Glucocorticosteroid receptor characteristics of peripheral blood mononuclear cells in oral steroid dependent asthma; utilization of an in vitro model of steroid resistant asthma to investigate mechanisms of resistance and functional consequences of altered receptor affinity.

By Elvis Malcolm Irusen

Dissertion in partial fulfillment for the degree of Doctor of Philosophy in the Department of Medicine at the Nelson R Mandela School of Medicine, University of Kwa Zulu Natal.

June 2007

I hereby declare that all experimental work and this dissertation is my own original work and has not previously in its entirety or in part been submitted to any other university for a degree.

Signature..... Date.....30-6-2007

Dedication & Acknowledgements

All the scientists in the department of Thoracic Medicine-Imperial College of Science, Technology and Medicine, especially Dr Ian Adcock, for expert assistance with the laboratory techniques and intellectual input.

Professors UG Lalloo and KF Chung for leadership, support and guidance.

Patients from the Royal Brompton Hospital and support by the South African Pulmonology Society through a Scholarship from Boehringer Ingelheim (S.A.).

Abstract

Background: Although glucocorticoids are the most effective treatment for asthma, some patients show a poor response. In such patients with steroid resistant asthma, this has been ascribed to altered glucocorticoid receptor (GR) ligand-binding affinity induced by IL-2 combined with IL-4 or IL-13 alone- all of which can also modulate glucocorticoid function in vitro.

Objective: We sought to assess the ligand-binding affinity in a distinct group of oral steroid-dependent asthmatic subjects and examine the mechanisms by which IL-2 and IL-4 (or IL-13) modify the ligand-binding affinity of the GR.

Methods: Using dexamethasone-binding assays, we examined PBMCs ex vivo from healthy subjects, subjects with controlled asthma, and oral steroid-dependent subjects with severe asthma. In addition, IL-2 and IL-4 were used to alter GR affinity in vitro. We used mediators or inhibitors of signal transduction to investigate the mechanisms of resistance. We also determined cytokine production of PBMC's by means of ELISA.

Results: GR ligand-binding affinity was significantly reduced in the nucleus but not in the cytoplasm of oral steroid-dependent asthmatic subjects compared with that seen in steroid-sensitive and healthy subjects (dissociation constant, 41.37 ± 17.83 vs. 25.36 ± 2.63 nmol/L vs. 9.40 ± 4.01 nmol/L, respectively [$p<.05$ for both in comparison to normals]).

This difference in ligand-binding affinity could be mimicked by IL-2 and IL-4 co-treatment and was blocked by the p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580. PBMC's rendered resistant in vitro demonstrated lower IL-10 and increased GM-CSF production following LPS or PMA & PHA stimulation compared to cells with normal GR affinity. Resistant cells also showed reduced dexamethasone repression of LPS-stimulated IL-10 release. These effects were also reversed by SB203580. Inhibition of the ERK MAPK pathway by PD098059 (10 μmol/L), phosphoinositol 3 kinase by wortmannin (5 nmol/L) or treatment with IL-10 (10 ng/mL) failed to modulate the effect of IL-2 and IL-4 on receptor affinity. Ro318220 (10 nmol/L), a specific protein kinase C inhibitor and theophylline, similarly, had no effect on affinity.

Conclusion: GR ligand binding affinity is tiered; compared to normal subjects; steroid responsive asthmatics have a mild reduction in ligand binding whereas oral steroid dependent asthmatics have greater reductions. When mononuclear cells are rendered resistant in vitro, cytokine production (low IL-10 and high GM-CSF) favours a pro-inflammatory state. Our data do not support the ERK MAPK, phosphoinositol 3 kinase, protein kinase C pathways in steroid resistance. Treatment with IL-10 and theophylline also failed to modulate the effect of IL-2 and IL-4 on receptor affinity. However, P38 MAPK inhibitors may have potential in reversing glucocorticoid insensitivity and re-establishing the beneficial effects of glucocorticoids in patients with severe asthma.

Table of Contents

CHAPTER 1: Literature Review 17

1.1. ASTHMA-THE DISEASE

A. The Immunologic Basis of Allergic Inflammation

IgE and Allergen Sensitisation

Cell-Mediated Responses

- Mast cells
- T Lymphocytes
- Eosinophils
- Macrophages
- Neutrophils

B. Mediators: Cytokines, Chemokines and their relevance to asthma 21

The Cytokine Network

- The Interleukins
- Tumour Necrosis Factor alpha
- Granulocyte Macrophage - Colony Stimulating Factor
- Interferon γ

Chemokines

Growth Factors

C. Confluence of Genes, the Environment and Adaptive immunity in the Pathogenesis of Asthma 31

The Th1/Th2 Paradigm

Genetics

The Hygiene Hypothesis

D. Asthma Pathogenesis-the future 33

1.2. THE GLUCOCORTICOID RECEPTOR IN THE PATHOGENESIS OF STEROID RESISTANT ASTHMA 34

Steroid Resistant Asthma- the beginning

Steroid Resistant asthma- the story continues

Peripheral Blood Mononuclear Cell studies

Structure and Activity of the Glucocorticoid Receptor

The Glucocorticosteroid splice variant: GR-β

Influence of signal transduction on GR

Glucocorticosteroid receptor numbers and affinity

1.3. SELECTIVE MEDIATORS AND CYTOKINES IN SIGNAL TRANSDUCTION 49

Phosphoinositide Hydrolysis

a) Phosphoinositide 3- kinase (PI-3k)

b) Protein Kinase C

The MAPKinases in Intracellular Signal Transduction

Experiments with PD 098059 of relevance to asthma

SB203580 – the p38 MAPK inhibitor

Role of p38 MAPK in proinflammatory cytokine expression

Theophylline

Interleukin-10

Granulocyte Monocyte –Colony Stimulating Factor

CHAPTER 2: Aims 65

CHAPTER 3: Glucocorticosteroid receptor characteristics in peripheral blood mononuclear cells in oral-steroid dependent asthma. 67

Rationale for using peripheral blood mononuclear cells as a reflection of the airway milieu

Methods
Patients and Subjects
Peripheral Blood Mononuclear Cell Isolation
Preparation of Tritiated Dexamethasone
Principle of the dexamethasone binding assay
Trial of high dose prednisone
Tritiated dexamethasone binding assay
Data analysis
Results
GR ligand binding of PBMCs
Glucocorticoid Receptor Numbers
GR Affinity
Response to Oral Prednisolone

CHAPTER 4: Characterisation of an in-vitro "Steroid Resistance Model" in Mononuclear Cells 89

4.1: Effect of incubating PBMC's with diminished GR affinity in medium alone for 48 hours. 89
Methods
Results
4.2: Establishing an in-vitro "Steroid Resistance Model" with mononuclear cells. 92
Methods
Experiments with Cells Incubated with Cytokines
Results
IL-2 and IL-4 or IL-13 alone Alter GR Ligand Binding In-vitro
4.3: Functional Consequences of Altered GR Affinity 97
Methods
GM-CSF and IL-10 Release: Experimental procedures
Enzyme-linked Immunosorbent Assay for IL-10 and GM-CSF
Results

Effect of IL-2/IL-4 on GM-CSF and IL-10 Release
Effect of Dexamethasone on IL-10 Release in cells co-incubated with IL-2,-4 or medium alone

CHAPTER 5: Signal-transduction Mechanisms and Functional Sequelae of Altered Affinity of the Glucocorticosteroid Receptor in-vitro. 104

5.1: Experiments with Cells Incubated with Cytokines and Inhibitors of Signal Transduction. 104
Methods
Results
p38 MAPK Regulates IL-2/IL-4 Alteration of GR Ligand-binding Affinity
Altered Nuclear GR affinity-effect of signal transduction inhibitors

5.2: Functional sequelae of restoration of GR affinity 113
Methods
Results

5.3: Demonstration of decreased activated p38 MAPK protein 115
Western Blot of activated p38 Mitogen Activated Protein Kinase (MAPK).

CHAPTER 6: Discussion 117
The Concept of Steroid Resistant asthma
Reasons for Sub-optimal Response to Corticosteroids
a) Glucocorticosteroid Receptor Number
b) Alteration in GR Affinity
Establishing the "Steroid Resistance Model"
Functional Sequelae of Steroid Resistance In-vitro
The Role of GM-CSF in Asthma
The Role of IL-10 in Asthma

Signal Transduction Pathways that are not Involved in Decreased GR Affinity

Combined IL-2, -4 or IL-13 Induced Diminished Glucocorticoid Receptor Affinity Normalises with the p-38 MAPK Inhibitor, SB203580, In-vitro.

Conclusion

Epilogue 139

References 140

List of Figures

Figure 1: Genomic and Complementary DNA and Protein Structure of the Human Glucocorticoid Receptor.

Figure 2: Traditional View of Corticosteroid Action.

Figure 3: Functional Domains and Sub-domains of the GR-α.

Figure 4: IP-3 Kinase and Diacylgycerol.

Figure 5: The Mitogen Activated Protein Kinase Cascades.

Figure 6: Graphical representation of the principle of radioligand binding studies showing saturable and non-saturable binding isotherms.

Figure 7a: Total Glucocorticosteroid receptor numbers in the individual patients in the different categories.

Figure 7b: Individual GR affinities in the three subject groups

Figure 7c: Mean Ligand Binding affinities in the Cytosolic and Nuclear Compartments of the Three Subjects groups.

Figure 8: The effect of prednisolone (40 mg/d for 7 days) on percent predicted FEV_1, GR affinity and receptor number in 9 oral-steroid-dependent and 7 SS subjects.

Figure 9: Normalisation of GR affinity (mean) in the nuclear compartment after 48 hours incubation of PBMC's in medium alone.

Figure 10: Establishment of the Steroid Resistance Model: Nuclear GR Affinity is Reduced (High Kd) in PBMC's Exposed to IL-2, -4 compared to Medium alone.

Figure 11: The Steroid Resistance Model: Increase in GR number in cells incubated with IL-2, -4 compared to Medium alone.

Figure 12: Example of preservation of high Kd in steroid dependant subject whose PBMC's were incubated with IL-2, -4.

Figure 13: Effect of PBMC's incubation in medium with or without IL-13.

Figure 14: Comparison of IL-10 production following LPS stimulation of PBMC's incubated with medium alone or with IL-2,-4.

Figure 15: Effect of Dexamethasone on LPS- induced IL-10 release in PBMC's incubated in medium alone or IL-2,-4.

Figure 16: Mean GM-CSF production after PMA, PHA stimulation after GR affinity had normalised or decreased with co-incubation with IL-2,-4.

Figure 17: Nuclear GR Affinity in Medium With and Without Cytokines and Inhibitors of Signal Transduction.

Figure 18a: Nuclear GR Affinity in PBMC's exposed to Media, IL-2, -4 with and without SB203580.

Figure 18b: Effect on glucocorticoid nuclear receptor number per cell in PBMC's incubated in medium, IL-2, -4 with and without SB203580

Figure 19: Effect of PBMC's incubation in medium and IL-13 with or without 1umol SB203580.

Figure 20: Absolute IL-10 production expressed as a percentage of medium with or without IL-2,-4 and SB203580.

Figure 21: Western Blot for activated p38 Mitogen Activated Protein Kinase.

Figure 22: Magnitude of the FEV_1 Response to Inhaled Corticosteroids

Figure 23: Contemporary View of Corticosteroid Action.

List of Tables

Table 1: Factors that might change the sensitivity of tissues to glucocorticoids.

Table 2: Comparative Characteristics of Asthma and Healthy subjects

Table 3: Glucocorticoid Receptor Number per cell and Affinity in the Oral Steroid Dependant Group

Table 4: Glucocorticoid Receptor Number per cell and Affinity in the Steroid Sensitive Asthmatic Group

Table 5: Glucocorticoid Receptor Number per cell and Affinity in Normal Subjects

Table 6a: Glucorticoid receptor affinity and numbers in the nuclear and cytoplasmic compartments before and after the prednisone course: Oral Steroid Dependent Group

Table 6b: Glucorticoid receptor affinity and numbers in the nuclear and cytoplasmic compartments before and after the prednisone burst: Steroid Sensitive Group

Table 7: Lung function, depicted as predicted % FEV_1 before and after the prednisone course in the Oral Steroid Dependent and Steroid Sensitive Groups

Table 8: Glucocorticoid Receptor Number and Affinity in the Nuclear compartment in PBMC's in the asthmatic subjects at baseline and after 48 hours incubation in medium alone

Table 9: Glucocorticoid Receptor Number and Affinity in the cytoplasmic compartment in PBMC's in the asthmatic subjects at baseline and after 48 hours incubation in medium alone

Table 10: Glucocorticoid Receptor Number and Affinity in the Nuclear compartment in PBMC's in the asthmatic subjects and normal subjects (N) after 48 hours incubation in medium alone and with IL-2 and IL-4.

