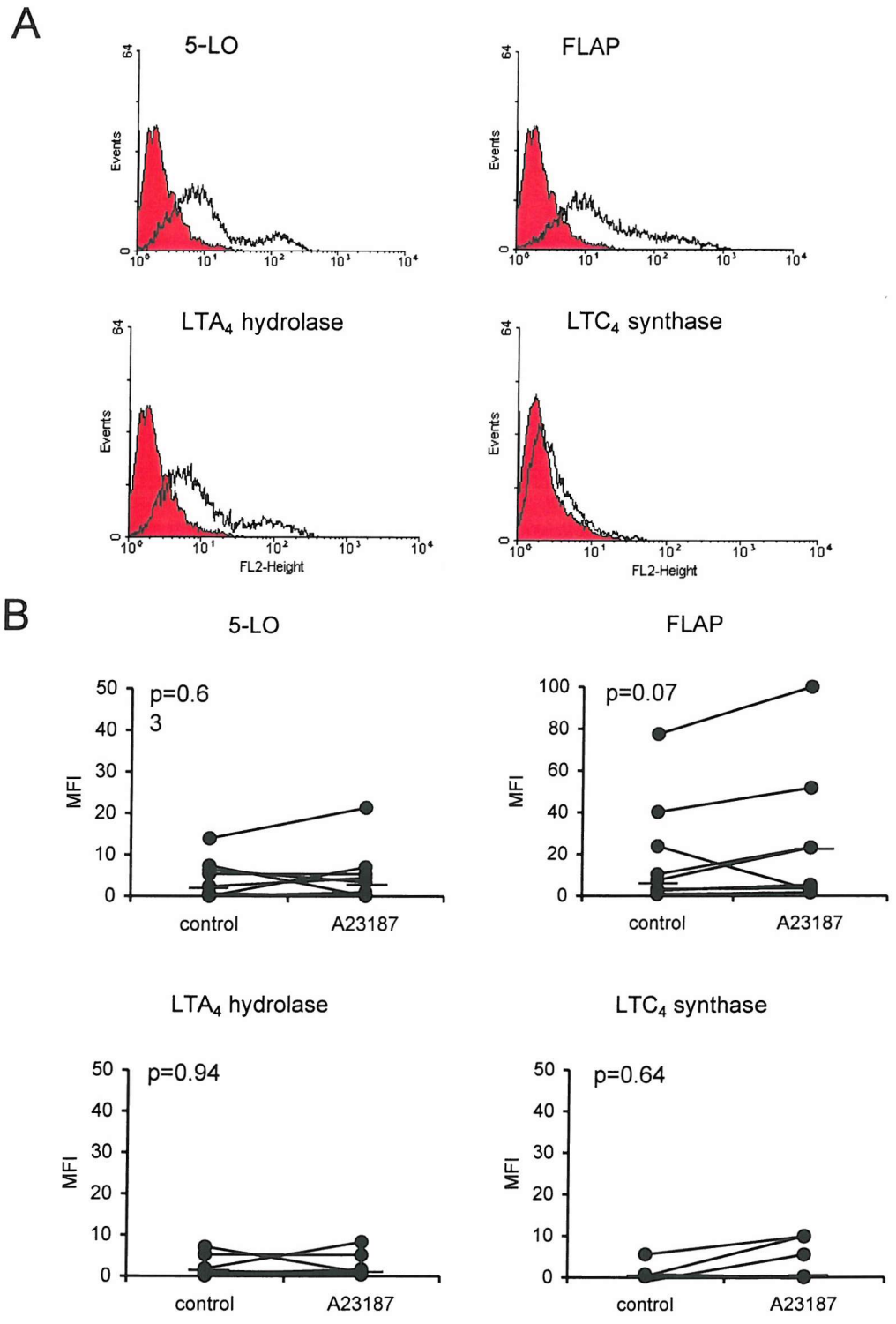


Figure 35. Effect of Ca ionophore A23187 on 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase expression in primary HBE cells as measured by FACS analysis. Cells were cultured for 6 hours in the presence or absence of A23187 (1μM), fixed, permeabilised and stained for 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase as described in methods. (A) FACS histograms show that 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase are detectable in primary cells but after stimulation with A23187 (B) only FLAP was increased by A23187 (p=0.076, n=9). Results are presented as individual data points with median bars and were analysed using Wilcoxon's signed rank test.

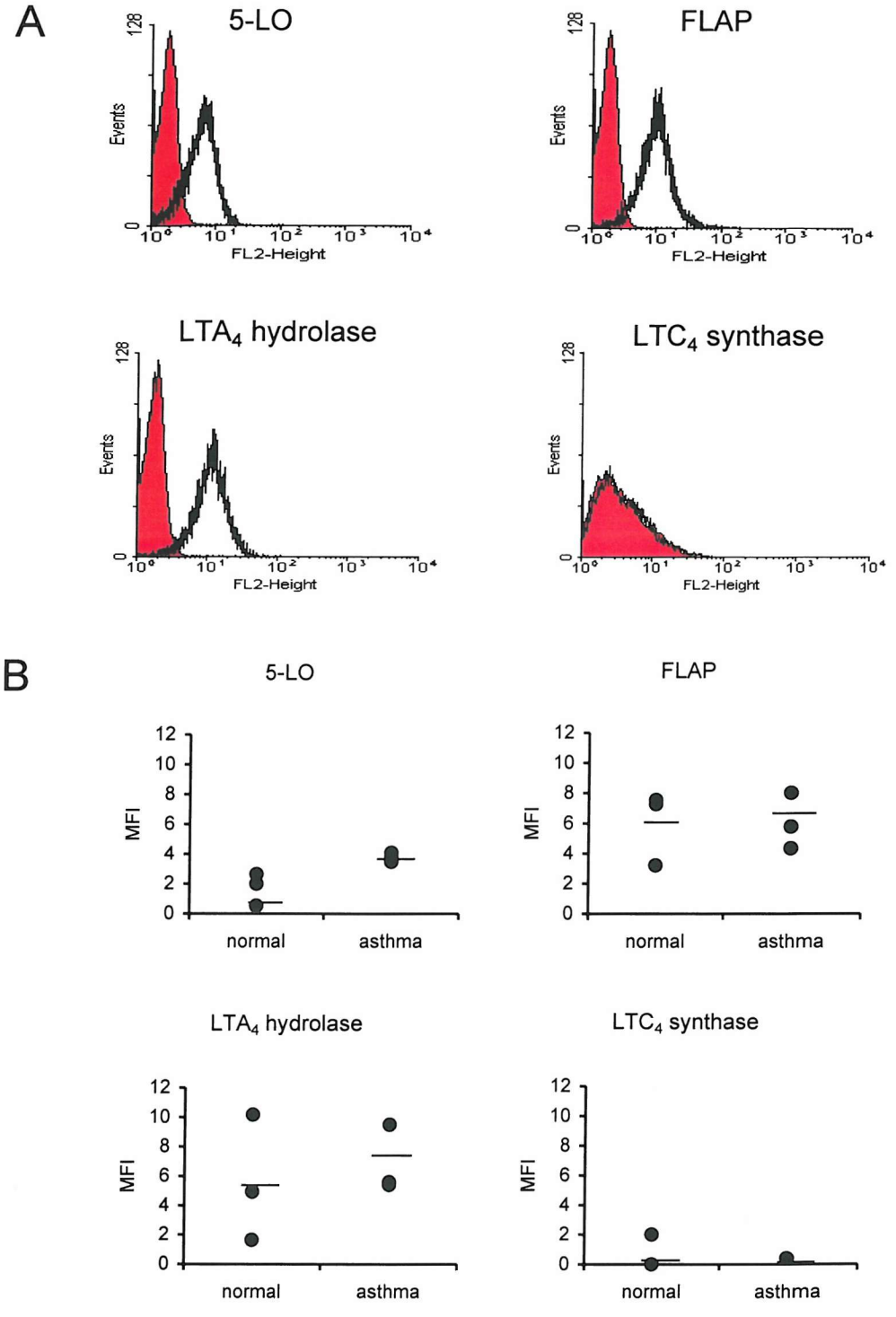


Figure 36. FACS analysis showing baseline expression of LT pathway enzymes in primary HBE cells from asthmatics and normals. Primary HBE cells were obtained by bronchial brushing of 3 asthmatics and 3 normals. Baseline 5-LO, FLAP, LTA_4 hydrolase and LTC_4 expression was measured by FACS analysis. There were detectable amounts of 5-LO, FLAP, LTA_4 hydrolase expression. LTC_4 synthase expression was minimal (A).Cell numbers were too small to test for differences between the groups (B).

3.7 The effect of LTs on the proliferation of 16-HBE cells.

Finally, as LTs are implicated in airway remodelling, the effect of LTD_4 and LTB_4 on the proliferation of 16-HBE cells was measured by methylene blue assay as described in Methods. 16-HBE cells were incubated up to 72 hours with either control medium, LTB_4 (0-100nM) or LTD_4 (0-100nM). In addition, the effect of LTB_4 (10nM) with the LTB_4 antagonist U75302 (10 and 100nM) and LTD_4 (10nM) with the $CysLT_1R$ antagonist MK571 (10 and 100nM) was investigated (**Figure 37**). Compared to basal proliferation levels, LTB_4 had no effect on the replication of 16-HBE cells and accordingly no significant blockade by U75302 was observable. However, comparing all time points, 1nM LTD_4 caused a very small but significant increase in 16-HBE proliferation ($p=0.028$) although LTD_4 at 10 and 100nM had no effect on the proliferation of 16-HBE cells.

It has also been suggested that cys-LTs may synergise with growth factors in other cell types to enhance growth factor induced mitogenesis. Therefore we examined the effect of EGF (10ng/ml) in combination with LTD_4 (1nM) or LTB_4 (1nM) (**Figure 38**). Surprisingly, EGF did not increase cell numbers, but instead significantly reduced them compared to baseline proliferation at all time points (ANOVA, $p=0.005$). LTB_4 did not further change this EGF induced reduction in proliferation, but it was inhibited by LTD_4 (EGF compared to EGF+LTD_4, ANOVA $p=0.044$).

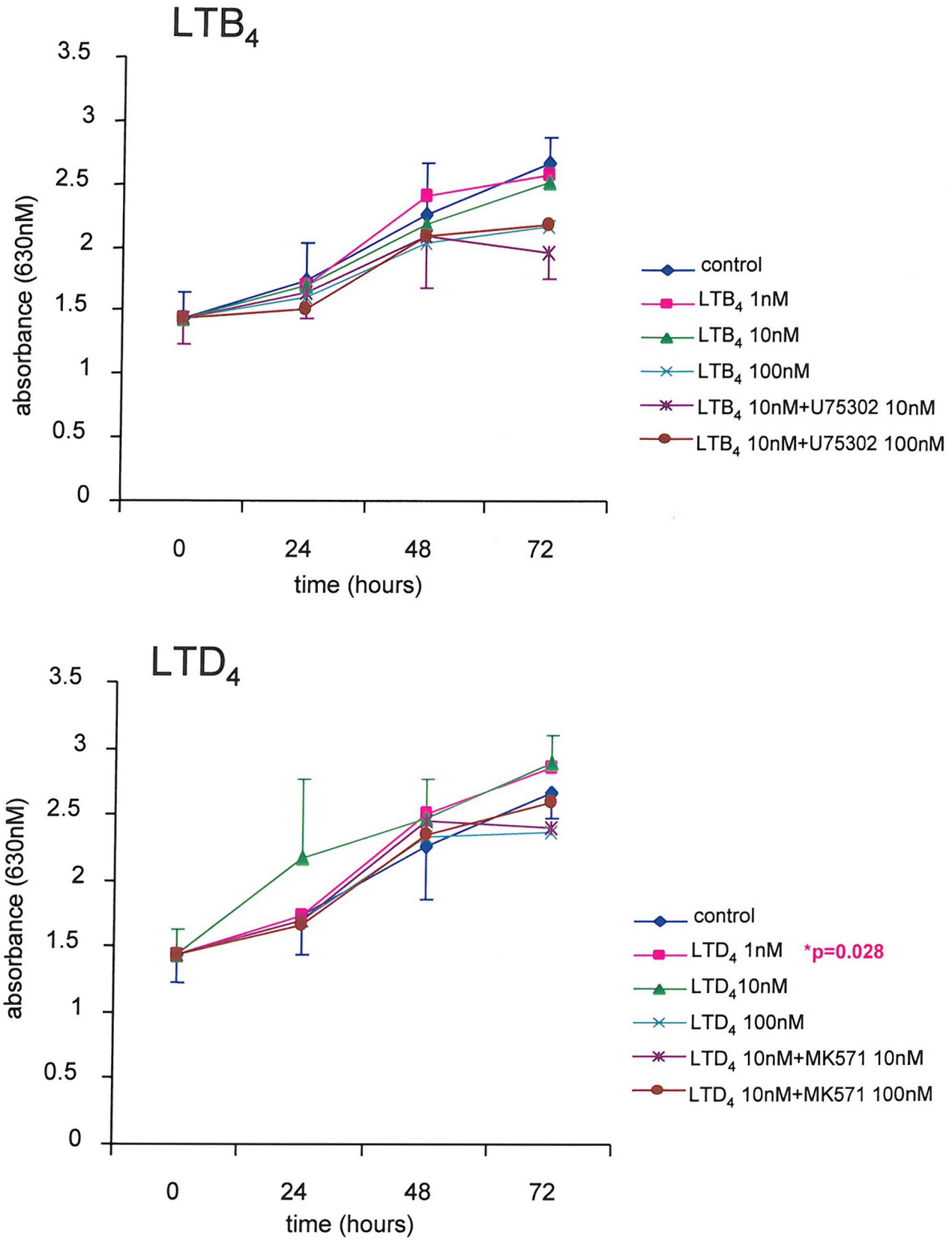
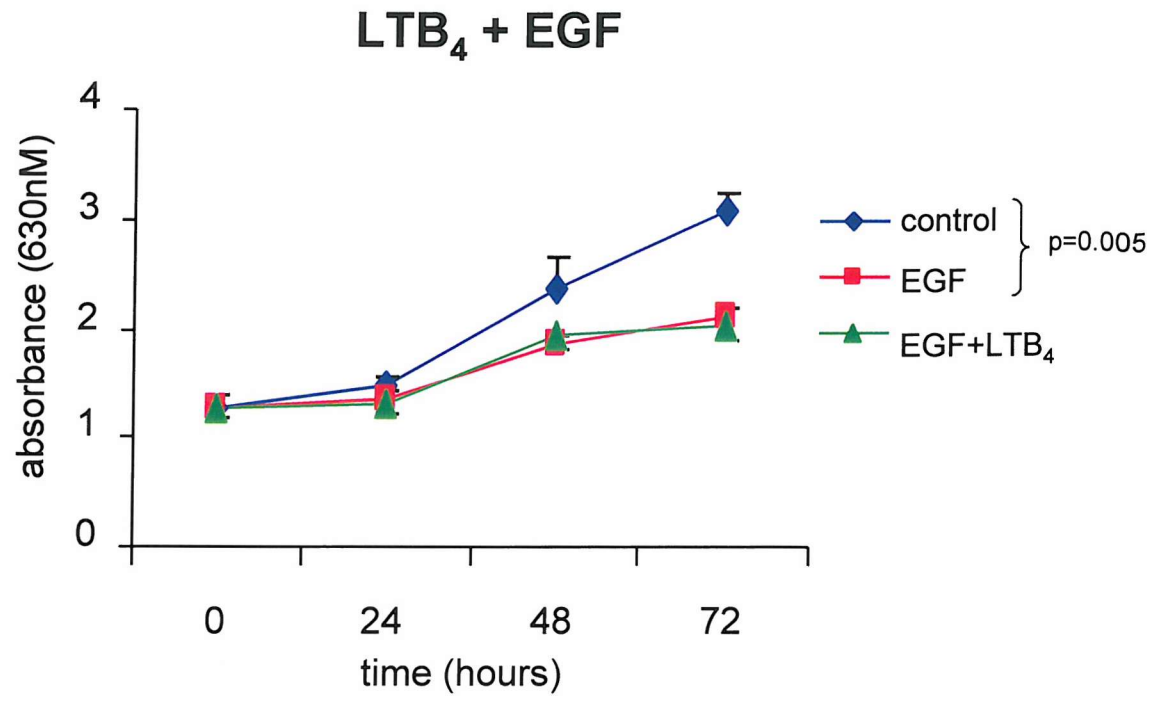


Figure 37. Proliferation assays showing response of 16-HBE cells to stimulation with leukotrienes. 16-HBE cells were incubated for 0-72 hours with LTB_4 (0-100nM), LTD_4 (0-100nM), LTB_4 (10nM) +U75302 (10 & 100nM), LTD_4 (10nM) + MK571 (10 & 100nM) or serum-free medium alone (control). Proliferation was measured by uptake of methylene blue dye as described in methods. Absorbance (630nM) was measured using a spectrophotometer and was directly proprtional to cell number. . Results are shown as mean±SEM and all time points were compared using ANOVA and paired t-tests. Overall, LTD_4 and LTB_4 did not cause marked changes in proliferation, although 1nM LTD_4 caused a small but significant increase in the proliferation of 16-HBE cells (p=0.028).



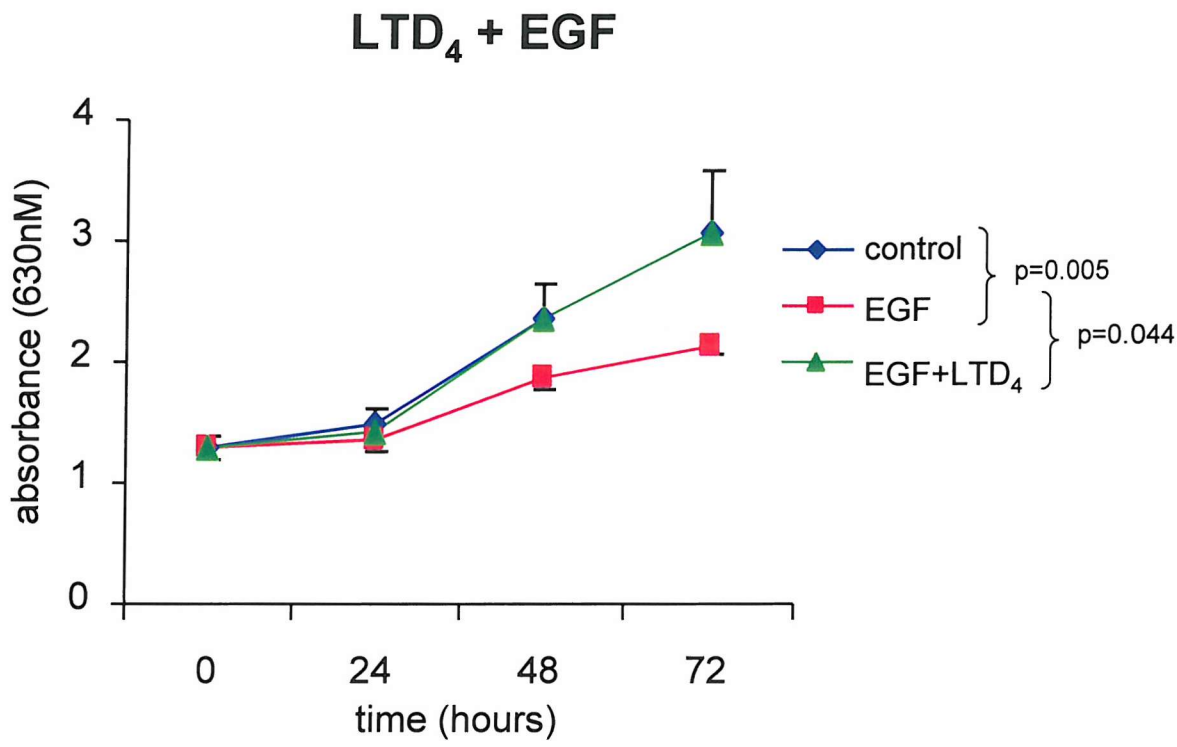


Figure 38. Proliferation assays showing response of 16-HBE cells to stimulation with EGF and leukotrienes. 16-HBE cells were incubated for 0-72 hours with either control medium, EGF (10ng/ml), EGF+LTB_4 (1nM), EGF+LTD_4 (1nM). Proliferation was measured by uptake of methylene blue dye as described in methods. Absorbance (630nM) was measured using a spectrophotometer and was directly proprtional to cell number. Results are shown as mean±SEM and all time points were compared using ANOVA. EGF significantly reduced proliferation (p=0.005) and LTD_4 significantly inhibted this reduction (p=0.044). LTB_4 was without effect.

3.8 Discussion

The leukotriene synthetic pathway in human bronchial epithelial cells

These results demonstrate for the first time that human bronchial epithelial cell lines constitutively express both mRNA and protein for the leukotriene pathway enzymes 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase. Stimulation with Ca ionophore increases the enzyme expression as well as cys-LT- and LTB_4-like immunoreactivity. Incubation of BEC with inflammatory mediators including certain concentrations of bradykinin and bacterial LPS, and IL-1β, TNFα and IFNγ showed some modest upregulation of 5-LO pathway enzyme expression. Treatment with IL-6 and TGFβ led to small, yet significant reductions in the expression of 5-LO and LTA_4 hydrolase respectively, although histamine, IL-3, IL-5, TNFα and GM-CSF had no effect on LT pathway enzyme expression. The corticosteroid dexamethasone and the $CysLT_1$ receptor antagonist MK571 were found to inhibit A23187-induced upregulation of 5-LO in 16-HBE cells. 5-LO pathway enzyme expression and leukotriene production was also confirmed in primary BEC, in which baseline leukotriene production was approximately 10 times greater than 16-HBE cells. Finally, a role for cys-LTs in the regulation of epithelial cell proliferation was implicated.

3.8.1 Baseline expression of LT pathway proteins in bronchial epithelial cells.

The finding that HBE cells express 5-LO, FLAP and the two downstream enzymes LTA_4 hydrolase and LTC_4 synthase at baseline, and generate both LTB_4 and LTC_4 contradicts the classical concept of LT production being restricted to cells of myeloid lineage. Reports of leukotriene synthesis by HBEC are few and varied, many relying solely on measurements of the released LTB_4 or LTC_4 product. Some reports describe the production of only 15-LO pathway products without significant LT production (Hunter et al. 1985), some detect LTB_4 production but only after transcellular donation of LTA_4 by 5-LO/FLAP expressing leukocytes (Bigby et al. 1989), and others detect cys-LT production but only after stimulation with, for example, ozone (McKinnon et al. 1993). Consistent with a complete LT synthetic pathway in BEC, this study shows both cys-LT and LTB_4 production in the absence of other cell types. Many previous studies suffered from important limitations. Many relied solely on cell-lines such as A549 type II alveolar epithelial cells, or the bronchial epithelial cell lines BEAS-S6 and BEAS-2B (Masubuchi et al. 1998, McKinnon et

al. 1993 and Behera et al. 1998), while the present work also investigated primary cells. One study used a radioimmunoassay that was clearly inadequate to detect the low levels of LTC_4 that may have been generated, rather than a more sensitive enzyme immunoassay (Tristram et al. 1998). In other studies, comparison of reported LT production is impossible because levels are not corrected for the numbers of cells generating them. For example, Behera et al. express LT levels as pg/ml, and not pg/10^6 cells. Furthermore, only one study attempted to investigate expression of 5-LO (Behera et al. 1998), while in the current study the complete enzymatic pathways for synthesis of LTB_4 and LTC_4 were examined, both at the mRNA and protein levels. Taken together, a critical appraisal of the existing literature on LT production by HBE cells would suggest that the constitutive capacity of HBE cells to generate LTs is absent or minimal, probably restricted to LTB_4, and that LTC_4 production remains very low even after activation by major inflammatory insults.

Expression of the LTB_4 biosynthetic pathway enzymes has been described in epithelial cells from several tissues other than lung. For example epithelial cells from the gut, middle ear and cornea all express 5-LO pathway enzymes (Cortese et al. 1995, Liminga et al. 2000, Lin et al. 1999). Therefore it is not surprising that epithelial cells from the bronchial airways have the capacity to produce LTB_4, although very few studies have examined the expression of 5-LO, FLAP and LTA_4 hydrolase. As mentioned briefly above, Behera et al. examined the expression of 5-LO mRNA and protein in two HBE cell lines but were not able to detect 5-LO at baseline, with expression becoming evident only after RSV infection (Behera et al. 1998). Bigby and colleagues describe the expression of LTA_4 hydrolase in transformed human tracheal epithelial cells and this enzyme was found to be different to leukocyte-derived LTA_4 hydrolase in that it did not show suicide inactivation and the activity of the enzyme had a slower time course (Bigby et al. 1994). In the present study, LTA_4 hydrolase was found to have the same molecular weight as that in leukocytes although the possibility of functional differences was not examined. Hong and colleagues found co-expression of 5-LO and FLAP mRNA in epithelial cells from lung and several other tissues, although the expression of LTA_4 hydrolase and LTC_4 synthase mRNA in these cells was not examined. As the aim of the Hong study was to investigate the growth of cancer cells, the cells used were tumour-derived and as a result may show altered characteristics (Hong et al. 1999). Therefore, to our knowledge this is the first study to demonstrate the constitutive expression of 5-LO, FLAP and LTA_4 hydrolase together, a complete LTB_4 synthetic pathway, in epithelial cell lines and primary BEC.

From previous studies, the enzyme whose expression in HBEC has been most uncertain is LTC_4 synthase. In favour of its expression, HBE cells produced detectable amounts of cys-LTs and LTC_4 synthase mRNA was present at baseline in 16-HBE and primary BEC. Additional controls were carried out by RT-PCR, in which the presence of LTC_4 synthase mRNA was confirmed in the basophilic cell line KU812F (positive control) and shown to be absent in HeLa cells (negative control). Also, DNA sequencing was carried out and results confirmed that that the base pair sequence of the PCR product corresponded to that of LTC_4 synthase. The use of an extremely sensitive enhanced chemiluminescent substrate for immunoblotting allowed detection of bands at 18 KDa when 16-HBE were stained for LTC_4 synthase at baseline. Immunocytochemistry showed that small percentages of 16-HBE cells were positive for LTC_4 synthase after A23187 stimulation and the enzyme was detectable by FACS in primary BEC and 16-HBE, although not in H292 cells, which also demonstrated a relative lack of 5-LO. These findings do point to the presence of LTC_4 synthase in BEC but at levels lower than those of the other three LT pathway enzymes.

LTC_4 synthase is a glutathione-S-transferase (GST) enzyme although it differs from other members of the GST family by its selectivity for LTA_4 and by its failure to conjugate xenobiotic drugs (Yoshimoto et al. 1985). Certain mammalian cytosolic and microsomal GST enzymes can conjugate reduced glutathione to either a xenobiotic or to LTA_4 (Jakobsson et al. 1996) but their role in the inflammatory response is not understood. Several GSTs have been found to be constitutively expressed in the bronchial epithelium of normal human subjects by immunohistochemical localisation (Anttila et al. 1993). Human umbilical vein endothelial cells contain microsomal GST2 and generate LTC_4 without expressing LTC_4 synthase (Sjöström et al. 2001). Cys-LTs produced by bronchial epithelial cells could be derived from a GST as opposed to LTC_4 synthase. We have confirmed the presence of LTC_4 synthase in BEC using a number of different techniques, but we cannot exclude a contribution of other enzymes to LTC_4 synthesis, such as MGSTII.

In order for LTC_4 to be transported out of bronchial epithelial cells the presence of a specific transmembrane transporter is required. The proteins responsible for LTC_4 export are members of the multidrug resistance-associated family of proteins, MRPs. As part of a larger investigation into the expression of MRP proteins in several different cell types, our group found positive immunocytochemical staining for MRP-1 and MRP-2 in 16-HBE and H292 cells. Bréchot et al. demonstrated that primary human bronchial epithelial cells

express MRP-1. In ciliated cells, immunostaining was restricted to the basolateral surface whereas in basal cells, MRP was present over the entire plasma membrane (Bréchot et al. 1998). These findings show that bronchial epithelial cells are able to export LTC_4 and also imply that export occurs mainly in the direction of the bronchial mucosa rather than into the airway lumen.

Differences were observed in baseline LT pathway enzyme expression between the different cell types. For example, 5-LO expression was greater in 16-HBE than in H292 and primary BEC, and LTC_4 synthase expression was detectable in primary cells and 16-HBE by FACS, but not in the H292 cell line. Differences may be due to the different genotypes and phenotypes of the cells. H292 cells are a mucoepitheloid carcinoma derived cell line. Their inherent genetic dysfunction may cause abnormal protein expression or produce anomalous responses to stimulation. The process of simian virus immortalisation in 16-HBE could also affect enzyme expression although these cells are considered to have characteristics closer to those of primary epithelial cells (Cozens et al. 1994). Cells that have been maintained in culture for long periods of time may also lose the expression of enzymes and proteins that are not essential to this *in vitro* environment. Differences in the level of enzyme expression have been shown in other similar cell types. Several T-cell lines express more LTA_4 hydrolase activity than normal human T-cells, and simian virus transformed fibroblasts have more LTA_4 hydrolase mRNA than non-transformed fibroblasts (Fu et al. 1988, Medina et al. 1990). Differences in the phenotypes of the cell types examined are also likely to explain the 10-fold difference in LT production observed between primary BEC and 16-HBE. Levels of LTs produced by HBE cells *in vivo* are most likely to resemble the high capacity observed in the cultured primary HBEC than the more modest synthesis seen in 16-HBE cells.

As well as expression of the leukotriene synthesising enzymes, we examined the baseline expression of the LTB_4 receptor, BLT and the cys-LT receptor $CysLT_1$ (**Figures 20&21, p94**). Despite the use of two different antibodies, BLT expression in 16-HBE cells was not detectable by flow cytometry. There is very little information regarding the actions of LTB_4 on bronchial epithelial cells. The impact of LTB_4 on epithelial ciliary beat frequency was examined but found to be without effect (Bisgaard et al. 1987) and in our studies LTB_4 was not shown to induce the proliferation of 16-HBE cells. However, the antibodies used in this study are now known to be specific for the BLT1 form of the receptor, not BLT2. Whereas the expression of BLT1, a high affinity LTB_4 receptor has been described as exclusive to

leukocytes, the low affinity receptor BLT2 is ubiquitously expressed (Yokomizo et al. 2000). Although we did not find evidence of BLT1 on bronchial epithelial cells, these cells may still possess BLT2 which could mediate as yet unknown LTB_4 actions.

Using RT-PCR and FACS analysis we showed that $CysLT_1R$ mRNA and protein was spontaneously expressed in 16-HBE and primary bronchial epithelial cells. To our knowledge, this is the first demonstration of the $CysLT_1$ receptor in isolated bronchial epithelial cells, although the presence of a cys-LT receptor on these cells has been suggested by several studies. LTD_4 is a potent mitogen for bronchial epithelial cells and also has effects on ciliary beat frequency and mucus secretion (Leikauf et al. 1990, Granbo et al. 1996, Marom et al. 1982). The presence of a cys-LT receptor on BEC as well as the capacity of these cells for cys-LT production raises interesting possibilities whereby LTs produced by BEC may have autocrine effects. Antagonism of the $CysLT_1$ receptor on bronchial epithelial cells may contribute to the clinical benefits of these drugs in asthma and further studies are required to examine $CysLT_1$ and $CysLT_2$ regulation in these cells.

3.8.2 Effect of A23187 on LT pathway enzyme expression in bronchial epithelial cells. Calcium ionophore A23187 is a pore-forming molecule that causes non-specific increases in intracellular $[Ca^{2+}]$. Short-term exposure can activate LT synthesis by activating the Ca^{2+}-dependent enzymes $cPLA_2$ and 5-LO, whereas long-term exposure may cause changes in enzyme expression by activation of transcriptional regulatory pathways. Increases in the expression of all four LT pathway enzymes induced by A23187 were shown by FACS analysis in 16-HBE cells. Increases in 5-LO, FLAP and LTA_4 hydrolase were also shown in H292 cells (**Figures 25&26, p100&101**) The effect of Ca ionophore was confirmed by immunocytochemistry in 16-HBE cells, in which A23187 caused dose- and time-dependent increases in 5-LO and FLAP expression, but was without effect on the high basal immunostaining for LTA_4 hydrolase (**Figure 22, p96**). A reason why Ca^{2+} ionophore was found to increase LTA_4 hydrolase expression by flow cytometry but not immunocytochemistry could be because the latter technique is not sensitive to intracellular changes in LTA_4 hydrolase expression. The increase in LTA_4 hydrolase expression detected by FACS may be due to increases in the levels of enzyme expression within cells, rather than an increase in the number of LTA_4 hydrolase positive cells. Although immunocytochemistry did not show a general time dependent increase in the percentage of LTA_4 hydrolase positive cells, the number of LTA_4 hydrolase positive cells was twice as

great in A23187 stimulated cells compared to unstimulated cells at the 12 hour time point alone. While this difference did not reach statistical significance (p=0.07), it may indicate that A23187 can increase the number of LTA_4 hydrolase positive cells although this effect begins to occur later than observed for 5-LO, FLAP, and LTC_4 synthase (>12 hours).

As well as the effect of Ca ionophore alone, the effect of A23187 in combination with PMA was examined by immunocytochemistry (**Figure 24, p98**). PMA has a structure analogous to diacylglycerol (DAG) an activator of protein kinase C, and can therefore initiate a cascade of cellular responses connected with but different to those of A23187. Results obtained for 5-LO and FLAP were similar to those observed with A23187 alone. However, the number of cells positive for LTA_4 hydrolase was increased almost 2-fold in samples with both A23187 and PMA, but no effect was observed by immunocytochemistry with 1μM A23187 alone. Also, whereas LTC_4 synthase staining was increased by A23187 alone, the combination of A23187 with PMA did not affect its expression. The promoter region of the LTA_4 hydrolase gene has a phorbol ester response element (AP-2) (Mancini et al. 1995) and PMA is able to double LTB_4 production in human neutrophils (McColl et al. 1987). PMA may have additional effects to A23187 causing further upregulation of LTA_4 hydrolase. In granulocytes, PMA can suppress LTC_4 production by stimulating LTC_4 degradation and inhibiting LTC_4 synthase activity (Sjölinder et al. 1995). It may be that PMA also reduces LTC_4 synthase expression which would explain why A23187 with PMA failed to increase the number of LTC_4 synthase positive cells.

In primary human bronchial epithelial cells from nine subjects, all four leukotriene pathway enzymes were detectable by flow cytometry (**Figure 35, p115**), but only FLAP expression was increased by A23187. Analysis of the FLAP promoter has shown that this gene is highly inducible, with binding sites for several nuclear transcription factors including AP-2 and the glucocorticoid response element (Kennedy et al. 1991), whereas the 5-LO gene promoter resembles a housekeeping gene (Funk et al. 1989). LT production cannot occur without FLAP in intact cells (Dixon et al. 1990) and if FLAP was a limiting factor in the LT pathway of primary BEC, then its upregulation alone may be sufficient to increase LT production. The failure of A23187 to upregulate 5-LO, LTA_4 hydrolase and LTC_4 synthase in primary cells may be due to subgroups in the population, with different characteristics in their epithelial LT pathway. The LTC_4 synthase results appear to show that 6 out of the 9 subjects have no LTC_4 synthase expression either before or after Ca ionophore stimulation,

but that 3 subjects have inducible LTC_4 synthase expression. Several polymorphisms have been discovered in the LTC_4 synthase gene promoter which could explain differences in LTC_4 synthase expression between individuals. For example, the C_{-444} polymorphism may be more common in aspirin sensitive asthmatics than aspirin tolerant asthmatics and explain why these subjects also express more LTC_4 synthase (Sanak et al. 1998, Cowburn et al. 1998). However, more recent findings have shown that the LTC_4S_{-444} allele is found in a significant proportion of normal subjects (approximately 30%) where it is thought to predispose for enhanced synthesis of cys-LTs when the 5-LO pathway is stimulated (Sampson et al. 2000). In asthmatics the LTC_4S_{-444} genotype may be a marker of a phenotype that responds well to treatment with LT antagonists, as preliminary results from Asano and colleagues show that while the LTC_4S_{-444} polymorphism was not associated with any subject group (asthmatic or normal), it was able to predict responsiveness to pranlukast (Asano et al. 2001). The ability of A23187 to upregulate leukotriene pathway enzymes in 16-HBE, H292 and primary BEC is most likely due to increases in transcription and suggests that stimuli causing increases in intracellular $[Ca^{2+}]$ of bronchial epithelial cells may increase LT enzyme expression and the capacity for leukotriene production by these cells.

In comparison to levels of 15-LO metabolites produced by airway epithelial cells (approximately $2000ng/10^6$ cells), levels of both LTB_4 and cys-LT immunoreactivity produced by 16-HBE and primary cells were at least 1000 times lower (Sigal et al. 1991). However, these levels are not directly comparable as the cells in the Sigal study were provided with exogenous arachidonate at high concentrations before measurement of 15-LO metabolites. 15-HETE and related mediators, although they have several physiological effects are not as biologically active as the leukotrienes. LTD_4 is 1000 times more potent than histamine, causing bronchoconstriction at picomolar concentrations (Dahlén et al. 1980) and LTB_4 is a potent neutrophil chemoattractant at concentrations as low as $10^{-8}M$ (Ford-Hutchinson et al. 1980). Although not directly comparable with other studies due to differences in incubation times and the use of methanol to extract intracellular LTs, it is clear that leukocytes produce greater amounts of leukotrienes than BEC. Amounts of LTB_4 produced by primary BEC in the absence and presence of A23187 are approximately 14% and 33% of those generated by A23187 treated mixed blood leukocytes (Sampson et al. 1990). Few studies report the amounts of LTs produced by leukocytes in the absence of A23187, although it appears that levels are similar to unstimulated BEC. Unstimulated

eosinophils release between 20 and 50 pg $LTC_4/10^6$ cells in 30 minutes (Tenor et al. 1996, Mahauthaman et al. 1988) which is broadly comparable with the 300 pg $LTC_4/10^6$ cells spontaneously released by primary BEC in 6 hours. However, unlike the HBEC in this study, LTC_4 production by eosinophils is increased by up to 100 times in response to 1 μM A23187 (Mahauthaman et al. 1988). In support of a biological role for BEC derived LT production however, there are far more epithelial cells than leukocytes in the airway. The total internal surface of the respiratory epithelium is approximately $100m^2$ so it is conceivable that even low level production of LTs by epithelial cells could have biological effects.