Table 11: Example of High Kd in OSD subject (JW) at baseline and normalisation in medium, IL-2 and IL-4 alone but preservation with this cytokine combination.

Table 12: Effect on nuclear GR Kd and number in PBMC's incubated in medium with and without interleukin 13 for 48 hours.

Table 13: Absolute Interleukin -10 production (IU/ml) by mononuclear cells at 60 hours incubation in medium with and without IL-2,-4 following LPS stimulation.

Table 14: Absolute Interleukin -10 production (IU/ml) by mononuclear cells at 60 hours incubation in medium with and without IL-2,-4 following LPS stimulation and the effect of varying concentrations of dexamethasone.

Table 15: Absolute levels of GM-CSF (pg/ml) following stimulation with PMA&PHA after PBMC incubation in medium alone or with IL-2,-4.

Table 16a: Results of GR binding experiments of PBMC's incubated in medium with or without IL-2,-4 and inhibitors of signal transduction: Wortmannin, IL-10 and Theophylline

Table 16b: Results of GR binding experiments of PBMC's incubated in medium with or without IL-2,-4 and inhibitors of signal transduction: PD98039 and Ro 318220.

Table 17: Results of GR binding experiments of PBMC's incubated in medium with or without IL-2,-4 and SB203580.

Table 18: Effect on nuclear GR Kd and number in PBMC's incubated in medium with or without IL-13 and SB203580 for 48 hours.

Table 19: Absolute Interleukin -10 production (IU/ml) by mononuclear cells at 60 hours incubation in medium with and without IL-2,-4 and SB203580 following LPS stimulation.

Abbreviations used (in alphabetical order):

A:	Adenosine
ADAM 33:	A Distintegrin and Metalloprotease 33
AP-1:	Activator Protein-1
ASM:	Airway Smooth Muscle
ASO:	Antisense Oligonucleotide
ATF-2:	Activating Transcription Factor-2
BAL:	Broncho-alveolar Lavage
BALF:	Broncho-alveolar Lavage Fluid
BHR:	Bronchial Hyper-responsiveness
C:	Complement
cAMP:	cyclic Adenosine Monophosphate
CBP:	CREB Binding Protein
CD:	Cluster of Differentiation
cGMP:	cyclic Guanosine Monophosphate
CREB:	cyclic AMP-responsive Element-binding Protein
CS:	Corticosteroid
DAG:	Diacylglycerol
DBD:	DNA Binding Domain
DNA:	Deoxyribonucleic Acid
DPP 10:	Dipeptidyl Preptidase 10
DRIP:	Vitamin D Receptor Interacting Protein
ECP:	Eosinophil Cationic Protein
EGF:	Epidermal Growth Factor
ELISA:	Enzyme Linked Immunosorbent Assay
ERK:	Extracellular Regulated Kinase
FEV_1:	Forced Expiratory Volume in 1 second
FGF:	Fibroblast Growth Factor
GC:	Glucocorticosteroid
GM-CSF:	Granulocyte Monocyte-Colony Stimulating Factor
GR:	Glucocorticoid Receptor
GR α and β:	Glucocorticoid Receptor α and β
GRE:	Glucocorticoid Response Elements
HAT:	Histone Acetyl-transferase
HLA:	Human Leukocyte Antigen
HDAC:	Histone Deacetylases
HR:	Hinge Region
ICAM:	Intercellular Adhesion Molecule
ICS:	Inhaled Corticosteroid
IFN-γ:	Interferon-γ
IL :	Interleukin
IL-R:	Interleukin Receptor
iNOS:	inducible Nitric Oxide Synthetase
IGF:	Insulin-like Growth Factor
JAK:	Janus Protein Kinase

JNK:	Jun N-terminal Kinase
Kd:	Dissociation Constant
LABA:	Long Acting β_2 Agonist
LBD:	Ligand Binding Domain
LPS:	Lipopolysaccharide
LT:	Leukotriene
MAPK:	Mitogen Activated Protein Kinase
MKK:	Mitogen Activated Protein Kinase Kinase
MBP:	Major Basic Protein
MCP-1:	Monocyte Chemoattactant Protein-1
MHC:	Major Histocompatibility Complex
MIP-1α:	Macrophage Inflammatory Protein-1α.
MMP:	Matrix Metallo-protease
mRNA:	messenger Ribonucleic Acid
NF-κB:	Nuclear Factor κB
NF-AT:	Nuclear Factor of Activated T cells.
OSD:	Oral steroid Dependant
PBMC's:	Peripheral Blood Mononuclear Cells
PDE:	Phosphodiesterase
PDGF:	Platelet-derived Growth Factor
PEF:	Peak Expiratory Flow
PGE:	Prostaglandin E
PHA:	Phytohemagglutinin
PI-3Ks:	Phosphoinositide 3-kinases
PKC:	Protein Kinase C
PLC:	Phospholipase C
PM:	Particulate Matter
PMA:	Phorbol 12-myristate 13-acetate
Rac:	ras-related C3 Botulinum Toxin Substrate
RANTES:	Regulated on Activation, Normal T Expressed and Secreted
RNA:	Ribonucleic Acid
SAPK:	Stress Activated Protein Kinase
SRA:	Steroid Resistant Asthma
SS:	Steroid Sensitive
STATs:	Signal Tranducers and Activators of Transcription
TGF-β:	Transforming Growth Factor-β
TNF:	Tumor Necrosis Factor
TRAF:	TNF Receptor Associated Factor
TRAP:	Thyroid Hormone Receptor Associated Protein
UDCA:	Ursodeoxycholic Acid
VCAM:	Vascular Cell Adhesion Molecule

CHAPTER 1: LITERATURE REVIEW

1.1 ASTHMA-THE DISEASE

Asthma is a chronic inflammatory disease of the airways characterised by bronchial hyper-responsiveness and reversible airways obstruction. Over the last 25 years, the prevalence has doubled for reasons that are not well understood. An analysis of this epidemiologic observation suggests that this represents a real increase and is not just due to heightened awareness or better diagnostic capabilities [1]. In this same period, the theories of asthma aetiology have moved through different paradigms. Initially an intrinsic airway smooth muscle abnormality was felt to be the primary problem; however, studies with cultured airway myocytes disproved this theory [1]. This was followed by the hypothesis that asthma was an autonomic dysfunction syndrome with excess activity in the cholinergic and tachykinin pathways- this was neither proven nor disproven[1]. Immunoglobulin E mediated responses then rose to prominence and when the abundant airway inflammatory cell infiltrate was detected, the inflammatory nature of the disease was accepted.

It has generally been presumed that asthmatics have episodic symptoms and attacks and that lung function is normal in between. This is not true; the inflammatory nature leads to ongoing structural damage with decreased reversibility and can lead to loss of lung capacity over time [2, 3].
The rapid growth of molecular medicine has led to major developments in the understanding of mechanisms of the allergic diathesis that underpin the disease[4]. Asthma is now considered a disease of gene – environment interaction with an intricate immunobiology[5].

An understanding of the various cells and mediators that orchestrate the process is essential to appreciate this complexity.

A. The Immunologic Basis of Allergic Inflammation.

IgE and Allergen Sensitisation

A large body of early research in asthma supported the notion that genetically susceptible individuals developed IgE responses that paralleled the clinical manifestations of asthma. A linear correlation was reported between the prevalence of asthma, bronchial hyper-responsiveness and IgE levels [6, 7].

Allergen sensitisation to specific inhalants in asthma can be demonstrated by skin tests, serum allergen specific IgE antibodies and bronchial challenge testing. The cross-linking of antigen and IgE bound to high affinity IgE receptors (FcεR1) on mast cells results in intracellular signalling cascades and a unique biphasic response [8]. The first- an immediate bronchospastic response is the consequence of the exocytosis and release of preformed mediators such as histamine, eicasanoids, free radicals, tryptase, chymase and cytokines [9]. In addition to tryptase potentiated histamine bronchoconstriction, these toxic products lead to loss of microvascular integrity with resulting exudative oedema [10]. Within a few hours this phase subsides and the original mediators induce a late phase response – associated with the influx of eosinophils, neutrophils and mononuclear cells into the inflamed bronchi [10].

Cell-Mediated Responses

Mast cells

Increased mast cells in the airways of atopic asthmatics have been documented by bronchoalveolar lavage [11]. Their preformed and newly generated mediators initiate and perpetuate the inflammatory response (as above).

T Lymphocytes

The late asthmatic response and sustained chronic inflammation is due to T lymphocytes, eosinophils and monocytes [12]. Increased numbers of activated T lymphocytes are found in peripheral blood and the airways of asthmatics [13]. These cells dictate the composition of the inflammatory response by secreting mediators-cytokines and chemokines- that stimulate proliferation and activate eosinophils and monocytes [14, 15]. T lymphocytes can further be divided into two subsets: Th1 and Th2. There has been particular interest in the latter as primary cytokines secreted by this type of cell are increased in bronchoalveolar lavage fluid of atopic asthmatics and production is also augmented following exposure to allergen [16 -18].

Eosinophils

Eosinophils are also increased in the peripheral blood and airways in both allergic and non-allergic asthma and correlate with severity of disease [19]. Their granules can cause tissue damage, smooth muscle contraction and increase vascular permeability leading to further mononuclear cell recruitment. Eosinophil derived proteins include major basic protein (MBP) and eosinophil cationic protein (ECP) that are toxic to respiratory epithelium [20]. Not all eosinophilic products are toxic; leukotrienes, kallikreins and neuropeptides promote mucus secretion and increase the contractile tone that may contribute to BHR [20, 21].

Macrophages

Macrophages are the most abundant resident cells in the bronchi and constitute more than 90% of cells detected in BALF in both normal and stable asthmatic subjects. As the surveillance cells of the airways, they are pivotal in host defence through phagocytosis and production of enzymes and reactive oxygen species. They are capable of up-regulating the inflammatory response by generating cytokines, leukotrienes, prostaglandins and thromboxane A_2 [22, 23]. Although alveolar macrophages can function as

antigen presenting cells, this is the primary responsibility of dendritic cells in the lung [24, 25].

Neutrophils

For many years, the notion of allergen mediated inflammation has resulted in the focus on the eosinophil with little attention to neutrophils. Of note, in the antigen challenge asthma model, is that neutrophils are the initial cells recruited to the airways and predominate for the first 6 hours; it is only later that other immune cells are preponderant [26, 27]. The principal chemotactants for neutrophils are IL-8, IL-6 and leukotriene B_4. Neutrophils may also contribute to pathogenetic mechanisms in asthma through production of lipid mediators, reactive oxygen species and proteases- myeloperoxidase and metalloproteinase [28]. Increased neutrophils in the bronchi have been detected during both infectious and non-infectious acute asthma exacerbations [29, 30]. Wenzel et al, [31] evaluated 14 severe high dose oral corticosteroid dependent asthmatics. Compared to controls and milder asthmatics, the severe group had a 2-fold higher concentration of neutrophils in BALF with similar results in endobronchial and transbronchial samples. The highest elevations of the eicosanoid mediators; thromboxane and leukotriene B_4, were also noted in steroid dependent asthmatics. A massive influx of neutrophils has also been noted in status asthmaticus and also documented in an autopsy study of fatal asthma [32, 33]. Thus neutrophilic inflammation appears to be particularly important in very severe asthma.

B. Mediators: Cytokines, Chemokines and their Relevance to Asthma.

The Cytokine Network

The proliferation, differentiation and effector functions of immune cells are regulated by a complex network of interactions involving cell- cell contact but, to a greater extent, by proteins secreted by activated cells- cytokines. Cytokines are pleiotropic- exhibiting multiple activities likely dependent on the inflammatory milieu and disease process.

INTERLEUKIN -1

There are 2 subtypes of IL-1 (α andβ) and although they only share a sequence homology of 20%, they attach to the same receptor and have almost identical properties [34].

IL-1 is produced by most immune and airway cells but the most abundant source is the monocyte-macrophage [22]. PGE_2 and CS can inhibit IL-1 at the transcriptional level [35].

Two IL-1R have been characterized that are widely distributed (IL-1RI & IL-1RII). Only IL-1R appears to be involved in signal transduction of IL-1 suggesting that IL-1R II may be a decoy receptor preventing IL-1 binding to IL-1R [36, 37]. Further signal transduction occurs via TNF receptor associated adapter proteins (TRAF) [38]. IL-1 also increases cAMP- the consequent activation of protein kinase A and PKC could result in phosphorylation of many substrates and transcription of cellular genes e.g. NF-κB [39, 40].

IL-1 is an important growth factor for T and B cells and induces many cytokines: IL-1 to IL-6, IL-8, RANTES, GM-CSF, IFN-γ and TNF [41]. It also co-promotes the expression of ICAM -1 and VCAM-1 on endothelial cells that could lead to increased eosinophil adhesion [42].

IL-1 expression is increased, particularly in symptomatic asthma (in both BAL cells and airway epithelium) and is reduced with CS treatment [43, 44].

INTERLEUKIN 2

The main source of IL-2 is activated T cells, especially Th0 and Th 1 T cells [45]; although it can also be produced by eosinophils and airway epithelial cells [46, 47]. This is followed by the upregulation of IL-2 receptors (IL-2R) of T cells themselves; binding of IL-2 to IL -2R stimulates T cell proliferation, cytokine secretion and growth factor receptor expression following which internalization of the IL-2R complex occurs. The IL-2R comprises α, β, and γ chains and belongs to the haemotopoeitic cytokine receptor group [48].The α and β chains bind the IL-2 with low affinity whilst the heterotrimer of α/ β/ γ constitutes a high affinity complex and α/γ, and β/γ heterodimers have intermediate affinity. The constitutively expressed β chain is essential for signal transduction [49]. IL-2 increases the production of GM-CSF in PBMC'S of asthmatics [50].

In ovalbumin sensitized Brown-Norway rats, allergen exposure and IL-2 caused a three fold increase in the late asthmatic response compared to those exposed to saline only [51, 52].

BAL cells from asthmatics express increased IL-2 mRNA and titres of IL-2 [16]. Cyclosporin A inhibits IL-2 gene expression in T lymphocytes through interference with transcription factors AP-1 and NF-AT causing decreased allergic airway eosinophilia and may explain the mechanism of benefit in oral steroid dependant asthma [53].

INTERLEUKIN -3

Activated Th cells and mast cells are the major source of IL-3 [54, 55]. The IL-3R comprises an α subunit (IL-3Rα) and a β unit shared with IL-5 and GM-CSF [56]. After IL-3 binding, there is rapid tyrosine and serine / threonine

phosphorylation of a host of cellular proteins [57, 58]. A monoclonal antibody to IL-3Rα was able to abolish its function [59]. IL-3 is a pluripotential haematopoietic growth factor. Increased IL-3 mRNA has been reported in bronchial mucosal biopsies and in BALF in asthma [16, 60].

INTERLEUKIN -4

Cross linking of the CD40 ligand on CD 4 + T cells generates a co-stimulatory signal that increases IL-4 synthesis [61]; a similar effect is seen with cross linking of the IgE Fc receptor on mast cells and basophils and stimulation of T cells [62].

The IL-4R consists of an α chain (that transduces growth promotion and activates transcription) and the IL-2Rγ chain that amplifies the signalling of IL -4R (denoted as the common γ chain: γc) [63].

Airway epithelial and immune cells express IL-4R and do so to a greater extent in asthma [64]. Upon stimulation, IL-4 induces phosphorylation of the IL-4 induced phosphotyrosine substrate associated with the p85 subunit of phosphotidylinositol 3- kinase and with Stat 6 and Janus protein kinase (JAK) [65] to effect signal transduction.

The RS67 allele of the IL-4Rα subunit has been associated with atopy [66].

IL-4 is important in B lymphocyte activation through increased expression of class II MHC molecules and also enhancing the expression of CD 23, the low affinity (Fc εRII) receptor, CD 40 and the α chain of the IL-2 receptor. It plays a pivotal role in immuglobulin class switching of activated B lymphocytes to the synthesis of Ig G4 and Ig E and also promotes the development of Th2 type cells whilst inhibiting the development of the Th1 type [67].

INTERLEUKIN 10

IL – 10 was originally discovered as a product of murine Th 2 clones that inhibited the antigen stimulated cytokine production of Th 1 clones [85]. In humans, Th 0, Th 1, Th 2, CD 8 + T cells and most cells are all capable of producing IL – 10 [86,87]. Monocytes in circulation however, produce more IL-10 than alveolar macrophages [88].

The IL-10 Receptor (IL-10 R) is a member of the IFN – receptor family [89]. Although the precise signalling cascade of IL-10 R has not been identified, the inhibitory effect on monocytes is dependent on NF-κB [90].

As regards its effects, although IL-10 can have an immunostimulatory effect, it is generally considered an immunosuppressive cytokine as it is a potent inhibitor of monocyte/macrophage function. IL-10 suppresses the production of most pro-inflammatory cytokines including TNFα, IL-Iβ, IL- 6, MIP-1α and IL 8 [91-93]. Indirectly, the expression of IL- 1ra, another anti-inflammatory cytokine, is upregulated in monocytes by IL-10 [94]. IL 10 also inhibits monocyte MHC class II, B7.1/ B7.2, CD 23 expression and the synthesis of superoxide anions and NO by activated monocytes [95, 96]. The production of RANTES, IL-8 [97, 98], IFN -γ and IL-2,-4,-5 can also be inhibited by IL-10 [99,100]. IL-10 also promotes B cell viability, proliferation and immunoglobulin secretion.

Bronchial asthma appears to be characterized by diminished IL10 production; this may in part be because of genetic defects in IL-10 production or a feature of asthmatic inflammation [101-103](discussed in detail later).

INTERLEUKIN 12

IL-12 stimulates T cells to produce IFN-γ and regulates the differentiation of T cells such that the balance between Th1 and Th2 is maintained [104]. In an animal model, IL-12 reduced allergen sensitisation and airway inflammation [105].

The PBMC's of atopic asthmatics also appear to have impaired IL-12 production [106].

INTERLEUKIN 13

IL-13 is produced by activated CD4+, CD8+T cells and all Th cell lines [107]. IL-13R shares the IL-4R α chain [108], demonstrating some economy in complexity and yet similarity of effects. It is a potent modulator of monocytes and B cell function. IL-13 upregulates the expression of β_1 intergrin, VCAM1, IL-6 and MCP-1 from lung fibroblasts [109]. In monocytes and macrophages however, IL -1β, IL-6, IL-8, IL-10, IL-12, 1FN- γ, GM-CSF, MIP-1^{α}, IL-1 and TNF- α are inhibited [109-110].

IL -13 further inhibits the release of IL-8 and RANTES from airway smooth muscle [110, 111]. IL-13 promotes the expression of CD23 on B cells and, like IL-4, causes isotype switching to Ig E synthesis [112]. Asthmatic patients exhibit an increased expression of IL-13 in RNA in airway mucosa [113,114].

INTERLEUKIN 15

IL-15 is also produced by activated CD4 + and CD8+T and can induce IL-18 and MCP- 1 production in monocytes [115, 116].

INTERLEUKIN 16

IL-16 is produced by activated CD8 + T cells, epithelial cells and mast cells [117]. Following allergen challenge in asthmatics, BALF contains high concentrations of IL-16 [118].

INTERLEUKIN 17

IL -17 is produced by CD4 +T cells and stimulates NF-κB, IL-6, IL-8, GM-CSF and PGE_2 in lung tissue [119].

INTERLEUKIN 18

IL-18 is a powerful inducer of IFN-γ and has an important role in Th1 responses [120]. It also induces IL-8, MIP-1α and MCP-1 in PBMC's. The synthesis of TNF-α from CD3+/ CD4+ T cells and NK cells is regulated by IL-18 [121]. NF- κB and MAPK can also be activated by IL-18 [122].

INTERLEUKIN 23

IL-23 is structurally related to and has similar biological properties to IL-12 [123].

INTERLEUKIN 25

IL-25 is released from mast cells via an IgE dependent transduction (thus is likely to have a role in allergy) and causes the release of Th2 type cytokines: IL-4,-5 and IL-13 [124,125].

TUMOUR NECROSIS FACTOR α

TNF exists in 2 principal forms: TNF-α and TNF-β and binds to similar receptors. Although primarily produced by macrophages, it can be also be secreted by T cells, mast cells and epithelial cells and is stimulated by IL-1, GM-CSF and IFN -γ [126].

TNF receptors- TNF –R55 and TNF –R75 are found on most cells and further signalling is mediated via TNF receptor associated factor (TRAF). TNF effects are similar to IL-1β [127].

TNF is widely expressed in the lung, increases airway hyper-responsiveness and probably amplifies inflammation [128-130].

GRANULOCYTE – MACROPHAGE COLONY STIMULATING FACTOR (GM-CSF)

GM- CSF regulates the growth, differentiation and activation of haematopoietic cells [131]. In the context of asthma it is produced by most airway cells that include macrophages, eosinophils, T cells, fibroblasts, endothelial, airway smooth muscle and epithelial cells [132].

GM-CSF RECEPTOR (GM- CSFR)

The GM-CSF receptor consists of an α chain and a β chain – the latter being shared by the IL-3 & IL-5 receptors [133] all of which signal via JAK, MAPK and IP-3K pathways [134]. These receptors are found on granulocytes, monocytes, endothelial cells and fibroblasts. Interestingly, airway biopsies have shown upregulation of the expression of the GM- CSFR α chain in non-atopic asthma but not in atopic subjects [135].

GM-CSF IN ASTHMA

GM-CSF may be involved in priming neutrophils and eosinophils, and can also enhance the release of superoxide anions and cys –LTs from eosinophils [136]. GM-CSF induces the synthesis and release of many cytokines including IL-1 and TNF α from monocytes. The expression of GM-CSF is increased in the bronchial epithelium of asthmatics and in T lymphocytes and eosinophils after allergen challenge [137-139].

In acute severe asthma, increased circulating GM-CSF has been noted [140] and PBMC from stable asthmatics also secrete increased quantities [141]. The transient expression of the GM-CSF gene in the epithelium of rats via an adenoviral vector caused an accumulation of eosinophils and macrophages and irreversible fibrosis suggesting that GM-CSF could be a factor implicated in persistent eosinophilia and airway remodelling that characterises asthma [142].

INTERFERON-γ

Interferon γ is produced by Th1 cells, has multiple immunoregulatory effects and is inhibitory to Th2 cells [143]. It is an immune modulator having both anti- and pro-inflammatory effects. Exogenous administration of IFN-γ inhibits allergic eosinophilia and airway hyper responsiveness [144] and IFN-γ levels also increase during allergen immunotherapy [145]. Corticosteroid therapy is also associated with an increased expression of IFN-γ in asthmatic airways [146].

IFN-γ can also amplify the immune response: following endotoxin exposure, TNF-α is released from alveolar macrophages [147] and it can also activate epithelial cells to release cytokines and express adhesion molecules [148].

Chemokines

Chemokines are small chemotactic compounds associated with inflammation. They are designated CC chemokines (when two cysteine residues lie adjacent to each) or CXC (when the residues flank another amino acid). There is much interest in CC chemokines as they are involved in eosinophil, monocyte and T lymphocyte chemoattraction.

CC Chemokines

Macrophage inflammatory protein 1 α (MIP-1 α) is inducible in human monocytes and attracts inflammatory cells to the site of inflammation [149]

Monocyte chemoattactant protein 1 (MCP-1) –as these name suggests, attracts monocytes and is an activating factor as well [150].

RANTES- Regulated on Activation, normal T cell Expressed and Selected, is expressed in IL-2 dependent cell lines [151].

EOTAXIN- is selective for eosinophil chemoattraction [152].

Chemokine Receptors- Although there may be some degree of specificity [153] it appears that most chemokines can bind to the same receptor [154].

There has been interest in blocking receptors to modify inflammation e.g. a monoclonal antibody directed to CCR3 (the eotaxin receptor) inhibited eosinophilia [155].

The chemokines are pluripotent; most can stimulate and attract eosinophils.

MCP-1 can promote exocytosis of basophils with release of large quantities of histamine [156]. Intracellular calcium release, respiratory burst and expression of β_2 integrins also occur [157].

Not surprisingly, increased chemokine messenger RNA and protein have been detected in all activated cells, tissues and BALF in asthma.

Growth Factors

Platelet –Derived Growth Factor (PDGF)

Although originally derived from platelets, it is in fact released by many airway cells. It is a principal mitogen, may activate fibroblasts to secrete collagen and stimulate airway smooth muscle proliferation and could play a role in airway remodelling [158].