The characteristics of LT release by HBE cells may suggest a different role for these LTs compared to those derived from inflammatory leukocytes. As mentioned, upon stimulation with Ca ionophore A23187 eosinophils respond with a rapid surge in LTC_4 release, between 30 and 100-fold greater than that observed in the absence of Ca ionophore, during a short 15 minute incubation (Mahauthaman et al. 1988, Burke et al. 1990). In 16-HBE cells, A23187 increased LTB_4 release by 25% and doubled cys-LT production although no significant effect was observed in the primary cells (**Figure 27, p102**). A23187 treatment in primary BEC caused a 2-fold increase in LTB_4 release but this did not reach statistical significance ($p=0.15$). Continuous low level LT production by BEC may be of greater importance in chronic disease than rapid increases, which may be more important, for example, in acute responses to allergen, such as during the EAR. Several agents caused upregulation of 5-LO pathway enzyme expression in HBEC, suggesting that there is a potential for increases in LT biosynthetic capacity, but that these increases may be associated with the more chronic effects of LTs such as proliferation and remodelling rather than acute bronchoconstriction. There could be a role for BEC derived LTs during the late airway response following inhaled allergen challenge. The late response may occur 3-4 hours after challenge and last for up to 24 hours, a time frame that could support transcriptional upregulation of 5-LO pathway enzymes and the associated increase in LT production in epithelial cells. The late response is characterised by an influx in inflammatory cells and subtle increases in BEC-derived LTC_4 and LTB_4, potent chemoattractants for eosinophils and neutrophils, could contribute to this effect.

Levels of the 5-LO pathway enzymes were compared between epithelial cells and mixed leukocytes using SDS/PAGE and Western blotting experiments (**Figure 18, p91**). When

equivalent numbers of 16-HBE and mixed leukocytes were electrophoresed, the epithelial cells appeared to express greater levels of 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase per cell than the leukocytes. This may be partly because epithelial cells are larger. When equal protein concentrations were used instead of cell number, enzyme expression was slightly higher in the leukocytes. An immunopositive band for LTC_4 synthase was not detectable in the mixed leukocytes. This is most likely due to the high composition of neutrophils (~60%) and lymphocytes (~30%) within the mixed leukocytes, which do not express LTC_4 synthase. Therefore, additional experiments were carried out using purified eosinophils which were found to express more LTC_4 synthase than the epithelial cells and mixed leukocytes.

3.8.3 Effects of bradykinin, histamine and LPS on the LT pathway in bronchial epithelial cells.

The inflammatory mediators bradykinin and histamine, and bacterial LPS are commonly used experimental stimuli of epithelial cells that might affect LT pathway enzyme expression. Bradykinin is generated in the respiratory tract during all forms of allergic, non-allergic and viral inflammation by kininogens (Proud et al. 1988). It has several effects on BEC mediated by its B2 receptor (Ricciardolo et al. 1998), including the stimulation of PGE_2 production (Aksoy et al. 1999). Lipopolysaccharide (LPS) is a toxic bacterial membrane product that has pro-inflammatory effects on most cell types. In BEC it has been shown to upregulate the expression of several enzymes and receptors associated with inflammation (Yao et al. 1996, Verheggen et al. 1996). Histamine is derived from mast cells and basophils and has been shown to increase inflammatory cytokine production in BEC (Plitt et al. 1999).

We showed that bradykinin upregulated 5-LO and LTA_4 hydrolase expression by approximately 40% and 60% respectively, while LPS increased FLAP and LTA_4 hydrolase expression by 90% and 50% (**Figures 28-30, p103-105**). Although these changes are relatively small, it is possible that they could increase the capacity for both LTB_4 and LTC_4 production. However, the physiological relevance of these sporadic changes should not be overestimated as they were not dose-dependent, a pharmacological characteristic one would normally expect to observe in experiments such as these. Histamine was not found to have any effect on expression of the LT enzymes. LTC_4 synthase expression was low in control samples and not significantly changed after simulation with bradykinin, LPS or histamine.

Differential regulation of LT pathway enzymes by bradykinin, LPS and histamine is consistent with the 5-LO, FLAP and LTA_4 hydrolase genes having different transcription factor binding sites, and suggests that different stimuli could specifically cause alterations in the production of either LTB_4 or LTC_4. An increase in LTB_4 production by BEC could be of particular relevance to bacterial infections and neutrophilic exacerbations of asthma (Fahy et al. 1995, Sur et al. 1993). LTB_4 is a potent neutrophil chemoattractant. Increases in neutrophils are also associated with allergen challenge and bacterial infections and treatment with LY293111 can reduce this neutrophilia (Evans et al. 1996, Crooks et al. 2000).

3.8.4 Effects of inflammatory cytokines on the LT pathway in bronchial epithelial cells.

The inflammatory cytokines investigated in this study were IL-3, IL-5, IL-6, TNFα, TGFβ and GM-CSF, chosen for their effects on LT pathway upregulation in leukocytes, or a mixture of IL-1β, TNFα and IFNγ, selected because of several known positive effects on HBE cells. BAL fluid from symptomatic asthmatics contains significantly increased levels of pro-inflammatory cytokines including IL-1β and TNFα (Broide et al. 1992). Also, TNFα and IFNγ, in combination with IL-1β, increase PGE_2 release and COX activity, and induce COX-2 and iNOS in pulmonary epithelial cells (Mitchell et al. 1994, Robbins et al. 1994). These studies found that IL-1β, IFNγ and TNFα used individually had no effect, but when used in combination caused significant changes. We found that this cytokine combination significantly increased the expression of FLAP by 64% in 16-HBE cells and by 33% in H292 cells (**Figures 32&33, p109&110**). Although further experiments are required to examine the effect of cytokine stimulation on LT production, this cytokine-induced FLAP expression could increase the capacity for LT production by BEC in an inflammatory environment.

Although bronchial epithelial cells do have receptors for IL-3, IL-5, IL-6, TNFα, TGFβ and GM-CSF (Levine 1995), these cytokines did not cause upregulation of either 5-LO, FLAP or LTA_4 hydrolase in 16-HBE (**Figure 31, p107**). Some very small (20-30%) but significant reductions in LTA_4 hydrolase and 5-LO expression were observed with TGFβ and IL-6 respectively. Downregulation of enzyme expression is feasible as the transcription factor binding sites in the 5-LO and LTA_4H enzyme promoters mediate both positive and negative regulation. IL-6 has several unique anti-inflammatory functions. It can terminate IL-1 and TNF synthesis and the overproduction of IL-6 in a murine transgenic model of bronchial

inflammation lessens bronchial hyperreactivity (DiCosmo et al. 1994). TGFβ also has anti-inflammatory effects, inhibiting Ig secretion by B lymphocytes and the cytotoxicity of mononuclear phagocytes (Borish 1998). The physiological relevance of such small reductions in enzyme expression may also be small, but these results do suggest that there is potential for both up and downregulation of LT pathway enzyme expression in HBEC.

3.8.5. The effect of anti-asthma drugs on LT pathway enzyme expression in bronchial epithelial cells.

Stimulation with dexamethasone was not found to have any effect on the baseline expression of 5-LO pathway enzymes in HBE cells. Therefore, dexamethasone was added in combination with A23187 to examine whether it caused any changes to the calcium ionophore-induced upregulation of 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase. Dexamethasone did not inhibit A23187 upregulation of FLAP, LTA_4 hydrolase and LTC_4 synthase but significantly reduced A23187 upregulation of 5-LO expression by approximately 40% (**Figure 34, p112**). Although moderate in size, this finding could be of physiological relevance. If epithelial 5-LO was upregulated in an inflammatory environment, corticosteroid treatment could reduce its expression and reduce the capacity for LT production. However, the general lack of effect of dexamethasone on epithelial LT pathway enzyme expression is not surprising as several *in vivo* studies have found that glucocorticosteroids are without effect on LT production. Short-term studies in both asthmatic and normal subjects have demonstrated that neither oral prednisolone nor inhaled budesonide and fluticasone propionate alter levels of urinary LTE_4, a marker of total body cys-LT production (O'Shaughnessy et al. 1993, Sebaldt et al. 1990, Manso et al. 1992).

The present findings are different from those in leukocytes where dexamethasone has been shown to increase the expression of both 5-LO and FLAP (Goppelt-Struebe et al. 1997, Pouliot et al. 1994, Colamorea et al. 1999). The physiological relevance of these surprising observations remains unknown but they appear to be specific to cells of myeloid origin. The activity of dexamethasone on LTA_4 hydrolase expression in another structural cell type, keratinocytes has been examined but was found not to change enzyme expression, even after incubation for 72 hours (3 times longer than the incubation time in this project). The effect of dexamethasone on basal LTC_4 synthase expression has been measured in mast cells and basophilic cells but was not found to alter levels of enzyme expression, although in basophilic cells dexamethasone did reduce LTC_4 production by a post-transcriptional

mechanism (Colamorea et al. 1999, Hamasaki et al. 1997). While it is possible that dexamethasone could reduce epithelial LT production by a mechanism other than regulation of enzyme expression, such as by inhibiting $cPLA_2$ generation of arachidonic acid via the induction of lipocortin (Croxtall et al. 1995), it would appear that the anti-inflammatory actions of glucocorticosteroids do not extend to an effect on LT synthesis.

The action of the $CysLT_1$ receptor antagonist, MK-571 on 5-LO pathway enzyme expression was also examined in 16-HBE cells and results were similar to those found with the corticosteroid. Although MK-571 appeared to completely inhibit the A23187-induced upregulation of 5-LO, this change was not statistically significant and MK-571 had no effect on the expression of FLAP, LTA_4 hydrolase and LTC_4 synthase, either basal or A23187-stimulated.

3.8.6 Effect of LTs on the proliferation of 16-HBE cells

Several of the structural changes that take place within the asthmatic airway are linked to epithelial cells. Asthma is associated with a characteristic shedding of the epithelium and as a result there is a continuous cycle of denudation and repair taking place within the lung. The processes of epithelial proliferation, differentiation and survival are of particular importance to this cycle. For example compared to normals, an increase in the number of mucus secreting goblet cells is found in asthmatic subjects (Aikawa et al. 1992). Along with their bronchoconstrictor and leukocyte chemoattractant actions, leukotrienes have been implicated in the proliferation of vascular and bronchial smooth muscle cells (Panettieri et al. 1998, Porreca et al. 1996). Therefore it was our aim to examine whether leukotrienes have a role in the regulation of epithelial cell proliferation. In our experiments, LTB_4 was without effect on the proliferation of 16-HBE. This finding was not surprising, as we were unable to detect the presence of BLT receptors in bronchial epithelial cells and no other reports of LTB_4 actions on HBE cells have been found. However, of the three concentrations of LTD_4 tested (1, 10 and 100nM), the lowest caused a very small, yet significant increase in the proliferation of 16-HBE, compared to cell numbers present in control medium alone. It is not uncommon to see bell shaped dose response curves in biological systems and it may be that low concentrations of LTD_4 are more effective in stimulating BEC proliferation. The effect of cys-LTs on the proliferation of airway epithelial cells has been examined in another study where LTC_4 was found to be more potent than LTD_4 and LTE_4, stimulating epithelial proliferation at concentrations as low as 10fM (Leikauf et al. 1990). Such findings may

suggest the involvement of a cys-LT receptor other than $CysLT_1$, at which LTD_4 is approximately ten fold more potent than LTC_4 (Lynch et al. 1999). However, in our studies the $CysLT_1$ antagonist MK571 was able to inhibit LTD_4 induced mitogenesis indicating the involvement of $CysLT_1R$. Further studies are required to examine the expression and function of $CysLT_1$ and $CysLT_2$ in bronchial epithelial cells.

The increases in 16-HBE division caused by LTD_4 were relatively small so we also studied the effect of LTs in combination with a growth factor (EGF) to see if proliferation could be increased further. In airway smooth muscle LTD_4 has been shown to synergise with growth factors such as IGF and EGF to increase proliferation to levels greater than those observed with either compound alone (Panettieri et al. 1998, Cohen et al. 1995). Our findings were unexpected and showed that EGF caused a significant decrease in the proliferation of 16-HBE compared to control. Even more surprising was the result that while LTB_4 was without effect, LTD_4 was able to prevent the EGF-induced reduction in proliferation (**Figure 38, p119**). There is overwhelming evidence that EGF (epidermal growth factor) is a mitogen of epithelial cells (Vignola et al. 1997) and literature searches have not revealed any conditions under which EGF is inhibitory. Therefore it is most likely that the EGF-induced inhibition observed here is an artefact of the experimental technique. Only one concentration of EGF was examined at a single time point. To clarify the reason for this downregulation, similar experiments need to be performed using different HBE cells as it is known that the processes of immortalisation may produce cells with altered characteristics (Medina et al. 1990, Fu et al. 1988). Different EGF concentrations should also be investigated as the authenticity of this effect would be supported if the inhibition was dose-dependent. In addition, the use of a longer time course could reveal a delayed mitogenic effect. Although the mechanism whereby EGF reduced 16-HBE proliferation is unknown and may be an artefact, the finding that LTD_4 was able to prevent this downregulation is consistent with a role for LTD_4 in regulating BEC proliferation, which could be of importance to airway remodelling in asthma. From these results, it is not possible to say that LTD_4 and EGF synergise to increase the proliferation of 16-HBE cells, although more work is required to clarify these findings.

3.8.7 Conclusion

Although specific IgE mediated mast cell degranulation is responsible for the acute bronchconstriction upon allergen exposure, the mechanisms of non-allergic asthma triggers are less well known. The finding that the LT pathway is expressed, and can be upregulated

in human bronchial epithelial cells could be of relevance to the mechanism of non-allergic asthma exacerbations. As bronchial epithelial cells are the first cells to come into contact with inhaled triggers of asthma such as pollutants and rhinoviruses, increases in LT production by these cells could contribute to impaired lung function during such exposure. Epithelial cells are the principal hosts for respiratory viruses and infection causes widespread in vivo damage, as well as increased production of a range of pro-inflammatory cytokines such as IL-6, IL-8, IL-11 and GM-CSF (Folkerts et al. 1998). Several bacteria such as *Streptococcus pneumoniae* adhere to HBEC and induce mediator release (Adamou et al. 1998). Studies have shown that experimental exposure of human BEC to nitrogen dioxide (NO_2), ozone (O_3), and diesel exhaust particles results in significant synthesis and release of pro-inflammatory mediators, including PAF, IL-6, IL-8 TXB_2 and PGE_2 (Mills et al. 1999). Exposure to certain allergens can also have an effect on BEC, not by IgE-mediated mechanisms, but instead due to the biochemically-active protease activity of allergens. The dust mite allergens Der p1 and Der p9 have cysteine and serine protease activity respectively and alter epithelial integrity and permeability, and induce cytokine and mediator production (Thompson 1998). They can also increase the expression of ICAM-1 and MHC class II expression.

In addition to pro-inflammatory cytokines, stimulation of HBE cells with inhaled asthma triggers leading to LT production could explain how non-allergic asthma precipitants augment inflammation. An increase in cys-LT production would increase mucus secretion, oedema and eosinophil migration and LTB_4 production would increase airway neutrophilia. As BEC do not rapidly increase LT production upon stimulation, it is unlikely that BEC derived cys-LTs contribute to immediate bronchoconstriction, but cumulative increases following 5-LO pathway enzyme induction may contribute to more chronic effects.

In summary, this is the first study to link LT immunoassays with RT-PCR, immunocytochemistry, Western blotting, and flow cytometry to characterise the expression of mRNA and protein for the enzymes of the LT synthetic pathway in human bronchial epithelial cells. Studies were performed in 16-HBE and H292 cell lines and in primary cells from normal lung, both at baseline and after treatment with a range of autacoids, cytokines, and growth factors. Our results demonstrate firstly that primary BEC and bronchial epithelial cell lines constitutively express 5-LO, FLAP, LTA_4 hydrolase, and LTC_4 synthase mRNA and protein in amounts broadly comparable to those in human blood leukocytes.

Secondly, these cells release both LTB_4- and cys-LT-like immunoreactivity, spontaneously and after stimulation with calcium ionophore A23187. Thirdly, treatment with lipopolysaccharide, inflammatory cytokines, A23187, and bradykinin can upregulate the expression of 5-LO pathway enzymes in 16-HBE cells, whereas dexamethasone and MK571 may downregulate enzyme expression. Fourthly, both 16-HBE cells and primary HBE cells expressed the $CysLT_1$ receptor on their surface, indicating that these cells can respond to cys-LTs as well as synthesise them, while the LTB_4 receptor BLT1 was not expressed in 16-HBE cells. Finally, preliminary experiments examining the effect of leukotrienes on BEC proliferation suggested a mitogenic role for LTD_4. Taken together with the relative abundance of HBE cells within the human airway compared to infiltrating leukocytes, our study suggests that the epithelium may contribute significantly to the total capacity of lung tissue to generate LTs and hence contribute to airway inflammation and remodelling.

4. Results

The leukotriene synthetic pathway in human airway smooth muscle cells

HASM cells are the principal cellular targets of the cys-LTs as bronchoconstrictor agents and are also implicated in leukotriene induced remodelling responses. However, the capacity of HASM cells to synthesise leukotrienes has never been investigated. The aim of experiments in this chapter was to examine:

1. The baseline expression and the effect of inflammatory stimuli on 5-LO pathway enzymes and leukotriene production in cultured human airway smooth muscle cells.
2. The expression and regulation of the BLT and $CysLT_1$ receptors on these cells.
3. Due to the characteristic increase in airway smooth muscle mass in asthma, whether leukotrienes have a role in the regulation of HASM proliferation.

4.1 Baseline expression of 5-LO pathway enzymes, Cys-LT_1R and BLT in HASM cells.

Baseline expression of 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase was examined by RT-PCR, SDS-PAGE/Western blotting, FACS analysis and immunocytochemistry in HASM cells prepared from explants of airway smooth muscle. RT-PCR showed detectable baseline mRNA levels for 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase (**Figure 39a**). SDS-PAGE/Western blotting of HASM showed immunopositive protein bands for 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase at approximately 80, 18, 70 and 18 kDa respectively (**Figure 39b**). Equivalent numbers of mixed leukocytes were used as a positive control for enzyme expression, and these showed immunopositive bands for 5-LO, FLAP and LTA_4 hydrolase. LTC_4 synthase was not detectable in the mixed leukocytes, most likely due to the high percentage of neutrophils (~60%) and lymphocytes (~30%) (which do not express LTC_4 synthase) in this mixed cell population.

Intracellular staining of HASM cells for FACS analysis confirmed the baseline expression of 5-LO, FLAP and LTA_4 hydrolase protein in HASM (MFI 20.7±5.5, 37.3±13.6 and 31.7±8.6 respectively, n=8) although LTC_4 synthase immnuofluorescence was low (0.3±0.1) (**Figure 40**). To confirm the phenotype of the cultured cells, HASM cells were also stained with antibodies for human smooth muscle actin and myosin, both of which were present. **Figure 41** shows representative photomicrographs of HASM cells immunostained for the LT pathway enzymes. Immunohistochemistry confirmed the FACS results, showing positive staining for 5-LO, FLAP, LTA_4 hydrolase and the positive controls actin and myosin, and small amounts of LTC_4 synthase.

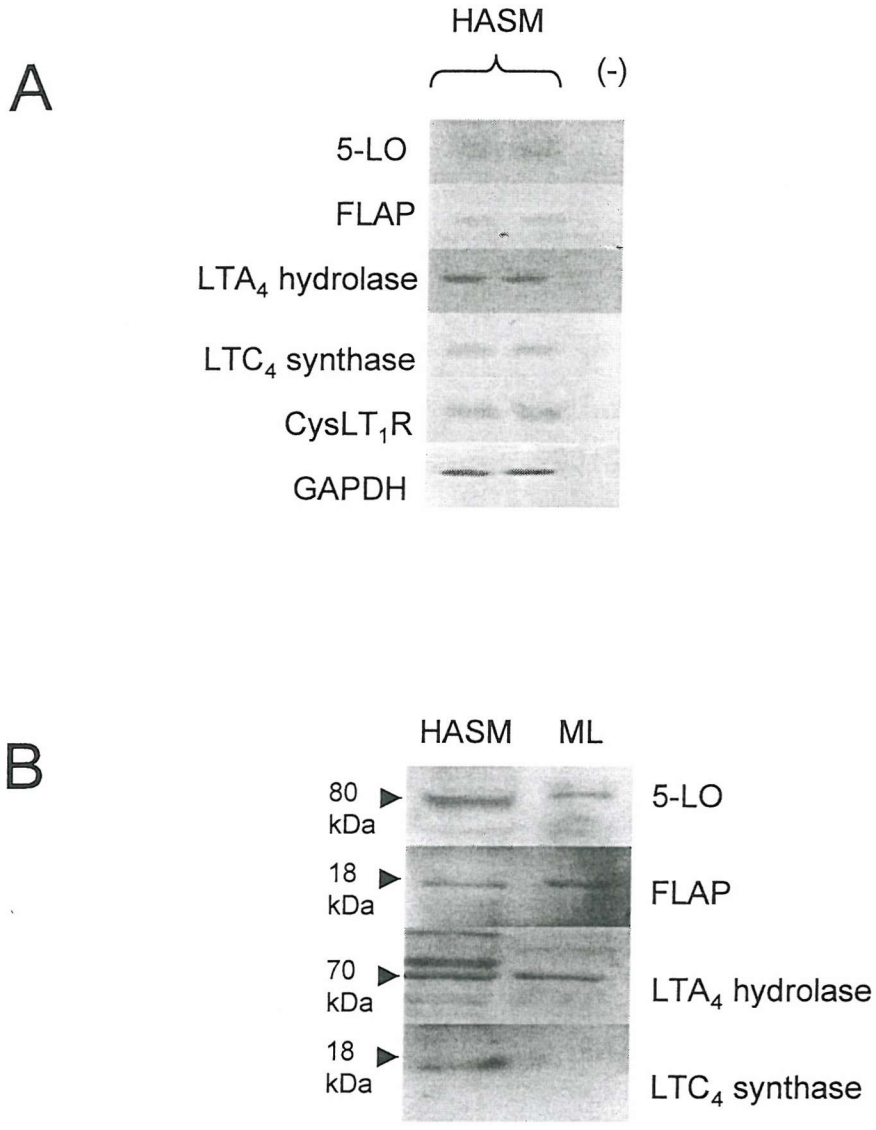


Figure 39. Expression of 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase mRNA and protein in HASM cells as shown by RT-PCR and SDS-PAGE/Western blotting. (A) Total RNA was isolated and RT-PCR was performed using gene specific primers. GAPDH was used as the internal positive control for PCR, and as a negative control, PCR was carried out in the absence of RT products. PCR products for 5-LO, FLAP, LTA_4 hydrolase, LTC_4 synthase and also $CysLT_1R$ were detected at 124, 210, 291, 155 and 250bp respectively. (B) Immunoblots for leukotriene pathway enzymes at baseline in HASM cells show immunoreactive bands for 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase at approximately 80, 18, 70 and 18 kDa respectively. Equal amounts of HASM protein lysates were resolved at 0.04A for 45 minutes on 10% polyacrylamide gels for 5-LO and LTA_4 hydrolase, and 12% gels for FLAP and LTC_4 synthase, using Bio-Rad mini protean II apparatus. Mixed leukocyte (ML) lysates, obtained by dextran sedimentation were used as a positive control for 5-LO, FLAP, LTA_4 hydrolase expression. Protein bands were visualised by Western blotting using a chemiluminescent chromagen as described in Methods.

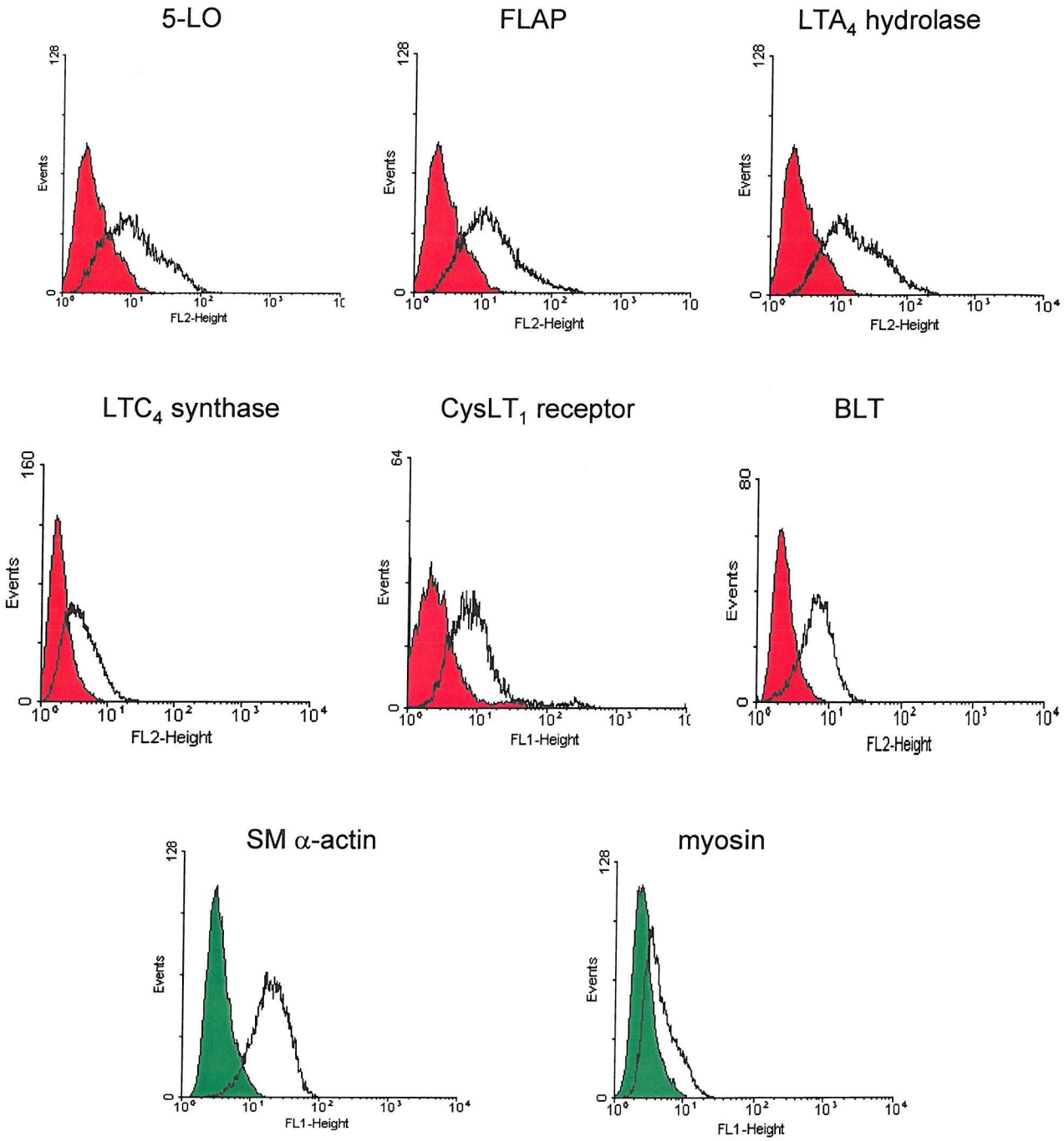


Figure 40. Representative FACS histograms showing baseline LT pathway enzyme and receptor expression in HASM cells. Cells were grown to confluence, detached and stained for 5-LO, FLAP, LTA_4 hydrolase, LTC_4 synthase, $cysLT_1R$ and BLT. As a positive control, cells were also stained for the intracellular contractile proteins α–actin and myosin. Filled curves show staining with the relevant isotype control and open curves show staining with the specific antibody. 5-LO, FLAP, LTA_4 hydrolase, LTC_4 synthase, $CysLT_1R$ and BLT all show detectable positive staining.

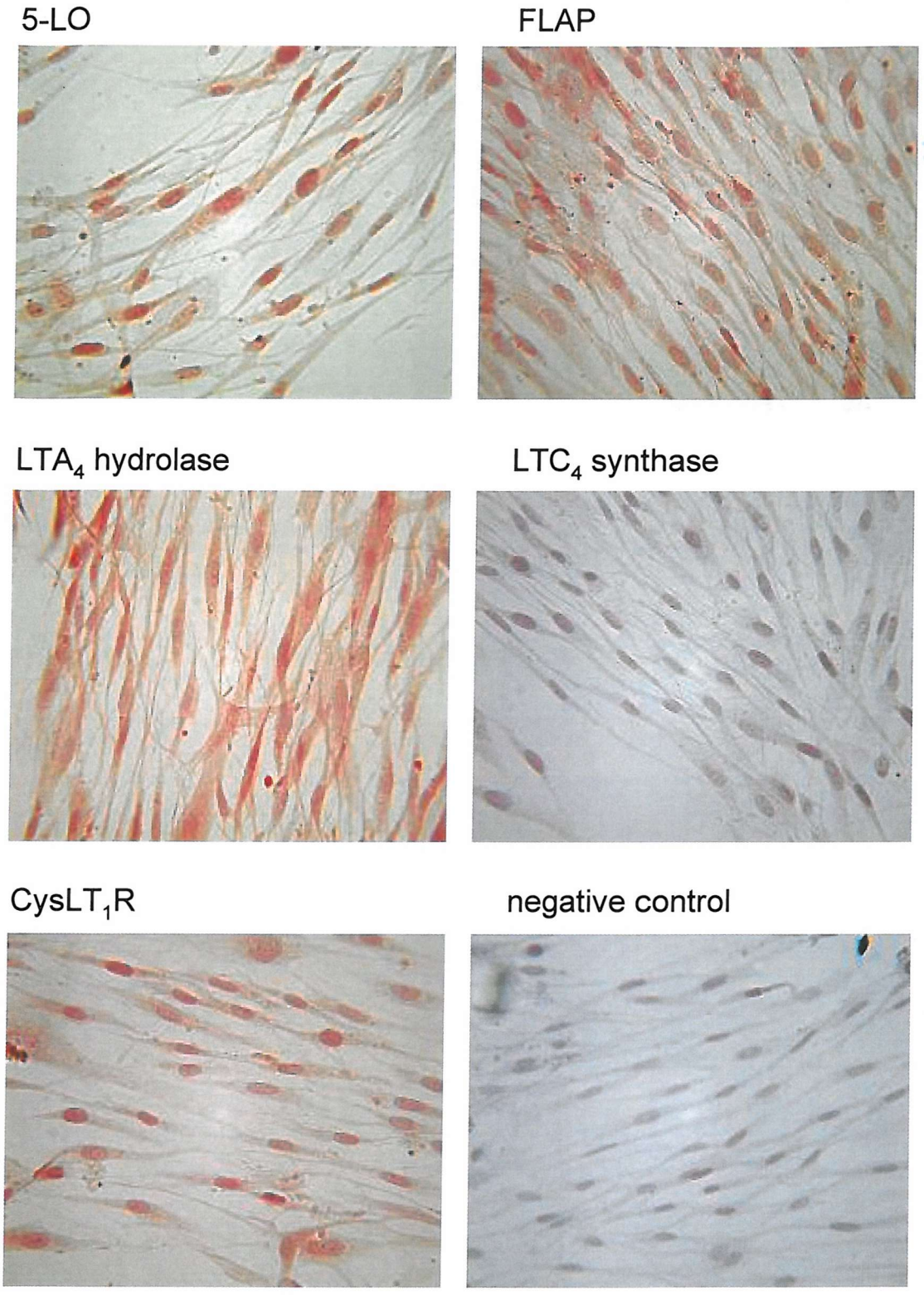


Figure 41. Representative photomicrographs (20x) showing baseline expression of LT pathway enzymes and $CysLT_1R$ in cultured HASM cells. HASM cells were grown to confluence on chamberslides and stained for 5-LO, FLAP, LTA_4 hydrolase, LTC_4 synthase and $CysLT_1R$. Primary antibody was omitted as a negative control. Positive AEC staining is red and nuclei are counterstained with Meyer's haematoxylin.

In addition to the LT pathway enzymes, the expression of Cys-LT_1R protein and mRNA in HASM cells was examined by FACS analysis, immunocytochemistry of cells grown on chamber slides and RT-PCR. FACS analysis showed positive staining for $CysLT_1$ on HASM (MFI 4.5±1.5) as did immunocytochemistry and RT-PCR (**Figures 39a, 40, and 41**). The baseline expression of the LTB_4 receptor, BLT was also measured by flow cytometry and results showed positive expression of this receptor, MFI 6.0±2.1 (**Figure 40**).

4.2 Effect of Calcium ionophore A23187 on the LT synthetic pathway in HASM cells

To investigate the effect of increased intracellular Ca^{2+} on the LT pathway in HASM, the expression of 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase was examined by FACS analysis in cells incubated for 6 hours with or without 1μM A23187. Compared to control MFI values in HASM cells (5-LO 18±4, FLAP 41±21, LTA_4 hydrolase 24±4, LTC_4 synthase 0.3±0.2), incubation with Ca ionophore had no significant effect on the expression of any enzyme (5-LO 15±4, FLAP 35±6, LTA_4 hydrolase 33±8 and LTC_4 synthase 0.7±0.2) (**Figure 42**).

To investigate the presence of an active LT biosynthetic pathway in HASM cells, production of LTB_4 and cys-LTs was measured by enzyme immunoassay of supernatants collected from cells incubated for 6 hours with or without Ca ionophore A23187 (1μM) (**Figure 43a**). There was baseline immunoreactivity for both LTB_4 and cys-LTs (1.9±0.2 ng/10^6 and 89±1.57 pg/10^6 cells respectively), although in accordance with the lack of effect on enzyme expression, A23187 did not significantly change the production of either LTB_4 or LTC_4 (1.6±0.3 ng/10^6 and 88.4±2.46 pg/10^6 cells respectively). The effect of passage number on leukotriene production was also examined and LTB_4 production showed a negative correlation with increasing passage number (ρ=-0.93, p=0.02, n=5) (**Figure 43b**).

4.3 Effect of pro-inflammatory mediators on the LT pathway in HASM

To investigate the possible regulation of the LT pathway enzymes by physiological agents, HASM cells were stimulated with a range of pro-inflammatory mediators (**Figures 44a and b**). Prior to FACS analysis of 5-LO, FLAP, LTA_4 hydrolase, LTC_4 synthase, $CysLT_1R$ and BLT expression, cells were incubated for 24 hours with either bradykinin (10μM), histamine (10μM), a cytokine mix consisting of IL-1β (20ng/ml), TNFα (200U/ml) and IFNγ (200U/ml), TGFβ (10ng/ml) or with LTB_4 (10nM) or LTD_4 (10nM). Compared to

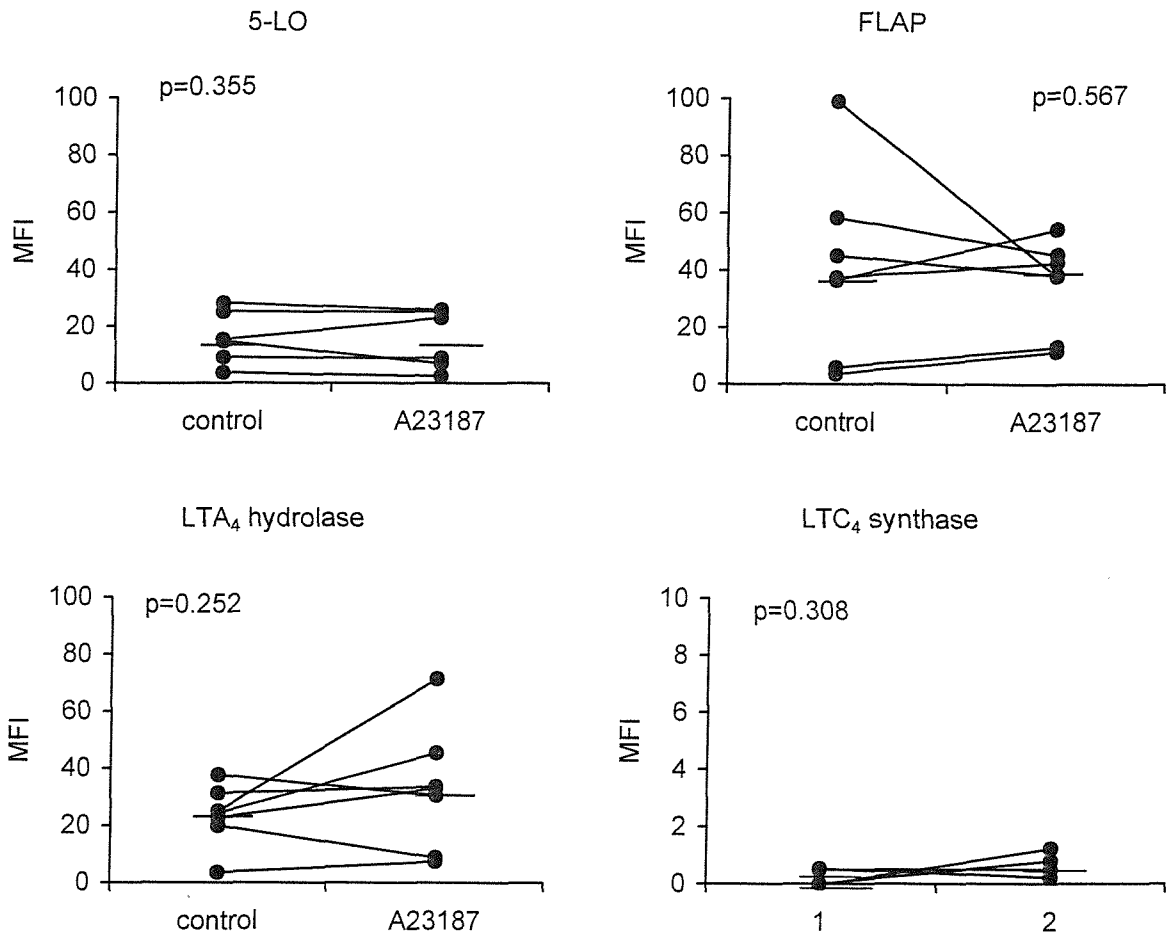


Figure 42. FACS analyses of the effect of Ca ionophore on 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase expression in HASM cells. Cells were cultured for 6 hours in the presence or absence of A23187 (1μM), fixed, permeabilised and stained for 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase as described in Methods. The fluorescence intensity of 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase staining was not significantly increased by Ca ionophore ($p>0.05$). Results are presented as individual data points with median bars and were analysed using Wilcoxon's signed rank test.