Transforming growth factor β (TGF- β)

Immune and constitutive cells in the lung can produce TGF-β and this is increased in asthma. Its role in the turnover of matrix proteins, epithelial repair and fibroblast stimulation may also contribute to remodelling [159].

Fibroblast and Epidermal growth factors

(FGF & EGF) are proliferative agents and thought to play a role in angiogenesis [160].

Insulin-like growth factor (IGF)

IGF is a potent mitogen, activates MAP Kinases and mediates LTD_4 induced smooth muscle proliferation [161].

C. Confluence of Genes, the Environment and Adaptive Immunity in the Pathogenesis of Asthma.

The Th1/Th2 Paradigm

As regards the biology of asthma, the introduction of allergen to the airways in childhood probably initiates allergic asthma. Antigen presenting cells, including dendritic cells, process these antigens and express them on the cell surface in the binding groove of the MHC II. These cells then migrate to the regional lymphoid tissue where they activate T cell receptors.

The consequent immune activation – in particular – cytokine expression – profoundly affects a process referred to as immune deviation. In the presence of IL -12, the cells have an IFN - γ expressing or Th1 – type phenotype [162]. The inability to express this phenotype is thought to promote the development of asthma. The influence of IL - 4 and IL-13 leads to the expression of the Th2 phenotype that appears to be required for the development of asthma [163]. Since this hypothesis has been proposed, it has become clear that it is an oversimplification. Th1 responses exclusively, can cause reversible airway inflammation and airway hyper responsiveness [164]. Asthma may also develop through non allergic mechanisms-with genetics, infections and environmental exposure contributing.

Genetics

Familial clustering clearly demonstrates the indisputable fact that there is a genetic component to asthma. Segregation analyses using multiple asthma traits (IgE, airway hyperesponsiveness, atopy, wheeze and asthma) have revealed that inheritance is probably polygenic [165].

To date, major susceptibility genes have not been identified [166]. Candidate gene/ loci studies have reported linkages to many chromosomes and novel regions of interest. Many of the relationships probably act as disease modifiers e.g. polymorphisms of β_2 adrenoceptor agonists in asthma:

- Gly 16: enhances down-regulation and is over-represented in nocturnal asthma
- Ile 164: decreased coupling, binding & sequestration
- Glu 27: resists β_2 adrenoceptor down- regulation [167]

However, doubts have been raised of the exclusive allergic aetiology of asthma because although polymorphisms of TNF - α are seen in asthma, there appears to be no relationship to atopy or IgE [168].

Also, genes thought to be important in asthma (IL-4, IL-13, T-bet and GATA 3- the latter a transcription factor necessary for IL-5 synthesis) did not actually correlate with the disease [5].

Genes have recently been identified that have hitherto been unknown in asthma pathogenesis. Some of these include a multifunctional gene- a distintegrin and metalloprotease (ADAM 33) [169] and dipeptidyl preptidase 10 (DPP 10) [170]. ADAM genes are important for cell adhesion, signaling and the activation and release of cytokines. The DPP 10 encodes a family of proteins that limit the activity of other proteins by cleaving terminal dipeptides from cytokines, chemokines and leukotrienes and has been associated with asthma and steroid- dependent asthma in children.

The mapping of the human genome and technological advances have allowed the identification of the expression of numerous individual genes using expression arrays. Following the application of clinically relevant standardized allergen solutions to airway epithelium, expression genomics revealed 141 sequences with increased expression and 8 with decreased

expression [5]. Amongst the 141 sequences were genes known to be associated with allergen exposure such as lipocortin, NF-κB, and the receptor subunits for IL-3, IL-4 and IL-5. However, the largest group of genes identified was those that are involved in growth, differentiation and proliferation. Therefore, over and above airway inflammation, asthma is a disease involving changes in growth and tissue responses; this adds credence to the concept of airway remodeling.

The Hygiene Hypothesis

The hygiene hypothesis of Strachan proposed that the immune system is skewed to a Th-2 cell construct at birth [171]. As children encounter infections and other environmental allergens, the immune system repositions in a Th-1 / Th-2 balance likely through the regulatory effects of IL-10 and TGF-β. Failure of exposure to these antigens by vaccination, frequent antibiotics, increased indoor activity and less antigenic food may prevent the re-channeling and back to a progressive Th-2 state and atopy. Evidence for this comes from the observation in the Tuscon Children`s Respiratory Study; the earlier children attended daycare and the presence of one or more siblings in the household, the lower the risk of developing asthma [172].

D. Asthma Pathogenesis: The Future.

Previous models of asthma have focused on airway smooth muscle dysfunction and inflammatory pathways. New models will also encompass the regulatory systems that affect the expression of mediators – subtypes, polymorphisms etc. including the allergen driven changes in airway resident cell growth and differentiation that allow us to understand asthma better in individuals. The confluence of the environment, genes and adaptive immunity will have been met.

1.2 THE GLUCOCORTICOID RECEPTOR IN THE PATHOGENESIS OF STEROID RESISTANT ASTHMA.

Glucocorticosteroids (GC) have a profound influence on human homeostasis and are used extensively in clinical medicine. GC mediate their effects via the glucocorticoid receptor (GR) –a member of the superfamily of ligand regulated nuclear receptors.

Since the cloning of GR in 1985 [173] there have been important developments of the molecular biology of GR that are crucial for improved understanding of asthma pathophysiology and therapeutics. Although glucocorticoids are effective in controlling asthma in the majority of patients, there is a minority of patients who respond less well, needing high doses of inhaled and or oral glucocorticoids, with a small proportion demonstrating extensive resistance [174]. These patients account for a large proportion of the high costs involved in treating asthma [175]. Impaired glucocorticoid responsiveness has been most extensively studied in asthma but has also been reported in other inflammatory diseases, including rheumatoid arthritis, inflammatory bowel disease, and transplantation rejection [176].

An appreciation therefore, of the factors that regulate GR expression, activity and responsiveness is the subject of intense research currently.

Steroid Resistant Asthma- the beginning

The concept of "steroid resistant asthma" was first proposed by Schwartz et al in 1968 [177]. They studied a group of six asthmatics who had responded inadequately to systemic steroids and noted a suboptimal eosinopaenic response to hydrocortisone. The administration of 40mg of intravenous hydrocortisone to the index cases resulted in a blunted eosinopaenic response in peripheral blood compared to a control group of asthmatics [177].

It was several years later-1981- that Carmichael formally extended these observations and the term "steroid resistant (SR) asthma," became entrenched [178]. They studied 58 chronic asthmatic patients characterized by a baseline FEV_1 of < 60% of predicted and a bronchodilator response of 30% or greater. However, the critical finding was the inability to increase the FEV_1 by more than 15% following a 7 day course of prednisolone 20 mg daily. By contrast, asthmatics who could respond to this steroid course with a FEV_1 increase of more than 30% were termed steroid sensitive, "SS".

It must be immediately appreciated that the term SR asthma is a misnomer. Firstly, endogenous steroids and the GR are essential for normal homeostasis and true steroid resistance would be incompatible with life. Secondly, Carmichael assessed the bronchodilator response by nebulisation with β_2 agonists- a practice which is not followed contemporarily as metered dose inhalers are employed. Thirdly, the dose and duration of CS are completely arbitrary; most clinicians (and patients) do not use higher doses or longer periods of administration because of the danger of systemic effects. The American Thoracic Society now defines a significant bronchodilator response as an increase in FEV_1 of 12% and 200ml of the pre-bronchodilator value [179].

A European Task team was set up by the European Respiratory Society and asked to look into the issue of SRA and noted that a variety of terms to describe this category were used: difficult acute, difficult chronic, chronic severe, acute severe, therapy resistant, difficult to control, corticosteroid resistant or corticosteroid dependent, symptomatic, life threatening and fatal. It must be remembered that each of these entities is specific with probable different pathogenetic mechanisms. The task team decided that the term difficult/ therapy resistant asthma would be a useful descriptive label for this category [180].

There follows a number of caveats before this label would strictly apply;

a) has the diagnosis of asthma been definitively established?
b) have asthmatic variants e.g. allergic bronchopulmonary aspergillosis and Churg Strauss Syndrome been excluded?
c) that other factors contributing to loss of control are excluded-

"i) poor compliance/adherence to therapy
ii) psychosocial and emotional factors
iii) inadequate medical facilities
iv) poor access to medical facilities
v) inadequate treatment
vi) exposure to allergens
vii) viral respiratory tract infections
vii) gastro-oesophageal reflux
vii) sinusitis
vii) genetic factors [181]"

The importance of the above cannot be adequately emphasized but will not be discussed further; instead I will concentrate on the disease process of asthma itself and where alternative terms for difficult asthma are used, they are referred to in precisely the same way as defined in the original reports.

Steroid Resistant asthma- the story continues

Could there be other reasons for an inadequate therapeutic response to CS? One aspect could be an enhanced clearance of CS. However, in studies of SR asthmatics as categorised by Alvarez [182] and Corrigan [183], pharmacokinetic abnormalities were examined and discounted as an explanation for steroid resistance. In the report by Alvarez, SR was defined as an FEV1 below 60% of predicted following a 2 week course of prednisone (mean dose: 45mg/d). Six of these subjects underwent a steroid pharmacokinetic evaluation where the rate and extent of absorption and metabolism of prednisone and its active metabolite, prednisolone, were measured; no abnormality could be detected.

PBMC's extracted from these patients were stimulated with PHA. The methyl prednisone dose response showed a significant increase in DNA synthesis consistent with DNA proliferation compared to steroid sensitive subjects. In vitro, this abnormality was reversed with 10μg/ml of troleandomycin, a macrolide with possible immunomodulatory properties, suggesting that this was an acquired reversible defect.

To further illustrate the lack of consensus on the definition of SRA, Corrigan used a definition of failure to increase the FEV_1 by 15% after oral prednisolone-20mg daily for 1 week followed by 40 mg daily for the second week in his cohort..

Peripheral Blood Mononuclear Cell studies

SR asthmatic subjects defined in many studies exhibited a number of cellular abnormalities:

- Kay et al reported that after GC therapy, PBMC's failed to reduce complement receptor expression as opposed to SS subjects [184]
- After exposure of PBMC to methylprednisolone, T lymphocyte proliferation was insignificantly inhibited whilst this was decreased by more than 60% in SS [185]
- No suppression of leukotriene B_4 production following hydrocortisone treatment [186]
- Unchanged TNF production compared to a significant reduction in SS [187]
- Increased expression of IL-2R, IFN-γ and HLA-DR activation antigens on circulating T cells [183, 188]

Lane and colleagues used the tuberculin test to investigate the in vivo responsiveness to oral prednisolone in SR and SS in a double-blind, placebo crossover fashion. The SS but not the SR subjects showed suppression in delayed type hypersensitivity and a decrease in the infiltration of macrophages, eosinophils and T cells [189]. Thus differential steroid sensitivity

can express itself at different sites in addition to the site of inflammation-the lung.

Structure and Activity of the Glucocorticoid Receptor

The GR is encoded for by a gene on a single locus on chromosome 5q31-32. Differential expression of the gene results in variation in GR signalling [190]. The 3 components of the first exon of the genomic structure exhibit the potential for functional sequelae as dexamethasone upregulates all 3 transcripts in acute lymphoblastic leukaemia T cells but depresses them variably in a B-cell line [191]. This and the expression of GR-β (see later) have crucial implications for the understanding of GR expression in disease. The GR consists of 3 domains (Fig1) - the amino N-terminal or immunogenic domain and the carboxy C-terminal or ligand binding domain flanking a DNA binding domain (DBD) [192]. The inactive GR is located in the cytoplasm as a hetero-oligomeric complex containing heat shock proteins 50, 70 and 90 and probably other proteins as well (Fig 2). After binding to GC, the GR undergoes conformational changes, dissociating from the chaperone proteins and the homodimerized complex is actively transported through the nuclear pore into the nucleus where the action of GC is mediated in at least 3 ways [193].

The first, a direct genomic mechanism occurs when the binding unravels two nuclear localisation sequences (NL1& 2) that enable translocation to the nucleus. Here *transactivation* by GR dimers requires specific palindromic sequences in the cis-regulatory regions of target genes called the GC response element-GRE. These are e.g. the mechanisms whereby β adrenoceptor regulation and the inhibitor-I- κBα of NF- κB, is controlled [194]. Gene repression is mediated by negative GRE's.

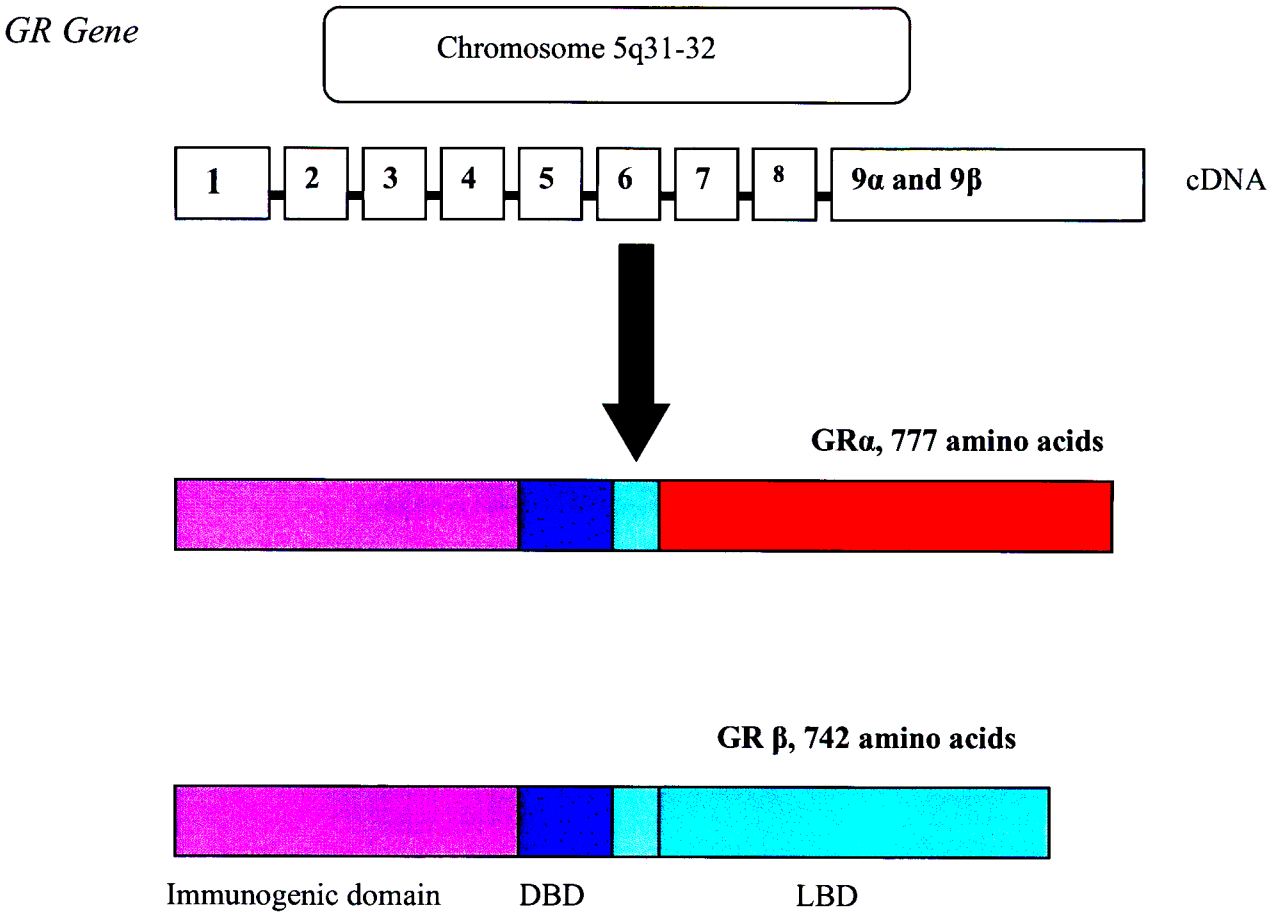


Fig 1. Genomic and complementary DNA and protein structure of the human Glucocorticoid Receptor. The human GR gene consists of 10 exons. Exon1 is an untranslated region whilst exon 2 codes for the immunogenic domain. Exon 3 and 4 code for the DNA-binding domain (DBD) and exons 5 to 9 code for the hinge region and the ligand-binding domain (LBD) respectively. The 2 terminal exons 9 (exon9α and 9β) are alternatively spliced to produce the classic GRα and the non-ligand binding GRβ.

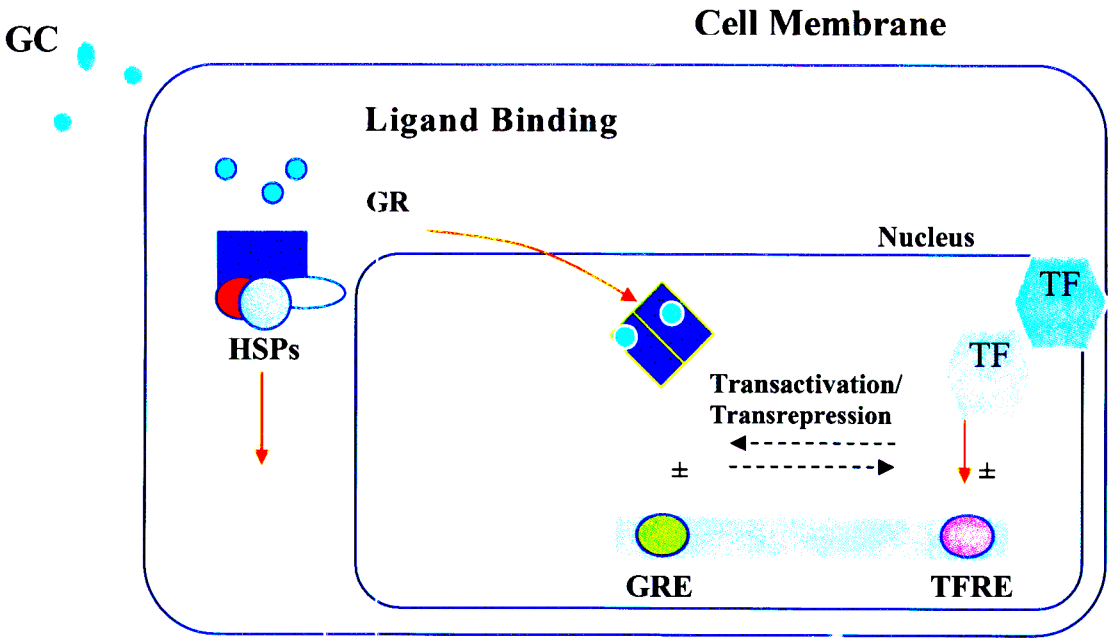


Fig 2. Traditional View of Corticosteroid Action

The Glucocorticoid Receptor (GR) is located in the cytoplasm in association with heat shock and other proteins. Upon binding to the glucocorticoid (GC), these proteins dissociate and the complex translocates to the nucleus. Here they interact with transcription factors and positive and negative glucocorticoid response elements (GRE's) to effect GC action.

TF: transcription factors

TFRE: transcription factor response element

However, many of the major pro-inflammatory genes do not possess GRE's suggesting that other mechanisms of inhibition exist [195]. *Transrepression* occurs when GR (probably in its monomeric form) engages in protein – protein interactions without DNA binding: examples include AP-1 and NF-κB binding- the latter probably related to a mechanism that involves deacetylation of acetylated core histones [193, 196]. Histone acetyl- transferases (HAT) and deacetylases (HDAC) are families of enzymes that regulate chromatin structure- a key pre-requisite for inflammatory gene expression. Acetylation of histones by coactivator proteins e.g. CBP- Creb binding protein, possess intrinsic HAT activity lead to unwinding of the DNA to allow transcription factors and RNA polymerase II to switch on gene transcription. In contra-distinction, deacetylation is associated with transcriptional repression. These represent the direct genomic effects of GC. In addition, glucocorticoids may also play a role in repressing the action of proinflammatory kinase cascade systems, such as the extracellular regulated kinase (ERK) and the Jun N-terminal kinase (JNK) mitogen-activated protein kinases (MAPKs) [197-200]. This occurs via non-genomic mechanisms. Thus directly or indirectly, the GR, functioning as a hormone activated transcription factor, is estimated to influence glucocorticoid target genes on approximately 10% of the human genome [201].

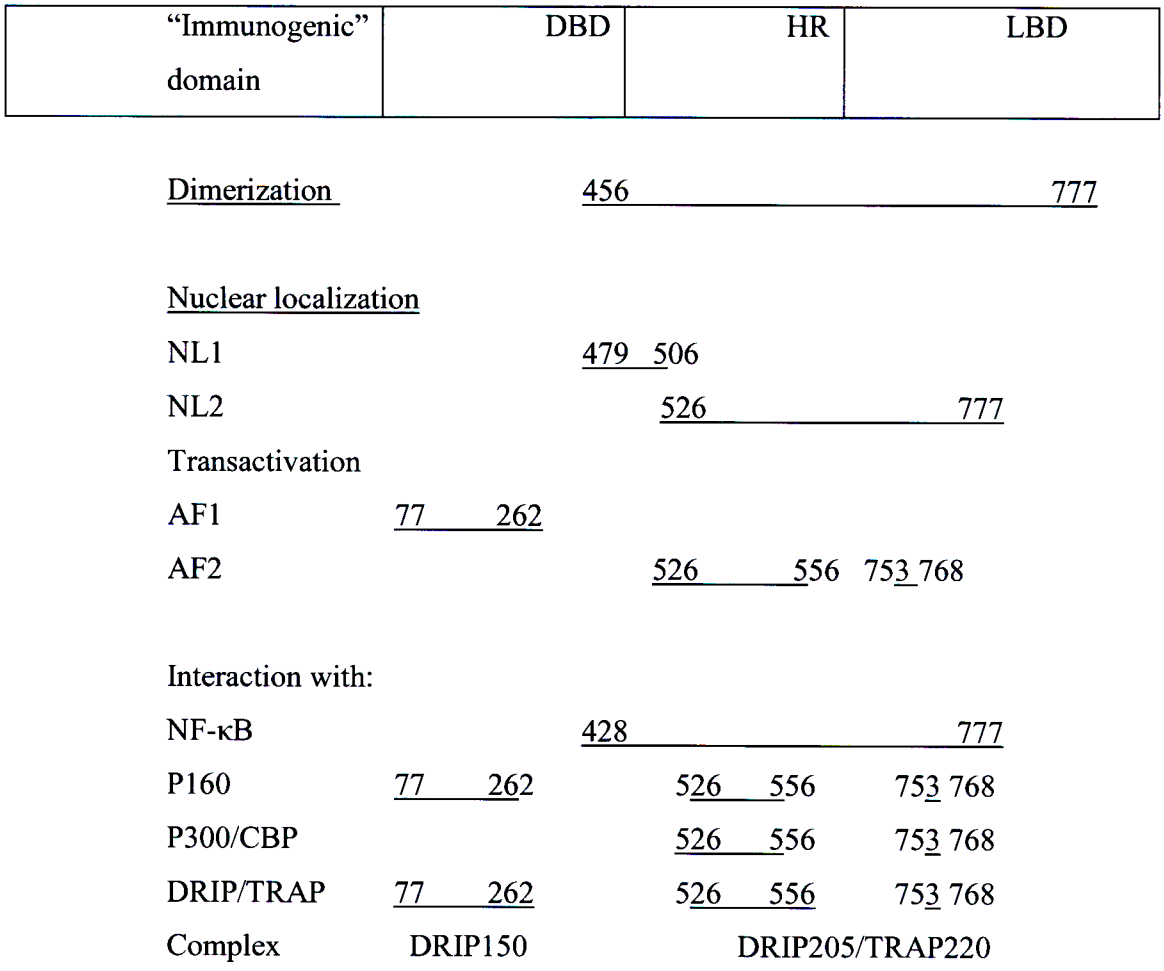


Fig 3: Functional domains and sub-domains of the GRα are indicated. DBD: DNA binding domain; HR, Hinge region; NL1 and NL2, nuclear translocation signals 1 and 2. Two transactivation domains, activation functions (AF) 1 & 2 located in the immunogenic domain and the LBD (ligand binding domain) respectively co-operate for full transcriptional activity of GR; deletion of either of them dramatically reduces the transactivational activity of GR.