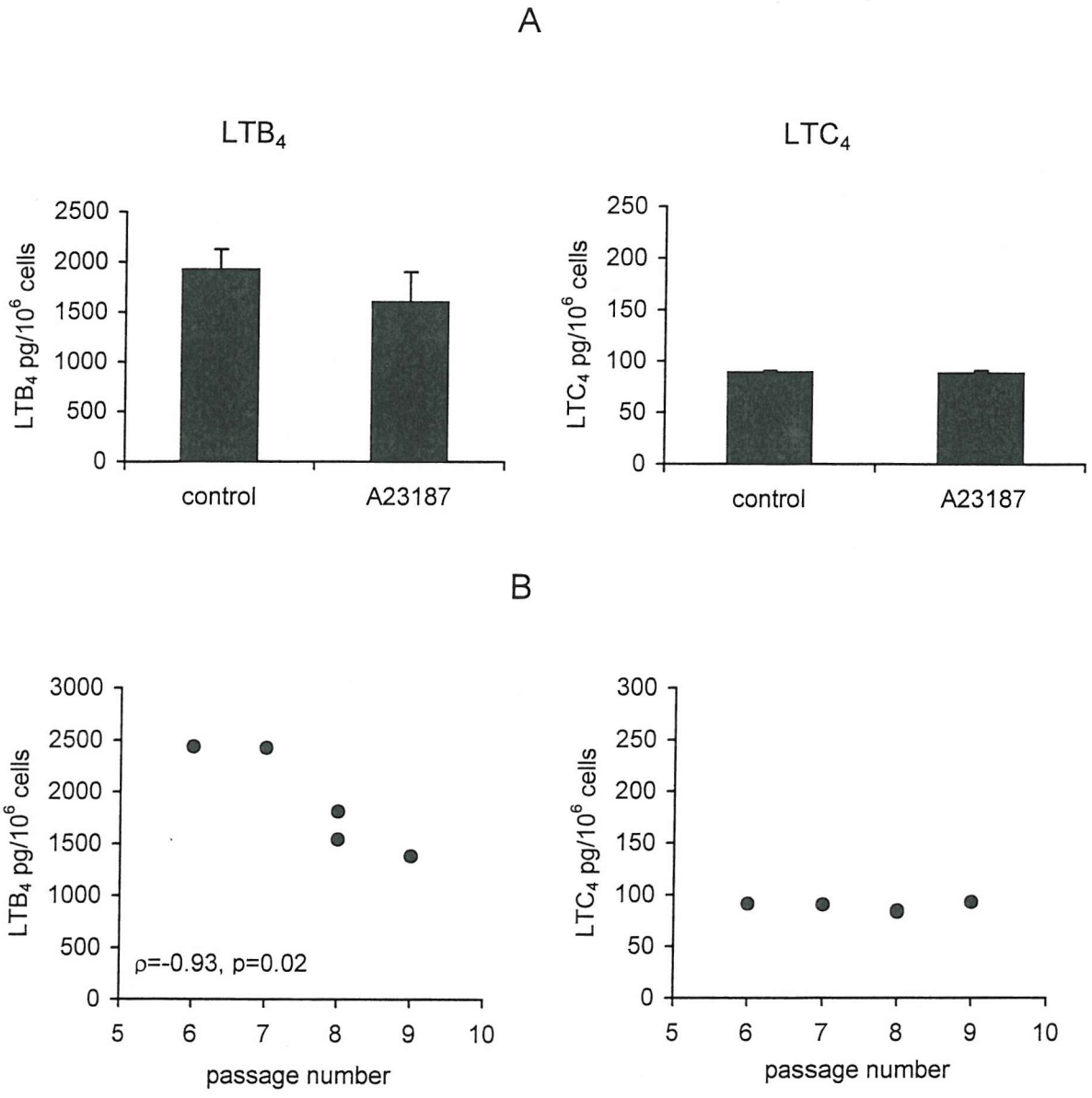


Figure 43. Effect of Ca ionophore A23187 on cys-LT and LTB_4 synthesis in HASM cells. Total cys-LTs (LTC_4,LTD_4 and LTE_4) and LTB_4 were measured by enzyme immunoassay of HASM cell supernatants incubated for 6 hours in the absence or presence of Ca ionophore (1μM) for 6 hours. (A) Leukotriene production (mean+/-SEM) is expressed as pg/million cells. Unstimulated HASM produced detectable amounts of both LTB_4 and cys-LTs. Incubation with Ca ionophore, however, did not significantly change either cys-LT or LTB_4 production in HASM. (B) Basal levels of LTB_4 and LTC_4 production are also plotted against cell passage number. LTB_4 production is significantly reduced as passage number increases (ρ=-0.93, p=0.02, Pearson correlation, n=5).

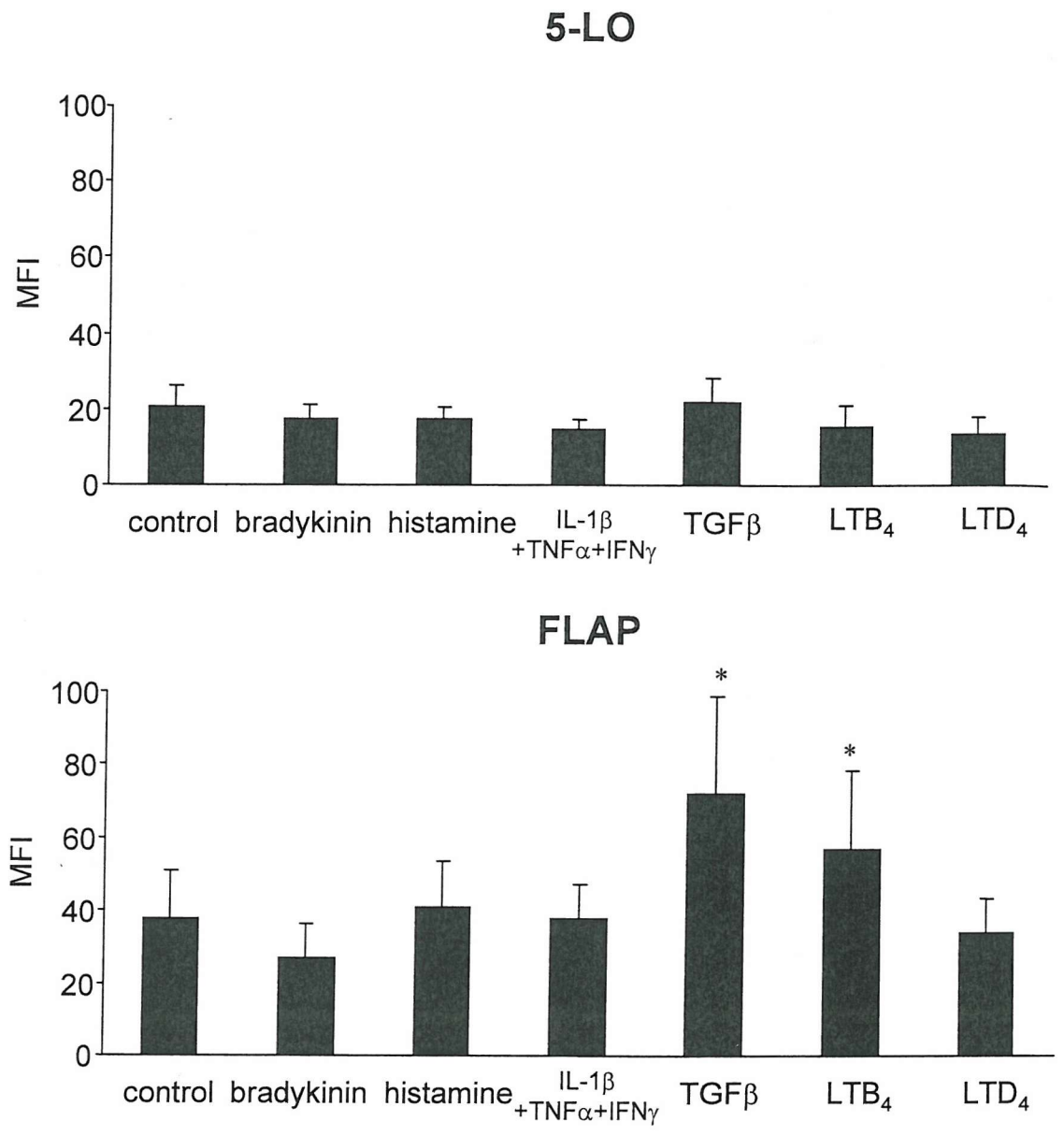


Figure 44a. FACS analyses showing the effect of pro-inflammatory cytokines on 5-LO and FLAP expression in HASM. Leukotriene pathway enzyme expression was examined in HASM cells incubated for 24 hours with either bradykinin (10μM), histamine (10μM), TGFβ (10ng/ml), LTB_4 (10nM), LTD_4 (10nM) or a cytokine mixture consisting of IL-1β (20ng/ml), TNFα (200U/ml) and IFNγ (200U/ml). Results show mean±SEM median fluorescence intensity and were compared using Student's paired t-test (*p=<0.05, n=6). Compared to control, 5-LO expression was not significantly changed by any of the stimuli. FLAP expression was significantly increased by TGFβ and LTB_4.

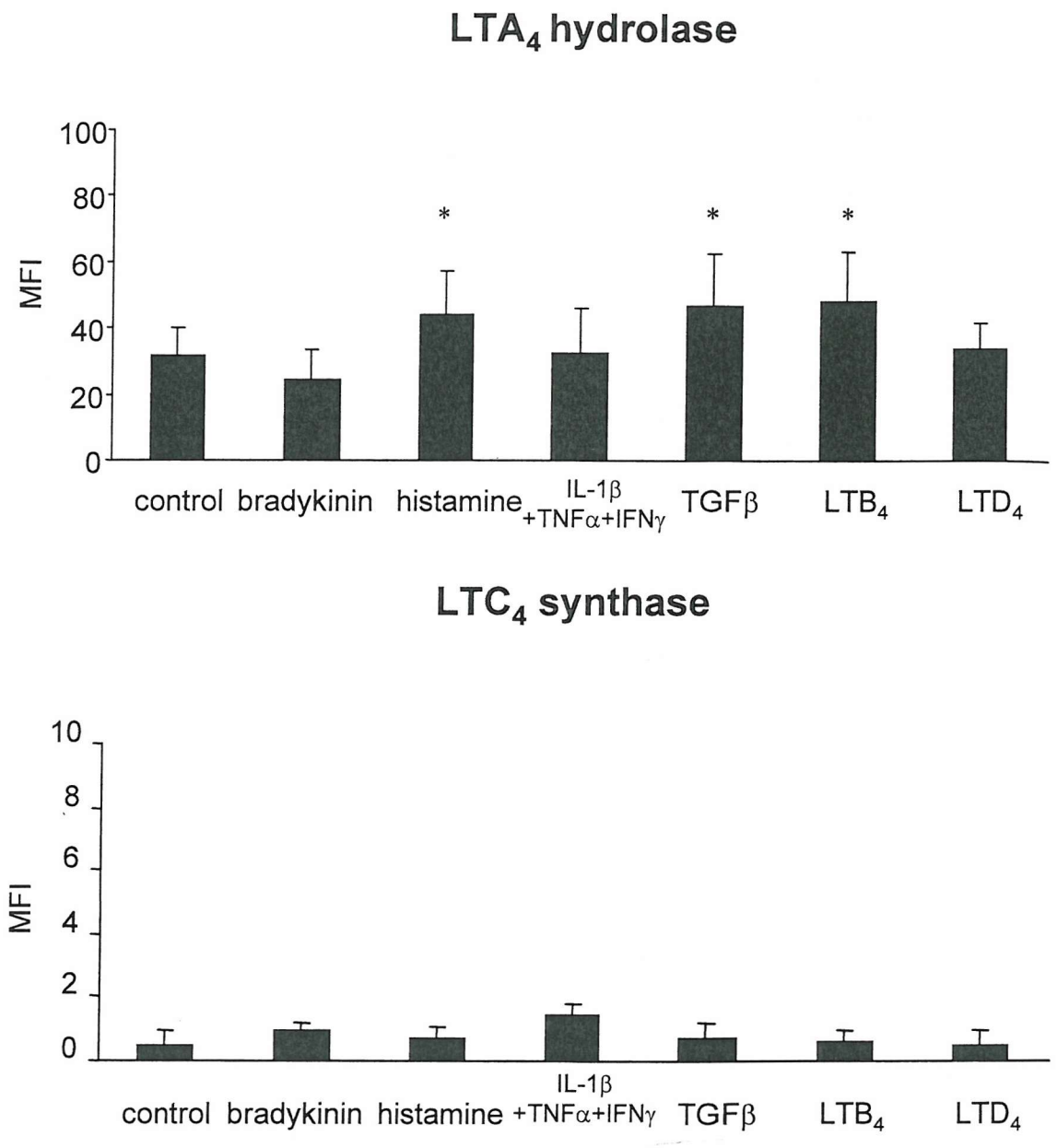


Figure 44b. FACS analyses showing the effect of pro-inflammatory cytokines on LTA_4 hydrolase and LTC_4 synthase expression in HASM. Leukotriene pathway enzyme expression was examined in HASM cells incubated for 24 hours with either bradykinin (10μM), histamine (10μM), TGFβ (10ng/ml), LTB_4 (10nM), LTD_4 (10nM) or a cytokine mixture consisting of IL-1β (20ng/ml), TNFα (200U/ml) and IFNγ (200U/ml). Results show mean±SEM median fluorescence intensity and were compared using Student's paired t-test (*p=<0.05, n=6). Compared to control, LTA_4 hydrolase expression was significantly increased by TGFβ, LTB_4 and histamine.

control (MFI 39.2±18.2), the expression of FLAP was significantly increased by TGFβ and LTB_4 (71.7±27.1, p=0.03 and 57±21.1, p=0.05 respectively, n=6). Compared to control (MFI 28.3±10.7), LTA_4 hydrolase expression was also significantly increased by TGFβ and LTB_4, as well as by histamine (46.7±15.8, p=0.05, 48.2±15.1, p=0.01 and 43.8±13.2, p=0.05, respectively, n=6). The expression of 5-LO was not significantly changed by any of the inflammatory stimuli and LTC_4 synthase expression remained low by FACS analysis, and showed no significant differences between control and stimulated cells.

The effect of these stimuli on $CysLT_1R$ expression in HASM was also examined by FACS analysis (**Figure 45**). Compared to control (MFI 4.5±1.5, n=7), expression of $CysLT_1R$ was significantly increased by TGFβ (7.7±2.3, p=0.03) and by the cytokine mix (14.3±5.1, p=0.05). Histamine, bradykinin, LTB_4 and the $CysLT_1R$ agonist LTD_4 had no significant effect on receptor expression. Compared to control (MFI 6.0±2.1), the expression of BLT was significantly increased by the cytokine mix (MFI 7.4±1.7, p=0.037). Bradykinin and histamine also increased BLT immunofluorescence (BK 9.3±2.6 and HIS 9.2±2.4) although these changes did not reach significance (p=0.063 and p=0.061 respectively) (**Figure 45**). LTB_4, LTD_4 and TGFβ had no significant effect on BLT receptor immunofluorescence.

The culture media of HASM cells incubated for 24 hours with histamine, bradykinin, TGFβ, LTB_4, LTD_4 and the cytokine mix were collected for enzyme immunoassay to measure LTB_4 and LTC_4 immunoreactivity (**Figure 46**) as described in Methods. There was spontaneous production of LTB_4- and LTC_4-like immunoreactivity as shown by their detection in supernatants collected from unstimulated HASM cells (LTC_4 245±10 pg/10^6 cells and LTB_4 473±150 pg/10^6 cells). Interestingly, compared to control, LTB_4 significantly increased the production of LTC_4 (335±20 pg/10^6 cells, p=0.05) and LTD_4 significantly increased levels of LTB_4 measured (765±139 pg/10^6 cells, p=0.017). Despite some induction of FLAP and LTA_4 hydrolase by TGFβ, LTB_4 and histamine, treatment of HASM with TGFβ, histamine, bradykinin or the cytokine mix did not significantly affect LTB_4 and LTC_4 production compared to control.

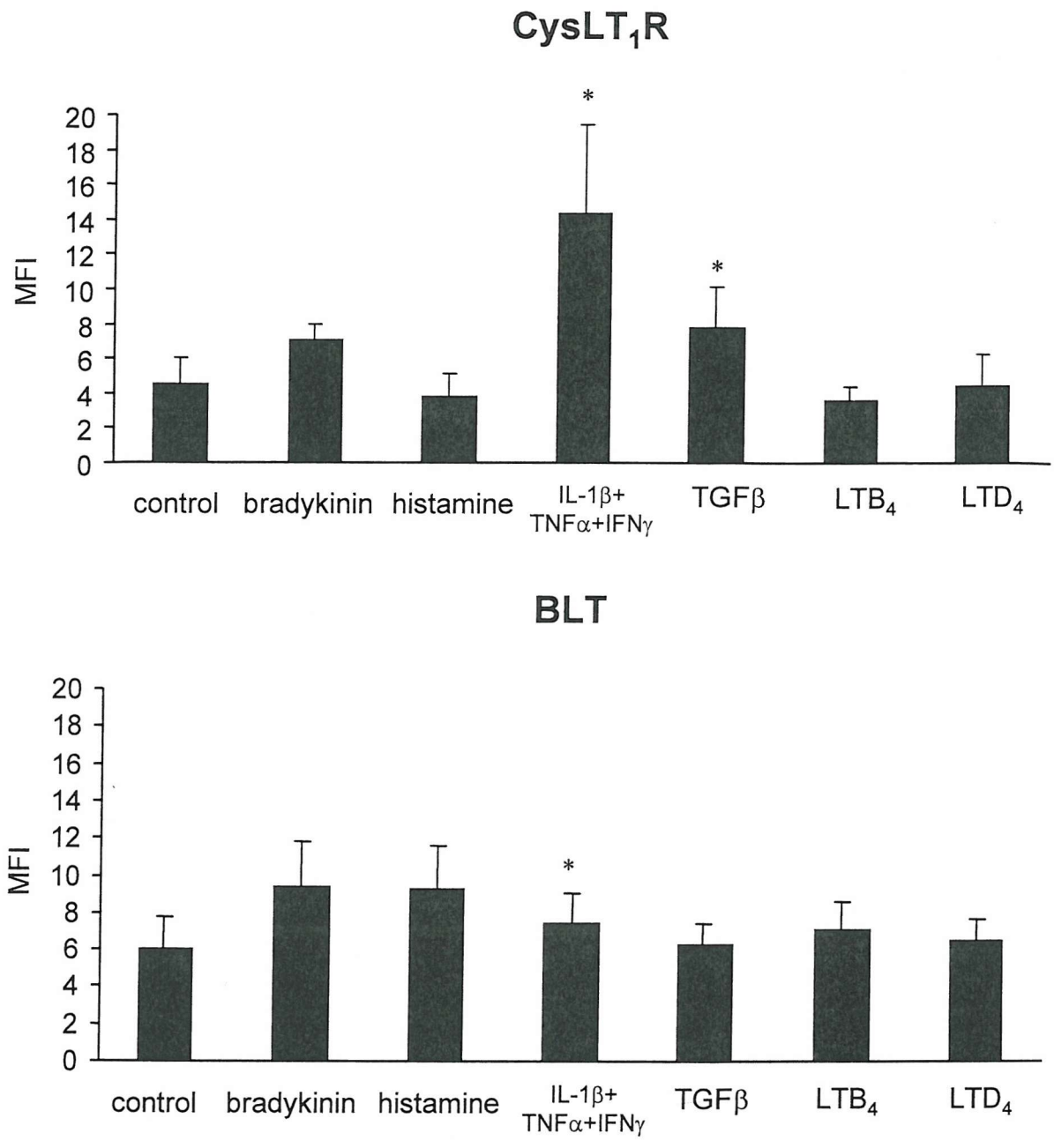


Figure 45. FACS analyses showing the effect of pro-inflammatory cytokines on $CysLT_1$ and BLT receptor expression in HASM. $CysLT_1$ and BLT receptor expression was examined in HASM cells incubated for 24 hours with either bradykinin (10μM), histamine (10μM), TGFβ (10ng/ml), LTB_4 (10nM), LTD_4 (10nM) or a cytokine mixture consisting of IL-1β, TNFα and IFNγ (each at 10ng/ml). Cells were fixed and permeabilised for BLT staining whereas $CysLT_1$ staining was carried out on live, unfixed cells. Results are shown as mean+SEM and receptor immunofluorescence in the stimulated samples was compared to control values using Student's paired t-test (*p=<0.05, n=6). MFI=Median Fluorescence Intensity.

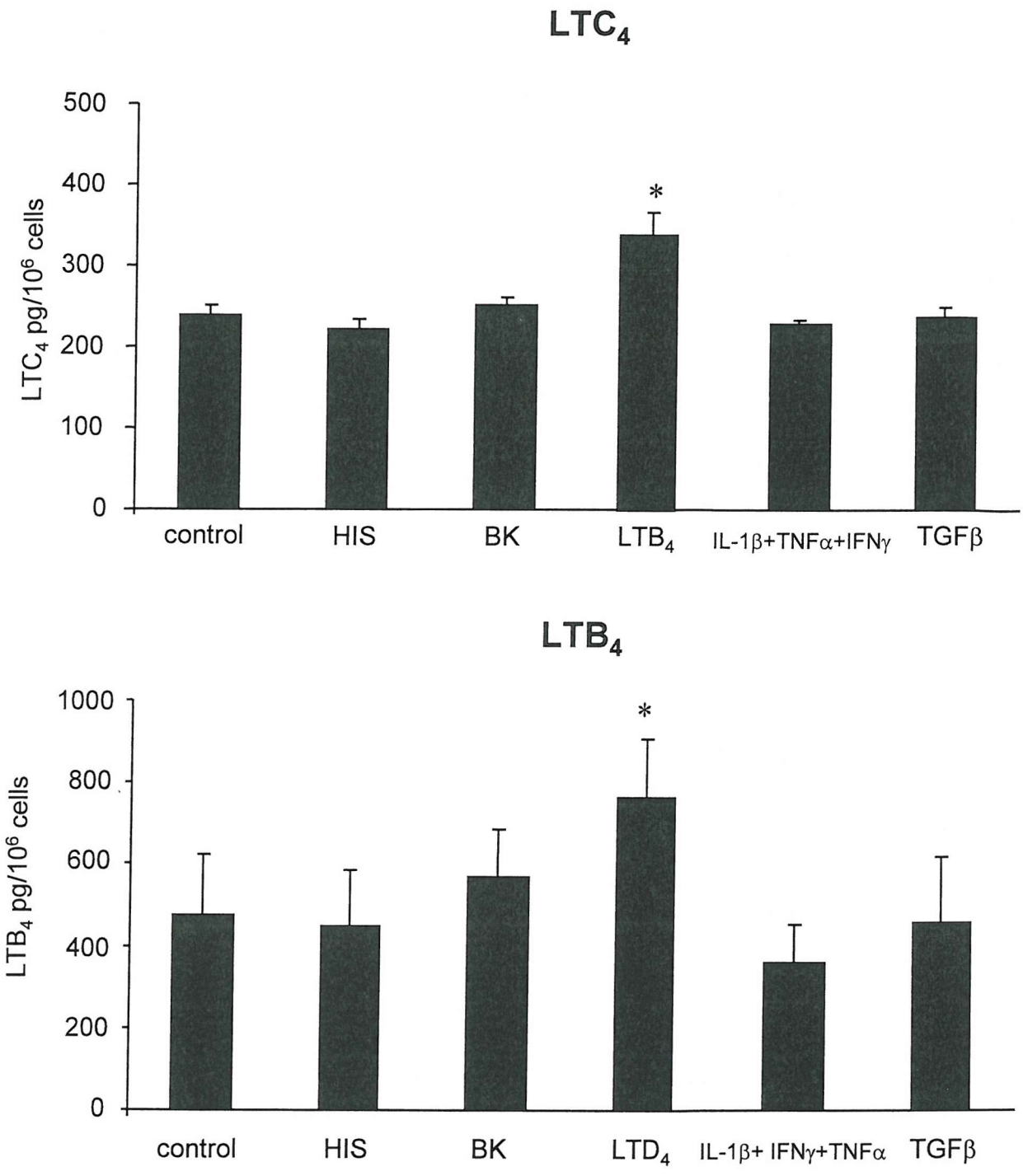


Figure 46. LT production by HASM in response to stimulation with inflammatory stimuli. LTC_4 and LTB_4 production by HASM was measured after incubation for 24 hours in either serum-free medium alone or EGF (10ng/ml), TGFβ (10ng/ml), LTB_4 (10nM), LTD_4 (10nM), bradykinin (10μM), histamine (10μM) or a combination of IL-1β, TNFα, and IFNγ (each at 10ng/ml). LT production was measured by enzymeimmunoassay as described in Methods. Levels of LTs produced by HASM after stimulation with inflammatory mediators were compared to levels present in control medium using Student's paired t-test (n=5, *$p<0.05$).

4.4 Effect of dexamethasone and MK571 on 5-LO pathway enzyme and CysLT$_1$R expression in HASM.

Corticosteroids and CysLT$_1$ receptor antagonists are two types of drug successfully used in the treatment of asthma. Because receptor antagonists may modulate expression of their receptors, and because corticosteroids may exert anti-inflammatory activity by modulating the expression of mediator-synthesising enzymes and their receptors, it was of interest to investigate the effect of the corticosteroid dexamethasone, and the CysLT$_1$R antagonist MK-571, on LT pathway enzyme and receptor expression in HASM cells. The expression of the 5-LO pathway enzymes and CysLT$_1$ receptor were examined by flow cytometry after treatment of HASM cells with dexamethasone (1μM) or MK-571 (10nM) for 24 hours. As shown in previous sections, none of the stimuli tested caused upregulation of all four LT pathway enzymes and CysLT$_1$, although TGFβ did increase the expression of FLAP, LTA$_4$ hydrolase and CysLT$_1$R. Therefore, the effect of dexamethasone and MK-571 on TGFβ (10ng/ml) stimulated cells was also examined.

Neither MK-571 alone nor dexamethasone alone affected baseline expression of the LT pathway enzymes (**Figure 47a and b**). Consistent with prior experiments, FLAP immunofluorescence was increased by TGFβ from 6.6±1.6 to 9.2±1.7 (p=0.06). Intriguingly, MK-571 significantly reduced TGFβ stimulated levels of FLAP expression from 9.2±1.7 to 7.4±1.6 (p=0.03). Dexamethasone also reduced TGFβ stimulated FLAP expression from 9.2±1.7 to 7.3±1.7 but this did not reach statistical significance (p=0.1). Neither dexamethasone nor MK-571 had any effect on LTA$_4$ hydrolase expression, either in control (MFI 5.0±1.3) or TGFβ treated cells (MFI 6.2±1.2). As expected from previous results, stimulation with TGFβ did not significantly regulate 5-LO and LTC$_4$ synthase expression and unsurprisingly, MK-571 and dexamethasone did not affect the expression of these enzymes in combination with TGFβ.

Compared to control (5.6±0.7), the fluorescence intensity of CysLT$_1$ receptor staining was significantly increased by TGFβ (7.4±0.8, p=0.02) (**Figure 47c**). MK-571 significantly reduced this TGFβ induced upregulation (MFI 5.8±1.0, p=0.05). Dexamethasone was without effect on TGFβ stimulated CysLT$_1$ expression but curiously, dexamethasone significantly increased the median fluorescence intensity of CysLT$_1$R at baseline from 5.6±0.7 to 7.8±0.5 (p=0.01) whereas MK-571 had no effect on basal levels of CysLT$_1$R.

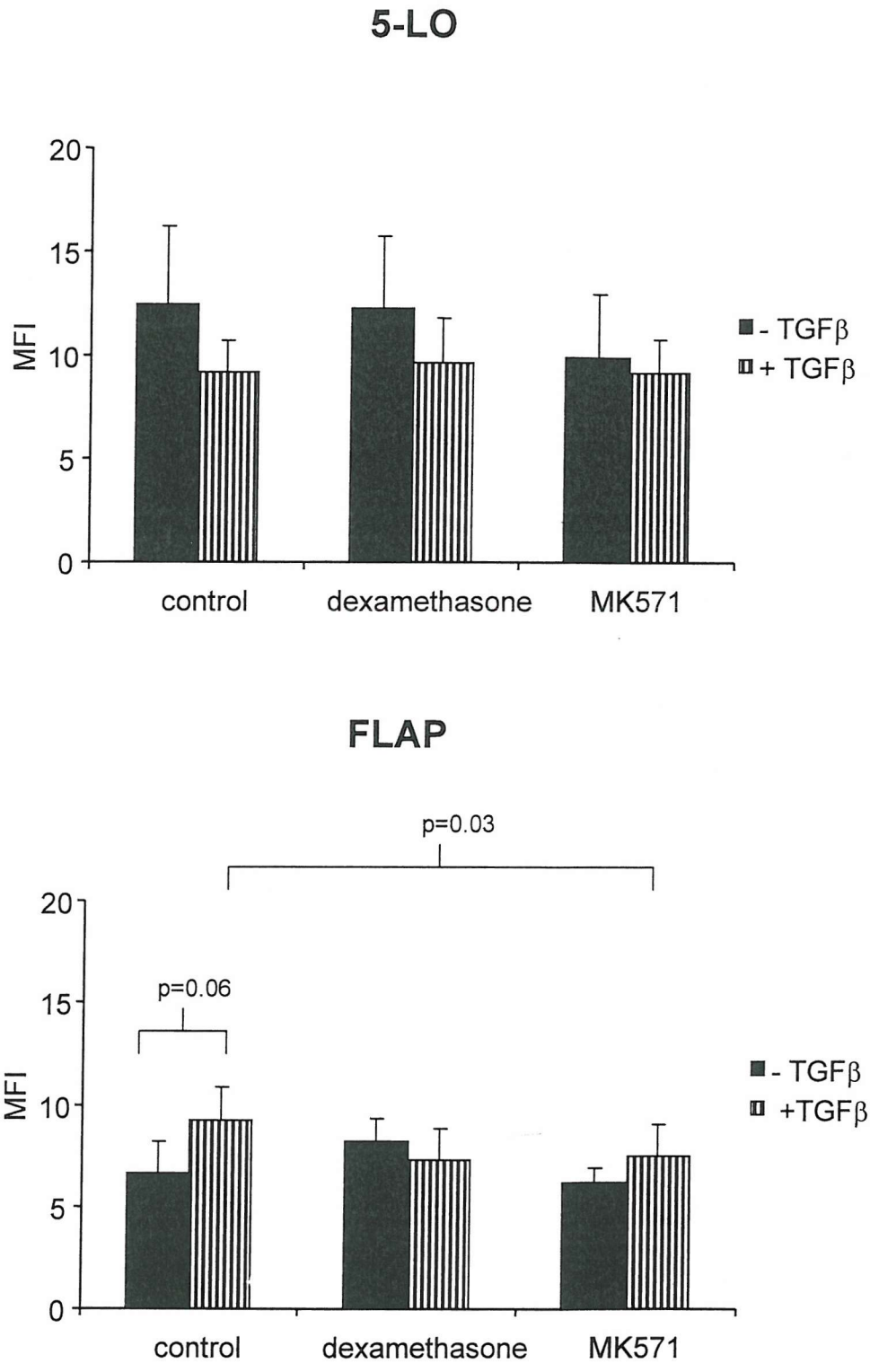


Figure 47a. Effect of MK-571 and dexamethasone on 5-LO and FLAP immunofluorescence in HASM cells incubated with or without TGFβ. HASM were cultured for 24 hours in either serum free medium, 1μM dexamethasone or 10nM MK-571. The effect of dexamethasone and MK-571 was also examined in cells stimulated with TGFβ (10ng/ml) for 24 hours. Cells were fixed, permeabilised and stained for 5-LO and FLAP. Results are shown as mean+SEM and were compared using Student's paired t-test (n=7).

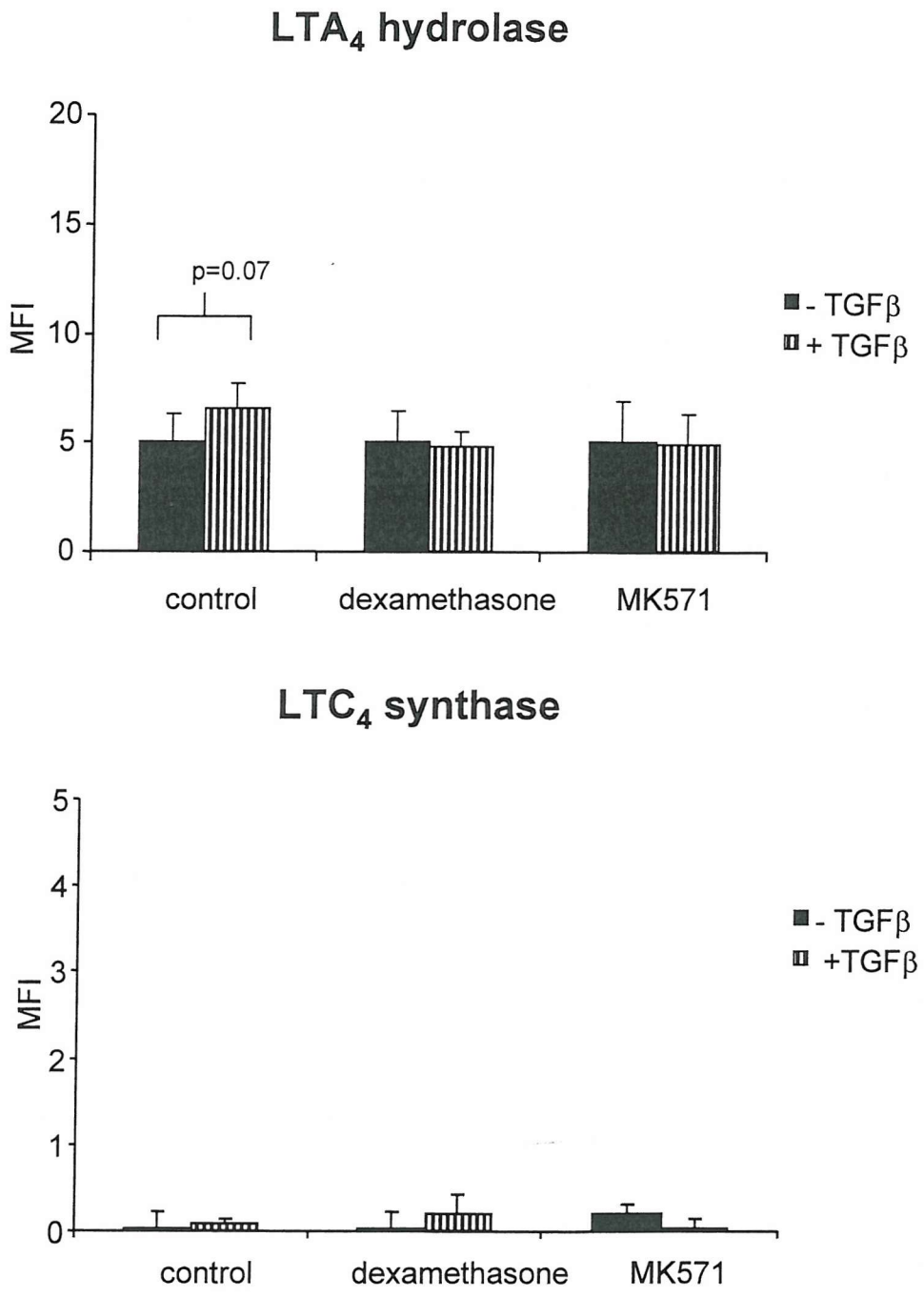


Figure 47b. Effect of MK-571 and dexamethasone on LTA_4 hydrolase and LTC_4 synthase immunofluorescence in HASM cells incubated with or without TGFβ. HASM were cultured for 24 hours in either serum free medium, 1μM dexamethasone or 10nM MK-571. The effect of dexamethasone and MK-571 was also examined in cells stimulated with TGFβ (10ng/ml) for 24 hours. Cells were fixed, permeabilised and stained for LTA_4H and LTC_4S. Results are shown as mean+SEM and were compared using Student's paired t-test (n=7 LTA_4H, n=4 LTC_4S).

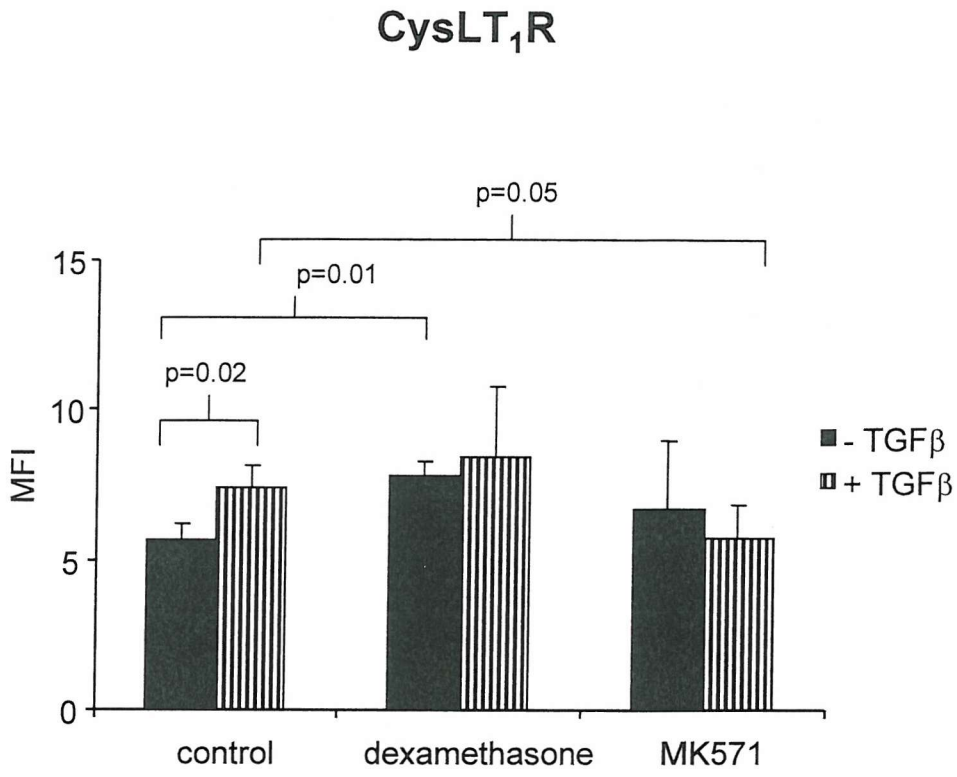


Figure 47c. Effect of MK-571 and dexamethasone on $CysLT_1R$ expression in HASM cells incubated with or without TGFβ. HASM were cultured for 24 hours in either serum free medium, 1μM dexamethasone or 10nM MK-571. The effect of dexamethasone and MK-571 was also examined in cells stimulated with TGFβ (10ng/ml) for 24 hours. Staining for the $CysLT_1$ receptor was carried out on live, unfixed cells. Results are shown as mean+SEM and were compared using Student's paired t-test (n=4).

4.5 Effect of receptor antagonism on Cys-LT_1R expression in HASM

As many drugs affect expression of the receptor at which they act, it was our aim to further investigate the effect of the $CysLT_1$ receptor antagonist MK-571 on $CysLT_1R$ expression in HASM, using more time points (0-72 hours) and more doses of LTD_4 (1, 10 and 100nM). As a control, we also examined the effect of the β_2-adrenergic receptor agonist salbutamol on β_2-AR expression. β_2 agonists are known to cause downregulation of β_2-adrenergic receptors on HASM cells (Danner et al. 1997). HASM cells were incubated for up to 72 hours with either control medium, salbutamol (0-10 μM) or MK-571 (0-100 nM). Cells incubated with salbutamol were immunostained for the β_2-AR and cells incubated with MK-571 were immunostained for $CysLT_1R$. Compared to control, FACS analysis showed a significant downregulation of β_2-AR expression after incubation with 1μM and 10μM salbutamol (n=5, p=<0.05, ANOVA, all time points) but no effect was seen with the lowest concentration of salbutamol, 0.1μM (**Figure 48**). Compared to control, cells incubated with MK571 showed no significant change in the expression of $CysLT_1R$ at any of the concentrations tested, and after incubation for up to 72 hours (**Figure 49**).

4.6 Proliferation of HASM cells in response to leukotrienes.

There are reports demonstrating the involvement of cys-LTs in the stimulation of airway smooth muscle proliferation (Panettieri et al. 1998, Cohen et al. 1995). Therefore we examined the proliferation of HASM cells in response to stimulation with both LTB_4 (0-100nM) and LTD_4 (0-100nM) alone and in combination with their respective receptor antagonists, U75302 (10 and 100nM) and MK571 (10 and 100nM). Cells were incubated with these stimuli for up to 72 hours and proliferation was measured by uptake of methylene blue dye, the intensity of which is directly proportional to cell number when absorbance is measured at 630nm using a spectrophotometer. **Figure 50** shows that neither LTB_4 nor LTD_4 at any concentration tested modified proliferation compared to that in control medium alone. Consequently, the BLT antagonist U75302, and the $CysLT_1$ antagonist MK571 were also without effect. However, statistical analyses showed that comparing all time points, there were significantly greater numbers of cells in samples stimulated with 10nM LTD_4 and 1 nM LTB_4 compared to control (p=0.032 and p=0.045 respectively, paired t-test). These increases are so small that they are not visible in **figure 50** and therefore may not be of great physiological relevance.

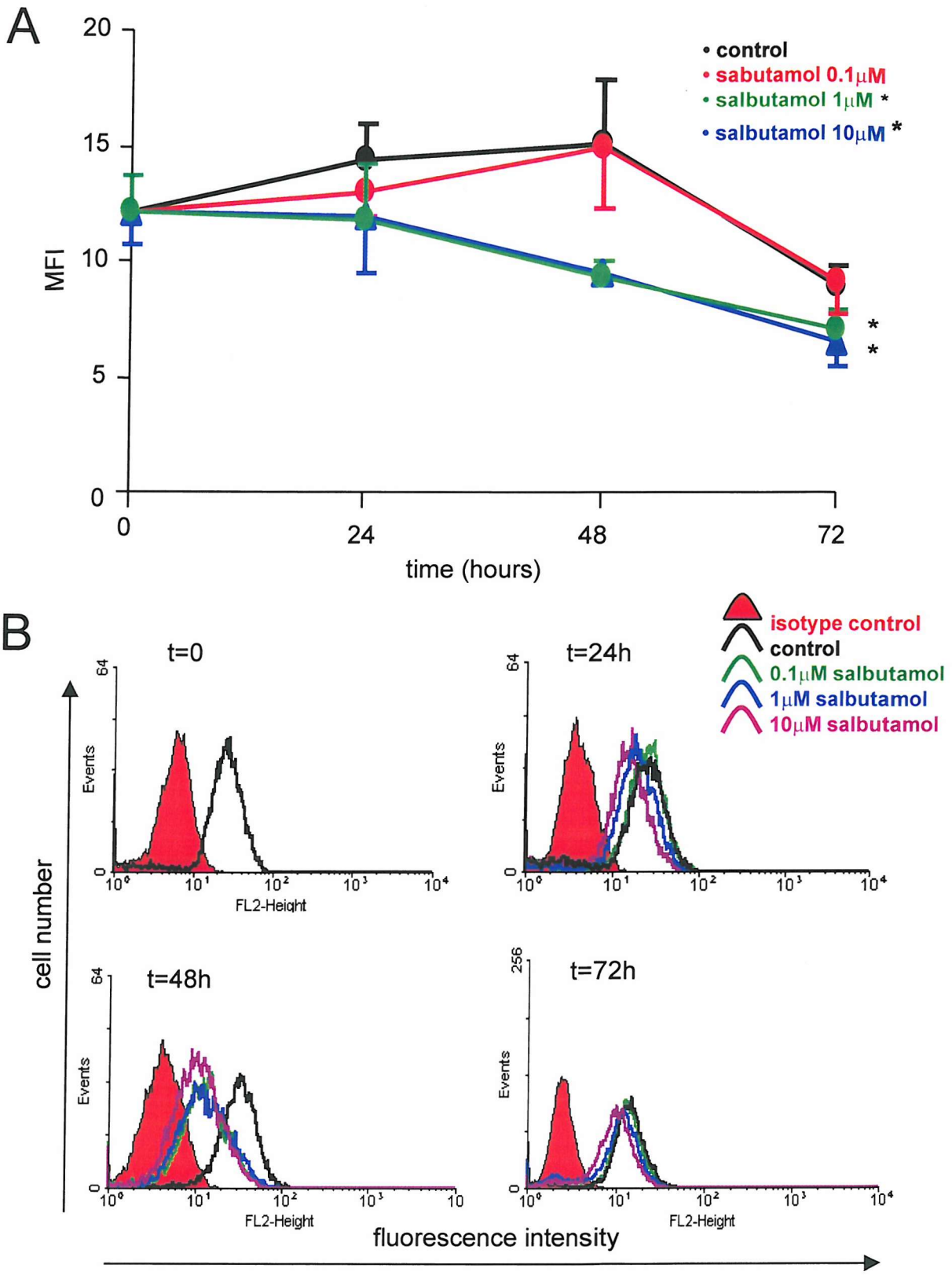


Figure 48. FACS analyses showing the effect of the β_2-AR agonist, sabutamol on β_2-AR receptor expression in HASM. HASM cells were incubated for up to 72 hours with either control medium or salbutamol (0.1-10μM). Cells were fixed and permeabilised for β_2-AR staining. FACS analysis was used to compare β_2-AR expression in control and salbutamol stimulated cells. Panel (A) shows mean+SEM of 5 experiments, panel (B) shows representative FACS plots of β_2-AR expression at 0-72 hours with 0-10μM salbutamol. Results were compared using ANOVA (*$p<0.05$). At 1 and 10μM salbutamol significantly downregulated β_2-AR expression in HASM. MFI=Median Fluorescence Intensity.

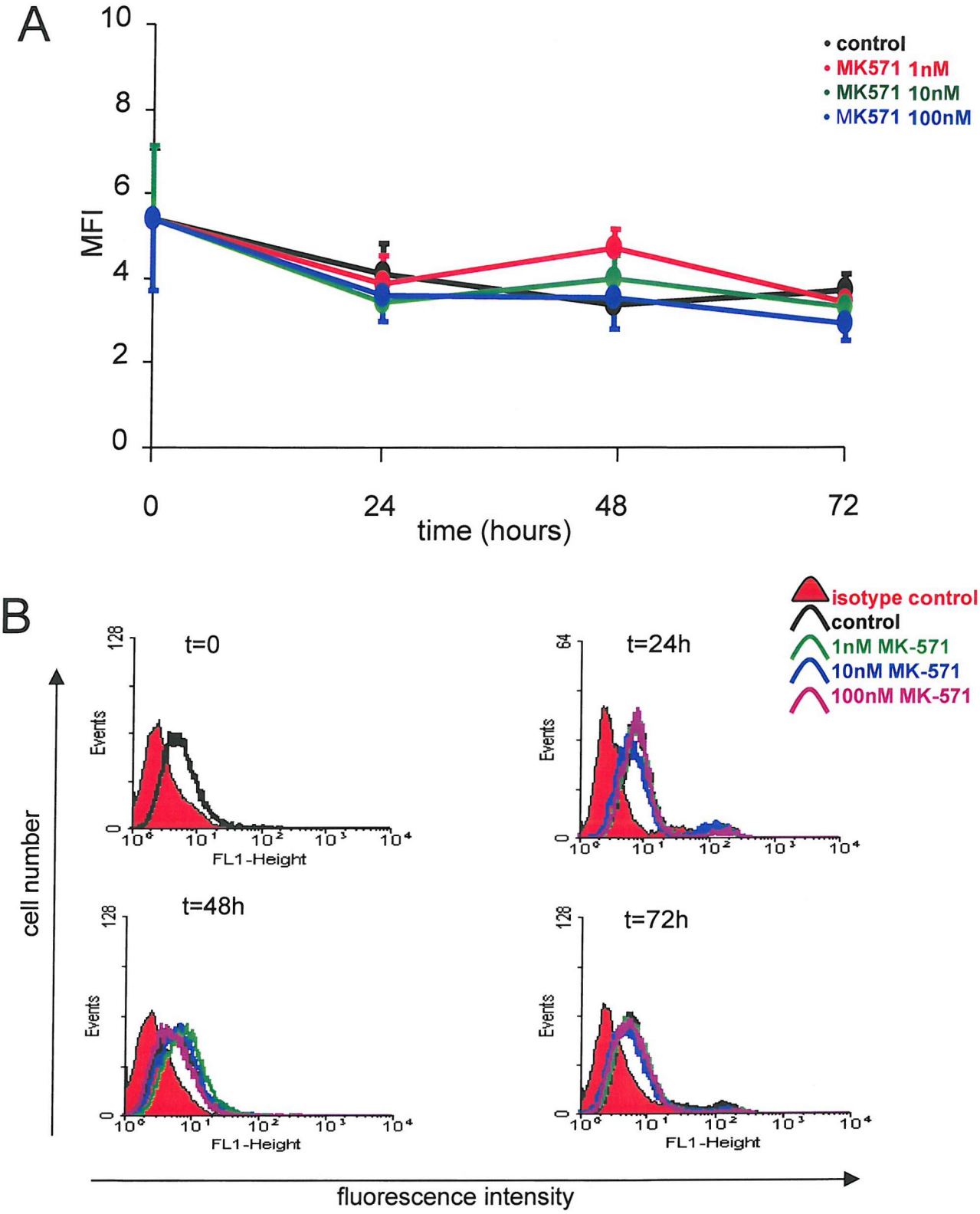


Figure 49. FACS analyses showing the effect of the CysLT$_1$ antagonist, MK-571 on CysLT$_1$ receptor expression in HASM. HASM cells were incubated for up to 72 hours with either control medium or MK-571 (1-100nM). CysLT$_1$ staining was carried out on live, unfixed cells. FACS analysis was used to compare CysLT$_1$ expression in control and MK-571 stimulated cells. Panel (A) shows mean+SEM of 5 experiments, panel (B) shows representative FACS plots of CysLT$_1$ expression at 0-72 hours with 0-100nm MK-571. Results were compared using ANOVA which showed that MK-571 had no significant effect on CysLT$_1$ expression in HASM. MFI=Median Fluorescence Intensity.

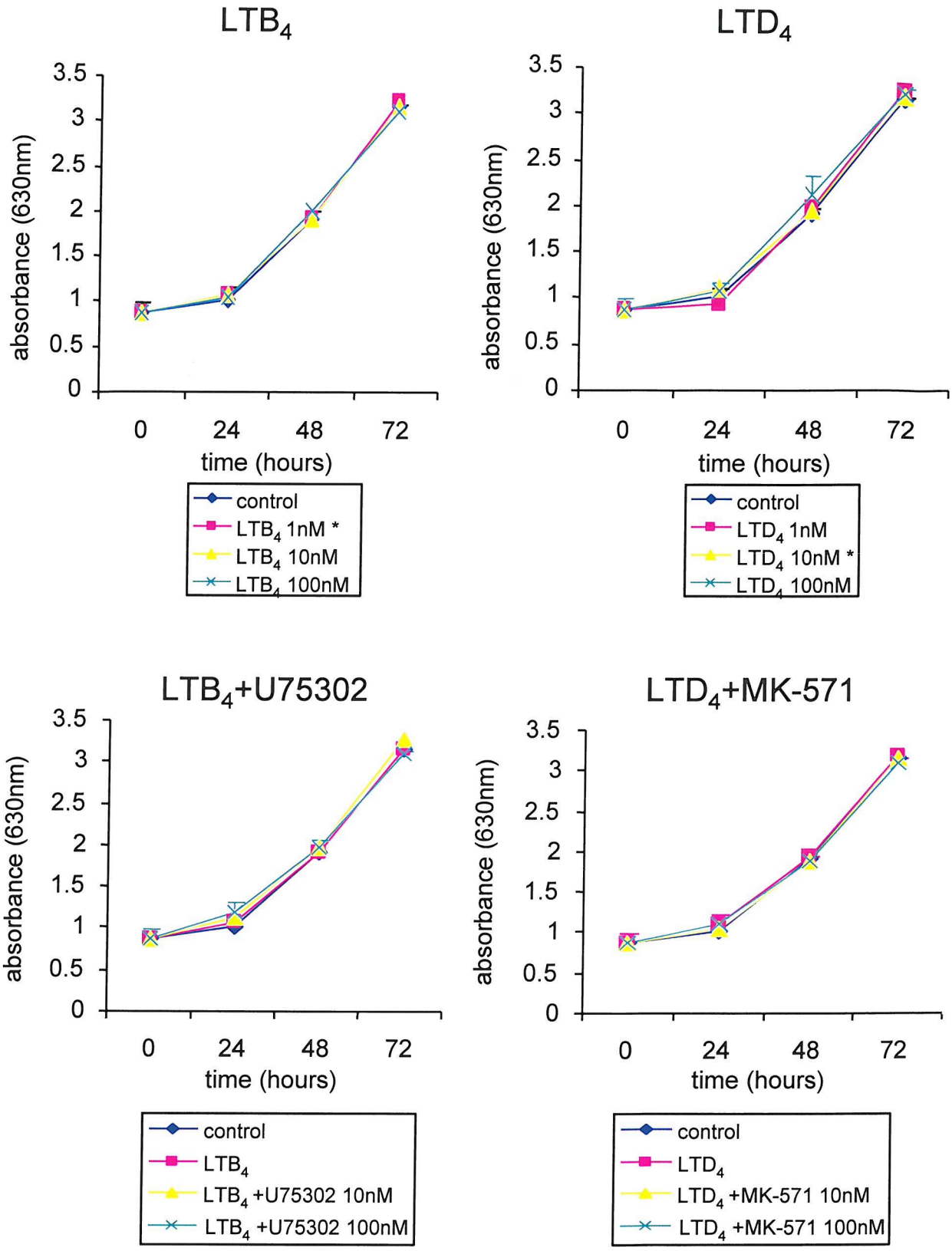


Figure 50. Proliferation assays showing response of HASM cells to stimulation with leukotrienes. 16-HBE cells were incubated for 0-72 hours with LTB_4 (0-100nM), LTD_4 (0-100nM), LTB_4 (10nM) +U75302 (10 & 100nM), LTD_4 (10nM) + MK-571 (10 & 100nM) or serum-free medium alone (control). Proliferation was measured by uptake of methylene blue dye as described in methods. Absorbance (630nm) was measured using a spectrophotometer and was directly proportional to cell number. Results are shown as mean±SEM and all time points were compared using paired t tests (*$p<0.05$). LTB_4 and LTD_4 caused no marked changes to HASM proliferation.

We also examined the effect of leukotrienes in combination with the growth factor EGF, as cys-LTs have been shown to synergise with growth factors and stimulate HASM proliferation, more so than levels seen observed with either compound alone (Cohen et al. 1995, Panettieri et al. 1998). EGF is a known mitogen of smooth muscle cells and at 10ng/ml it significantly increased HASM proliferation compared to basal levels at all time points ($p=0.002$). However, in these experiments when EGF was added in combination with LTB_4 (1nM) or LTD_4 (1nM) there were no further increases in the rate of HASM proliferation (**Figure 51**).

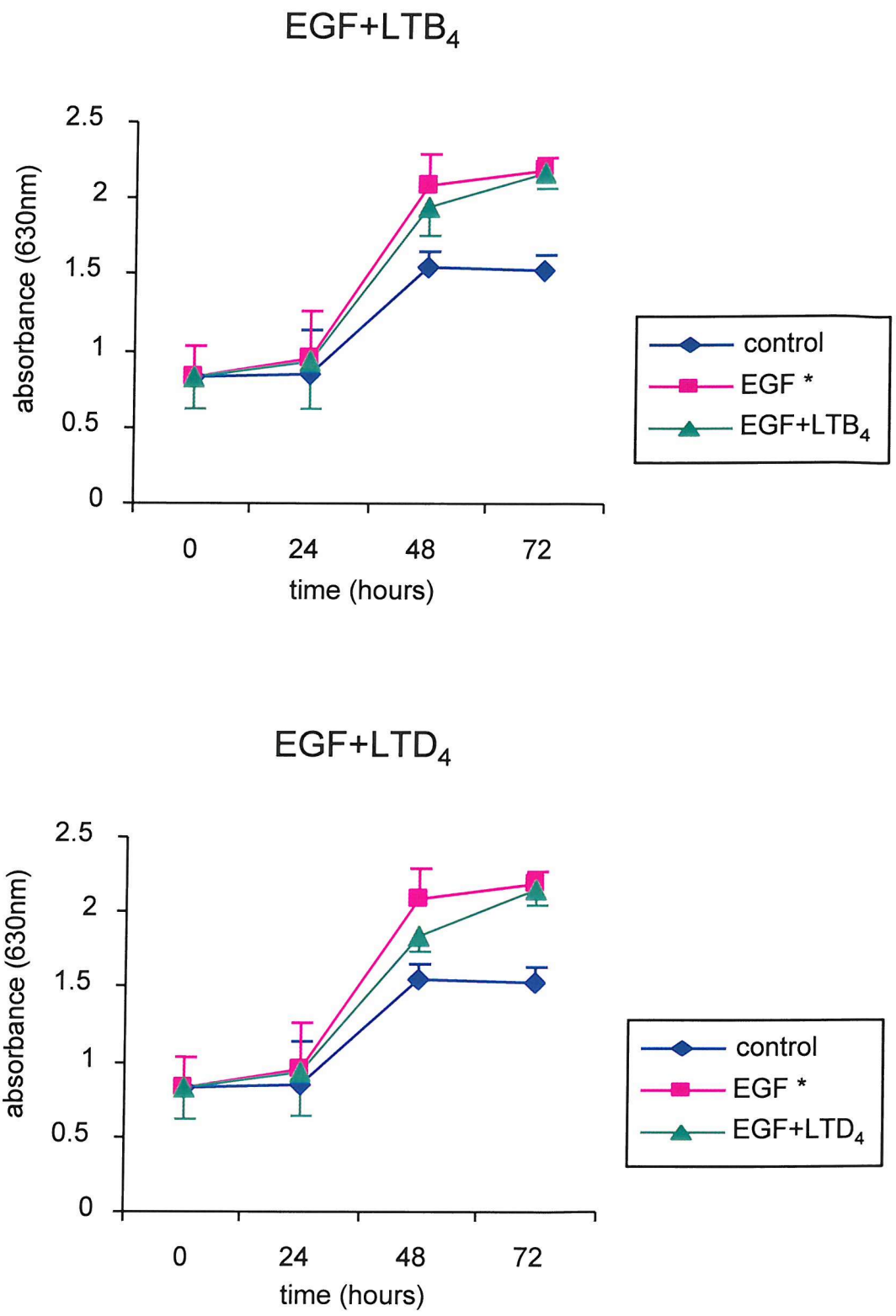


Figure 51. Proliferation assays showing response of 16-HBE cells to stimulation with leukotrienes and EGF. HASM cells were incubated for 0-72 hours with EGF (10ng/ml), EGF+LTB_4 (1nM), EGF+LTD_4 (1nM) or serum-free medium alone (control). Proliferation was measured by uptake of methylene blue dye as described in methods. Absorbance (630nm) was measured using a spectrophotometer and was directly proprtional to cell number. . Results are shown as mean±SEM and all time points were compared using ANOVA (*$p<0.05$). EGF significantly increased HASM proliferation ($p=0.02$) but LTD_4 and LTB_4 had no signifiacnt effect in combination with EGF.

4.7 Discussion

The leukotriene synthetic pathway in human airway smooth muscle cells

The aim of experiments using human airway smooth muscle cells was to establish whether these cells have the enzymatic machinery that would enable them to produce LTB_4 or LTC_4 or both. The expression of 5-LO pathway enzymes was examined both at baseline and after incubation with inflammatory stimuli and anti-inflammatory drugs. In addition to enzyme expression, leukotriene production by HASM cells was measured after stimulation with inflammatory cytokines and mediators. It was also the aim of this work to examine the expression of the $CysLT_1$ receptor and the LTB_4 receptor, BLT on HASM cells, and to investigate the effect of receptor agonists and antagonists as well as inflammatory stimuli on $CysLT_1R$ and BLT expression. Finally, proliferation assays were carried out to investigate whether leukotrienes can regulate smooth muscle proliferation.

4.7.1 Baseline expression of leukotriene pathway enzymes and receptors in HASM cells.

There are no published reports of LT production by HASM cells, although a synthetic function of these cells is well recognised. ASM cells of both human and animal origin are able to produce several inflammatory cytokines and growth factors (Johnson et al. 1997). The presence of the 5-LO pathway in HASM cells is conceivable as HASM cells express COX-1, COX-2, 15-LO and 12-LO, and produce lipid mediators of these pathways (Pang et al. 1997). As in epithelial cells, HASM were found by RT-PCR to spontaneously express 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase mRNA, and immunoblotting experiments confirmed the presence of 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase protein at their expected molecular weights (**Figure 39, p137**). However, while constitutive expression of 5-LO, FLAP and LTA_4 hydrolase was confirmed by FACS analysis, LTC_4 synthase immunofluorescence was found to be lower (**Figure 40, p138**). As LTC_4 synthase mRNA and protein were detectable by RT-PCR and Western blotting, and HASM cells spontaneously released both LTB_4- and LTC_4-like immunoreactivity this could reflect a problem with the sensitivity of flow cytometry for LTC_4S. A possible reason may be that LTC_4 synthase expression is low but also relatively inaccessible. LTC_4 synthase is a membrane-associated protein (Jakobsson et al. 2000) and whereas intact cells are stained for FACS analysis, cells are lysed for SDS-PAGE, which could make LTC_4 synthase more accessible for antibody binding. However, FLAP is also membrane-associated yet this

protein is readily detectable by flow cytometry. It is unlikely that the 18 KDa bands observed in Western blotting experiments are due to crossreactivity of the LTC_4 synthase antibody with FLAP as Penrose at al. have shown that the LTC_4 synthase antibody does not bind to FLAP (1995). The enzyme immunoassay used is very specific for LTC_4 and sensitive, detecting levels of LTC_4 as low as 15 pg/ml. As in epithelial cells, it appears that the LTC_4 synthase enzyme is expressed, but at considerably lower levels than the other LT pathway enzymes. However, there are possible explanations for the lack of LTC_4 synthase immunofluorescence that cannot be excluded, such as the production of LTC_4 in the absence of LTC_4 synthase by another microsomal glutathione transferase (mGST) enzyme (Jakobsson et al. 1996). For example, while LTC_4 synthase is absent in HUVEC cells, the presence of GST2 enables LTC_4 synthesis to take place (Sjöström et al. 2001). There are several classes of mGST enzymes and immunocytochemistry has shown that mGST3 is expressed in airway smooth muscle cells (Anttila et al. 1993). Although this work demonstrates that HASM cells express LTC_4 synthase it is a possibility that mGST enzymes also contribute to LTC_4 synthesis.

The contraction of airway smooth muscle in response to cys-LTs provided overwhelming pharmacological evidence for the expression of a cys-LT receptor on airway smooth muscle cells (Dahlén et al. 1980). In addition, blockade by $CysLT_1R$-specific antagonists confirms that such contractions are modulated by $CysLT_1$ (Buckner et al. 1986). However, the expression of the $CysLT_1R$ protein on isolated HASM cells has not been examined previously. Baseline expression of Cys-LT_1R on HASM may mediate cys-LT induced effects such as contraction and proliferation (Dahlen et al. 1980, Panettieri et al. 1998). Using flow cytometry, immunocytochemistry and RT-PCR this study confirmed constitutive expression of $CysLT_1$ protein and mRNA in HASM cells. The LTB_4 receptor, BLT was also found to be spontaneously expressed on HASM cells by FACS analysis. The functional relevance of a BLT receptor on HASM is not clear, as very few LTB_4 mediated effects have been reported in these cells. Although LTB_4 has been shown to cause bronchoconstriction in guinea pigs (Silbaugh et al. 2000), LTB_4 inhalation does not affect lung function in humans (Sampson et al. 1997). LTB_4 can induce chemotaxis of fibroblasts, another mesenchymal cell type (Mensing et al. 1984), but such experiments using HASM cells have not been published. This study has shown that LTB_4 can increase the expression of FLAP and LTA_4 hydrolase, which confirms that HASM cells may express functional LTB_4 receptors, although more BLT mediated functions may become apparent in the future.

4.7.2 The effect of Ca ionophore on LT pathway enzyme expression and LT production in HASM cells.

By FACS analysis, Ca ionophore (A23187) did not show any significant effects on 5-LO, FLAP, LTA_4 hydrolase or LTC_4 synthase expression in HASM cells (**Figure 42, p141**), in contrast to epithelial cells in which A23187 stimulation increased expression of all four leukotriene synthetic proteins. Stimulation with Ca ionophore also had no significant effect on the expression of the $CysLT_1R$ receptor in HASM cells (results not shown). It may be that a 6-hour incubation with A23187 is too short to observe changes in enzyme expression in HASM cells. Indeed, changes in 5-LO pathway enzyme expression in HASM cells following stimulation with inflammatory mediators occurred over a 24-hour incubation period. Alternatively, signalling pathways that do not involve acute changes in intracellular calcium may regulate LT pathway enzyme expression in HASM.

LT production was measured in supernatants from HASM cells incubated with or without A23187 for 6 hours. LTC_4 and LTB_4 immunoreactivity was detectable in these cells although Ca ionophore did not significantly increase the amounts generated (**Figure 43, p142**). The release of LTB_4 (~2 ng/10^6 cells) and LTC_4 (~100pg/10^6 cells) confirms the presence of a complete and functional LT pathway. There are reports of LTB_4 release by A23187-stimulated neutrophils being as great as 50 or 100 ng/10^6 cells (Weller et al. 1983, Dessein et al. 1986) and LTC_4 production by A23187-stimulated eosinophils appears to range from 10 to 30 ng/10^6 cells, levels several fold greater than LT production in A23187 treated HASM cells (Cowburn et al. 1998, Weller et al. 1983). However, a better comparison would be with baseline levels of LT production by unstimulated leukocytes. Although there are few reports of leukocyte LT production in the absence of stimulation, there are reports of eosinophils spontaneously producing between 20 and 50 pg LTC_4/10^6 cells in 30 minutes, and neutrophils between 100 and 500 pg/10^6 cells (Tenor et al. 1996, Mahauthaman et al. 1988, Sampson et al. 1990). These amounts become comparable to those produced by HASM when adjusted for the different incubation times. Therefore the major difference between LT production in HASM cells and leukocytes may be the amounts produced in response to stimulation which suggests different biological roles for the low levels of LTs produced continuously by HASM cells and the high levels produced acutely by stimulated leukocytes. The LTC_4 concentration measured from each well in a six well plate after 6 hours was 0.6nM and stimulatory effects on HBEC proliferation have been observed at concentrations as low as 10fM. In addition the Kd value of LTD_4 for $CysLT_1R$

is ~0.4nM, so LT production by HASM is likely to be biologically effective (Leikauf et al. 1990, Lynch et al. 1999).

4.7.3 Effect of inflammatory mediators on the expression of 5-LO, FLAP, LTA_4 hydrolase, LTC_4 synthase, Cys-LT_1R and BLT, and the production of leukotrienes in HASM cells.

Regulation of LT pathway enzyme expression in HASM could have important consequences. In an inflammatory environment such as the asthmatic airway there is an increase in mediators such as LTs (Arm et al. 1993), cytokines (Broide et al. 1992), bradykinin and histamine (Holgate 1994, Gawlik et al. 1995). The presence of such mediators could upregulate LT pathway enzymes, increase the capacity of HASM to produce LTs which could then contribute to perpetuating the inflammatory response, bronchoconstriction and possibly airway remodelling. FLAP and LTA_4 hydrolase expression was increased approximately 2-fold by TGFβ and LTB_4 (**Figure 44, p143**). TGFβ is a growth factor produced primarily by mesenchymal cells (Borish 1998). It is thought to be involved in the remodelling of asthmatic airways as it is produced by myofibroblasts, epithelial cells and HASM. It causes increased collagen production by fibroblasts (Holgate et al. 2000) and its expression is increased in areas of pulmonary fibrosis (Proud et al. 1998). TGFβ increases cytokine production by cultured HASM (Fong et al. 2000) and causes LTC_4 synthase upregulation in monocytes (Riddick et al. 1999). Due to its involvement in remodelling, TGFβ induced LT enzyme upregulation may be involved in potentiation of the mitogenic response. Although LTD_4 does not have a direct effect on the proliferation of HASM cells in vitro, it greatly augments that produced by epidermal growth factor (EGF) (Panettieri et al. 1998). TGFβ may increase the production of cysLTs, both of which could then interact to control HASM proliferation. However, as discussed further below, it is not clear how meaningful the TGFβ-induced increase in FLAP and LTA_4 hydrolase are, as treatment with TGFβ for 24 hours was not found to increase the release of either LTB_4 or LTC_4.

The effect of LTB_4 on the expression of 5-LO pathway proteins has not been studied previously but our findings suggest a positive feedback loop whereby LTB_4 could increase its own production, and possibly that of LTC_4, by upregulating FLAP and LTA_4 hydrolase. LTB_4 causes activation of the transcription factor NFκB, immediate early gene transcription and AP-1 DNA binding in monocytes (Aoki et al. 1998, Brach et al. 1992 and Stankova et

al. 1992) and hence could influence FLAP and LTA_4 hydrolase gene transcription. As mentioned later, treatment with LTB_4 did infact increase LTC_4 release by HASM.

This study showed that histamine significantly increased the expression of LTA_4 hydrolase by 60% in HASM cells. Histamine is an intensively studied inflammatory mediator released by mast cells and basophils. Histamine levels are increased in the BAL fluid of asthmatics (Schwartz et al. 1998). Effects on airway smooth muscle include contraction and increased proliferation (Hirst 2000). As histamine is released upon mast cell contact with allergen, the increased levels during exposure to allergen could increase HASM LTA_4 hydrolase expression and LTB_4 production and contribute to an infiltration of neutrophils and other leukocytes into the airways.

The lack of effect of histamine, bradykinin, LTD_4, LTB_4, TGFβ and the cytokine mix on 5-LO expression in HASM is consistent with the housekeeping characteristics of its gene promoter. Nor did any of the stimuli tested have any effect on LTC_4 synthase expression. LTC_4 synthase can be upregulated in other cell types, for example by TGFβ in monocytes and by PMA in erythroleukaemia cells (Riddick et al. 1999, Söderström et al. 1992). It may be necessary to investigate LTC_4 synthase regulation in HASM by another technique (e.g. SDS-PAGE/Western blotting), as LTC_4 synthase detection by FACS analysis was poor and this approach may not be sensitive enough to detect changes in expression. In HASM, regulation of FLAP and LTA_4 hydrolase may be sufficient to alter LT production. FLAP is readily upregulated in leukocytes by stimuli such as GM-CSF and IL-5 (Pouliot et al. 1994, Cowburn 1999). FLAP is essential for leukotriene synthesis (Dixon et al. 1990) and an increase in its expression could affect both LTC_4 and LTB_4 production. The capacity for LT production is greater in alveolar macrophages than in their monocyte precursors. It has been shown that while alveolar macrophages express 7-times more 5-LO and 2-times more LTA_4 hydrolase, they express 40-times more FLAP than monocytes which correlates well with the ability of alveolar macrophages to generate LTs from arachidonic acid (Coffey et al. 1994). The promoter region of the LTA_4 hydrolase gene has several transcription factor binding sites including the phorbol ester response element AP-2 (Mancini et al. 1995) and recently LTA_4 hydrolase expression was shown to be increased by stimulation of human polymorphonuclear leukocytes with IL-4 and IL-13 (Zaitsu et al. 2000).

Regulation of the leukotriene receptors by inflammatory stimuli could enhance the effects of cys-LTs and LTB_4 on HASM. $CysLT_1R$ expression was increased almost 2-fold by TGFβ and 3-fold by a combination of IL-1β, TNFα and IFNγ. This cytokine combination is implicated in asthma and has several known effects on HASM such as the induction of COX-2 (Pang et al. 1997). As discussed above, the effects of TGFβ appear to be related to remodelling. $TGF\beta_1$ immunoreactivity is increased in the epithelium of asthmatics (Vignola et al. 1997) and in the BAL fluid of stable asthmatics compared to normals (Redington et al. 1997). It is expressed in areas of pulmonary epithelium where there is advanced fibrosis (Khalil et al. 1996). As TGFβ increases the expression of LT synthesising enzymes and increases the expression of $CysLT_1$ in HASM, it may not only increase LT production by HASM, but also increase the sensitivity of these cells to cys-LTs.

Compared to control, histamine, bradykinin and the cytokine mix (IL-1β, TNFα and IFNγ) all moderately increased the expression of BLT. To date there is no evidence of BLT regulation by cytokines or inflammatory mediators in other cell types (Owman et al. 2000) although BLT is differentially expressed in different cell types and the promoter region of its gene has several transcription factor binding sites, characteristics which could represent an inducible protein (Yokomizo et al. 2000). As previously mentioned, the functional significance of BLT expression on HASM cells is unclear, so it is not possible to determine which biological effects are likely to be affected by BLT upregulation in HASM. It is interesting that histamine upregulated both BLT and LTA_4 hydrolase expression and therefore could increase both the synthesis and effects of LTB_4. Histamine, bradykinin and the cytokines IL-1β, IFNγ and TNFα are all involved in inflammation and it is possible that the upregulation of BLT is somehow involved in the potentiation of LTB_4 mediated pro-inflammatory effects. The effect of a BLT antagonist (LY293111) on the early and late response to inhaled allergen has been examined in 12 asthmatic subjects but while treatment with LY293111 reduced BAL neutrophils and LTB_4 compared to placebo, it was of no measurable physiological benefit (Evans et al. 1996). This may suggest that BLT mediated actions are not of great importance to the pathogenesis of asthma although this study only examined the effect of LY293111 following experimental allergen challenge, and confirmation is required from long-term clinical trials examining the effect of a BLT antagonist on asthma symptoms.

The measurement of leukotriene-like immunoreactivity in HASM supernatants after incubation with inflammatory stimuli further suggested the production of LTB_4 and LTC_4 by these cells, although stimulation with TGFβ, histamine, bradykinin and the cytokine mix had no effect on LT production. Surprisingly, LTB_4 significantly increased the production of LTC_4 whereas LTD_4 significantly increased the production of LTB_4. These findings raised questions about crossreactivity in the enzyme-immunoassay kits, for example, LTD_4 may appear to increase LTB_4 production by binding to the anti-LTB_4 antibody used in the kit. However, according to the manufacturer's information, cross-reactivity between LTB_4 and the LTC_4 antibody and LTD_4 and the LTB_4 antibody was 0.3 and 0.1 % respectively. Calculations showed that if LTD_4 was added at a concentration of 10nM, 0.3 pg/well would be measured as LTB_4, which represents only 4.6 % of the total LTB_4 measured, and does not account for the increased levels of LTB_4 measured in LTD_4 stimulated samples. Both $CysLT_1$ and BLT receptors respond to agonist binding with increases in intracellular calcium (Lynch et al. 1999, Yokomizo et al. 2000). Increased intracellular calcium can lead to the translocation and activation of $cPLA_2$ and 5-LO, resulting in arachidonic acid release and leukotriene production (Peters-Golden et al. 2000). Such a mechanism could explain the cross-stimulation observed here. We also found that LTB_4 increased the co-expression of FLAP and LTA_4 hydrolase, changes that could increase both cys-LT and LTB_4 production. However, due to difficulties in separating exogenous leukotriene added from leukotriene produced, it was not possible to measure whether incubation with LTB_4 or LTD_4 affected LTB_4 and cys-LT production respectively.

Although TGFβ and histamine both had stimulatory effects on LT pathway enzyme expression they did not stimulate leukotriene production above that measured in control samples. This suggests that levels of LT pathway enzymes expressed may not necessarily correlate with amounts of LT produced. The limiting factor in the regulation of leukotriene production may be upstream in the synthetic pathway, for example at the level of substrate availability or $cPLA_2$ expression. Compared to the far greater capacity for leukotriene production in leukocytes compared to HASM cells, it is interesting that by FACS analysis the expression of 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase is relatively similar in the two cell types (Seymour ML. PhD thesis, University of Southampton 2000). Western blotting experiments also showed that immunopositive bands for 5-LO pathway proteins were comparable between HASM cells and mixed leukocytes. Alternatively, changes in

enzyme expression may be responsible for more subtle changes in LT production that are not apparent during a 24-hour incubation.

4.7.4 The effect of MK-571 and dexamethasone on 5-LO pathway enzyme and $CysLT_1R$ expression in HASM cells.

$CysLT_1$ antagonists and corticosteroids are two classes of drug used in the treatment of asthma due to their anti-inflammatory actions. It was of interest to examine whether their anti-inflammatory effects extended to regulation of the synthesis and effects of leukotrienes in HASM cells (**Figure 47, p149**). MK-571 had no effect on the baseline expression of LT pathway enzymes in HASM although it significantly inhibited TGFβ induced upregulation of FLAP. This suggests that the mechanism of TGFβ induced upregulation of FLAP expression involves $CysLT_1$ activation, although the effect of MK-571 was modest in size and therefore may not be meaningful *in vivo*. Leukotrienes have been found to be involved in cellular responses to growth factors. For example, EGF has been shown to activate $cPLA_2$ causing arachidonic acid release and the generation of leukotrienes, which are involved in the EGF-mediated effect of intracellular actin reorganisation (Peppelenbosch et al. 1999). However, TGFβ stimulation for 24 hours did not significantly increase cys-LT production by HASM and stimulation with LTD_4 did not increase FLAP expression. It is possible that MK-571 could have effects independent of $CysLT_1$ that could underlie this effect. For example, MK-571 has been shown to increase IL-6 production by human monocytes but the mechanism was found not to involve the synthesis and effects of leukotrienes (Vellenga et al. 1999).