Table 1. Factors that might change the sensitivity of tissues to glucocorticoids

Cellular factors
Ligand export system
Ligand activation-inactivation (eg.IIβ -HSD and 5α -reductase)
Chaperones-cochaperones
Transcription factors (e.g. NF- κB, AP-1, CREB, STATs,
C/EBPB, p53, and GATA-1)
Receptor isoform (GRβ)
Coregulators
Coactivators (+)
Corepressors (-)
Viral coactivators (HIV-1 Vpr, Tat)
Chromatin modifiers (SWI/SNF)
DRIP/TRAP components (DRIP150 and DRIP205/TRAP220)
Phosphorylation status
Nitrosylation status
Thioredoxin
Extracellular factors
Small molecules (UDCA)
Inflammatory cytokines
Drugs (RU-486)

11B –HSD, IIBHydroxysteroid dehydrogenase; AP-1, activator protein1; CREB, cyclic AMP-responsive element-binding protein; STATs, signal tranducers and activators of transcription; C/EBPB, CAAT/enhancer binding Protein B; DRIP150; vitamin D receptor-interacting protein150; TRAP220, thyroid hormone receptor - associated protein 220; UDCA, ursodeoxycholic acid.

Adapted from Reference 202.

The Glucocorticosteroid receptor splice variant: GR-β

One of the major discoveries in the last few years, particularly for asthma, has been the recognition of GR-β [203]. Alternative splicing of GR pre-mRNA gives rise to 2 variants of GR: GRα, the 777 amino acid moiety and active form of the GR and GR-β- which differs only in the carboxy terminal end by being deficient in 35 amino acids. However, being the crucial ligand- binding domain, this isoform is unable to bind CS. Interestingly GR-β is synthesized in tissues of normal individuals; but its production is increased in PBMC's and BALF cells in asthma and the highest levels are found in severe and near fatal asthma [204, 205]. In severe steroid dependent and resistant asthmatics, GR-β expression is increased as well [206-208].

Clearly, this is one mechanism why these patients are poorly responsive to CS treatment – they have a receptor that cannot bind CS and therefore cannot mediate the anti-inflammatory effects! Worse still- GR-β inhibits the GR-α DNA binding capacity– further diminishing the possibility of a good therapeutic outcome [209].

Some of the mechanisms whereby this occurs include GR-β competitive binding to GRE- DNA and the formation of transcriptionally impaired GR-α GR-β heterodimers instead of the active homodimer [203]. An important line of investigation has opened up regarding the control of GR-β expression and whether this can be influenced beneficially. We do not know what these factors are presently but what has been demonstrated is that inflammation increases GR-β expression- thus far TNF-α and IL-1 have been shown to upregulate GR-β levels [210]. This over-expression can also exert effects on pro-inflammatory transcription factors e.g. AP-1 and NF-κB and thus contribute to the regulation of inflammatory responses [211].

GR-β is also increased at night providing yet another mechanism and explanation for the characteristic diurnal symptomatology of asthma [212]. Fortunately GR-β has a negligible effect on the binding of other steroid hormones.

Influence of signal transduction on GR.

Cell stimulation by a variety of mediators e.g.cytokines and hormones result in the activation of intracellular enzyme systems and the generation of a number of signal transduction proteins. These have the capacity to interact with GR and enhance / inhibit its function (table 1).

Glucocorticosteroid Receptor Numbers and Affinity.

The effectiveness of a ligand in stimulating GC actions is dependent on its ability to bind to the receptor and the number of receptors present. The inability of some patients to respond to GC has been proposed to be due to altered affinity for GR & or number [213,214]. One would expect that if there is a sub-optimal response to GC, that a reduction in the number of corticosteroid numbers may be an explanation. Contrary to this expectation, several groups that have studied SRA for some time have actually described an increase in the number of receptors [183,215, 216]. GR numbers can be acutely downregulated following high dose corticosteroids [183] because of unique intragenic regulatory sequences in GR itself. These were confirmed by transfection studies by Burnstein et al, who were able to show downregulation of human GR via decreased mRNA and protein [217]. Following experiments using RT-PCR for the α and β isoforms in respiratory epithelial cells, Pujols et al were able to conclude that transcriptional, post-transcriptional and post-translational mechanisms were involved in the regulation of GR expression [218].

To test whether a poor glucocorticoid response could be due to altered affinity, Sher et al [216], studied a group of asthmatics designated SR by failure

to increase FEV1 by 15% after a 7 day course of prednisolone- 20mg twice daily. PBMC's from these patients were subjected to [^{3}H] dexamethasone ligand binding assay and Scatchard analysis; the SR patients had a significant increase in their GR Kd (implying *decreased binding affinity* for CS) and an increased receptor number only in the nuclear fraction compared to SS patients. This poor affinity was also observed by Corrigan et al [183], using a similar methodology and corroborated by Spahn [219] who also demonstrated a decreased binding affinity in poorly controlled asthmatics. Although Lane [215] also detected a higher Kd in corticosteroid-resistant subjects compared to cortico-steroid sensitive subjects, this was not statistically significant (possibly due to small numbers of patients)

In an attempt to further elucidate the reason for diminished affinity for GR, Leung et al, performed bronchoalveolar lavage in these subjects and detected an increased number of cells expressing positive hybridisation signals for IL-2, IL-4, IL-5 [220] and (later) IL-13 mRNA [221]. Subsequent *in vitro* studies revealed that altered GR affinity could be induced in peripheral blood mononuclear cells (PBMC) from normal subjects with a combination of IL-2 and IL-4 or IL-13 alone [222]! Incubation of cells with IL-1, IL-5 and IFN-γ could not alter GR affinity [222]. Diminished affinity has also been documented in PBMC's co-incubated with allergens [223].

Using these data, we developed an *in vitro* model for steroid resistance to study the mechanisms underlying this altered affinity and the functional consequences thereof.

One must appreciate that GR affinity is not static but is altered by disease states and inflammation. This binding affinity changes diurnally (the decrease at night being yet another reason for more nocturnal symptoms) and over time [224]. The fact that the alteration in Kd is due to inflammation was well demonstrated in a study where GR affinity decreased with the onset of the

allergen season and importantly, before the onset of symptoms [223, 226]! In fact, GC are capable of normalising affinity as inflammation is brought under control [225]; the more severe the asthma the more potent the CS needed to normalise affinity to control symptoms.

A spectrum of glucocorticoid responsiveness in airway inflammatory diseases may exist, reflecting several mechanisms caused by either disease activity itself or by the effects of therapy, with the glucocorticoid-resistant asthmatic subjects at one extreme of this spectrum. At a molecular level, resistance to the anti-inflammatory effects of glucocorticoids can be induced by several mechanisms [227]. The reduction in glucocorticoid responsiveness observed in cells from these subjects has been ascribed to a reduced number of GRs, altered affinity of the ligand for GRs, reduced ability of the GR to bind to DNA, or increased expression of inflammatory transcription factors, such as activator protein 1, that compete for DNA binding.

It is now prudent to look at signal transduction pathways that might interact with GR.

1.3 SELECTIVE MEDIATORS AND CYTOKINES IN SIGNAL TRANSDUCTION.

Phosphoinositide Hydrolysis

Phosphoinositide hydrolysis is one of several important mechanisms of signal transduction [228]. The activation of airway cell surface receptors e.g., muscarinic or cysteinyl – leukotriene results in the further activation of the enzyme phospholipase C (PLC). This in turn converts phosphoinositide 4, 5 – biphosphate (PIP2) to two second messengers: myoinositol 1, 4, 5-triphosphate (IP3) and 1,2- diacylglycerol (DAG). These products are intimately linked to intracellular Ca^{2+} and airway smooth muscle contraction but additionally, to pathways of inflammation in these and other immune cells.

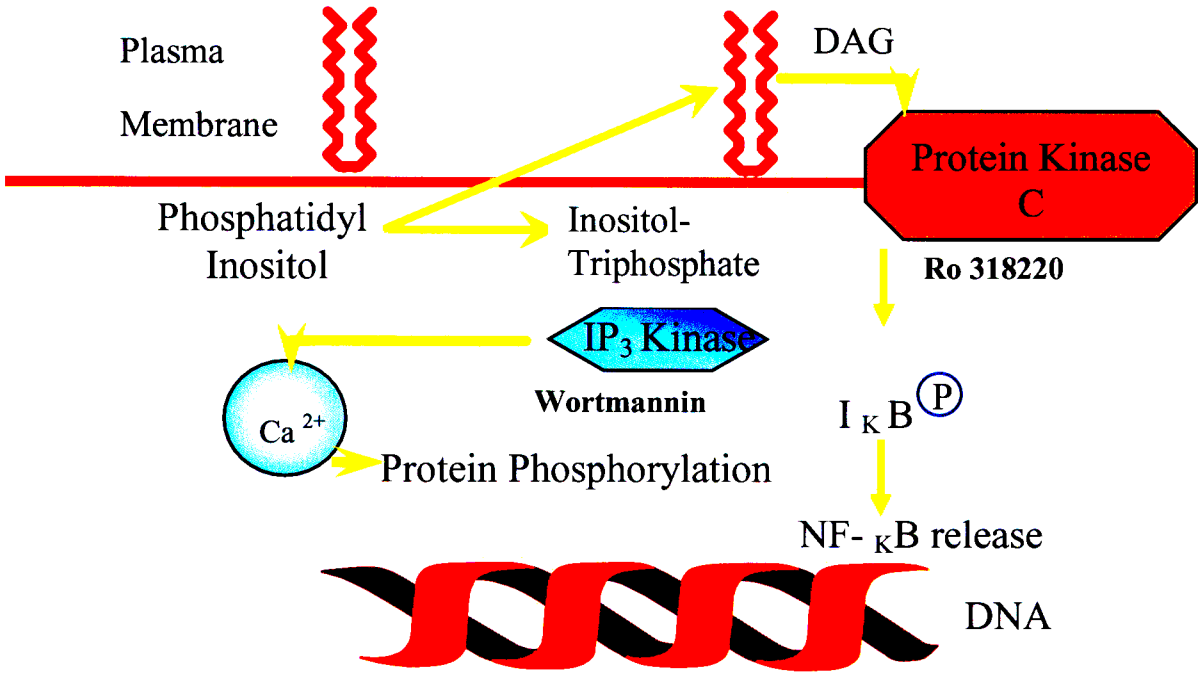


Fig 4. The IP-3 kinase and Diacylglycerol pathways showing the inhibitors Wortmannin for IP-3 Kinase and Ro318220 for Protein Kinase 3.

a) PHOSPHOINOSITIDE 3- KINASE (PI-3K)

Phosphoinositide 3 kinases are lipid kinases (phosphorylating membrane lipids of the phosphoinositide family) that recruit and activate downstream targets involved in mitogenesis, apoptosis, differentiation and activation, cytoskeletal remodelling and vesicular trafficking.

Structure, substrate specificity and regulation have resulted in 3 classes of PI-3K being recognized [229]. Class II consist of monomers of uncertain significance (as yet) in mammals. Class III appear unregulated and are thought to be involved in intracellular "housekeeping" e.g. protein and vesicular trafficking. Class I are heterodimers of catalytic and regulatory subunits that subserve principal intracellular functions.

Airway smooth muscle proliferation has been linked to PI-3K. A characteristic feature of remodelled airway is the increase in airway smooth muscle bulk and number. In two animal models, a three fold increase in ASM DNA has been demonstrated [230,231]. The availability of a PI-3K inhibitor –Wortmannin- was subsequently shown to decrease ASM DNA synthesis by more than 90% [232] and PI-3K may be important in the remodelling process.
PI-3K are also key regulators of neutrophil recruitment and activation [233] (cells important in severe and fatal asthma)

Eosinophils are also not normally resident in the lung. Their presence in asthma together with their toxic products ECP & MBP damages airway epithelium and contributes to BHR. Wortmannin was shown to inhibit IL-5 induced eosinophil release by the bone marrow [234] and blocked eosinophil migration in response to chemoattractants [235, 236].

The induction of cytokine gene expression in monocytes is also sensitive to PI-3K inhibition [237].

b) Protein Kinase C

DAG, the other product of PI hydrolysis is the endogenous activator of another important regulatory enzyme- protein kinase C (PKC).

The Protein kinase C (PKC) is an increasingly diverse family of serine/threonine kinases that are involved in multiple cellular processes as well [238]. Presently, the PKCs comprise twelve different isoforms that are grouped into three subfamilies based on their unique structure:

- classical PKCs (α, β1, β2 and γ) – activated by (DAG) and calcium,

- novel PKCs (δ, ε, η, and θ) – activated by DAG, and the

- atypical PKCs (ζ and λ/ι) that do not respond to either DAG or calcium.

PKC isozymes appear to play distinct, and in some cases opposing roles in the transduction of intracellular signals and are often over expressed in disease states.