No work has been carried out to investigate the effect of dexamethasone on 5-LO pathway enzymes in structural cell types apart from one study which described a lack of effect of dexamethasone on LTA_4 hydrolase expression in keratinocytes (Iversen et al. 1996). Several studies have examined the effect of dexamethasone on LT production in leukocytes *in vitro* and *in vivo*. In HL-60 cells dexamethasone suppressed the induction of LTC_4 synthesis but induced LTB_4 production (Zaitsu et al. 1998) and in human alveolar macrophages and neutrophils, dexamethasone was without effect on the A23187 induced release of lipoxygenase products (Azevedo et al. 1995, Schleimer et al. 1989). *In vivo,* LTs are detectable in the BAL fluid from asthmatic subjects despite corticosteroid therapy (Lam et al. 1988). Neither oral prednisolone nor inhaled budesonide taken for approximately one

week altered urinary LTE_4 levels in normal subjects (Sebaldt et al. 1990, Manso et al. 1992). Similarly, fluticasone propionate taken for two weeks had no effect on urinary LTE_4 in mildly atopic asthmatics (O'Shaugnessy et al. 1993) and in patients with COPD, fluticasone proprionate treatment reduced BAL fluid prostaglandin levels but had no effect on levels of LTB_4 or cys-LT measured (Verhoeven et al. 2001). Taken together, these results suggest that the anti-inflammatory action of dexamethasone is not due to a reduction in leukotriene production. Dexamethasone neither increased nor decreased the expression of any 5-LO pathway enzymes in HASM cells, findings that further support a lack of effect of dexamethasone on leukotriene production.

A surprising result concerning dexamethasone was the finding that it significantly increased $CysLT_1$ receptor expression in HASM. This result could have important clinical implications. Many asthma sufferers rely on long term inhaled corticosteroid treatment. If this increased $CysLT_1$ expression on HASM, the major target cells of cys-LT mediated bronchoconstriction (and remodelling), corticosteroids could in fact increase the responsiveness of the airways to the pathogenic effects of the cys-LTs, and hence explain the additive benefits of corticosteroid and LT-antagonist combination therapy on lung function in asthmatics (Laviolette et al. 1999). The beneficial anti-inflammatory effects of corticosteroids in the treatment of asthma are well documented (Adcock 2000). Asthmatics experience an accelerated rate of respiratory function deterioration with age (Elias 2000). It has been shown that corticosteroid therapy is not able to protect against this progressive deterioration (CAMP study, 2000), thought to be due to structural changes within the airway. Recent evidence suggests that Cys-LTs are involved in airway remodelling (Baud et al. 1987, Leikauf et al. 1990, Panettieri et al. 1998, Wang et al. 1993) and an increase in $CysLT_1$ expression after corticosteroid treatment could explain, at least in part why corticosteroids fail to protect asthmatic airways from structural changes.

4.7.5 The effect of Salbutamol on β_2-AR and MK-571 on $CysLT_1R$ expression in HASM cells.

Tachyphylaxis to asthma medications may be a problem and can be caused by drug-induced downregulation or internalisation of specific receptors. Downregulation of the β_2-adrenoceptor by prolonged exposure to β_2 agonists has been demonstrated in several cell types and by several different mechanisms. For example, metaproterenol inhalation reduces both β_2-AR expression and function on bronchial epithelial cells and macrophages (Turki et

al. 1995). In the BEAS-2B cell line, isoproterenol downregulates β_2-AR by increasing the rate of receptor degradation (Kelsen et al. 1997). Clinically, this means that β_2 agonists, the most common reliever medication for asthma, become less effective with prolonged use (Barnes 1995) although curiously, their expression is restored by the concomitant use of steroids (Hauck et al. 1997). In clinical studies, leukotriene antagonists do not appear to cause tachyphylaxis or rebound effects on withdrawal (Leff et al. 1995). This study examined the effect of the $CysLT_1R$ antagonist MK-571 on *in vitro* $CysLT_1$ expression in HASM . MK-571 at 1, 10 or 100nM had no effect on receptor expression after incubation for up to 72 hours (**Figure 49, p154**). Downregulation of the β_2-adrenoceptor by salbutamol was demonstrated in parallel experiments and found to be significant at agonist concentrations of 1 and 10μM. The lack of effect of MK-571 on $CysLT_1R$ supports the clinical observations and contributes to the advantages of using LTRAs in the treatment of asthma.

4.7.6 The proliferation of HASM cells in response to leukotrienes and EGF.

While we were able to show a significant increase in the proliferation of HASM cells in response to EGF, LTB_4 and LTD_4 were without effect, either alone or in combination with EGF (**Figures 51&51, p155&157**). The lack of effect of LTB_4 was not surprising as it has been found not to affect the proliferation of fibroblasts or vascular smooth muscle cells (Baud et al. 1987, Modat et al. 1987), however, there are no reports examining its effect on HASM cells. The effects of cys-LTs on the proliferation of HASM cells have been studied before and in accordance with our findings, LTD_4 was found not to stimulate proliferation when added alone (Panettieri et al. 1998). However, LTD_4 has been shown to markedly augment the proliferation of airway smooth muscle induced by EGF and IGF (Panettieri et al. 1998, Cohen et al. 1995), a result not confirmed in the present study. A likely explanation for the lack of effect of LTD_4 in our experiments could be the concentration of LTD_4 used. Panettieri et al. used HASM cells obtained in a similar way to those used in the present study, and used a similar concentration of EGF, but showed significant effects of LTD_4 at 1 and 10μM, over 1000 times greater than the single dose of 1nM used in our experiments (Panettieri et al. 1998). Concentrations of 1 and 10μM are very high, the Kd value for $CysLT_1R$ is ~0.3nM (Lynch et al. 1999) and for $CysLT_2R$ is ~4.8nM (Heise et al. 2000). Therefore the concentrations used by Panettieri et al. are 2500- to 25000-fold excessive and a dose of 1nM could be considered more realistic. Despite the negative findings of this

study, a role for cys-LTs in airway remodelling is becoming increasingly evident from their mitogenic effects on epithelial cells (Leikauf et al. 1990), fibroblasts (Baud et al. 1987), airway smooth muscle (Panettieri et al. 1998, Cohen et al. 1995, Wang et al. 1993), vascular smooth muscle (Porreca et al. 1996, Porreca et al. 1995) and endothelial cells (Modat et al. 1987). In addition, cys-LTs can stimulate fibroblast collagen production and goblet cell mucus secretion, both of which are also considered to be features of remodelling (Phan et al. 1988, Marom et al. 1982). Further studies are required to build upon the preliminary data obtained in our HASM cell proliferation assays, to investigate the mechanisms whereby $CysLT_1$, $CysLT_2$ and growth factor receptors may interact and also to examine the role of anti-leukotriene therapy in the prevention of such structural changes.

4.7.7 Summary

In summary, these results provide the first evidence that primary human airway smooth muscle cells in culture are capable of releasing LTB_4- and cys-LT-like immunoreactivity. The enzymes responsible for LT production are upregulated by TGFβ, LTB_4 and histamine and may be downregulated by MK-571. Cys-LTs produced by HASM could act in an autocrine fashion on the $CysLT_1$ receptor, which this study has directly shown to be expressed on these cells. The expression of $CysLT_1R$ is increased by TGFβ, pro-inflammatory cytokines and surprisingly, the corticosteroid dexamethasone. These effects could potentiate the actions of cys-LTs on HASM, including bronchoconstriction and mitogenesis. The LTB_4 receptor BLT was also found to be present on HASM cells and its expression was increased by histamine, bradykinin and a combination of IL-1β, TNFα and IFNγ. To date, the functional relevance of BLT expression and regulation in HASM is unknown. In addition, using salbutamol-induced β_2-AR downregulation as a control, we confirmed clinical observations by showing that $CysLT_1R$ antagonists do not cause receptor downregulation.

5. Results

The leukotriene pathway in bronchial fibroblasts from asthmatic and normal subjects

As part of the wider hypothesis that structural airway cells may contribute to leukotriene production within the lung, the principal aim of experiments using primary human lung fibroblasts was to investigate whether these cells express the 5-LO pathway at baseline and/or after stimulation with pro- and anti-inflammatory mediators. The second aim was to compare the characteristics of LT pathway enzyme expression in fibroblasts with differentiated myofibroblasts. Thirdly, although effects of LTs on fibroblasts have been described, there is no published information concerning the expression of LT receptors on these cells so the expression of BLT and $CysLT_1$ was examined. Finally, the effect of LTs on the proliferation of human lung fibroblasts was investigated. The availability of bronchial fibroblasts from both asthmatic and normal subjects, allowed comparison between these two groups.

As described in Methods, fibroblasts were grown as explants from bronchial biopsy tissue and were characterised both morphologically and by immunostaining of contractile proteins. Fibroblasts are spindle-shaped, elongated cells with a large oval nucleus and can be distinguished from smooth muscle cells by expressing less actin and myosin (**Figure 11, p75**).

5.1 Baseline expression of LT pathway proteins in human bronchial fibroblasts

To examine whether human bronchial fibroblasts constitutively express leukotriene pathway enzymes, baseline levels of 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase mRNA and protein were assessed by RT-PCR, Western blotting, immunocytochemistry and FACS analysis.

RT-PCR was carried out using mRNA derived from normal fibroblasts and results showed that these cells had detectable mRNA for all four LT pathway proteins (**Figure 52a**). Amplicons generated were consistent with the expected number of basepairs for 5-LO (124), FLAP (210), LTA_4 hydrolase (291) and LTC_4 synthase (155). SDS-PAGE/Western blotting experiments confirmed that as well as mRNA, fibroblasts also spontaneously express 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase protein, with immunopositive bands corresponding to the expected molecular weights of approximately 80, 18, 70 and 18 kDa respectively. **Figure 52b** shows a representative immunoblot using fibroblasts from one normal subject and one asthmatic subject, with mixed leukocytes as a positive control. Equal protein concentrations of each cell preparation were used. 5-LO, FLAP, LTA_4H and LTC_4S

were also present in the mixed leukocyte positive control at levels comparable to those in the fibroblasts.

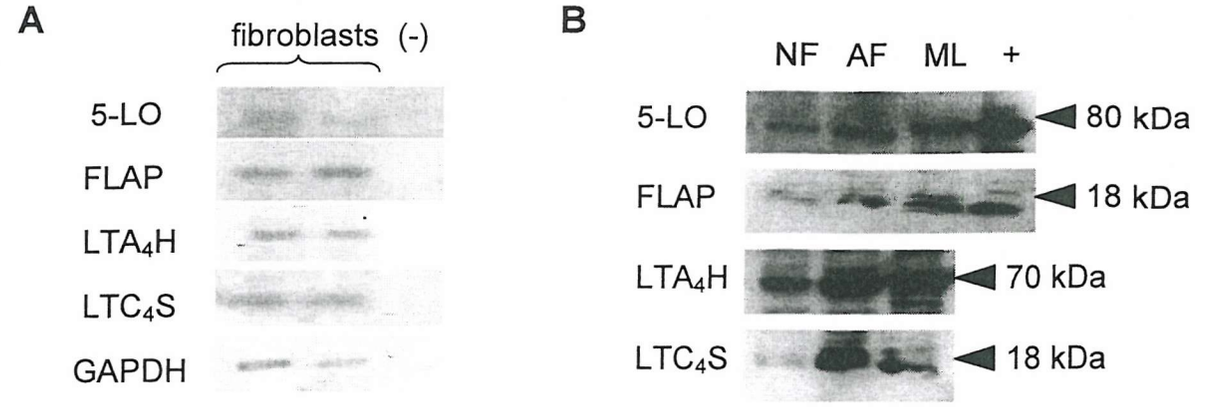


Figure 52. Basal expression of LT pathway enzyme mRNA and protein in human bronchial fibroblasts as shown by (A) RT-PCR and (B) SDS-PAGE/Western blotting. (A) After RNA extraction, RT-PCR was performed in two normal fibroblast samples. GAPDH was the internal positive control and as a negative control, PCR was carried out in the absence of RT product. (B) For immunoblotting, equal amounts of cell lysates from normal and asthmatic fibroblasts (NF and AF) and mixed leukocytes (ML) were resolved on 10 and 12% polyacrylamide gels for 5-LO & LTA_4H and FLAP & LTC_4S respectively. Recombinant protein controls were also added for 5-LO and FLAP (+). Protein bands were visualised using a chemiluminescent chromagen.

Photomicrographs showing LT pathway protein immunostaining in normal fibroblasts are shown in **Figure 53**. The strongest positive staining appears to be for LTA_4 hydrolase with weaker 5-LO, FLAP and LTC_4 synthase staining. In addition cells were also stained for the LTB_4 receptor (BLT1) and the $CysLT_1$ receptor. Positive staining was apparent for both receptors.

FACS analysis was also performed to examine basal 5-LO, FLAP, LTA_4H and LTC_4S expression in fibroblasts obtained from normal and asthmatic subjects. There was positive staining for all four enzymes in both subject groups **(Figure 54 and Table 11)**. In addition, the expression of BLT and $CysLT_1R$ was examined and FACS analysis shows constitutive expression of both receptors on asthmatic and normal human airway fibroblasts. In both asthmatics and normals, 5-LO, FLAP and LTA_4 hydrolase immunofluorescence was significantly greater than that of LTC_4 synthase (**Table 11**). There were no significant differences at baseline between the normal and asthmatic groups. Prior to bronchial biopsy, the lung function and bronchial responsiveness of each subject was measured. While there

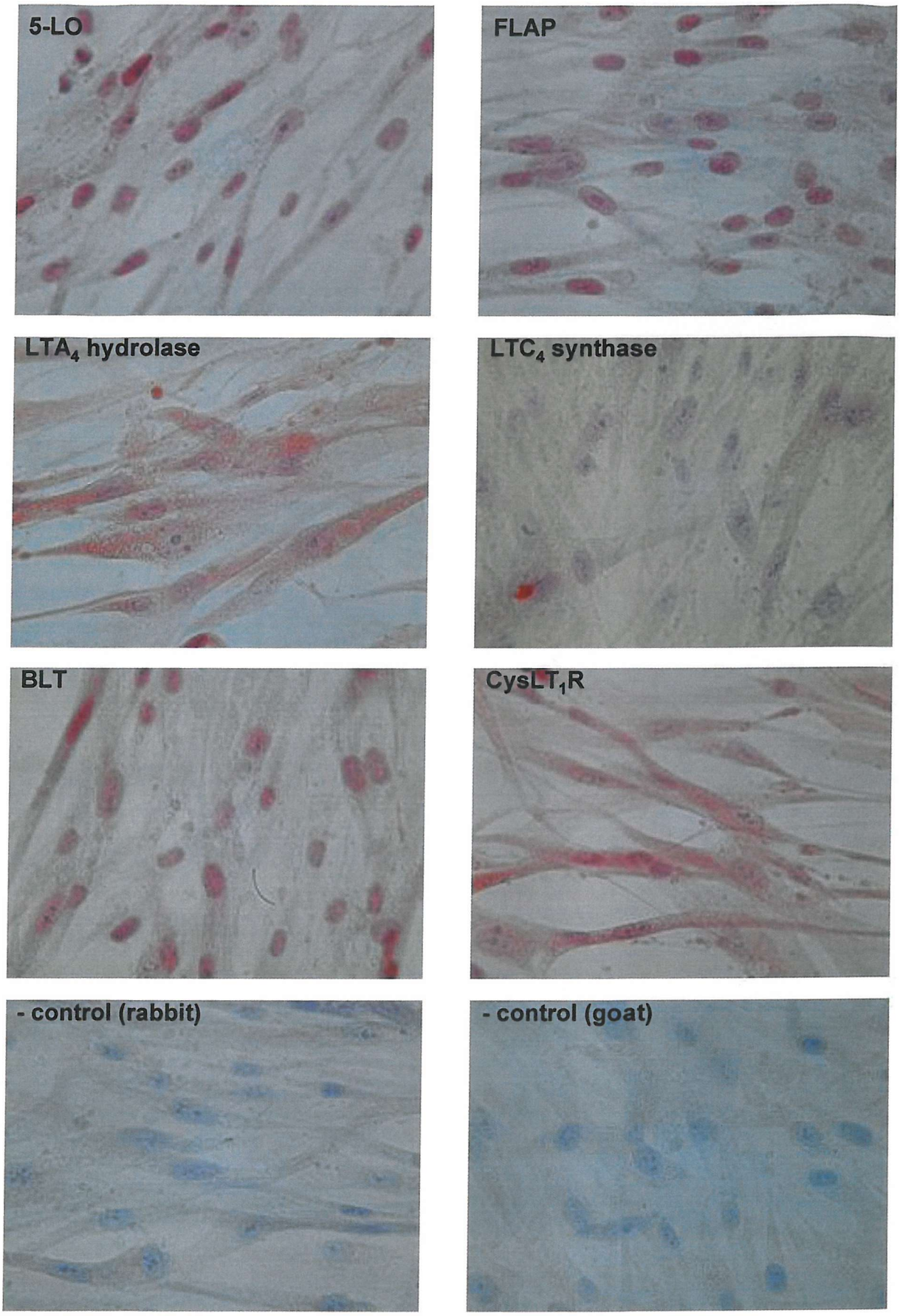


Figure 53. Representative photomicrographs (40x) showing LT pathway enzyme and receptor immunostaining in human bronchial fibroblasts. Fibroblasts were grown to confluence on coverslips and stained using antibodies for 5-LO, FLAP, LTA_4hydrolase, LTC_4 synthase, and the receptors BLT and $CysLT_1R$. For negative controls primary antibody was omitted and photomicrographs show staining with secondary anti-rabbit and anti-goat antibodies. Nuclei were counterstained using Meyer's haematoxylin and positive AEC staining is red.

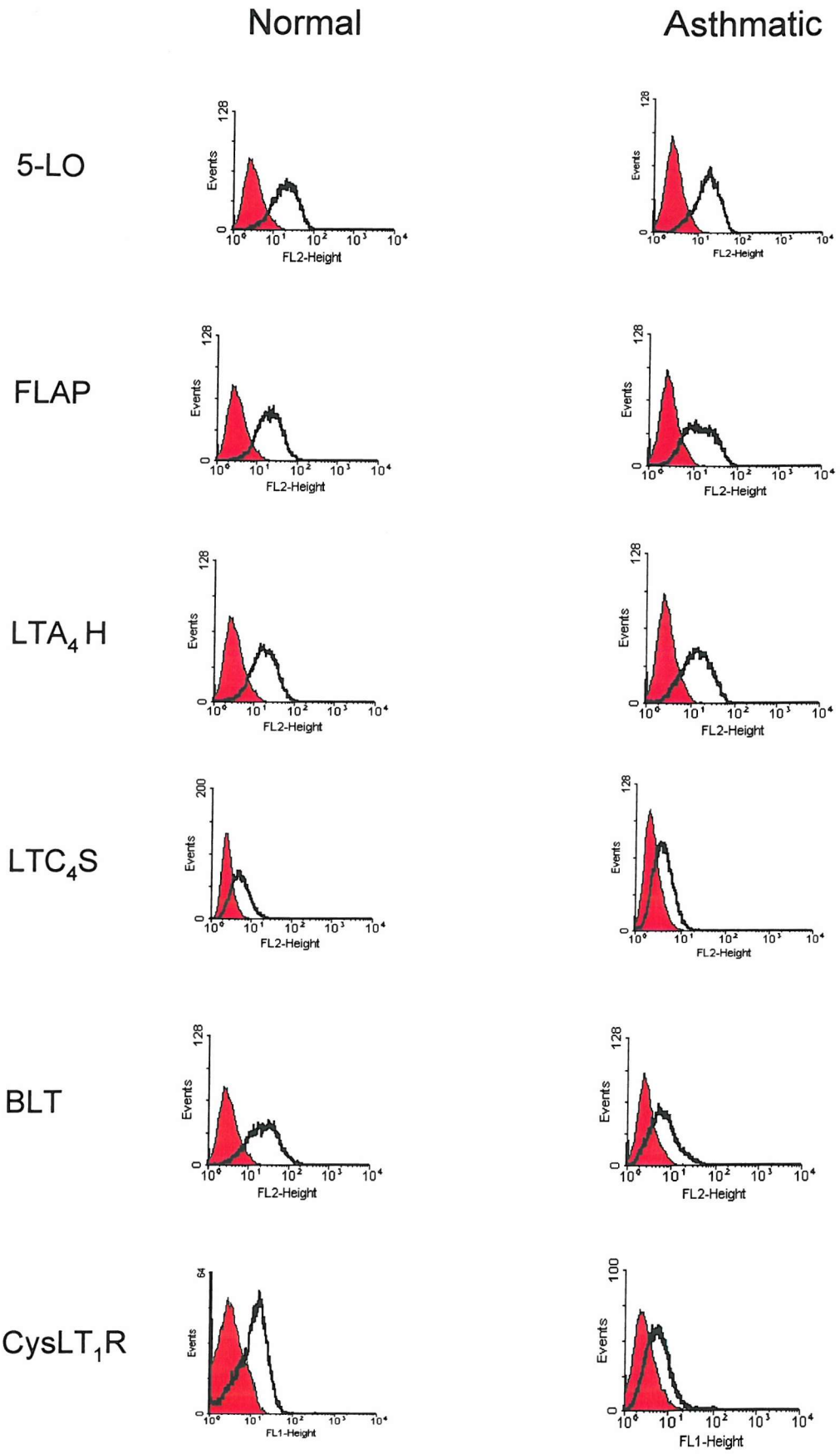


Figure 54. Representative FACS histograms showing baseline expression of LT pathway enzymes and receptors in bronchial fibroblasts from a normal and asthmatic subject. Cells were fixed, permeabilised and stained for 5-LO, FLAP, LTA_4 hydrolase, LTC_4 synthase and the BLT receptor. $CysLT_1R$ staining was carried out on live cells. Filled graphs show fluorescence intensity of isotype control and open graphs show staining with specific antibody.

was a significant correlation between PC_{20} and FEV_1 (ρ=0.7, p=0.03) there were no significant correlations between either FEV_1 or PC_{20} and the basal levels of any of the LT pathway enzymes or receptors (**Table 7, p74**).

Table 11. Basal immunofluorescence (MFI) of LT pathway proteins in asthmatic and normal bronchial fibroblasts.

	Normal Fibroblasts	Asthmatic Fibroblasts
5-LO	9.7±2.8	8.5±3.1
FLAP	12.3±3.8	10.5±4.5
LTA_4 hydrolase	7.8±1.9	8.6±3.7
LTC_4 synthase	0.97±0.4	2.1±1.4
BLT	10.5±3.1	6.7±3.6
$CysLT_1R$	7.9±1.4	5.5±2.4

Mean±SEM, n=5 for each group, Mann Whitney test, ***p<0.01, **p<0.05, *p<0.1

5.2 The effect of inflammatory stimuli on LT pathway protein expression in bronchial fibroblasts from asthmatics and normals.

Several pro-inflammatory stimuli are able to affect mediator production by airway fibroblasts so the effect of such stimuli on the expression of LT pathway enzymes and receptors in bronchial fibroblasts was assessed by FACS analysis. Fibroblasts from 5 asthmatic and 5 normals were incubated for 24 hours in the absence or presence of cytokines (TGFβ, EGF or a combination of IL-1β, TNFα and IFNγ all at 10ng/ml) and autacoids (10mM bradykinin, 10mM histamine, 10nM LTB_4 and 10nM LTD_4). Levels of 5-LO, FLAP, LTA_4 hydrolase, LTC_4 synthase, as well as BLT and $CysLT_1R$ were measured by FACS and results are shown in **Figure 55**. Compared to control values, none of the stimuli tested produced any significant changes in the expression of the 5-LO pathway enzymes or BLT and $CysLT_1R$, nor were there any significant differences between levels of immunofluorescence in the asthmatic and normal groups.

5.3 LT production by human bronchial fibroblasts from asthmatic and normal subjects in response to pro-inflammatory stimuli.

To investigate expression of an active LT biosynthetic pathway in the human bronchial fibroblasts, production of LT-like immunoreactivity was measured by enzyme immunoassay. Fibroblasts from normal and asthmatic subjects were exposed for 24 hours to

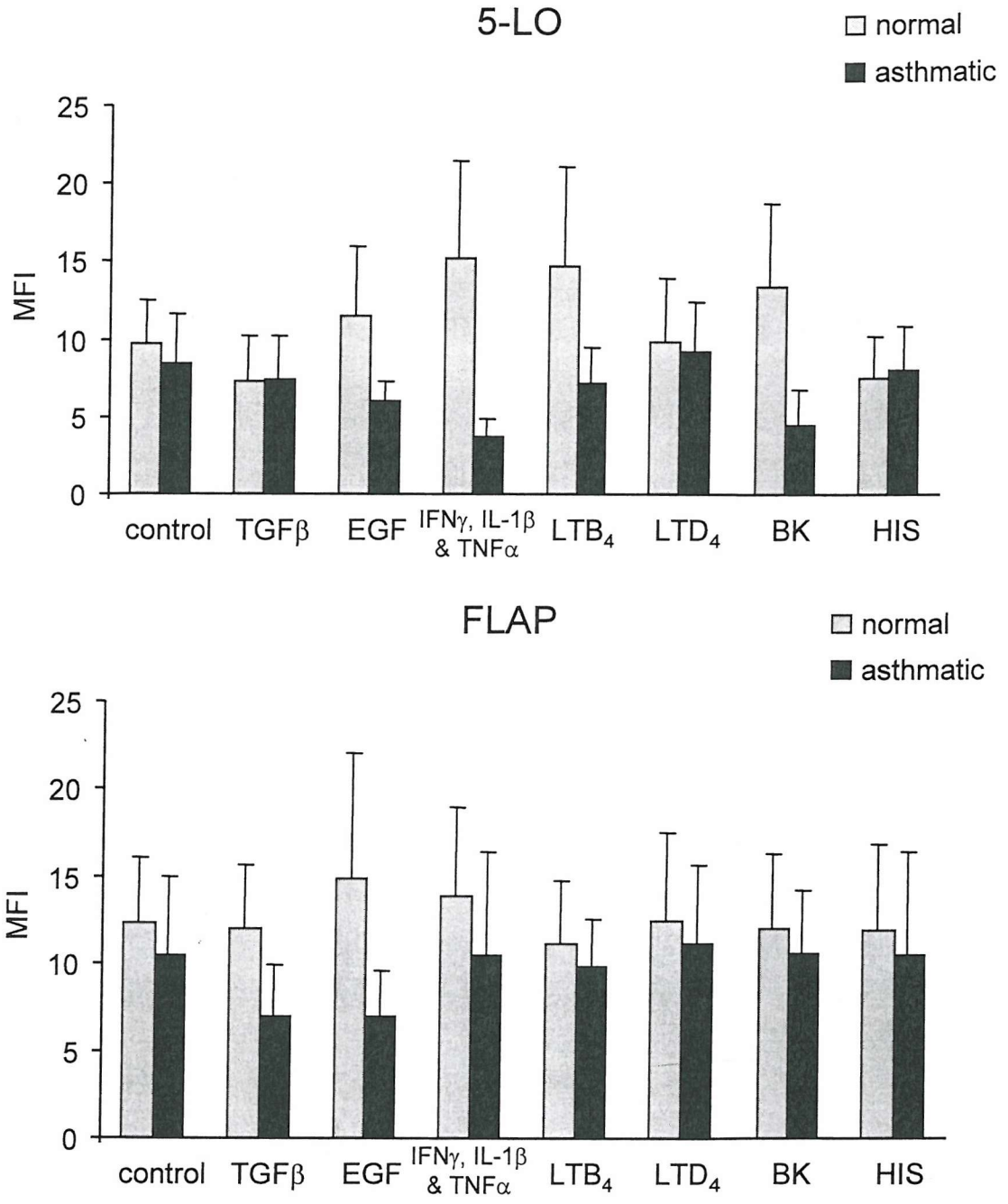


Figure 55a. Effects of stimulation with inflammatory mediators on 5-LO and FLAP expression in bronchial fibroblasts from asthmatic and normal subjects. Cells were incubated for 24 hours with or without EGF (10ng/ml), TGFβ (10ng/ml), a combination of IL-1β, TNFα and IFNγ (each at 10ng/ml), bradykinin (10μM), histamine (10μM), LTB_4 (10nM) or LTD_4 (10nM). Cells were fixed, permeabilised and stained for 5-LO and FLAP prior to FACS analysis. Results are shown as mean+SEM and results were compared using Student's paired t-test for intragroup differences or the Mann Whitney U-test for intergroup differences (n=5 for each subject group).

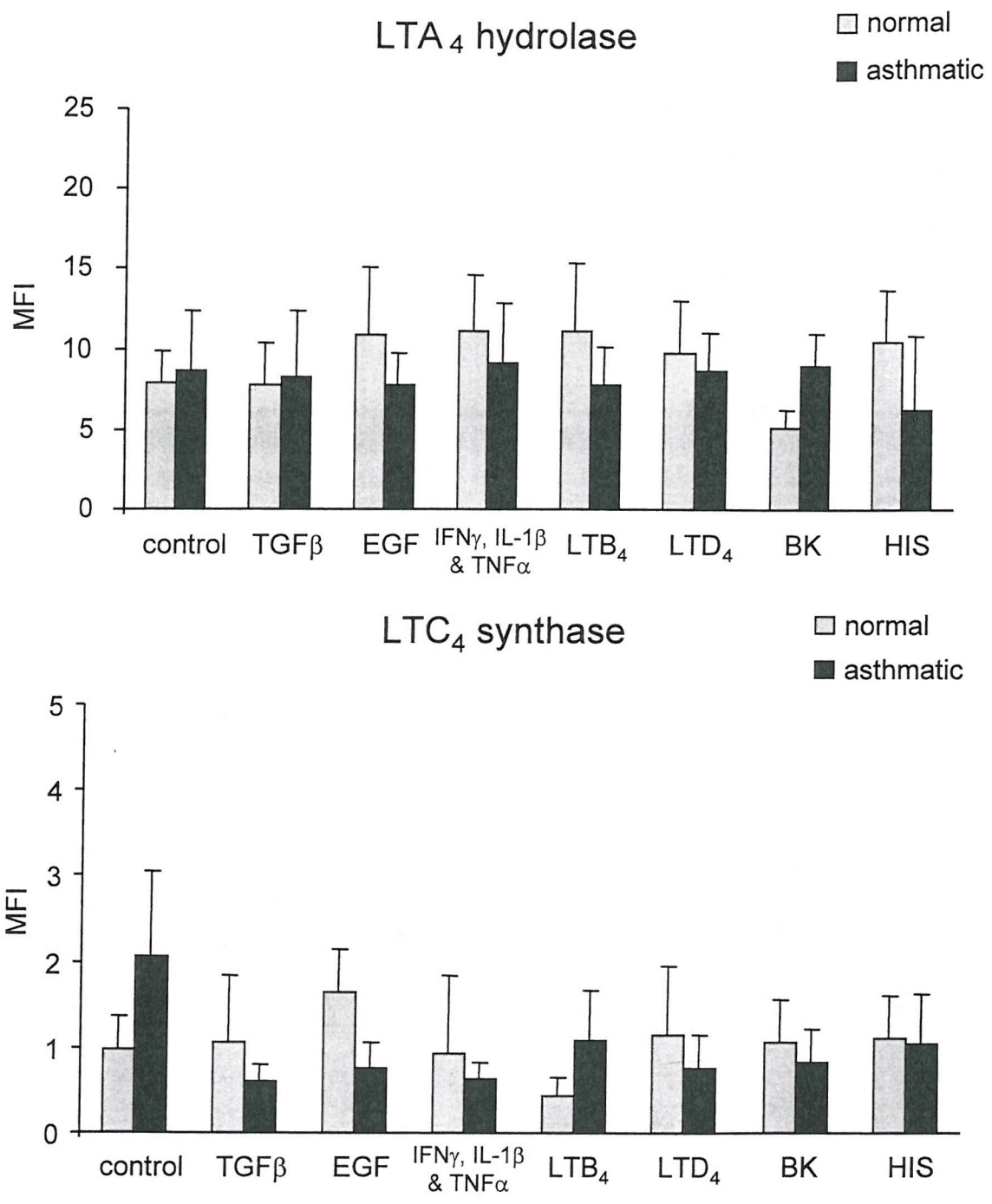


Figure 55b. Effects of stimulation with inflammatory mediators on LTA_4 hydrolase and LTC_4 synthase expression in bronchial fibroblasts from asthmatic and normal subjects. Cells were incubated for 24 hours with or without EGF (10ng/ml), TGFβ (10ng/ml), a combination of IL-1β, TNFα and IFNγ (each at 10ng/ml), bradykinin (10μM), histamine (10μM), LTB_4 (10nM) or LTD_4 (10nM). Cells were fixed, permeabilised and stained for LTA_4 hydrolase and LTC_4 synthase prior to FACS analysis. Results are shown as mean+SEM and results were compared using Student's paired t-test for intragroup differences or the Mann Whitney U-test for intergroup differences (n=5 for each subject group).

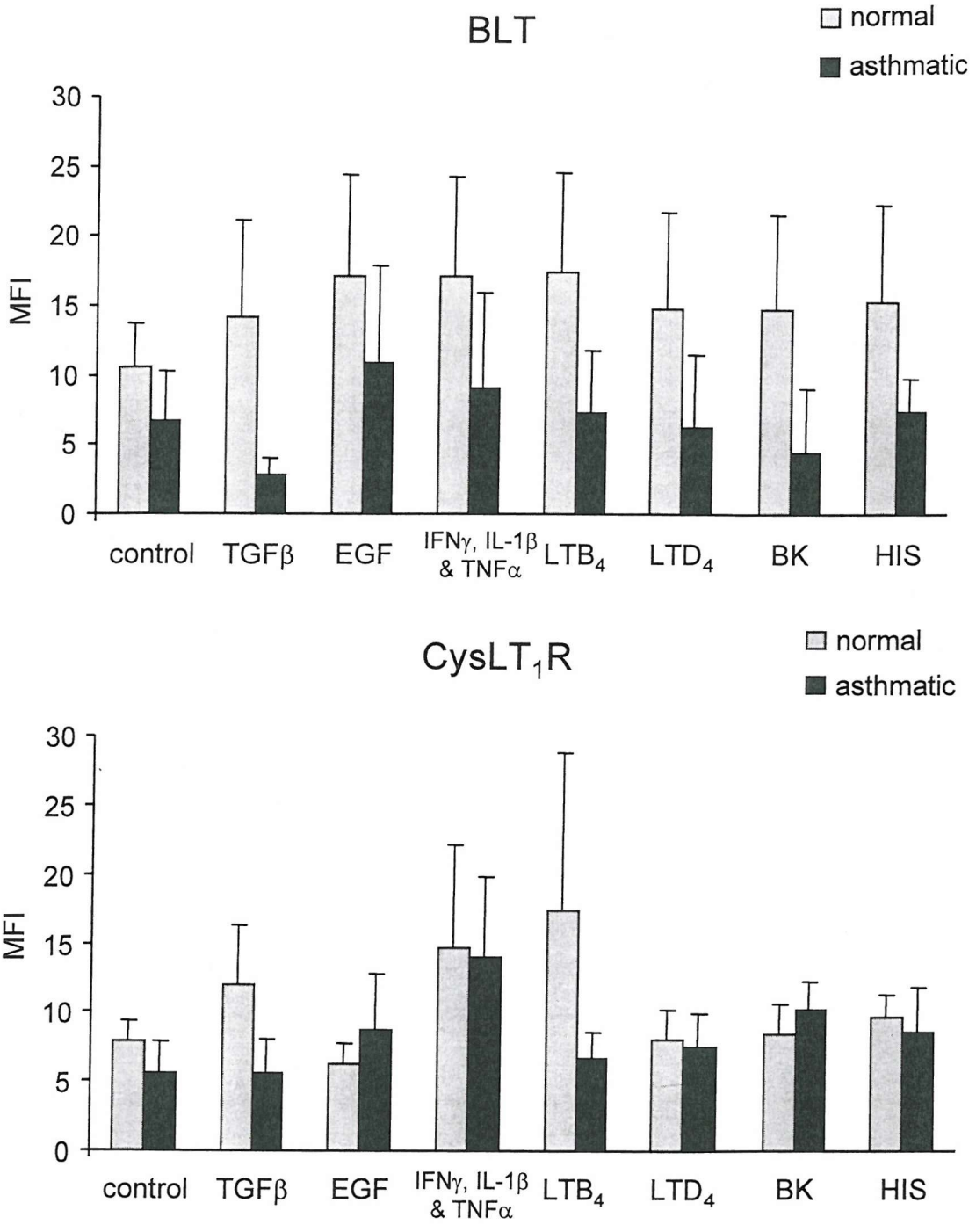


Figure 55c. Effects of stimulation with inflammatory mediators on $CysLT_1$ and BLT receptor expression in bronchial fibroblasts from asthmatic and normal subjects. Cells were incubated for 24 hours with or without EGF (10ng/ml), TGFβ (10ng/ml), a combination of IL-1β, TNFα and IFNγ (each at 10ng/ml), bradykinin (10μM), histamine (10μM), LTB_4 (10nM) or LTD_4 (10nM). Prior to FACS analysis cells were fixed, permeabilised and stained for BLT whereas $CysLT_1$ R staining was carried out on live cells. Results are shown as mean+SEM and results were compared using Student's paired t-test for intragroup differences or the Mann Whitney U-test for intergroup differences (n=5 for each subject group).

the same inflammatory mediators as mentioned above (10ng/ml TGFβ, 10ng/ml EGF, 10nM LTB_4, 10nM LTD_4, 10μM histamine, 10μM bradykinin, or a combination of IFNγ, TNFα and IL-1β, each at 10ng/ml). As a control, fibroblasts were incubated for 24 hours in experiment medium alone. As shown in **Figure 56**, LTC_4 and LTB_4 immunoreactivity was detectable in supernatants collected from unstimulated cells, both from normals and asthmatics. In 24 hours, normal fibroblasts released 275±66 pg $LTB_4/10^6$ cells and 157±34 pg $LTC_4/10^6$ cells, while fibroblasts from asthmatic subjects released 194±56 pg $LTB_4/10^6$ cells and 262±75 pg $LTC_4/10^6$ cells. In accordance with the lack of effect of the inflammatory stimuli on LT pathway enzyme expression, these agents did not significantly affect LTC_4 and LTB_4 production by either asthmatic or normal cells compared to control levels. Mean levels of LTB_4 production were consistently greater in normal compared to asthmatic fibroblasts, and these differences reached statistical significance in cells stimulated with EGF, TGFβ and histamine where LTB_4 production was 6-, 4- and 2-fold greater in the normal samples ($p=0.04$, $p=0.04$ and $p=0.05$ respectively).

5.4 Effect of MK-571 and dexamethasone on expression of LT pathway enzymes and receptors in human bronchial fibroblasts.

The effect of two types of anti-asthma drug on the expression of 5-LO pathway enzymes and receptors was examined by FACS analysis in bronchial fibroblasts from asthmatic (n=5) and normal (n=5) subjects. Cells were incubated for 24 hours with either the $CysLT_1$ receptor antagonist MK-571 (10nM), or the corticosteroid dexamethasone (1μM). As shown in **Figure 57**, neither MK-571 nor dexamethasone had any significant effect on basal immunofluorescence for 5-LO (6.9±2.8), FLAP (8.5±4.2), LTA_4 hydrolase (6.1±1.6), LTC_4 synthase (0.2±0.1), BLT (4.6±0.9) or $CysLT_1R$ (10.0±3.8) in the asthmatic fibroblasts. Dexamethasone and MK-571 were also without statistically significant effects on LT pathway enzyme expression in normal fibroblasts. Dexamethasone reduced 5-LO immunofluorescence from 8.3±3.5 to 5.3±3.0 ($p=0.057$), FLAP from 10.0±5.2 to 5.4±3.4 ($p=0.079$) and LTA_4 hydrolase from 9.2±3.6 to 4.5±1.7 ($p=0.059$) although these changes were not significant. MK-571 also non-significantly reduced the expression of FLAP in the normal fibroblasts from 10.0±5.2 to 6.0±2.9 ($p=0.059$). Compared to controls, dexamethasone and MK-571 were without significant effects on the immunofluorescence of LTC_4 synthase (MFI 0.4±0.3), BLT (MFI 4.9±2.0) and $CysLT_1R$ (MFI 13.1±5.8) in normal fibroblasts. There were no differences in LT pathway enzyme and receptor expression

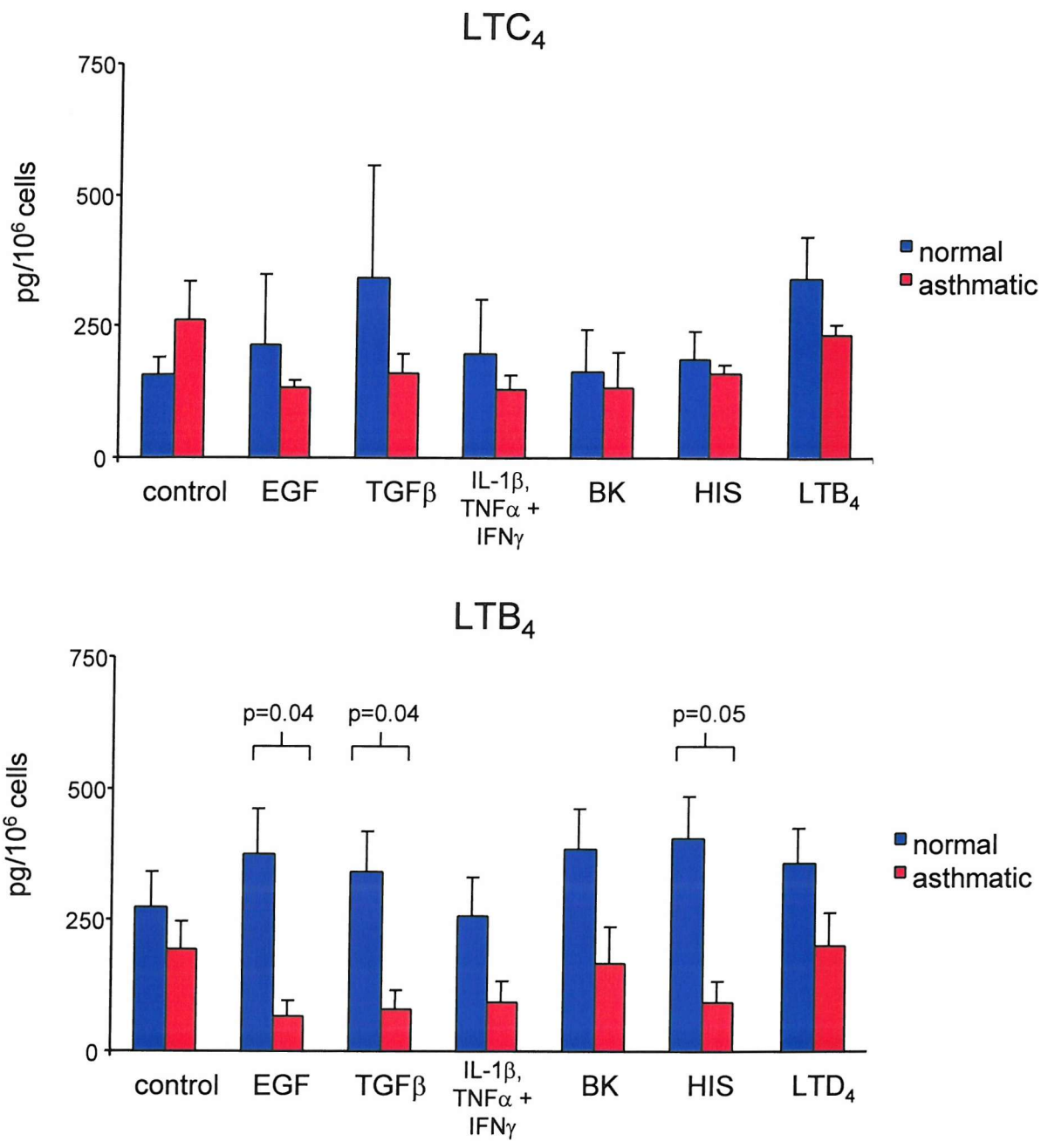


Figure 56. LTC_4 and LTB_4 production by asthmatic and normal fibroblasts in response to inflammatory stimuli. Cells were incubated for 24 hours with or without EGF (10ng/ml), TGFβ (10ng/ml), a combination of IL-1β, TNFα and IFNγ (all at 10ng/ml), bradykinin (10μM), histamine (10μM), LTB_4 (10nM) or LTD_4 (10nM). Cell supernatants were collected and LT prduction was measured using enzyme immunoassay kits for LTB_4 and $LTC_4/D_4/E_4$. Results are shown as mean + SEM and were compared using Student's paired t-test or Mann-Whitney for differences between asthmatics and normals. The asthmatic fibroblasts produced significantly less LTB_4 than the normal cells in response to EGF, TGFβ and histamine.

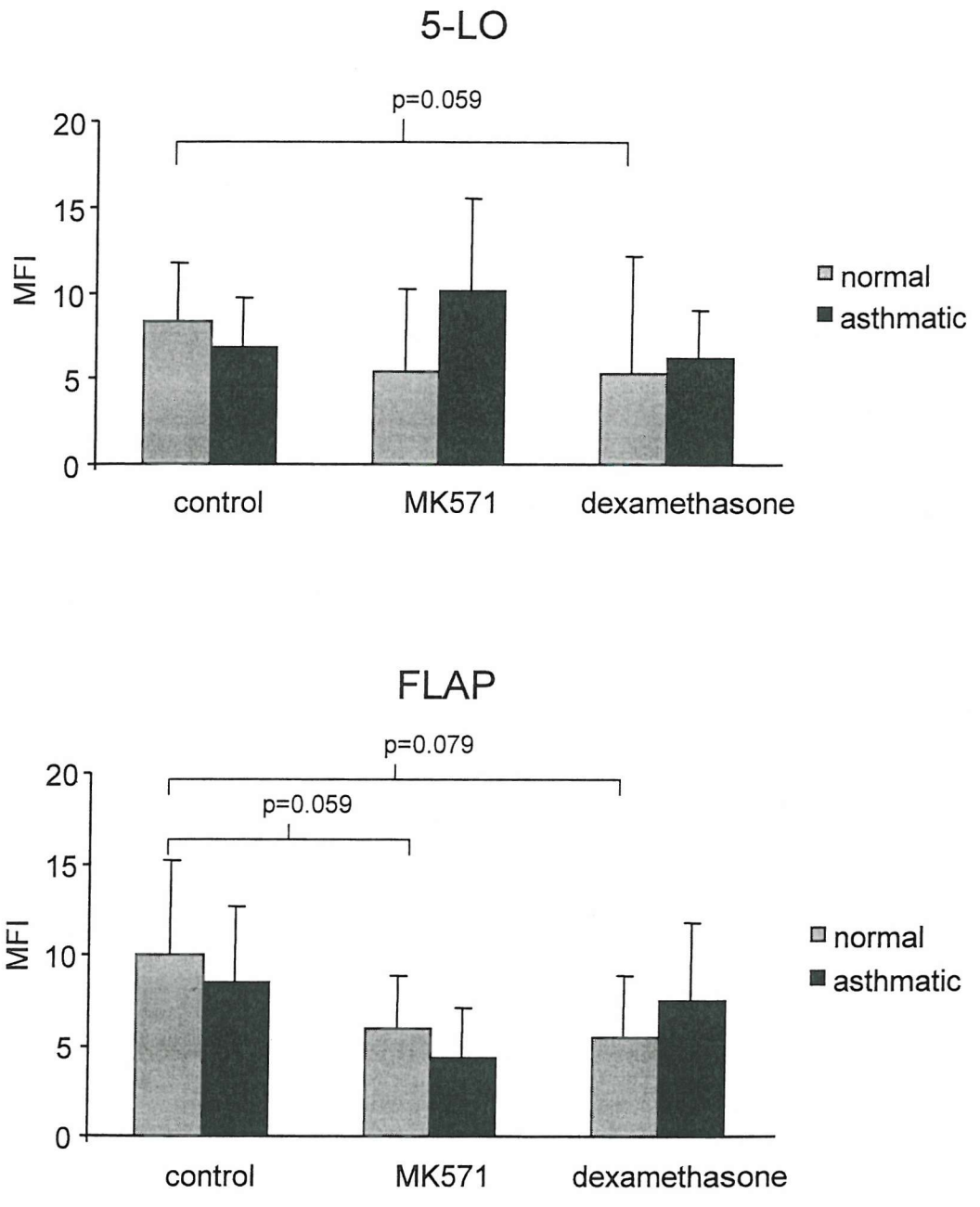


Figure 57a. Effect of MK-571 and dexamethasone on 5-LO and FLAP expression in asthmatic and normal fibroblasts as determined by FACS analysis. Cells from asthmatic (n=4) and normal (n=5) subjects were incubated for 24 hours with 10nM MK-571, 1μM dexamethasone or control medium. Cells were fixed, permeabilised and stained for 5-LO and FLAP. Data are shown as mean+SEM and results were compared using Student's paired t-test. Neither MK-571 nor dexamethasone caused significant changes to 5-LO and FLAP immunofluorescence.

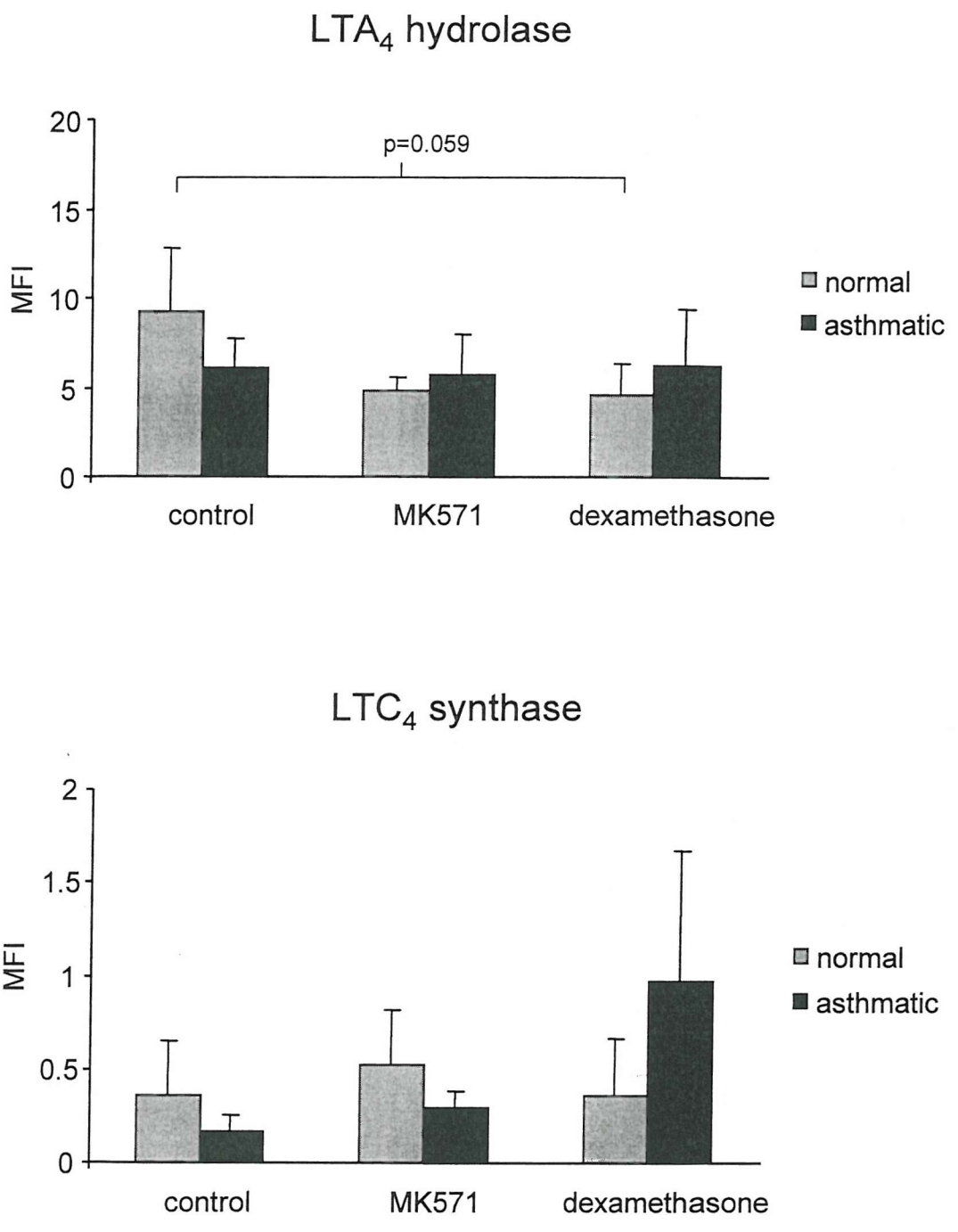


Figure 57b. Effect of MK-571 and dexamethasone on LTA_4 hydrolase and LTC_4 synthase expression in asthmatic and normal fibroblasts as determined by FACS analysis. Cells from asthmatic (n=4) and normal (n=5) subjects were incubated for 24 hours with 10nM MK-571, 1μM dexamethasone or control medium. Cells were fixed, permeabilised and stained for LTA_4 hydrolase and LTC_4 synthase. Data are shown as mean+SEM and results were compared using Student's paired t test. Neither MK-571 nor dexamethasone caused significant changes to LTA_4 hydrolase and LTC_4 synthase immunofluorescence.

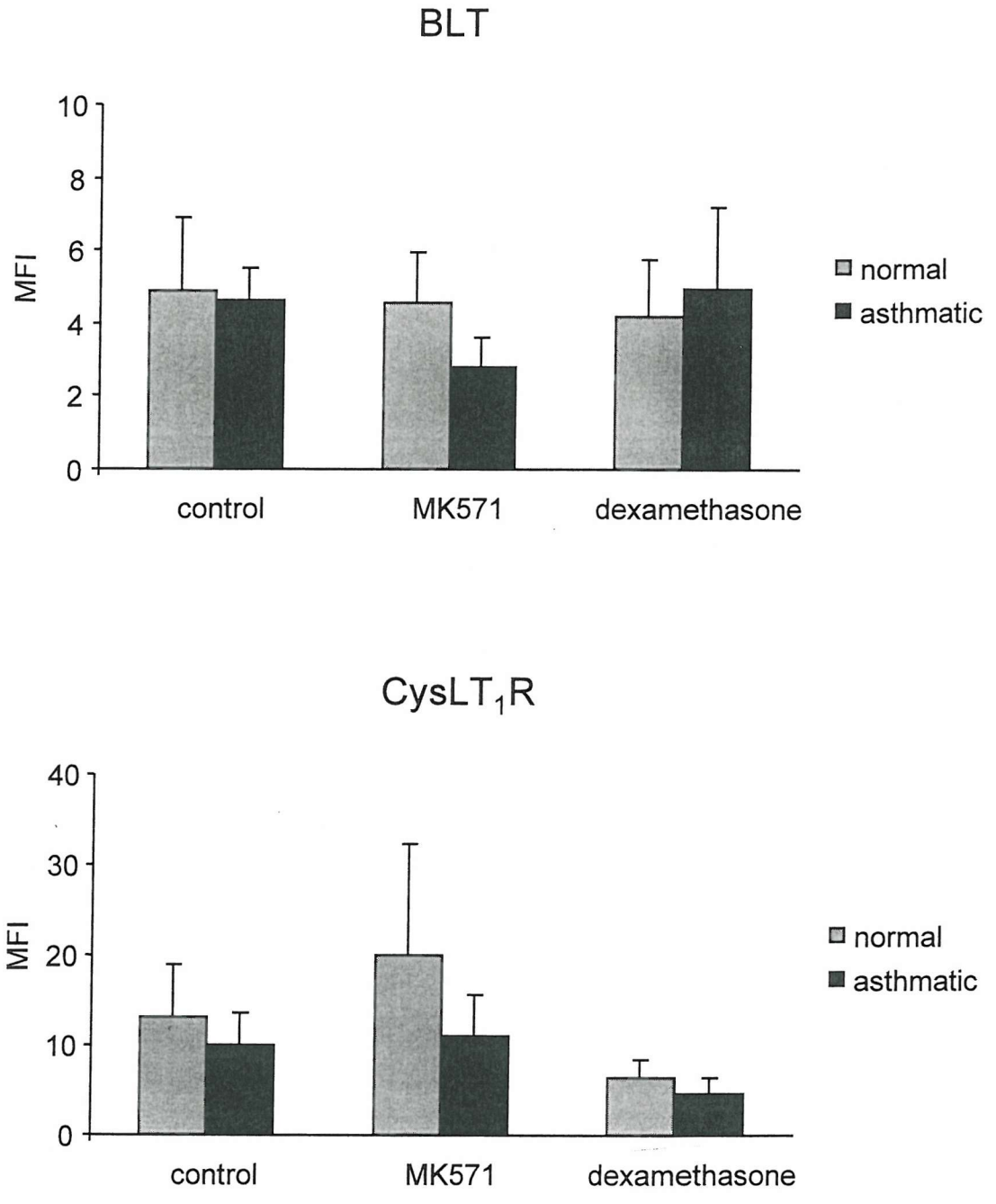


Figure 57c. Effect of MK-571 and dexamethasone on BLT and $CysLT_1$ receptor expression in asthmatic and normal fibroblasts as determined by FACS analysis. Cells from asthmatic (n=4) and normal (n=5) subjects were incubated for 24 hours with 10nM MK-571, 1μM dexamethasone or control medium. Cells were fixed, permeabilised and stained for BLT. $CysLT_1R$ staining was carried out on live cells. Data are shown as mean+SEM and results were compared using Student's paired t test. Neither MK-571 nor dexamethasone caused significant changes to BLT and $CysLT_1$ immunofluorescence.

between the normal and asthmatic groups, either in control samples or after treatment with dexamethasone and MK-571.

5.5 Expression of LT pathway proteins in fibroblasts and myofibroblasts.

Normal human bronchial fibroblasts can be transformed into a more contractile phenotype by stimulation with TGFβ (Chaudhary et al. 2001). We examined the expression of 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase, as well as BLT and $CysLT_1$ receptors in fibroblasts from asthmatics and normals that had been incubated for 72 hours with or without 10ng/ml TGFβ1. In accordance with the findings of other groups, stimulation with TGFβ transformed the fibroblasts into a more contractile phenotype (myofibroblasts) as demonstrated by increased expression of the contractile proteins, α-smooth muscle actin and myosin. No significant differences were found between the asthmatic and normal fibroblasts, with cells from both groups responding in a similar way to TGFβ stimulation. Therefore statistical tests were performed on the normal and asthmatic groups together. TGFβ stimulation increased actin immunofluorescence from 15.1±1.1 to 126.0±38.5 ($p=0.006$) and myosin from 1.8±0.3 to 4.1±1.2 ($p=0.014$) (**Figure 58**). Treatment with TGFβ did not significantly alter the immunofluorescence of 5-LO (5.5±3.3) or FLAP (7.8±5.0) in fibroblasts (**Figure 59a**). The immunofluorescence of LTC_4 synthase rose from 0.02±0.05 to 0.3±0.1 with TGFβ treatment ($p=0.058$), while $CysLT_1R$ rose from 7.6±1.4 in the fibroblasts to 32.7±8.4 in the myofibroblasts ($p=0.008$). Conversely, expression of LTA_4 hydrolase and the BLT receptor were significantly reduced by treatment with TGFβ. The immunofluorescence of LTA_4 hydrolase was reduced from 5.4±2.6 in fibroblasts to 1.8±0.7 in myofibroblasts ($p=0.014$), and BLT was reduced from 6.9±2.6 in fibroblasts to 2.03±1.2 in myofibroblasts ($p=0.009$) (**Figure 59b and c**).

5.6 Proliferation of asthmatic and normal fibroblasts in response to LTs.

To investigate a possible role for leukotrienes in the regulation of fibroblast replication, methylene blue proliferation assays were performed as described in Methods. The proliferation of fibroblasts from asthmatic and normal subjects was measured in response to LTB_4 (1, 10 and 100nM), LTD_4 (1, 10 and 100nM) and the known mitogen for structural cells EGF (10ng/ml). In addition, the effect of LTB_4 (10nM) was examined in the presence of the BLT antagonist, U75302 (10 and 100nM), and effects of LTD_4 in combination with the $CysLT_1$ antagonist, MK-571 (10 and 100nM). Results showed that in serum-free

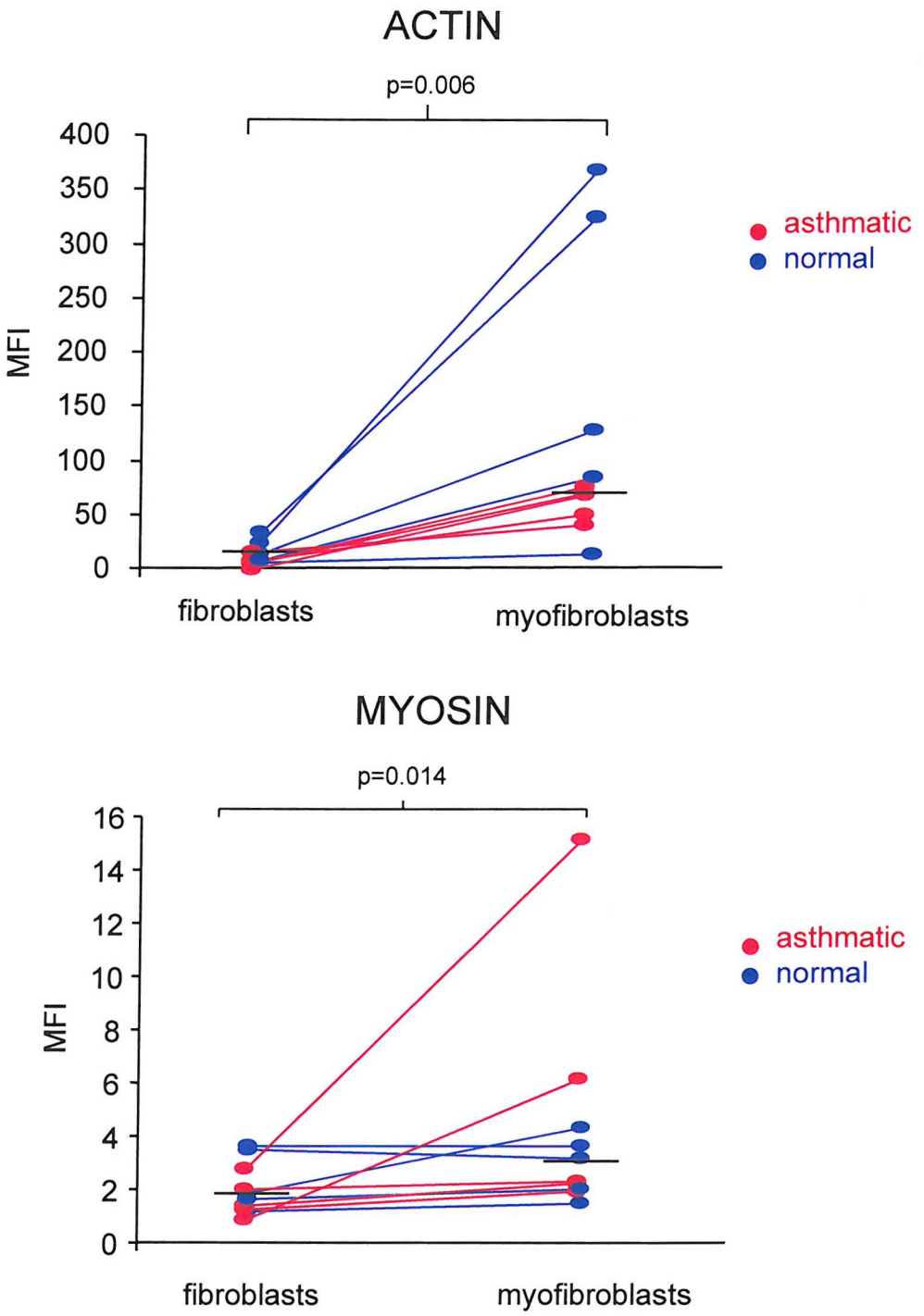


Figure 58. FACS analyses showing expression of Actin and Myosin in fibroblasts and myofibroblasts from asthmatic and normal subjects. Asthmatic and normal fibroblasts were incubated with or without TGFβ (10ng/ml) for 72 hours for transformation into myofibroblasts. Cells were fixed, permeabilised and stained for actin and myosin. Results are shown as individual data points with median bars. Cell samples from asthmatic and normal samples are shown in red and blue respectively. Data sets were compared using Mann-Whitney and Wilcoxon signed rank tests (n=5 for each subject group).

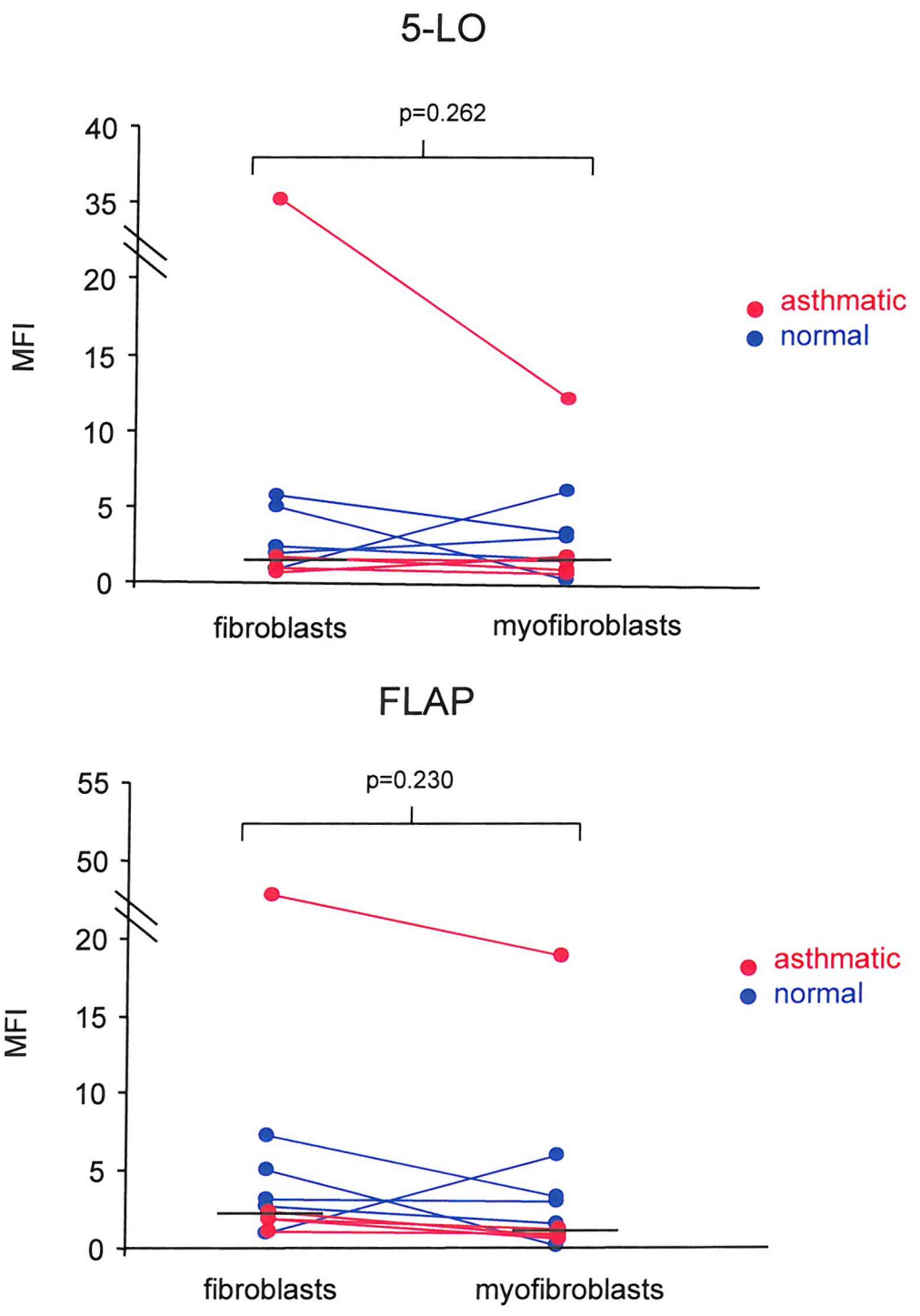


Figure 59a. FACS analyses showing expression of 5-LO and FLAP in fibroblasts and myofibroblasts from asthmatic and normal subjects. Asthmatic and normal fibroblasts were incubated with or without TGFβ (10ng/ml) for 72 hours for transformation into myofibroblasts. Cells were fixed, permeabilised and stained for 5-LO and FLAP. Results are shown as individual data points with median bars. Cell samples from asthmatic and normal samples are shown in red and blue respectively. Data sets were compared using Mann-Whitney and Wilcoxon signed rank tests (n=5 for each subject group).

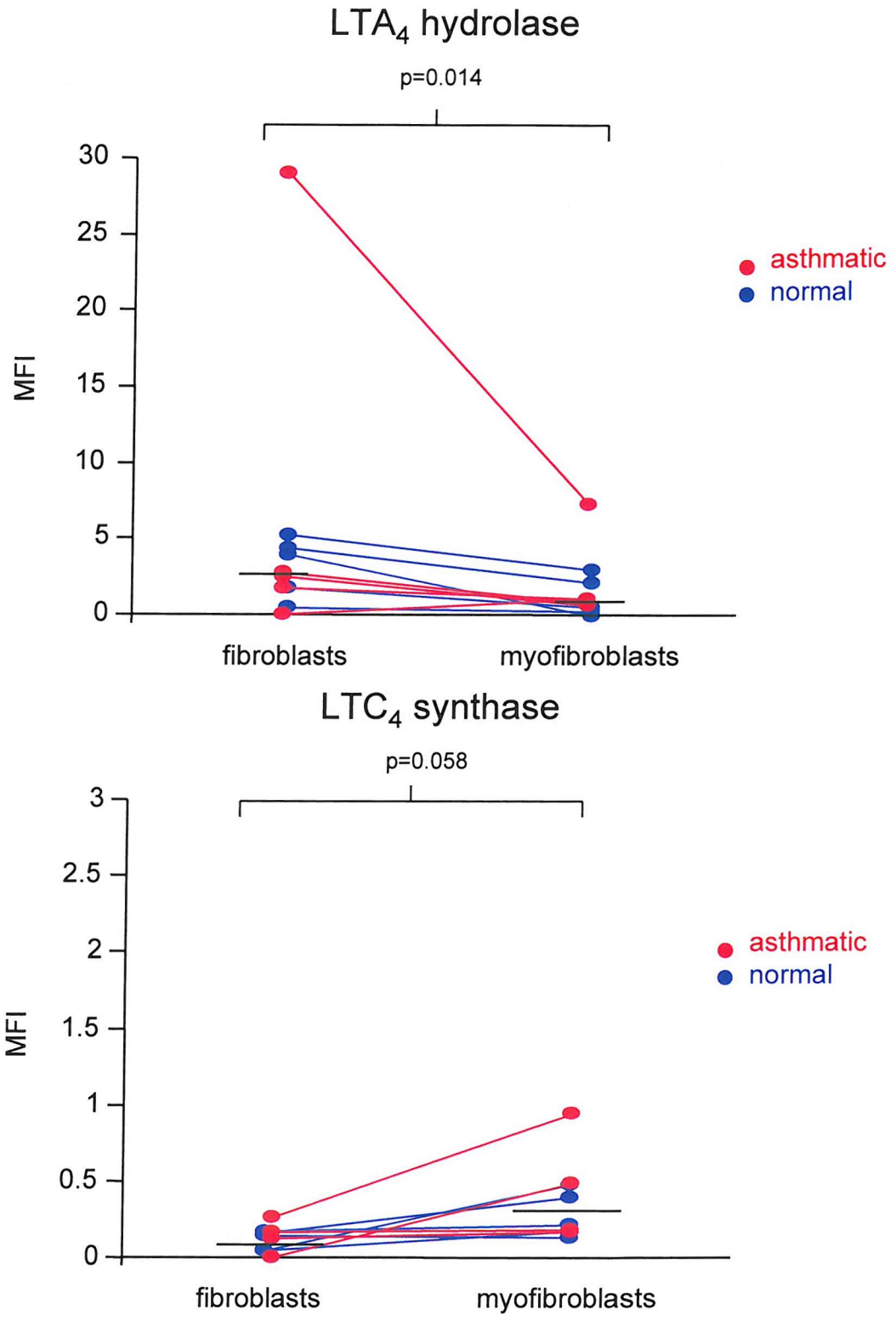


Figure 59b. FACS analyses showing expression of LTA_4 hydrolase and LTC_4 synthase in fibroblasts and myofibroblasts from asthmatic and normal subjects. Asthmatic and normal fibroblasts were incubated with or without TGFβ (10ng/ml) for 72 hours for transformation into myofibroblasts. Cells were fixed, permeabilised and stained for LTA_4H and LTC_4S. Results are shown as individual data points with median bars. Cell samples from asthmatic and normal samples are shown in red and blue respectively. Data sets were compared using Mann-Whitney and Wilcoxon signed rank tests (n=5 for each subject group).

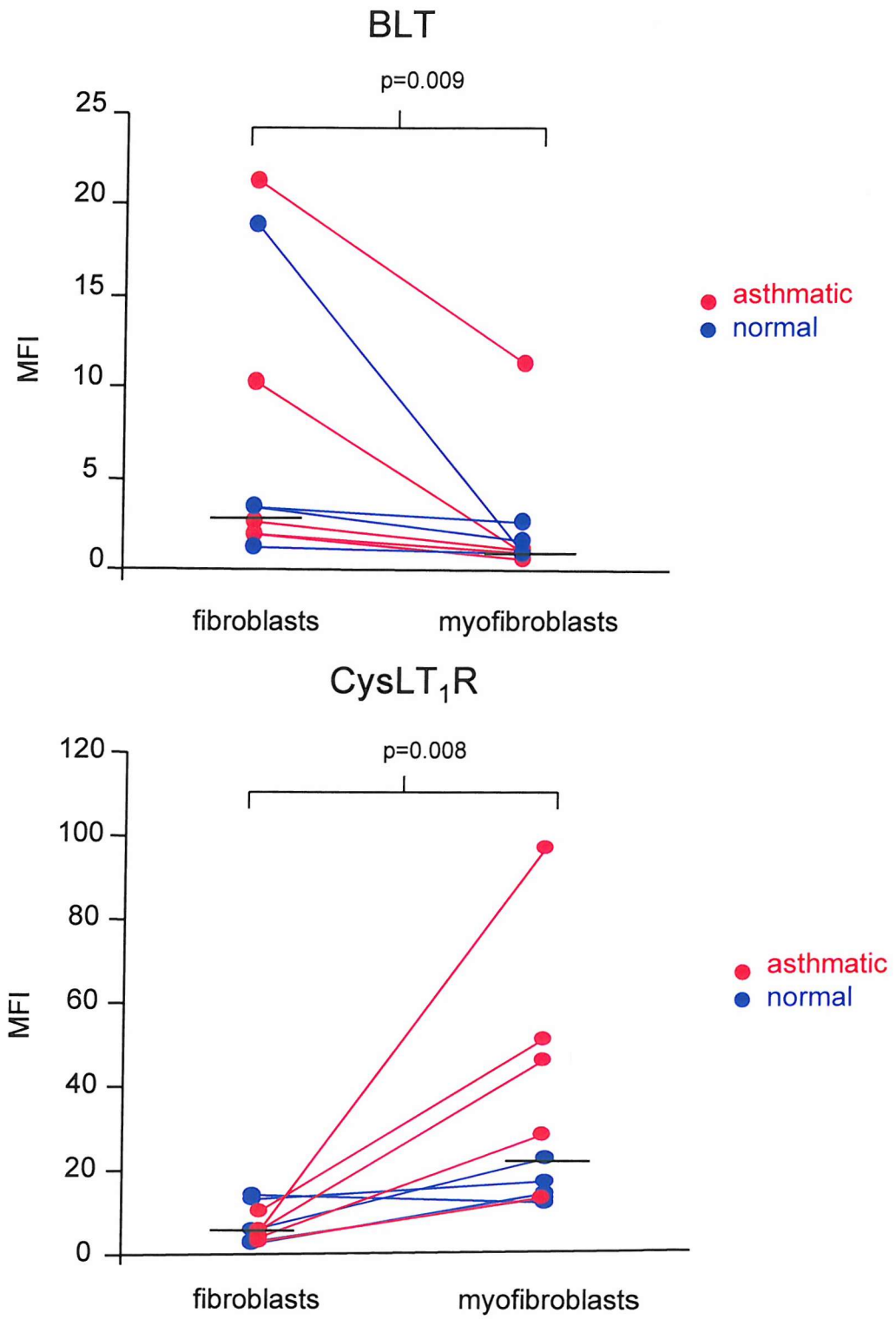


Figure 59c. FACS analyses showing expression of BLT and $CysLT_1$ receptors in fibroblasts and myofibroblasts from asthmatic and normal subjects. Asthmatic and normal fibroblasts were incubated with or without TGFβ (10ng/ml) for 72 hours for transformation into myofibroblasts. Cells were fixed, permeabilised and stained for BLT. $CysLT_1R$ staining was carried out on live unfixed cells. Results are shown as individual data points with median bars. Cell samples from asthmatic and normal samples are shown in red and blue respectively. Data sets were compared using Mann-Whitney and Wilcoxon signed rank tests (n=5 for each subject group).

medium alone, asthmatic fibroblasts proliferated at a significantly higher rate than normal fibroblasts ($p=0.004$). However, compared to basal levels of proliferation, neither LTB_4 nor LTD_4 at any concentration tested modified the proliferation of asthmatic or normal fibroblasts (**Figures 60-62**). Accordingly, U75302 and MK571 were also without effect in both normal and asthmatic cells. Compared to control levels of replication, stimulation with EGF significantly increased numbers of normal and asthmatic cells ($p=0.009$ and $p<0.001$ respectively) (**Figure 60**).