Evans et al demonstrated an increased expression and activation of the PKCξ isoform in sputum eosinophils that occurred in the late phase following allergen challenge in asthmatics [239].

However, Vachier et al, reported abnormal regulation of PKC activity in alveolar macrophages and PBMC's of asthmatic patients and suggested that this was one mechanism for the functional hyperreactivity of inflammatory cells in asthma [240].

Furthermore, activation of PKCδ in an airway epithelial cell line enhanced IL-8 expression via an effect on NF-κB suggesting that PKCδ plays a key role in the regulation of NF-κB-dependent gene expression in these cells [241].

Endothelin is a small peptide that is probably involved in the pathogenesis of asthma. It is a potent bronchoconstrictor, a mitogen factor for airway smooth muscle and a powerful stimulator of the extracellular regulated kinase 2 (ERK2) subgroup of MAP kinases. It was established that the endothelin signal transduction pathway that culminates in ERK2 activation was dependent on PKC [242].

Histamine –another mediator of inflammation- induces the release of many cytokines including IL-8 and GM-CSF. This action was found to dependent on Histamine 1 receptors acting via PKC [243].

The Mitogen Activated Protein Kinases in Intracellular Signal Transduction.

The bronchial epithelial and immune cells are exposed to numerous inhaled environmental stimuli that could influence intracellular signalling pathways and a variety of cellular responses. Of these, the mitogen-activated protein kinases are felt to play a major role in these responses as they regulate intracellular signal transduction by many agonists, growth factors, hormones, cytokines, oxidants and environmental stress factors [244]. The MAPK system is highly conserved in all organisms [245].

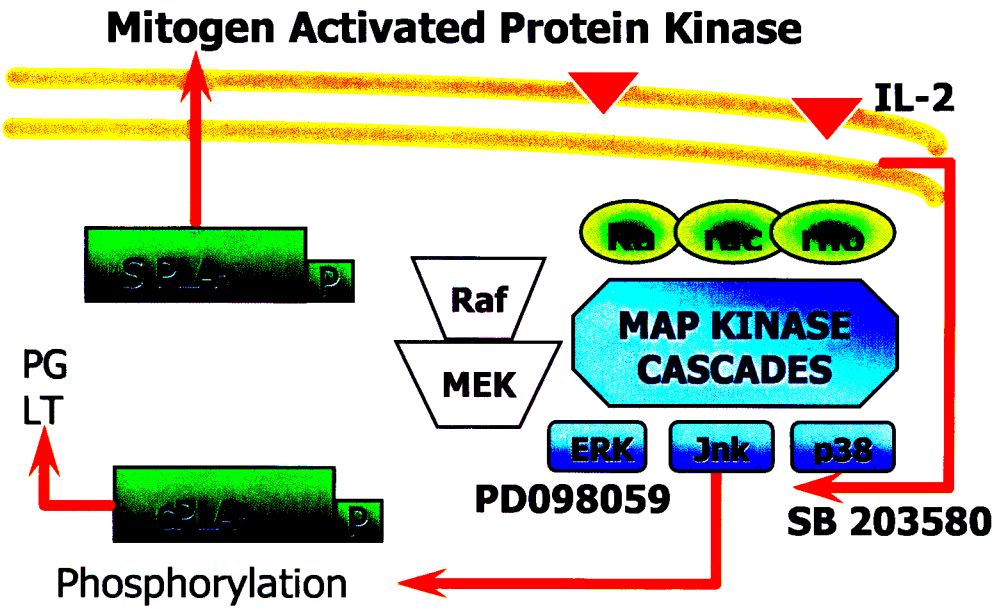


Fig.5. The Mitogen Activated Protein Kinase Cascades. IL-2 signals via p38MAPK –the latter being inhibited by SB 203580; PD098059 is a specific inhibitor of ERK.

The three best characterised cascades are:

i) the extracellular regulated kinases (ERK)

ii) the p38 MAPK

iii) the c-Jun NH_2 –terminal kinases (JNK) (the stress activated protein kinases)

MAPK are activated by phosphorylation and in turn phosphorylate cytoplasmic signalling proteins, cytoskeletal proteins or modulate transcription factors and kinases [245].

The MAPK / ERK cascade is activated following mitogen exposure and downstream gene induction leads to the appropriate mitogenic or differentiation response [244].

The JNK / SAPK and p38 MAPK are the two most important systems and are activated by stress, LPS, UV radiation, pro-inflammatory cytokines (1L1-β, TNF-α) but respond weakly to growth factors [245-247]. Inflammatory cytokines activate the cascade via the Rho family of small GTPases (Rho, Rac and Cdc 42) and p21 Ras- activated kinase (PAK) [248].

Although there is some degree of upstream specificity of the MAPK at the level of the MKK's, there is a lot of cross - talk [249]. The MAPK stress pathways are closely related to NF-κB – dependant gene expression [249].

The study of MAPK modules has been enhanced by the availability of 2 cell permeable MAPK inhibitors – PD 098059 (a flavone) [250] and SB 203580.
PD 098059 binds to the inactive form of MKK1 with ERK being its specific downstream effector [250].

Experiments with PD 098059 of Relevance to Asthma.

Vanadium pentoxide causes occupational asthma and chronic bronchitis through unknown molecular mechanisms. A study utilizing rat pulmonary myofibroblasts demonstrated activation of the extracellular signal-regulated kinases 1 and 2 (ERK-1/2) with Vanadium but not by the inert metal titanium dioxide. Vanadium induced ERK-1/2 activation was abolished by pretreatment with PD98059, indicating a dependence on the mitogen-activated protein (MAP) kinase kinase pathway [251].

Endothelin, a small peptide, is a potent bronchoconstrictor and mitogen for airway smooth muscle and is likely involved in the pathogenesis of asthma. Endothelin is also a potent stimulator of the extracellular regulated kinase 2 (ERK2) subgroup of MAP kinases and ERK2 activation is closely linked to smooth muscle cell proliferation. Whelchel reported that PD98059 was found to significantly inhibit the ability of endothelin to activate ERK in cultured airway smooth muscle cells [242, 252].

IL-13 dysregulation plays a pathogenetic role in inflammatory and remodelling diseases with STAT 6 probably mediating its tissue effects. Because signalling cascades involving MAPKs have been linked to inflammation and remodelling, Lee et al, administered PD98059 to mice and studied the consequences. They found that IL-13-induced inflammation and alveolar remodelling was inhibited with an associated decrease in IL-13-induced chemokines (MIP-1alpha, MIP-1beta, MIP-2, RANTES, MMP-2, -9, -12, and -14, and cathepsin B and increased levels of alpha1-antitrypsin [253].

Respirable particulate matter (PM) is responsible for lung problems and airway epithelium exposed to PM secretes pro-inflammatory cytokines. Blanchet et al [254] demonstrated that human bronchial epithelial cells exposed to PM with an aerodynamic diameter < 2.5 micron (PM2.5) or diesel exhaust particles, upregulated the expression of amphiregulin (AR), a ligand of the

epidermal growth factor receptor (EGFR) that is capable of inducing GM-CSF. They further reported that amphiregulin was inhibited by PD098959 during these studies [254].

Finally PD098059 may block tissue eosinophilia as MAPK are responsible for eotaxin 2 mediated expression of VCAM; when blocked by the inhibitor, cells shift their adhesion molecule usage away from VCAM dominated pathways to ICAM pathways [255].

SB203580 – the Specific p38 MAPK Inhibitor.

SB203580 belongs to a class of pyridinylimidazole compounds initially investigated for their ability to inhibit inflammatory cytokine synthesis [256]

SB203580 is a specific inhibitor of p38α, p38β and p38-2MAP kinases that prevent activation of its downstream effector MAPK-activating protein kinase 2 [257]. P38MAPK (p38 /Mpk2/ CSBP/ RK) is part of the ras pathway and responds primarily to stressful and inflammatory stimuli e.g. tumour necrosis factor- alpha, IL-1 and lipopolysaccharide. Potential cellular targets of MAPK include PLA_2 and p90 s6 kinase [258].

Role of P38 MAPK in Pro-inflammatory Cytokine Expression.

p38 MAPK activation leads to the production and activation of inflammatory mediators that play a role in leucocyte recruitment and activation [259]. Additionally, p38 MAPK regulates the expression of a number of genes involved in inflammation e.g. those coding for TNF-α , IL1-β, IL-6, IL-8 cyclooxygenase 2 and collagenase-1, -3 [260].

SB 203580 inhibits p38 activation and

- IL- 6 and GM-CSF transcription in TNFα stimulated fibroblasts [261],
- IL- 8 transcription in IL -1 stimulated monocytes [261]
- translation of IL -1 and TNF-α in LPS stimulated monocytes and inhibits NFκB – dependant gene transcription (indirectly) [262]

- decreases RANTES and GM – CSF production of TNF-α or IL-1β treated bronchial epithelial cells (although high concentrations were used in this report that could have inhibited other MAPKs) [263].
- causes a reduction in TNF-α mRNA in human macrophages [264]

p38 MAPK is also involved in iNOS expression and the inflammatory response in macrophages [265].

The therapeutic potential of p38 MAP kinase inhibition has also been investigated in animal studies. Antisense pharmacodynamic activity was demonstrated by Duan et al who characterized a potent and selective p38 MAP kinase antisense oligonucleotide, aerosolized, and then administered it by inhalation in a mouse model of asthma [266]. BALF from the mice showed significant reductions of ovalbumin-induced increases in total cells, eosinophils, and IL-4, IL-5 and IL-13 levels. This correlated further with a dose-dependent inhibition of airway hyper responsiveness in allergen-challenged mice. The p38alpha-ASO significantly reduced p38alpha MAPK mRNA expression in mononuclear cells from BALF and peri-bronchial lymph node tissue. A control 6- base mismatched oligonucleotide did not exhibit any of these effects.

Thus in the context of asthma pathogenesis, the synthesis of many inflammatory mediators such as TNFα, IL-4, IL-5, IL-8, RANTES and eotaxins, are all regulated through activation of p38 MAPK [259] .

A significant report that p38 MAPK was crucial for the mitogenic response of IL-2 prompted us to test SB203580 in the steroid resistance model [267].

We were aware that glucocorticosteroid receptor resistance in vitro was dependent on the co-incubation of IL-2 and IL-4 and was not possible with either cytokine alone. The availability of antibodies against these cytokines/receptors would have allowed us the opportunity of performing experiments to see the effect of blocking these cytokines. The next best was to attempt to block their signal transduction pathways.

It was known that one of the members of the mitogen-activated protein kinase (MAPK) family, p42/44MAPK (ERK2/1), is activated by IL-2. Crawley et al, investigated the response to IL-2 of two other members of the MAP kinase family, p54MAP kinase (stress-activated protein kinase (SAPK)/Jun-N-terminal kinase (JNK)) and p38MAP kinase, in a T cell line [267]. They showed that IL-2, and another T cell growth factor, IL-7, activated both SAPK/JNK and p38MAP kinase. Importantly, inhibition of p38MAP kinase activity with SB203580 prevented activation of its downstream effector, MAPK-activating protein kinase-2, and correlated with the suppression of IL-2- and IL-7-driven T cell proliferation.

Theophylline.

Theophylline has been used for decades in asthma. However, its narrow therapeutic range and adverse event profile saw its use decline as the safety and superior efficacy of LABA+ ICS emerged to become first line therapy in symptomatic asthma.

Its exact mechanism of action has been debated for decades. The current views are the inhibition of cAMP and cGMP phosphodiesterases and adenosine receptor antagonism [268, 269]. cAMP is an important second messenger, inhibition of which will decrease inflammatory responses in many cells [270]. The predominant PDE isoenzyme is PDE4 in human eosinophils and although at high theophylline concentrations (IC_{50} 300–661 mM) this enzyme can be inhibited, at therapeutic concentrations of 27–80 mM there is almost no inhibition [271]. However, the highly specific PDE4 inhibitor-Rolipram-failed to inhibit eosinophil degranulation, suggesting that PDE inhibition alone is not the only prerequisite [272].

Apart from A1 and A2 receptors, adenosine exerts its effect via A3 receptors; these are abundant on eosinophils [273] and when stimulated, inhibit degranulation and chemotaxis [274, 275].

Ezeamuzie showed that theophylline at therapeutic concentrations was able to suppress C5a induced eosinophil degranulation via an A3 agonist effect [276]. Other effects of theophylline at therapeutic doses include the inhibition of the late asthmatic response by decreasing the inflammatory response to allergen and concomitant airway hyper-responsiveness [277-279], the latter effect however, was not noted by other investigators [280].