To examine whether LTB_4 and LTD_4 modulated fibroblast proliferation in combination with EGF, further experiments were carried out with cells cultured for up to 72 hours in the presence of either EGF (10ng/ml), EGF with LTD_4 (10nM) or EGF with LTB_4 (10nM). Neither normal nor asthmatic fibroblasts treated with the combination of LTB_4 and EGF showed levels of proliferation significantly different to EGF alone (**Figure 63**). However, the combination of LTD_4 and EGF significantly increased cell numbers, compared to EGF alone, in the asthmatics ($p=0.019$), with a similar trend in the normals ($p=0.07$).

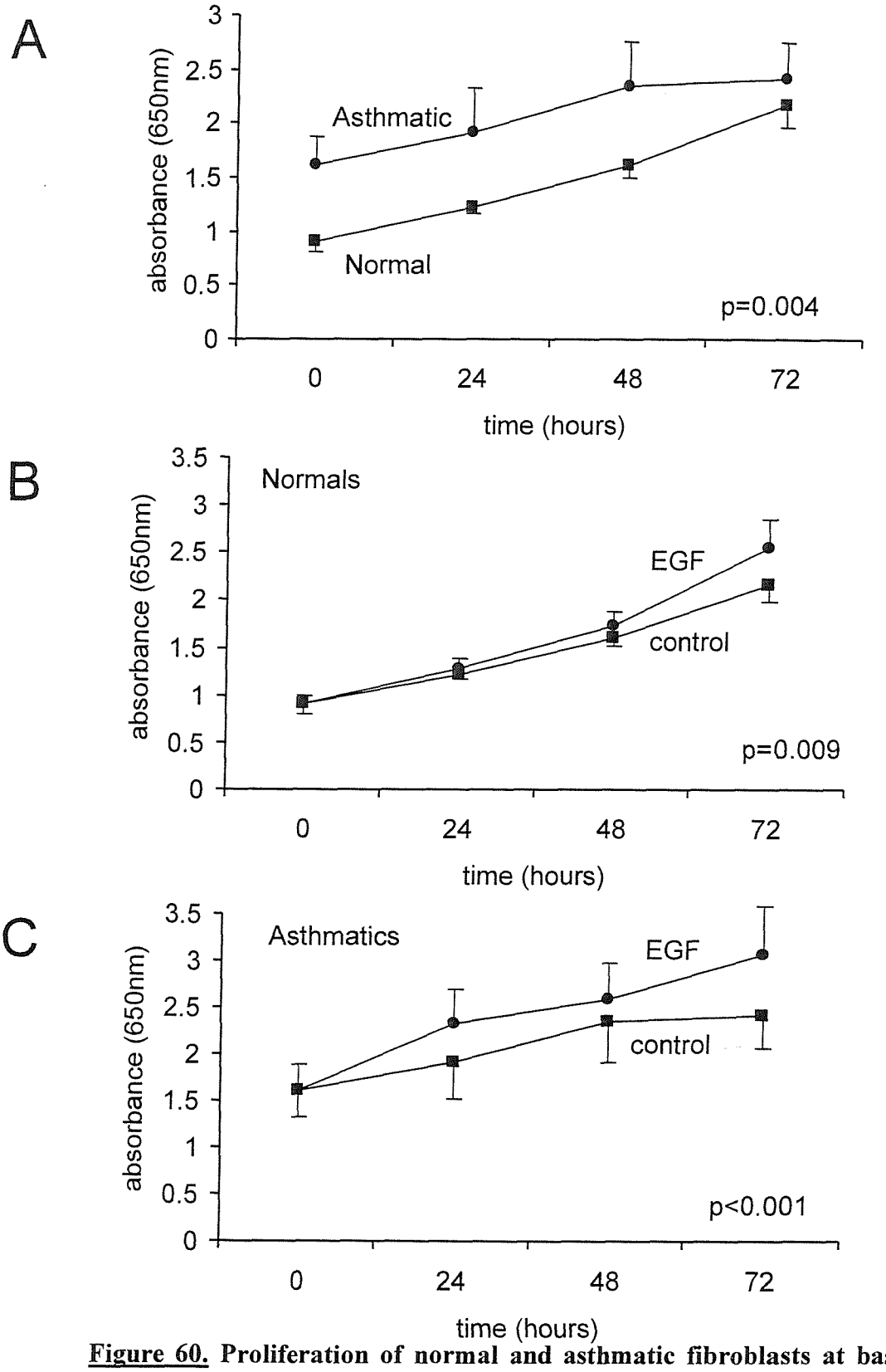


Figure 60. Proliferation of normal and asthmatic fibroblasts at baseline and following stimulation with EGF. Fibroblasts obtained from normal and asthmatic subjects were incubated for 0-72 hours with or without EGF (10ng/ml). Methylene blue proliferation assays were carried out as described in methods. Graphs show (A) Baseline proliferation of asthmatic and normal cells, (B) response of normal fibroblasts to EGF and (C) response of asthmatic fibroblasts to EGF. Results are shown as mean±SEM and were compared using ANOVA.

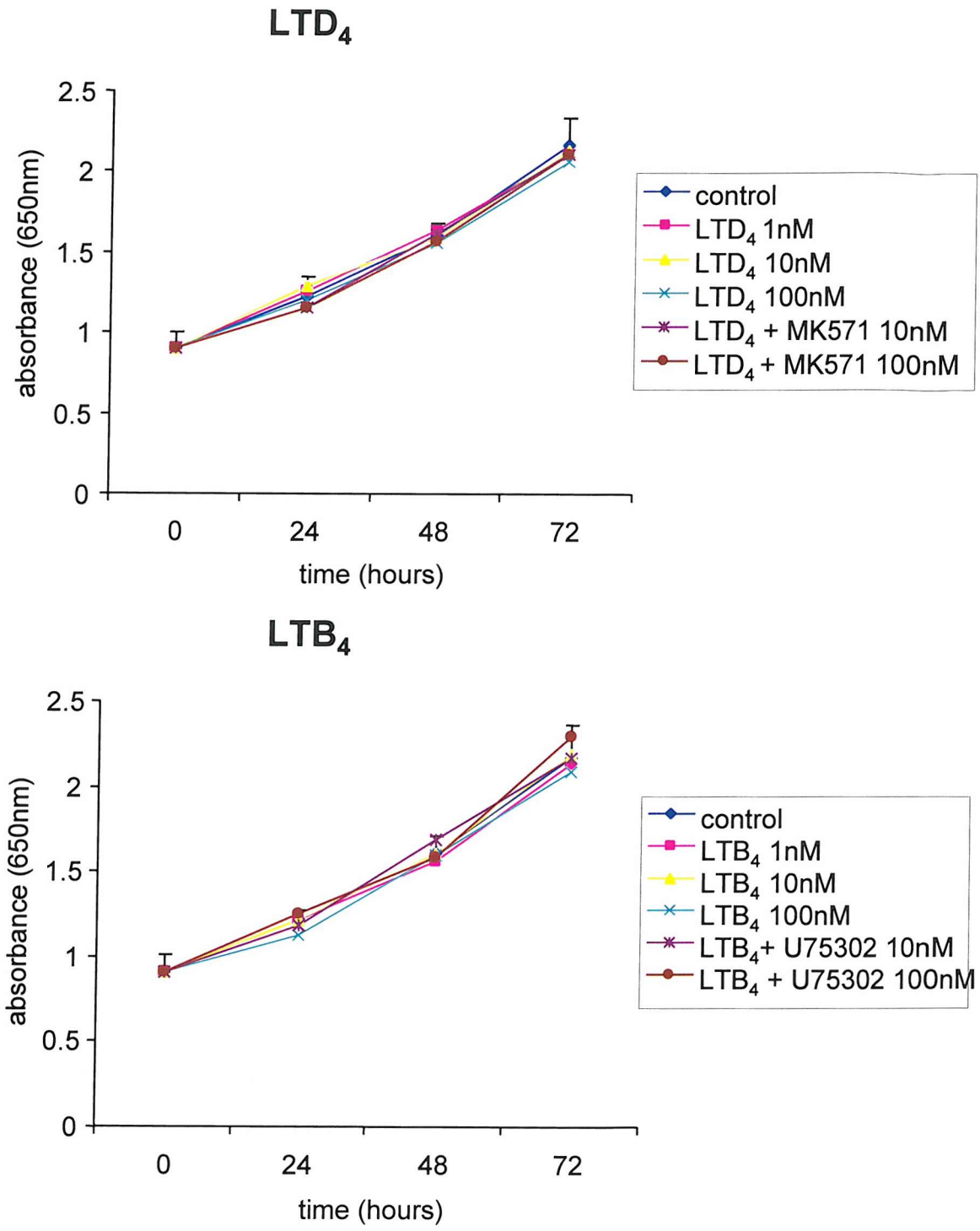


Figure 61. Proliferation assays showing response of normal human bronchial fibroblasts to stimulation with leukotrienes. Fibroblasts obtained from normal subjects were incubated for 0-72 hours with LTB_4 (0-100nM), LTD_4 (0-100nM), LTB_4+U75302 (10 & 100nM), LTD_4 + MK571 (10 & 100nM) or serum-free medium alone (control). Methylene blue proliferation assays were carried out as described in methods. Results are shown as mean±SEM and were compared using ANOVA and paired t-tests. LTB_4 and LTD_4 had no signifiacnt effect on fibroblast proliferation.

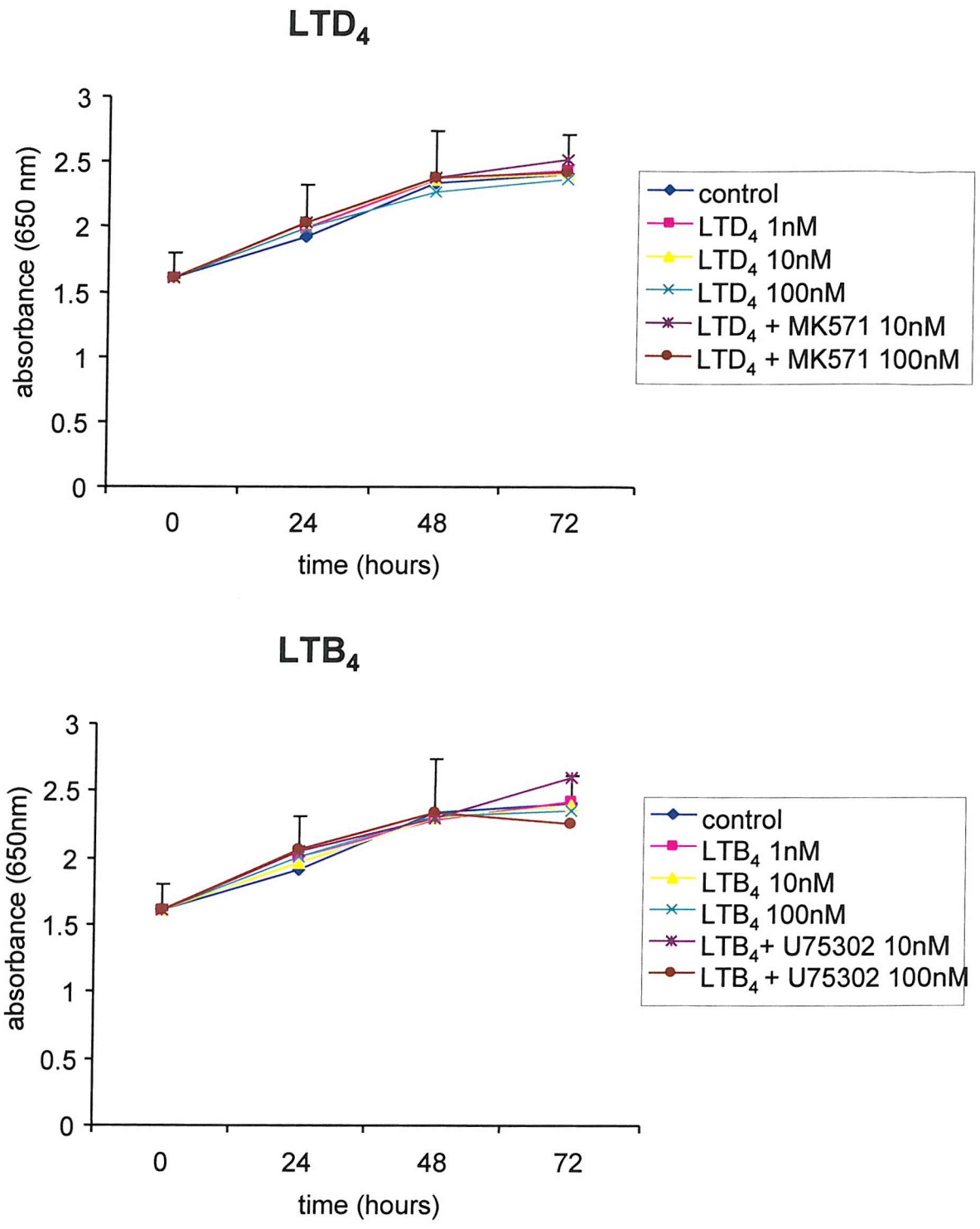


Figure 62. Proliferation assays showing response of human asthmatic bronchial fibroblasts to stimulation with leukotrienes. Fibroblasts obtained from asthmatic subjects were incubated for 0-72 hours with LTB_4 (0-100nM), LTD_4 (0-100nM), LTB_4+U75302 (10 & 100nM), LTD_4 + MK571 (10 & 100nM) or serum-free medium alone (control). Methylene blue proliferation assays were carried out as described in methods. Results are shown as mean±SEM and were compared using ANOVA and paired t-tests. LTB_4 and LTD_4 had no signifiacnt effect on fibroblast proliferation.

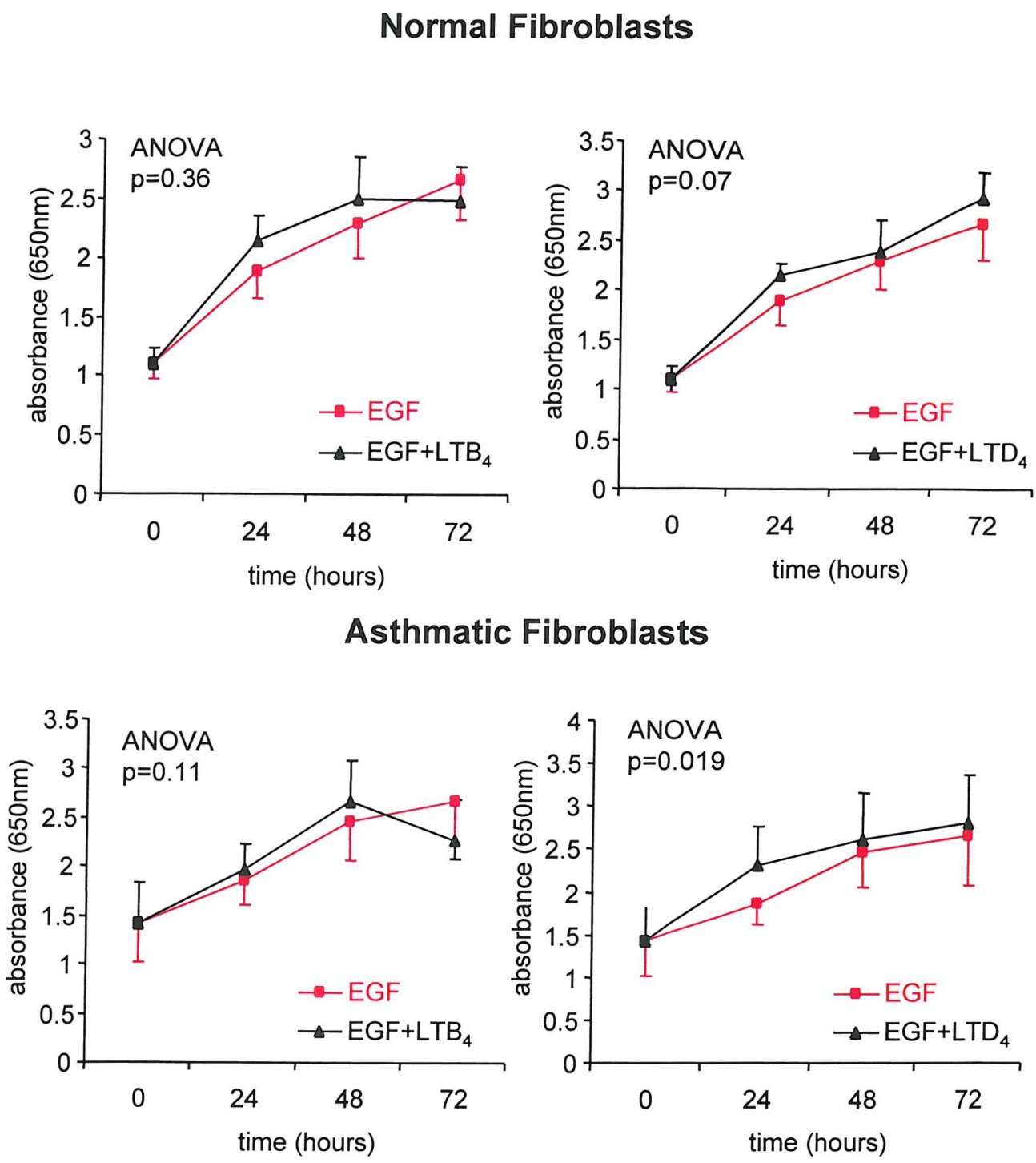


Figure 63. Proliferation of asthmatic and normal fibroblasts in response to EGF in combination with LTB_4 or LTD_4. Fibroblasts obtained from normal and asthmatic subjects were incubated for 0-72 hours with EGF alone (10ng/ml) or EGF with LTB_4 (1nM) or LTD_4 (1nM). Methylene blue proliferation assays were carried out as described in methods. Results are shown as mean±SEM and values at all time points were compared using ANOVA. LTD_4 caused a very small but significant increase in EGF induced proliferation in asthmatic fibroblasts (p=0.019), and a similar trend was observed in the normal subjects (p=0.07).

5.7 Discussion

5.7.1 Baseline expression of LT pathway proteins in human lung fibroblasts

Fibroblasts are a source of many different cytokines (IL-1, IL-6, GM-CSF) and peptides (ET-1) that are important in normal and pathological processes within the airway (Roche et al. 1991). However, despite the importance of LTs in asthma pathogenesis, relatively little is known about the production of these mediators by airway fibroblasts. Several other lipid mediators are produced by fibroblasts such as the cyclooxygenase-derived TXA_2, PGI_2, $PGF_{2\alpha}$ and PGE_2, and the 12-lipoxygenase-derived 12-HETE (Feinmark et al. 1982, Glasgow et al. 1992, Goerig et al. 1988, Gu et al. 2001). As will be discussed below, there are some immunoassay-based reports of leukotriene production by fibroblast cell lines, although there are few studies examining the mechanism of leukotriene synthesis and the enzymes involved. While 5-LO from Syrian hamster embryo fibroblasts has been cloned and characterised (Kitzler et al. 1996), the only LT pathway enzyme reported to be expressed in human cells is LTA_4 hydrolase. The expression of LTA_4 hydrolase mRNA and protein has been demonstrated in both primary and SV40 transformed human fibroblasts by Northern and Western blotting, in combination with the measurement of LTB_4 production by HPLC (Medina et al. 1990). This study also showed that SV40 immortalisation resulted in a 40-fold increase in the expression of LTA_4 hydrolase (Medina et al. 1990), emphasising the importance of the primary cells used in this thesis. Immunohistochemistry of biopsy material has revealed intense LTA_4 hydrolase staining in cardiac myofibroblasts and dermal fibroblasts (Ikai et al. 1994, Ishizaka et al. 1999), although there are no reports of FLAP or LTC_4 synthase expression in human fibroblasts to date.

This study demonstrates for the first time constitutive expression of 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase mRNA and protein in normal and asthmatic bronchial fibroblasts (**Figure 52, p171**). Mixed leukocytes were used as a positive control for the expression of 5-LO pathway enzymes in Western blotting experiments; the intensity of 5-LO, FLAP and LTA_4 hydrolase staining was comparable in the two cell types. Flow cytometry showed positive immunofluorescence for all four LT pathway proteins in fibroblasts but, as in BEC and HASM, 5-LO, FLAP and LTA_4 hydrolase immunofluorescence was greater than that of LTC_4 synthase in both asthmatic and normal fibroblasts. This finding could suggest that either fibroblasts are a specialised source of LTB_4 rather than LTC_4, or that LTC_4 synthase is not the rate limiting enzyme in LTC_4

production and only relatively low levels of this enzyme are necessary for LTC_4 synthesis. Indeed experiments in our group have shown that eosinophils, which produce large quantities of cys-LTs, show low LTC_4S immunofluorescence with a median fluorescence intensity of ~2 which is similar to fibroblast levels (Michelle Seymour, PhD thesis, 2000).

In accordance with the constitutive expression of 5-LO, FLAP, LTA_4H and LTC_4S, both asthmatic and normal fibroblasts generated significant levels of LTC_4- and LTB_4-like immunoreactivity over a 24-hour period. Unlike BEC and HASM, which both produced more LTB_4 than LTC_4, asthmatic and normal fibroblasts generated approximately equal amounts of LTC_4 and LTB_4 (approximately 250 pg/10^6 cells). As LTA_4 hydrolase immunofluorescence is higher in the fibroblasts than LTC_4 synthase, low levels of LTC_4 synthase expression do not necessarily correlate with low LTC_4 production.

The possibility that fibroblasts may generate leukotrienes by a transcellular mechanism has been investigated (Claesson et al. 1988). Endothelial cells were able to convert leukocyte derived LTA_4 into cys-LTs and LTB_4, but co-culture of leukocytes with a rat fibroblast cell line did not result in increased leukotriene generation. However, fibroblasts obtained from different tissues may behave differently, as using another rat fibroblast cell line, Peppelenbosch and colleagues were able to measure both LTB_4 and cys-LT production by enzyme immunoassay (Peppelenbosch et al. 1999). In similar studies using human lung fibroblasts, three Japanese studies measured approximately 250 pg/ml LTB_4 by radioimmunoassay after a 72-hour incubation, although cys-LTs were not measured (Koyama et al. 2000, Sato et al. 1999, Takamizawa et al. 1999). A study using the same Amersham $LTC_4/LTD_4/LTE_4$ enzyme immunoassay kit as used in the present study, was able to detect cys-LT release by human lung fibroblasts after a 16 hour incubation (Aoshiba et al. 1999), but again the units (pg/well), incubation time and lack of information regarding cell numbers, prevent direct numerical comparisons with our data. Unlike the primary human bronchial fibroblasts used in the present study, these four studies all used human lung fibroblast cell lines. The phenotypes of cells that have been immortalised or cultured for long periods of time may be different to those of primary cells. As mentioned above, simian virus-transformed fibroblasts express 40-fold more LTA_4 hydrolase mRNA than their non-transformed counterparts (Medina et al. 1990). The characteristics of the primary lung fibroblasts used in this project are more likely to resemble those of cells *in vivo*. To our knowledge this is the first study to report constitutive 5-LO, FLAP, LTA_4 hydrolase and

LTC_4 synthase expression along with LTC_4 and LTB_4 release, in primary human bronchial fibroblasts.

Baseline FACS analysis also showed positive expression of the LTB_4 receptor, BLT1 and the cys-LT receptor, $CysLT_1$. The BLT antibody used in these experiments is for the high affinity BLT1 receptor, re-named as such due to the recent discovery of the low affinity BLT2 receptor (Yokomizo et al. 2000). While BLT1 expression has been described as exclusive to leukocytes, BLT2 is ubiquitously expressed. Therefore one may predict that BLT2, rather than BLT1 would be expressed on fibroblasts. Although the two BLT receptors share 45% sequence homology (Yokomizo et al. 2000), it is unlikely that the BLT1 antibody cross-reacts with BLT2, as this antibody was raised against a synthetic peptide from the BLT1 receptor that shows no homology with BLT2. While expression of $CysLT_1$ on lung fibroblasts was not specifically described in the first study to characterise this receptor, high levels of $CysLT_1$ mRNA were found in the lung, and *in situ* hybridisation showed high levels of $CysLT_1$ expression on smooth muscle cells which share many of the characteristics of myofibroblasts (Lynch et al. 1999). As there are reports of both cys-LT and BLT-mediated actions on human fibroblasts (Medina et al. 1994, Mensing et al. 1987), the presence of $CysLT_1$ and BLT1 was not entirely unexpected. Neither BLT nor $CysLT_1$ showed different levels of expression in asthmatic and normal fibroblasts, but this does not preclude differing functional responses to LTB_4 or LTC_4 exposure.

In this study, there were no marked differences in the basal immunofluorescence for 5-LO pathway enzymes, and $CysLT_1$ and BLT1 receptors, between fibroblasts from normal and asthmatic subjects. However, differences in the behaviour of cultured asthmatic and normal fibroblasts do exist, demonstrating that even outside the environment of the asthmatic airway, these cells are not phenotypically identical. For example, even in the absence of stimulatory factors, cultured asthmatic fibroblasts proliferate more rapidly than normal cells, synthesise more procollagen, have a decreased capacity to degrade collagen and express more TGFβ (Dube et al. 1998, Vignola et al. 1997, Laliberte et al. 2001).

5.7.2. Effect of inflammatory stimuli on the LT pathway in lung fibroblasts

The effects of several pro-inflammatory mediators on the LT pathway in fibroblasts were examined (**Figure 55, p175**). These mediators have known actions on cells of mesenchymal origin and are of particular relevance to asthma. For example, EGF is a mitogen of lung

fibroblasts (Vignola et al. 1997) and is also involved in actin remodelling and repair processes (Peppelenbosch et al. 1999). It has been shown to activate $cPLA_2$ and lead to arachidonic acid release in a number of cell types (Peppelenbosch et al. 1993). TGFβ is involved in the differentiation of myofibroblasts by inducing the expression of contractile proteins such as actin and myosin, and by increasing collagen production (Richter et al. 2001). Levels of TGFβ are increased in the BAL fluid of asthmatics (Redington et al. 1997). The combination of IFNγ, IL-1β and TNFα increases proliferation, chemotaxis and metalloproteinase production by human lung fibroblasts (Sasaki et al. 2000). BAL fluid from symptomatic asthmatics contains elevated amounts of IL-1β and TNFα (Broide et al. 1992). Bradykinin and histamine have several effects on lung fibroblasts ranging from increased cytokine, peptide and collagen production to increased proliferation (Ricupero et al. 2000, Sato et al. 2000, Jordana et al. 1988). LTD_4 increases fibroblast collagen and collagenase production and is implicated in the proliferation of fibroblasts, whereas LTB_4 is chemotactic for human lung fibroblasts (Abe et al. 2000, Medina et al. 1994, Baud et al. 1987, Mensing et al. 1984).

The promoter regions of 5-LO, FLAP, LTA_4 hydrolase, LTC_4 synthase and BLT genes have binding sites for nuclear transcription factors (Silverman et al. 1998, Kennedy et al. 1991, Mancini et al. 1995, Penrose et al. 1996 and Kato et al. 2000). Several different cytokines are able to regulate expression of LT pathway proteins. For example, GM-CSF upregulates 5-LO in polymorphonuclear leukocytes (Pouliot et al. 1997), IL-5 increases the expression of FLAP in eosinophils (Cowburn et al. 1999), IL-4 and IL-13 increase LTA_4 hydrolase in PMNs (Zaitsu et al. 2000) and the expression of LTC_4 synthase is increased by TGFβ in monocytes (Riddick et al. 1999). In addition the IL-5-induced upregulation of $CysLT_1$ transcription has recently been shown in eosinophils (Thivierge et al. 2000). However, despite regulation in other cell types, none of the stimuli at the concentrations tested and after a 24 hour incubation caused any changes to the basal immunofluorescence of LT pathway proteins, in either asthmatic or normal fibroblasts. This finding suggests that if there is regulation of LT production in fibroblasts, it occurs by a mechanism other than expression of 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase, for example by modulating substrate availability, the expression of $cPLA_2$ or even subcellular translocation.

Treatment with EGF, TGFβ, bradykinin, histamine, or a combination of IL-1β, TNFα and IFNγ did not significantly change the basal amounts of LTB_4 and LTC_4 released by normal and asthmatic fibroblasts (**Figure 56, p179**). Although rat fibroblasts have been shown to generate greater amounts of LTB_4 and cys-LTs following treatment with EGF (Peppelenbosch et al. 1999), studies using human fibroblasts have not shown regulation of LTB_4 production. Incubation of human lung fibroblasts with bradykinin, smoke extract or bleomycin for 72 hours had no effect on LTB_4 release, compared to levels produced by untreated samples (Koyama et al. 2000, Sato et al. 1999, Takamizawa et al. 1999). Along with our results, these findings point to a role for continuous LT production by lung fibroblasts as opposed to the acute increases in LT production seen after the stimulation of leukocytes. Direct comparisons between levels of LTs produced in leukocytes and fibroblasts are difficult, but levels produced appear to be greater in the cells of myeloid origin. While neutrophils can release up to $48ng/10^6$ cells LTB_4, and eosinophils can release up to $30ng/10^6$ LTC_4 during a short incubation with A23187 (Burke et al. 1990, Weller et al. 1980), Ca ionophore is not a physiological stimulus. Compared to A23187, eosinophils release up to 100-fold less LTC_4 following treatment with zymosan, PAF or fMLP (Mahauthaman et al. 1988, Takafuji et al. 1991, Tamura et al. 1998). Levels of LTC_4 produced in response to these stimuli range from approximately 700 to 1600 $pg/10^6$ cell (Mahauthaman et al. 1988, Takafuji et al. 1991, Tamura et al. 1998). Although these concentrations are closer to those produced by fibroblasts, none of the inflammatory stimuli were found to alter LT release by fibroblasts and a better comparison would be with LT production by unstimulated leukocytes. When adjusted for incubation times, spontaneous LTC_4 production by human blood eosinophils is only four times greater than fibroblast LTC_4 production (Tenor et al. 1996) and therefore, as in HBE and HASM cells, the greatest difference between the characteristics of LT release in leukocytes and fibroblasts, is in the response to stimulation.

There were no significant differences between asthmatic and normal fibroblasts in the amounts of LTC_4 generated. Both produced approximately $200pg/10^6$ cells irrespective of stimulus. Normal fibroblasts tended to produce more LTB_4 than asthmatic cells although this difference only reached statistical significance in samples treated with EGF, TGFβ and histamine. While these differences could highlight a difference in the response of normal and asthmatic cells to these mediators, their effects are not significant compared to the basal

levels of LTB_4 produced in either normal or asthmatic fibroblasts. Therefore it is unlikely that these changes represent meaningful biological effects.

5.7.3 Effect of dexamethasone and MK-571 on the LT pathway in fibroblasts

Dexamethasone is one of the most potent glucocorticoid steroids with anti-inflammatory and immunosuppressive actions. Corticosteroids, both inhaled and oral are commonly used in asthma management. Although there was a trend for 5-LO, FLAP and LTA_4 hydrolase immunofluorescence to be reduced following dexamethasone treatment in normal fibroblasts, these changes were not statistically significant (**Figure 57, p180**). Overall, dexamethasone did not significantly alter the expression of 5-LO pathway enzymes, or $CysLT_1$ and BLT receptors in either normal or asthmatic fibroblasts. The paradoxical lack of effect of dexamethasone on the LT pathway in fibroblasts is confirmed in several other cell types. For example, in this study dexamethasone had no effect on the basal expression of 5-LO pathway enzymes in HBE or HASM cells. Iversen and colleagues demonstrate a lack of effect on LTA_4 hydrolase expression in keratinocytes (Iversen et al. 1996). In mast cells and basophilic cells, dexamethasone has not been found to alter the expression of LTC_4 synthase (Colamorea et al. 1999, Hamasaki et al. 1997). Surprising effects on 5-LO and FLAP have been demonstrated in other myeloid cells. In differentiated THP-1 cells, neutrophils and monocytes, dexamethasone has been found to increase levels of FLAP mRNA (Goppelt-Struebe et al. 1997, Pouliot et al. 1994) and more recently the same effect has been shown with 5-LO in mast cells (Colamorea et al. 1999). The biological relevance of this effect remains unknown, although studies *in vivo* suggest an overall lack of effect on leukotriene production. Short-term treatment with oral or inhaled corticosteroids has been shown not to reduce urinary LTE_4 levels in normal and asthmatic subjects (O'Shaugnessy et al. 1993, Sebaldt et al. 1990, Manso et al. 1992). Dexamethasone induced 5-LO and FLAP upregulation was not confirmed either in the normal or asthmatic fibroblasts which is most likely due to differences between structural and myeloid cell types.

Montelukast is a $CysLT_1$ receptor antagonist successfully used in the treatment of asthma with both bronchodilator and anti-inflammatory activity. MK-571 is a racemic mixture of R and S enantiomers of which montelukast is structurally similar to the R enantiomer. It is not inconceivable that cys-LTs could regulate the transcription of their own synthetic enzymes. We have shown that fibroblasts release LTC_4, and activation of cys-LT receptors is known to increase intracellular Ca^{2+} which could lead to activation of transcription factors such as

NFκB. Indeed, it has been shown that LTB_4 is able to activate NFκB (Aoki et al. 1998) which may explain the mechanism whereby LTB_4 treatment increased FLAP and LTA_4 hydrolase expression in HASM cells. MK-571 was not found to significantly regulate the expression of 5-LO, FLAP, LTA_4 hydrolase or LTC_4 synthase in either normal or asthmatic fibroblasts. As previously described, certain drugs such as β_2 agonists downregulate their receptors (β_2-ARs) (Kelsen et al. 1997). The $CysLT_1$ agonist LTD_4 had no effect on the expression of $CysLT_1$ in fibroblasts but of particular interest is the lack of effect of MK-571 on $CysLT_1$. While MK-571 is an antagonist, not an agonist, and may not be expected to have functional effects at this receptor, our findings provide further support to the clinical lack of tachyphylaxis or rebound worsening following drug withdrawal observed with $CysLT_1$ antagonist therapy (Leff et al. 1995).

5.7.4 The LT pathway in differentiated fibroblasts

Myofibroblasts are a phenotypic intermediate between fibroblasts and smooth muscle cells with specialised characteristics such as contractility and matrix deposition which make them important cells in wound healing processes. Several different agents have been shown to control the differentiation of fibroblasts into myofibroblasts, such as the Th2 type cytokines IL-4 and IL-13 (Hashimoto et al. 2001) and the BEC derived peptide ET-1 (Sun et al. 1997) which both increase contractile protein expression. Fluticasone propionate and the glycoprotein vitronectin both downregulate actin expression in fibroblasts (Scaffidi et al. 2001, Cazes et al. 2001). Several studies have shown that by far the most potent inducer of myofibroblast differentiation is TGFβ (Richter et al. 2001, Morishima et al. 2001). We used TGFβ1 to transform fibroblasts from normal and asthmatic subjects into myofibroblasts, and confirmed previous observations, that expression of the contractile proteins α smooth muscle actin and heavy chain myosin was induced in the cells incubated with TGFβ. Asthmatic and normal cells responded to TGFβ treatment in a similar way, with no significant differences in actin and myosin expression between the two groups. This finding implies that the signalling pathway involved in myofibroblast differentiation is functionally intact in both normal and asthmatic fibroblasts. The TGFβ signalling pathway involves the binding of TGFβ to a TGFβ receptor with intrinsic serine/threonine activity that leads to phosphorylation of Smad proteins and the assembly of Smad complexes which translocate to the nucleus and regulate the transcription of target genes (Nakao et al. 2001). We

hypothesised that such genes might include 5-LO, FLAP, LTA_4 hydrolase, LTC_4 synthase, $CysLT_1R$ or BLT.

Treatment with TGFβ produced no significant change in expression of 5-LO or FLAP in fibroblasts differentiated into myofibroblasts, but caused a 4-fold increase in $CysLT_1R$ and 3-fold decrease in LTA_4 hydrolase and BLT expression (**Figure 59, p185**). The immunofluorescence of LTC_4 synthase was 15-fold greater in the myofibroblasts than the fibroblasts although this change narrowly failed to reach statistical significance ($p=0.058$). These findings imply that there may be a switch from the synthesis and/or actions of LTB_4 in myofibroblasts to those of the cys-LTs, a change that could be of importance to the remodelling and contractility of asthmatic airways.

A unique feature of asthma is the increased deposition of interstitial collagens, (especially myofibroblast derived types III and I) in the lamina reticularis, the thickness of which correlates with the elevated numbers of myofibroblasts in asthmatic airways, pointing to a key role for these cells in airway remodelling (Brewster et al. 1990). TGFβ is a potent inducer of myofibroblast differentiation, it can prolong myofibroblast survival and its levels are increased in BAL fluid from asthmatic subjects (Redington et al. 1997). A suggested model for increased collagen production and increased myofibroblast numbers is that epithelial damage results in the production of growth factors such as TGFβ, which causes increased collagen synthesis through myofibroblast induction (Morishima et al. 2001). While LTB_4 mainly has effects on neutrophils, cys-LTs are increasingly regarded as playing a role in remodelling responses. LTD_4 can increase collagen production by dermal fibroblasts and increase the collagenase expression of lung fibroblasts (Abe et al. 2000, Medina et al. 1994). LTD_4 can also increase fibroblast proliferation when the endogenous production of the anti-proliferative PGE_2 is blocked by indomethacin (Baud et al. 1987). Therefore, a switch from the production of LTB_4 to LTC_4, associated with an increase in the numbers of $CysLT_1$ receptors, could amplify the structural changes that occur in asthma and possibly even contribute to bronchoconstriction. Future studies will need to confirm that these changes in enzyme expression translate to production of less LTB_4 and more LTC_4. A similar A23187-induced increase in LTC_4 synthase was associated with a doubling of LTC_4 release by HBE cells, although in this case the contribution of 5-LO and FLAP, which were also increased, cannot be discounted.

5.7.5 Proliferation of bronchial fibroblasts in response to LTs

A prominent characteristic of asthma is the increased numbers of (myo)fibroblasts within the airway. This may be due to increased proliferation or reduced apoptosis and therefore factors controlling these responses have been widely studied. Several agents have been described as regulating fibroblast proliferation including TGFβ, bFGF, IL-4, IL-13, tryptase and histamine (Silvestri et al. 2001, Kraft et al. 2001, Ahers et al. 2001). Even dexamethasone has been shown indirectly to induce fibroblast proliferation by its activational effects on the PDGFα receptor (Warshamana et al. 1998). PGE_2 has inhibitory effects on fibroblast replication (McAnulty et al. 1997). It is unclear whether LTs have an effect on human lung fibroblast proliferation. LTB_4 has not been found to increase fibroblast proliferation (Pollo et al. 1985, Baud et al. 1987) although the cys-LTs may have mitogenic effects. LTC_4 and LTD_4 were able to increase the replication of skin fibroblasts when prostaglandin production was blocked by indomethacin (Baud et al. 1987) although in a similar system, LTD_4 alone was not capable of stimulating proliferation (Abe et al. 2000).

Our results showed that neither LTB_4 nor LTD_4 alone at any of the concentrations tested had stimulatory (or inhibitory) effects on the proliferation of asthmatic and normal fibroblasts (**Figure 61&62, p190&191**). The lack of effect of LTD_4 alone on fibroblast proliferation is consistent with other studies in fibroblasts where cys-LTs alone were without effect (Abe et al. 2000, Phan et al. 1988, Baud et al. 1987). However, the effects of cys-LTs on cell proliferation appear to vary between cell types. While airway smooth muscle cells do not proliferate in response to cys-LTs alone (Cohen et al. 1995, Panettieri et al. 1998), cys-LTs alone are mitogenic to bronchial and glomerular epithelial cells (Leikauf et al. 1990, Baud et al. 1985).

As it has been suggested that cys-LTs interact with growth factor activated signalling pathways (IGF and EGF) to increase the proliferation of HASM (Panettieri et al. 1998, Cohen et al. 1995) some preliminary experiments were carried out to examine the effects of LTB_4 and LTD_4 (1nM) in combination with EGF (10ng/ml). With EGF, LTB_4 did not have any additional effect on the proliferation of EGF stimulated fibroblasts. However, LTD_4 caused a very small, but significant, increase in fibroblast proliferation above that seen with EGF alone. Pannettieri et al observed a much greater effect in HASM cells although the concentration of LTD_4 used was between 100- and 10000-fold greater than the 1nM LTD_4 used here. However, 1nM is closer to physiological levels likely to be found *in vivo*. Little is

known about the mechanisms whereby cys-LT receptors and growth factor signalling pathways interact. In IGF stimulated HASM it was shown that LTD_4 enabled more free IGF to interact with its receptors by reducing levels of an IGF binding protein (IGFBP) (Cohen et al. 1995). A mechanism underlying the potentiating influence of LTD_4 on EGF stimulated HASM has not been suggested, although the inhibitory effects of pranlukast and pobilukast suggest a $CysLT_1$ receptor mediated phenomenon (Panettieri et al. 1998).

The effect of LTD_4 observed here is relatively small and therefore may not be of great biological significance. However, our findings may underrepresent the full capacity of LTD_4 and EGF synergism. Only one dose each of EGF (10ng/ml) and LTD_4 (1nM) were used and it may be necessary to increase or decrease these concentrations to observe optimal effects. Also, EGF is only one of several growth factors (e.g. IGF, TGF, PDGF) that stimulate fibroblast division and effects may be more pronounced in conjunction with other members of this family.

A highly significant result in these experiments was the confirmation that asthmatic fibroblasts proliferate at a greater rate than normals even in serum free medium alone, as has been observed before by Chaudhary (2001) and Xu (1997). One explanation is that asthmatic cells do not show the contact inhibition that prevents cells from replicating further when they are touching neighbouring cells. This finding is likely to underlie the greater numbers of fibroblasts seen in asthmatic airways. Asthmatic fibroblasts were also significantly more responsive to EGF than normal cells. A possible reason may be that most mitogenic effects become apparent when cells reach higher densities and as the asthmatic fibroblasts proliferated at a greater rate than normals, their cell density is always higher and so they may be more responsive to mitogenic stimuli (Richter et al. 2001).

5.7.6 Summary

Using RT-PCR, Western blotting, flow cytometry, immunocytochemistry and enzyme immunoassay, this study has greatly extended previous reports of fibroblast production of LTs, by showing that human lung fibroblasts constitutively express 5-LO pathway enzymes as well as generating both LTC_4- and LTB_4-like immunoreactivity. Neither the expression of LT pathway enzymes nor LT-like immunoreactivity was affected by a 24 hour incubation with EGF, TGFβ, IL-1β, TNFα, IFNγ, bradykinin, histamine, LTB_4 or LTD_4. However, when fibroblast differentiation to myofibroblasts was induced by TGFβ, there appeared to

be a significant switch from LTB_4 synthesis and responsiveness of to that of LTC_4. This finding could be relevant to the role of myofibroblasts in asthma as cys-LTs may affect myofibroblast contractility, proliferation and collagen deposition. Overall, neither dexamethasone nor MK-571 altered the expression of the 5-LO pathway enzymes, or the $CysLT_1$ and BLT receptors. Preliminary results suggest that LTD_4 may synergise with EGF to increase fibroblast proliferation.

While the 5-LO pathway in lung fibroblasts does not appear to be markedly different in normals and asthmatics, nor strikingly responsive to stimulation with acute inflammatory autacoids, the finding that differentiation into a contractile myofibroblast phenotype may be associated with increased LTC_4 synthase activity and $CysLT_1R$ expression suggests an increased readiness of these cells to contribute to cys-LT synthesis in asthma and to the hyperresponsiveness of the contractile elements of the airway to these mediators.

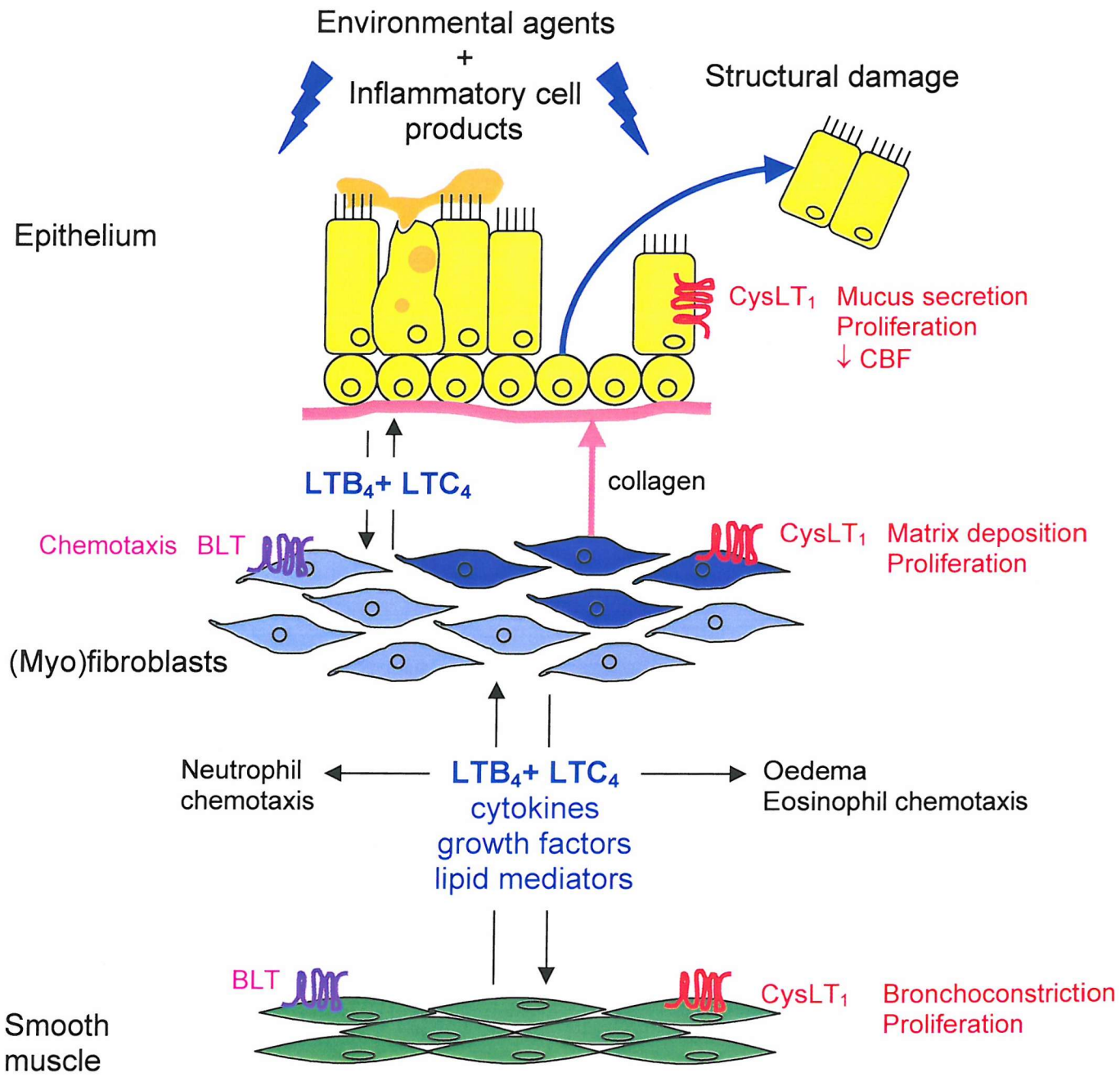
6. General Discussion

6.1 General Discussion

The presence of a complete synthetic pathway for the production of LTB_4 and LTC_4 in HBEC, HASM and fibroblasts, three structural cell types increasingly recognised as participating in the inflammatory process, may have important physiological consequences. **Figure 64** suggests a model by which LT production by BEC, HASM and fibroblasts fits into the vicious cycle of airway inflammation and remodelling. The nature of LT production by airway structural cells suggests a particular role in the long term symptoms of asthma rather than acute bronchoconstriction. BEC, HASM and fibroblasts expressed LT pathway enzymes and produced LTs spontaneously. However, in contrast to eosinophils and neutrophils, stimulation with Ca ionophore or inflammatory mediators did not cause marked increases in LT-like immunoreactivity by any cell type. If this is representative of their behaviour *in vivo*, there may be a role for low level baseline LT production by structural airway cells, with subtle changes in production caused by regulation of LT enzyme expression. Cys-LTs are chronically over-produced even in stable asthmatics compared to normals (Asano et al. 1995) and treatment with the LT antagonists significantly improves baseline lung function (Spector et al. 1995). In asthma, chronic overproduction of cys-LTs by structural cell types in the airway may contribute to chronic impairment of lung function, airway inflammation and airway remodelling.

Remodelling has been postulated to be a characteristic of asthma of great importance to the disease. Several structural changes are associated with chronic asthma, such as epithelial damage, goblet cell metaplasia, increases in airway smooth muscle mass, afferent nerves, microvessels and the deposition of matrix proteins under the epithelium wall (Holgate et al. 2000). These changes are thought to be linked to bronchial hyperresponsiveness, a physiological characteristic of asthma that is defined by enhanced responses of the airways to experimental stimuli such as methacholine. Reductions in bronchial hyperresponsiveness are observed with long term anti-leukotriene treatment (Fischer et al. 1995, Taki et al. 1994) and several lines of evidence point to a role for leukotrienes in airway remodelling. Cys-LTs increase the proliferation of smooth muscle cells and fibroblasts and stimulate bronchial epithelial cell proliferation at concentrations as low as 10fM (Leikauf et al. 1990, Baud et al. 1987, Cohen et al. 1995, Panettieri et al. 1998). Nanomolar concentrations of LTC_4 induce the expression and activity of collagenase both in primary human lung fibroblasts and in cell lines (Medina et al. 1994). Cys-LTs may also be involved in the vascular changes that take

Figure 64. Possible mechanisms of LT synthesis and effects in HBEC, HASM and fibroblasts



place in the asthmatic airway as they can stimulate mitogenesis in vascular smooth muscle (Porreca et al.1996, Porreca et al. 1995) and endothelial cells (Modat et al. 1987). Cys-LTs can also contribute to the mucus plugging of the airways by their stimulatory effects on goblet cell mucus secretion and inhibitory effects on ciliary beat frequency (Marom et al. 1982, Granbo et al. 1996). Although there are no human in vivo studies examining the effects of anti-leukotriene therapy on airway remodelling, studies in rats have shown that the increase in smooth muscle mass observed after allergen exposure is reduced after pranlukast and MK-571 therapy (Salmon et al. 1999, Wang et al. 1993). In addition to ASM thickness, MK571 also reduced bronchial responsiveness (Wang et al. 1993). These studies strongly suggest a role for cys-LTs in remodelling which would require the continuous production of elevated LT levels, a function that could be carried out by LTs generated chronically by structural cells such as HBEC, HASM and fibroblasts. Cys-LTs generated by these cells could act on neighbouring tissues and also in an autocrine manner at the $CysLT_1$ receptor, shown here to be expressed on HBEC, HASM and fibroblasts.

The effects of LTB_4 are mainly restricted to leukocytes, in particular neutrophils and monocytes (Hoover et al. 1984). LTB_4 production by HBEC, HASM and fibroblasts could contribute to the influx of leukocytes into the airway, as LTB_4 is chemotactic for eosinophils and neutrophils at concentrations as low as 10^{-8}M (Spada et al. 1994, Ford-Hutchinson et al. 1980). However, although there are reports of elevated LTB_4 levels in the BAL fluid of asthmatics compared to normals (Wenzel et al. 1995), and neutrophils are implicated in nocturnal and sudden onset asthma (Fahy et al. 1995, Martin et al. 1991), treatment with the BLT antagonist LY293111 was without clinical benefit during experimental allergen challenge (Evans et al. 1996). In this project the LTB_4 receptor, BLT was found to be present on fibroblasts and HASM cells but not bronchial epithelial cells. This is consistent with the lack of reports of LTB_4 mediated effects on bronchial epithelial cells, while LTB_4 is chemotactic for fibroblasts (Mensing et al. 1984). Such an effect could be of relevance within the airway. Fibroblasts have been shown to migrate to areas beneath damaged epithelium where they secrete collagen and other matrix proteins (Holgate et al. 2000). Many mediators are produced by BEC which affect fibroblast function such as IGF, TGFβ and FGF (Holgate et al. 2000). LTB_4 production by BEC could provide the signal required for fibroblast migration to the site of injury.

The functional relevance of a BLT receptor on HASM is not clear. In guinea pig lung parenchyma LTB_4 has contractile activity due to the secondary release of TxA_2 (Dahlén et al. 1983, Piper et al. 1983) but LTB_4 is inactive on human bronchi (Björck et al. 1993). HASM, HBE cells and fibroblasts contain a functional synthetic pathway for LTB_4 production and the presence of the BLT receptor on fibroblasts and HASM cells suggests that LTB_4 could have autocrine effects. In HASM cells pro-inflammatory stimuli may increase both the synthesis and effects of LTB_4 although the effect of LTB_4 on human airway smooth muscle is unknown. More LTB_4 and BLT mediated functions may become apparent in the future.

While we have shown that HBE, HASM cells and bronchial fibroblasts all have the capacity to produce LTs, it is difficult to determine which of these three cell types may have the greatest role in leukotriene synthesis in vivo. Comparing enzyme expression between the cell types (**figure 65**), the expression of 5-LO, FLAP and LTA_4 hydrolase was greater in HASM than in the epithelial cells and fibroblasts, and LTC_4 synthase expression appeared to be greatest in the asthmatic fibroblasts. However, spontaneous release of LTC_4- and LTB_4-like immunoreactivity was greatest not in HASM cells or fibroblasts, but in the primary bronchial epithelial cells. Intriguingly, primary BEC produced approximately 6 and 11 times more LTB_4 and cys-LTs than 16-HBE cells but had significantly lower levels of 5-LO expression. Experiments carried out in our group examining the expression of LT pathway enzymes in blood leukocytes are also shown. Although these studies were not carried out in parallel and therefore cannot be directly compared, it appears that 5-LO, FLAP and LTA_4 hydrolase immunofluorescence is greater in the structural cells than blood leukocytes. This further suggests that baseline enzyme expression does not necessarily correlate with LT release and raises the possibility that LT synthesis is regulated at another level than enzyme expression, such as substrate availability. The changes in enzyme expression demonstrated here, following stimulation with inflammatory mediators, may seem irrelevant as they did not result in large changes to LT production. However, it is possible that they may increase the capacity for LT production following activation of LT synthesis by another stimulus. This process of priming has been described in eosinophils, in which IL-5 increases the percentage of cells immunostaining for FLAP and also increases cys-LT production, but only when A23187 is used to initiate LT production (Cowburn et al. 1999).

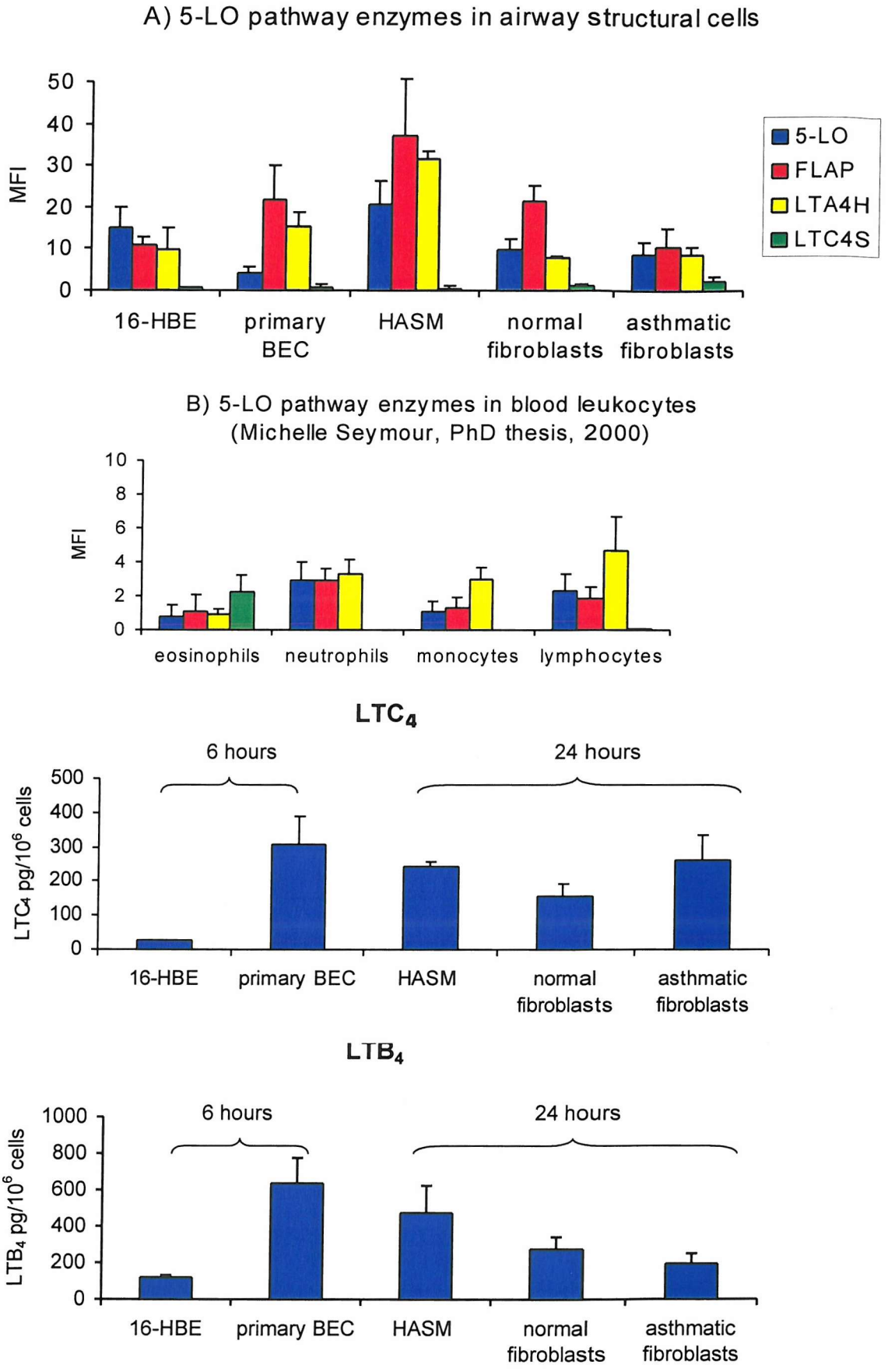


Figure 65. Comparison of LT pathway enzyme expression and LT production in HBEC, HASM and fibroblasts. Top panel shows baseline flow cytometric analysis of 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase immunofluorescence in 16-HBE, primary BEC, HASM and fibroblasts (mean+SEM). 5-LO pathway enzyme expression is also shown in blood leukocytes courtesy of Michelle Seymour (PhD thesis, 2000). Lower panels show spontaneous LTB_4 and Cys-LT production in structural cells (pg/10^6 cells). Note that 16-HBE and primary BEC supernatants were collected after **6** hours and HASM and fibroblast supernatants were collected after **24** hours. LT production was measured by enzyme immunoassay.

A range of inflammatory mediators was incubated with the HBEC, HASM and fibroblasts in order to examine the effects of stimulation on LT pathway enzyme expression. Different agents were responsible for regulation of the LT pathway enzymes in the three cell types and no single mediator caused an effect common to HBEC, HASM and fibroblasts. For example, bradykinin upregulated 5-LO pathway enzyme expression in 16-HBE cells but had no effect on HASM and fibroblasts, whereas TGFβ had more effects on enzyme expression in HASM and fibroblasts than epithelial cells. While there was regulation of enzyme expression in all three structural airway cell types, the variation in their responses to stimulation confirms that these are distinct cell types and suggests that epithelium, smooth muscle and fibroblast-derived LTs could have different biological roles. Taken together, the effects of dexamethasone in 5-LO pathway enzyme expression in HBEC, HASM and fibroblasts were relatively few. Dexamethasone had no significant effect on baseline LT pathway enzyme expression in HBEC, HASM or fibroblasts.

The overall lack of effect of dexamethasone on 5-LO pathway enzyme expression in HBEC, HASM and asthmatic fibroblasts was not unsurprising as there is much evidence to suggest that corticosteroids do not affect LT production both *in vivo* and *in vitro* (Verhoeven et al. 2001, Azevedo et al. 1995, Schleimer et al. 1989). In fact, as discussed previously, dexamethasone has been shown to increase 5-LO and FLAP expression in leukocytes (Colamorea et al. 1999, Goppelt-Struebe et al. 1997). Particularly interesting observations regarding the effect of dexamethasone came from the experiments on $CysLT_1$ expression in HASM, which dexamethasone was found to increase. Considering the stimulatory effects of dexamethasone on 5-LO and FLAP expression, such a result is not inconceivable and could explain clinical observations, such as the inability of inhaled corticosteroids to prevent airway remodelling. The findings of the Childhood Asthma Management Program Research Group (CAMP), show that the beneficial effect of an inhaled corticosteroid on the post-bronchodilator improvement in airway function observed during the first year of treatment in 5-11 year old children, was lost over the following three years (CAMP 2000). This effect is most likely explained by the continuous remodelling of the airways, even in the presence of the corticosteroid.

In general MK-571 was also without effect on the baseline expression of 5-LO pathway enzyme expression in HBEC, HASM and fibroblasts although it was able to prevent stimulus-induced increases in 5-LO and FLAP expression in HBEC and HASM cells

respectively. Such effects could contribute to the clinical efficacy of leukotriene antagonists by reducing LT production within an inflammatory environment. Although MK-571 did not affect baseline $CysLT_1$ expression in either HASM or fibroblasts, it significantly inhibited the TGFβ-induced upregulation of $CysLT_1$ expression in HASM. Such an effect could be of particular importance in asthma therapy, especially due to the finding that dexamethasone upregulated $CysLT_1R$. If LTRAs are able to prevent overexpression of their own receptor, they may be able to reduce Cys-LT induced bronchoconstriction and remodelling responses, both by blocking $CysLT_1$, and also by regulating its expression. LTRAs have been shown to prevent allergen-induced thickening of ASM in animal models (Wang et al. 1993, Salmon et al. 1999), although their effect in humans remains to be seen.

As described above, we found that the fibroblasts and HASM cells used in this project did behave differently in terms of responses to pro- and anti-inflammatory stimuli. However, as cultured smooth muscle cells and fibroblasts share several characteristics and in culture appear similar, it was our aim to examine whether the HASM and fibroblasts used in this project were sufficiently different to allow classification as two different cell types. The expression of several contractile proteins was measured by flow cytometry in smooth muscle and fibroblast cells (**figure 11, p76**). The literature states that although fibroblasts in culture will stain weakly positive for the contractile protein actin, it is generally accepted that only smooth muscle cells will stain for both actin and myosin using smooth muscle specific antibodies (Hirst 1996). Desmin and vimentin are both intermediate filament proteins. Vimentin is expressed in all mesenchymal cell types and to a greater extent in synthetic proliferating tracheal smooth muscle cells than in contractile non-proliferating cells (Tom-Moy et al. 1987). Desmin is thought to be expressed in smooth muscle cells but not fibroblasts (Chaudhary et al. 2001). In our experiments, HASM cells were found to contain significantly greater levels of actin, myosin, desmin and vimentin. While these results confirm that our cultured HASM are different from the fibroblasts, they also emphasise the difficulty in separating these two types in culture. We found low levels of myosin and desmin immunofluorescence in fibroblasts, proteins not thought to be present in these cells. Furthermore, when fibroblasts were converted to myofibroblasts by stimulation with TGFβ, actin levels were increased to levels greater than in the HASM cells at baseline. It has been shown that over successive passages, smooth muscle cells in culture become increasingly non-contractile (Campbell et al. 1993). The finding that no protein marker was exclusive to either fibroblasts or smooth muscle suggests that in culture, the phenotype of

these cells represents a balance of features common to both cell types and care should be taken when interpreting studies using cultured mesenchymal cells such as (myo)fibroblasts and smooth muscle.

To summarise, this project has demonstrated, using a range of different techniques that examined mRNA, protein, and lipid levels, that human bronchial epithelial cells, smooth muscle cells and fibroblasts all have the enzymatic machinery required for the generation of LTB_4 and LTC_4, and that this may be regulated by pro-inflammatory stimuli. These cell types also express $CysLT_1R$, levels of which could also be increased in an inflammatory environment to increase cys-LT mediated effects such as bronchoconstriction and remodelling. The BLT receptor was also found to be expressed on fibroblasts and smooth muscle cells. Previously, these three cell types have all been shown to produce a range of cytokines, growth factors and lipid mediators, although there are no other detailed reports of leukotriene production in HBEC, HASM and fibroblasts. This work reveals new leukotriene sites for the synthetic pathway that may contribute to the vicious circle of inflammation and remodelling that exists in the asthmatic airway.

6.2 Future work

This project has confirmed the presence of complete and functioning pathways for the synthesis of leukotrienes in airway epithelial cells, smooth muscle cells and fibroblasts, yet while this work has answered many questions it has raised many more. There are several ways in which the validity of the work performed here can be strengthened and also further developed to increase our understanding of the synthesis and effects of leukotrienes in structural cell types of the human airway.

As LTC_4 synthase expression was poorly detectable by flow cytometry in HBEC, HASM cells and fibroblasts, as well as in eosinophils (Michelle Seymour, PhD Southampton University, 2000), further experiments may be required to confirm the expression and activity of this enzyme. For example, measurement of LTC_4 production could be made by HPLC or mass spectroscopy. These techniques do not rely on antibodies and would therefore eliminate the possibility of crossreactivity in the enzyme immunoassay. Another way to confirm LT production would be to block LT synthesis, for example, at the level of 5-LO by MK-886. Also, it has been shown that LTC_4 production can occur in some cell types by microsomal glutathione transferases (Sjöström et al. 2001) and the presence and

relative contribution of these enzymes to LTC_4 production in HBEC, HASM and fibroblasts may need to be examined. However, it is unlikely that further experiments would contradict our initial findings as despite the minimal LTC_4 synthase immunofluorescence measured by FACS analysis, the other techniques used to detect this enzyme all confirmed its presence. RT-PCR showed mRNA for LTC_4 synthase in all three cell types and DNA sequencing confirmed that the PCR product corresponded to that expected for LTC_4 synthase. Western blotting using a highly sensitive chemiluminescent substrate allowed detection of immunopositive bands for LTC_4 synthase at the correct molecular weight of 18 kDa. Cys-LT-like immunoreactivity was also measured by enzyme immunoassay in epithelial, smooth muscle and fibroblast supernatants. The relatively low levels of LTC_4 synthase immunofluorescence in all the structural cell types and blood leukocytes examined to date may accurately reflect relatively low levels of enzyme expression required for cys-LT synthesis or a low sensitivity of the LTC_4 synthase antibody.

The sub-cellular localisation of the LT biosynthetic enzymes has been studied in leukocytes and location was shown to have effects on enzyme activity. For example, to become active, 5-LO undergoes a Ca^{2+} dependent redistribution from a soluble compartment (cytoplasm or nucleoplasm) to the nuclear membrane (Peters-Golden et al. 2000). In some cells 5-LO shows a migration from the cytoplasm to the nucleoplasm that is associated with cell maturation and an increased capacity for LT generation (Peters-Golden et al. 2000). As we were able to detect spontaneous leukotriene production by HBEC, HASM and fibroblasts it would be of interest to examine, using confocal microscopy, the intracellular location of the LT pathway enzymes to find out if they show the same subcellular arrangement as required for LT production in leukocytes. It would also be of interest to show whether there is redistribution of the 5-LO pathway enzymes upon cell stimulation. The cellular location of the LT receptors could also be examined. If $CysLT_1$ and BLT receptors are present within the cell as well as on the surface a new range of functions for the LTs may be suggested.

This study has shown that 5-LO pathway enzymes are constitutively expressed in bronchial epithelial cells and that these enzymes are upregulated by agents including Ca ionophore, bradykinin and LPS. There are several types of inhaled stimuli, known to precipitate asthma exacerbations and as BEC are the first cells to come into contract with such agents, it would be of interest to examine their effects on leukotriene production. Allergens including DerP1 increase cytokine production by HBE cells, not by an allergic process but by the intrinsic

protease activity of these molecules (Thompson et al. 1999). Cigarette smoke, ozone, asbestos and NO_2 have all been shown to stimulate cytokine production by bronchial epithelial cells. Also, damage to the epithelial layer is a characteristic of asthma and may be caused by the release of cytotoxic mediators from infiltrating inflammatory cells, as well as by direct allergen induced proteolysis. Cultured HBE cells could be stimulated with eosinophil and mast cell derived products such as ECP, neutrophil elastase and tryptase to induce deformation and detachment. Another way of damaging monolayers of epithelial cells is by mechanical injury, such as scraping. Due to the stimulatory effect of LTC_4 and LTD_4 on epithelial cell proliferation (Leikauf et al. 1990), it is conceivable that cys-LTs may have a role in epithelial repair following damage. To follow up experiments that have shown regulation of LT enzymes, and to further our understanding of the mechanism whereby regulation occurred, simple experiments could be carried out using inhibitors of transcription such as actinomycin D, G-protein blockers such as pertussis toxin and the relevant antagonists of the stimulus itself.

As described throughout this project, LTs have been shown to have a role in the regulation of structural cell mitogenesis. Cys-LTs can increase the proliferation of bronchial epithelial cells, EGF or IGF-stimulated smooth muscle cells and fibroblasts (Leikauf et al. 1990, Cohen et al. 1996, Panettieri et al. 1998, Baud et al. 1987). We also carried out preliminary experiments to examine the effects of leukotrienes on structural cell proliferation. Our results suggested that LTD_4 may have mitogenic effects on epithelial cells and fibroblasts, but much more work is required on this topic, for example using a wider range of LT and growth factor concentrations and several different types of $CysLT_1$ antagonist. Also, as DNA synthesis can take place without changes in cell number, a more sensitive proliferation assay technique may be required. In this project cell numbers were measured by the uptake of methylene blue dye, although it may be better to use a technique such as the detection of tritiated thymidine which is incorporated into dividing cells. Future work could also take these studies a step further by examining the effect of other anti-asthma drugs on LT induced proliferation.

The mechanism whereby LTD_4 synergises with growth factors to enhance airway smooth muscle proliferation is most likely due to the liberation of growth factors from inhibitory growth factor binding proteins (IGFBP). LTD_4 has been found to induce the expression of matrix metalloproteinase-1 (MMP-1) in smooth muscle cells and MMP-1 is known to cause

proteolysis of IGFBPs (Rahah et al. 1996). MMPs can also release soluble forms of growth factors from their membrane bound precursors (Streuli 1999). The finding that $CysLT_1$ activation can activate MMP-1 raises the possibility that cys-LTs may be involved in a wide range of growth factor mediated processes that may be of importance in asthma, and in particular, remodelling responses. For example, as shown in this project, TGFβ induces the transformation of fibroblasts into myofibroblasts. Myofibroblasts are increased beneath the epithelium of subjects with asthma and are involved in the deposition of repair-like matrix proteins (Holgate et al. 2000). MMPs are directly involved in extracellular matrix (ECM) remodelling by their ability to cleave all proteins constituting the ECM. Interestingly, MMP-9 staining has been shown to be increased in the submucosa of subjects with asthma, and to correlate with the number of infiltrating eosinophils, effects which may both be related to the overproduction of cys-LTs (Hoshino et al. 1998). The range of growth factor and MMP mediated effects is vast, and the possibility that cys-LTs could affect these processes requires much further work. However, the results could provide exciting findings and new therapeutic targets.

The recent characterisation of a second cys-LT receptor, $CysLT_2$ and the development of a $CysLT_2$ antibody (Cayman Chemicals) will enable further studies to be carried out to examine the expression and regulation of this receptor on structural cell types. Although the lack of a specific receptor antagonist has hindered the discovery of $CysLT_2$ mediated responses, several lines of evidence point to the involvement of a receptor other than $CysLT_1$ in cys-LT mediated effects. Cysteinyl-LTs are highly effective mitogens for airway epithelial cells, but LTC_4 is markedly more potent in inducing their proliferation than either LTD_4 and LTE_4 (Leikauf et al. 1990), suggesting that this response is mediated by $CysLT_2$ receptors. Similarly, LTD_4 synergises with epidermal growth factor (EGF) to induce proliferation of airway smooth muscle cells; this response is inhibited by pranlukast and pobilukast, but not by zafirlukast, perhaps indicating differential affinities of these drugs for a third, unidentified CysLT receptor (Panettieri et al. 1998). Wang and colleagues showed that airway smooth muscle mass is increased in response to ovalbumin challenge in sensitised Brown-Norway rats. This increase in smooth muscle mass was reduced by about half following pre-treatment with the experimental $CysLT_1$ antagonist MK-571 indicating the prominent involvement of $CysLT_1$ receptors, but not excluding a contribution of $CysLT_2$ receptors or of other mediators (Wang et al. 1993). In human umbilical vein endothelial cells (HUVECs), LTC_4 and LTD_4 cause a rapid upregulation of P-selectin, an early adhesion

molecule involved in leukocyte rolling prior to diapedesis. The P-selectin response in HUVECs is clearly not mediated by $CysLT_1$ receptors as it is unaffected by pre-treatment with pranlukast or zafirlukast, or by pobilukast (SKF 104,353) at concentrations that effectively blocked cysteinyl-LT-induced contractions of human bronchial smooth muscle (Pedersen et al. 1997). These data suggest that specific blockade of $CysLT_2$ receptors may provide anti-inflammatory effects additional to those seen with $CysLT_1$ antagonists, and even prevent remodelling of bronchial smooth muscle, epithelium, and other airway tissues in chronic asthma.

There are many as yet unanswered questions regarding the functions of the leukotrienes and it is likely that more functions will become apparent with time. For example, the functional relevance of LT synthesis occurring at the nuclear membrane is still unknown. One relatively recent discovery is the involvement of leukotrienes in the mechanisms that control apoptosis. In fibroblasts, leukotrienes are involved in apoptosis induced by thiol depletion. Apoptosis was found to be induced initially by oxidant accumulation, which activated leukotriene production which in turn activated MAPK (mitogen activated protein kinase) signalling pathways (Aoshiba et al. 1999). The translocation of 5-LO to the nuclear membrane of rat fibroblasts is also a characteristic of this MAPK/leukotriene cell death pathway (Eom et al. 2001). Inhibition of the lipoxygenase pathway protects against radiation induced thymocyte apoptosis (Korystov et al. 1996) and TNF mediated apoptosis of fibrosarcoma cells (O'Donnell et al. 1995). These effects are receptor mediated as they are blocked by both LT synthesis inhibitors and leukotriene receptor antagonists (FPL 55712 and ONO1078) (Aoshiba et al. 1999). Interestingly, leukotrienes also have a role in the inhibition of apoptosis as the LTB_4 antagonist SB201146 can abolish LPS-, GM-CSF- and dexamethasone-induced neutrophil survival (Lee et al. 1999). Control of cell apoptosis could be important to structural changes within the airway, for example, the increased numbers of fibroblasts and myocytes within the airway could be due not only to increased proliferation but also reduced apoptosis.

All of the experiments in this study were carried out in vitro and to complete work on the leukotriene pathway in structural cells of the human airway, the expression of 5-LO pathway enzymes and receptors should ideally be examined in bronchial biopsies, either by immunohistochemistry or in situ hybridisation. Such experiments could provide a wealth of of information such as the effects of drug treatments, differences in expression between

different subject groups and the in situ localisation of protein expression. For example, immunohistochemistry has shown that the EGF receptor is highly expressed in areas of damaged epithelium, highlighting its role in the mechanism of epithelial repair (Holgate et al. 2000). In vivo expression of the LT pathway enzymes in airway epithelium has not been rigorously examined in GMA embedded bronchial biopsies. This may be complicated by a number of factors such as epithelial mucus production that attracts antibody due to its negative charge, the "edge effect" seen with staining and also loss of epithelium due to shedding in asthma or artefactual damage due to biopsy collection. However, if these problems were overcome, staining for the 5-LO pathway enzymes would provide valuable confirmation of the in vitro results of this thesis.

Finally, to determine whether LTs have remodelling effects *in vivo*, clinical trials need to be performed to examine the impact of anti-LT therapy on airway remodelling as detected in bronchial biopsies. The long time-scale required for such studies, and the ethical implications of a control group untreated with anti-inflammatory therapy, may make such studies difficult to perform.

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