The suggestion that it has anti-inflammatory activity at lower doses [281, 282] and two studies where clinical efficacy was noted at this lower dose with ICS rather than doubling doses of ICS saw a renewed interest in the drug [283, 284].

Additionally, the following anti-inflammatory effects have been noted in experimental and in vitro studies:

- prevention of expression and release of TNF-α and IL-1β by monocytes and alveolar macrophages [285,286]
- non-specific suppression of lymphocyte activity [287]
- blocks IL-2 dependent T cell proliferation and IL-2 production by T cells [288]
- inhibition of eosinophilic degranulation and release of mediators (high dose) and low dose causing a decrease in eosinophilic inflammation but no change in NO [279, 289].

Finally, in the setting of GR related transcription, was the finding that theophylline at therapeutic levels also inhibited NF-κB in human mast cells stimulated with TNF [290].

Since the precise mechanism of action of theophylline was uncertain and the description of experimental evidence of anti-inflammatory effects that may be indirectly related to the GR, we decided to test theophylline in our steroid resistance model.

Interleukin-10.

As cytokines were investigated and their functions elucidated, it became apparent that although most were pro-inflammatory, a few regulated immune cells and were anti-inflammatory [291]. In this latter category, interleukin-10 is one of the most important. Prior to full characterization, it was quite clearly an inhibitor of cytokine synthesis [85]. It is a late release cytokine produced by lymphocytes, monocytes and macrophages. It inhibits production of iNOS, the cytokines IL-1, IL-6/-8, TNF-α, GM-CSF, G-CSF, IFN-γ and chemokines at the transcriptional level and also induces humoral responses [91-100, 292, 293].

When studying its applicability to asthma it appeared that there was a defect in the expression of IL-10 which may represent an endogenous reason why inflammation persists [294]. Production of IL-10 is decreased in alveolar macrophages and PBMC's in asthma [295].

One of the other factors contributing to this may be due to genetic polymorphisms. IL-10 is transcriptionally regulated and there are 3 single base pair substitutions between - 1117 and -627 at the 5' flanking region that produce three different haplotypes: ATA, GCC and ACC [296]. These polymorphisms are in proximity to several transcription factors that might interfere with transcription [297]. Using sequence-specific oligonucleotide probes with a dot blot technique, Lim et al looked for the six possible alleles. They found that significantly fewer severe asthmatics than controls had the putative high IL10- producing haplotype GCC and more had the putative low IL10-producing haplotype, ATA [102].

Defective IL-10 expression

Tomitka et al utilised the technique ofLPS stimulation of whole blood cultures and analysis by flow cytometry [298]. Unlike mild asthmatics and controls, there was a significant reduction in intracellular staining for IL-10 (and IL-12) in severe persistent asthma.

Beneficial effect of IL-10

In animal models, IL-10 can effectively suppress the immune response to inhaled allergen [299] and in another novel report; CD4+ cells engineered to increase IL-10 secretion also controlled airway reactivity and inflammation[300].

During specific immunotherapy, Th cells secrete more IL-10, offering a possible explanation for the success of this modality [301].

Recombinant human IL-10 has been utilized successfully in Crohn's disease and psoriasis (although haematological side-effects raised safety issues)
ICS therapy is associated with increased IL-10 production by alveolar macrophages whilst pro-inflammatory cytokine production decreases [302].

These data on IL-10 prompted us to study its effect in the steroid resistance model both as a modulator of altered GR binding and its secretion after resistance was induced in vitro.

Granulocyte Monocyte - Colony Stimulating Factor.

There is increased expression of GM-CSF in the airways and peripheral blood in asthma. It enhances the production of a host of cytokines and is chemotactic for immune cells especially eosinophils.

Deleterious Effect of Elevated GM-CSF

La Grutta et al, [303] from an analysis of GM-CSF and other inflammatory/signal transduction molecules, have postulated that biological heterogeneity may exist in children with asthma that has clinical consequences. They studied steroid –naïve and moderate asthmatics and found that the moderate group had 2 phenotypes of GM-CSF production: low and high producers. This latter group experienced more exacerbations than the low producers. Moreover, the p65 nuclear factor-kappaB subunit and phosphorylated IkB alpha expression by PBMC's was also higher in the moderate asthmatics suggesting greater inflammatory activation.

The adhesion molecules ICAM-1 and VCAM-1 are members of the immunoglobulin superfamily adhesion molecules on vascular endothelium and play a key role in eosinophil accumulation in allergic inflammation. Although VCAM- 1 has been reported to cause spontaneous eosinophil adhesion, [304] GM-CSF was required and augmented ICAM-1 eosinophil adhesion. In this in vitro experiment, GM-CSF also increased eosinophil superoxide anion (O2-) generation and eosinophil –derived neurotoxin release.

Decreased GM-CSF is associated with Asthma Improvement

To establish the role of GM-CSF in asthma, a group of asthmatics were given beclomethasone dipropionate 800µg/day for 1 month and subjected to the sputum induction technique before and after therapy [305]. The ICS course was associated with an increase in the mean peak expiratory flow and with a decrease in the diurnal variation of PEF accompanied by a significant decrease in the mean GM-CSF level after treatment.

Superior clinical asthma outcomes have also been described with LABA+ ICS combination therapy[306, 307]. A number of synergistic molecular mechanisms have been demonstrated in vitro to explain the observation of the potentiation of the anti-inflammatory effect of ICS by the LABA:

increased inhibition of TNF-α induced eotaxin release, thus limiting eosinophil accumulation in the lung [308]

ligand independent translocation of GR to the nucleus [309]

activation by GR by formoterol [310].

A report by Spoelstra et al described decreased GM-CSF production by lung fibroblasts as a further explanation of the benefits of combination treatment [311]. The cells were pre-incubated with Budesonide and then stimulated with (IL)-1beta in the presence of Formoterol. Supernatants were assayed and the cells subjected to a cell surface ELISA technique. Formoterol had an additive effect in decreasing GM-CSF production and inhibiting the expression of ICAM-1 and VCAM-1 as well.

CHAPTER 2: AIMS

1. Review the history and experimental procedures in Steroid Resistant Asthma.
2. a. Define a cohort of oral-steroid resistant asthmatics as a surrogate for steroid resistant asthmatics to explore Glucocorticosteroid Receptor (GR) characteristics and compare to steroid sensitive patients and normal subjects.
   b. Test whether decreased GR number and/or affinity are responsible for diminished corticosteroid effects.
   c. Administer a corticosteroid burst and re-examine GR parameters.

3. Confirm cytokine induced altered GR affinity in vitro utilizing IL-2 and IL-4 primarily- use as "steroid resistance model".

4. Attempt to normalize GR affinity with inhibitors of signal transduction in vitro.
   a. IL-4 induces phosphorylation of the IL-4 induced phosphotyrosine substrate associated with the p85 subunit of phosphotidylinositol 3-kinase: block with Wortmannin
   b. PKC is associated with phosphorylation and the regulation of NF-κB: block with Ro318330
   c. ERK MAPK : block with PD098059
   d. P38 MAPK- transduces inflammatory stimuli and essential for mitogenic response of IL-2: block with SB203580.
   e. Theophylline: mechanism of anti-inflammatory action not fully understood; interacts with transcription factors- possible interaction with GR.
   f. IL-10 is a major regulatory cytokine and inhibits synthesis of most pro-inflammatory cytokines; co-incubate with Il-2,-4.

5. Explore functional consequences of altered GR affinity in terms of cytokine production by cells- hypothesis: cytokine profile should favour a pro-inflammatory state. Measure IL-10, anti-inflammatory/ regulatory cytokine and GM-CSF- a pro-inflammatory cytokine.

6. Attempt to demonstrate steroid resistance in vitro by measuring cytokine expression and the possible effects of dexamethasone.

CHAPTER 3: Glucocorticosteroid receptor characteristics in peripheral blood mononuclear cells in oral-steroid dependent asthma.

RATIONALE FOR USING PERIPHERAL BLOOD MONONUCLEAR CELLS AS A REFLECTION OF THE AIRWAY MILIEU

Peripheral blood mononuclear cells (PBMC's) have been used extensively to study pathogenetic mechanisms in asthma. One of the reasons is their relative ease of access; studying the airway milieu is relatively invasive and can be complicated in difficult asthmatics with labile and pre-existing narrowed airways. I chose to study PBMC's as it was non-invasive, had already been thoroughly researched and my data would be comparable to existing data on the subject.

The intuitive question therefore is: to what extent do circulating mononuclear cells reflect inflammation in the lung? One must remember that airway cells are derived from the circulation. Thus inflammatory stimuli attract and activate cells in the bronchi in asthma. These stimuli, including local airway mediators, also activate immune cells trafficking through the lung circulation and they in turn, together with circulating mediators, activate cells in the systemic circulation. This process extends all the way to the bone marrow [10].

Once mononuclear cell diapedesis has occurred, they become resident in the lung. Whilst these cells do change phenotypically, they retain many of the properties of their circulating counterparts' e.g.

IL-10 inhibits cytokine production by both alveolar macrophages [91] and PBMC's [92]

IL-13 inhibits expression of MIP1-α from lung macrophages and blood mononuclear cells [110]

altered GRβ expression is seen in both BAL cells & PBMC's in acute severe asthma [204, 205]

There is thus a large body of evidence to suggest that PBMC's do reflect the behaviour of cells in the airways [10, 12, 13, 77, 88, 90-92, 110, 204, 205].

RESEARCH QUESTION

The research question in phase 1 was: Is there a difference in corticosteroid receptor number and or affinity that is responsible for decreased corticosteroid effectiveness in oral-steroid dependent subjects?

METHODS

Patients and subjects.

The study was approved by the Ethics committee of the Royal Brompton Hospital, and written informed consent was obtained from all subjects.

Nineteen oral steroid-dependent (OSD) asthmatic subjects (poorly controlled on inhaled and oral steroids) were carefully selected from a group of difficult asthmatics attending the asthma clinic at the Royal Brompton. A correct diagnosis of asthma had been ascertained and known factors contributing to difficult asthma had been excluded in all subjects. Cessation of oral steroids had been attempted without success and alternate immunosuppressive therapy had also been tried in many of them in the past.

By way of comparison, ten asthmatic subjects whose symptoms were controlled (designated steroid sensitive, SS) with ICSs, and 11 healthy, non-asthmatic subjects (normal, N) were also studied. Their demographic characteristics are depicted in Table 2. All subjects were matched in terms of age and atopic status. No patients were current smokers. Asthmatic subjects were defined as patients who had shown a 15% increase in FEV1 to 400 μg of inhaled salbutamol administered through a metered-dose inhaler. All patients

were taking inhaled salbutamol on an as-required basis. The mean daily doses of salbutamol administered through a metered-dose inhaler were 0.4 ± 0.1 mg/d and 1.3 ± 0.6 mg/d for the SS and steroid-dependent groups, respectively.

The SS group had a mean baseline FEV1 of 2.31 ± 0.3 L (76% ± 5% of predicted value) and were on a mean inhaled fluticasone equivalents steroid dose of 0.8 g/d [range, 0.2-1.2 g/d].

Steroid-dependent patients, however, had a baseline FEV1 of 1.39 ± 0. 2 L (52% ± 3%) of predicted value and were receiving high-dose ICS therapy (1200 – 1800 μg of fluticasone or equivalent) together with a mean maintenance dose of oral prednisolone of 20 (range, 15-30) mg per day for the control of their asthma.(Table 2). This group had been attending the asthma clinic at the Royal Brompton for many years, were well categorized and had been on sustained oral prednisone. The diagnosis of asthma was established and asthma mimics and other treatable factors for difficult asthma had been excluded.

Eleven healthy volunteers were selected as control subjects. One would have preferred age-matched subjects, however, donating 100 mls of blood for research purposes could lead to reservations and I had to settle for willing volunteers of as close an age as possible. There is no suggestion in the literature that GR number or affinity is age related-the primary comparison.

Table 2: Comparative Characteristics of Asthma and Healthy subjects

	Oral –Steroid Dependant Subjects	ICS subjects	Healthy subjects
No. of subjects	19	10	11
Age (y), mean	46 (40 - 52)	48 (38 - 58)	32 (27 - 38)
Sex (M/F)	4/15	5/5	6/5
Baseline FEV_1 pre BD (% predicted)	1.39 ± 0. 2 L 52 (± 3) §≈	2.31 ± 0.3 L 76 (± 5) †	3.42 ± 0.3L 91.6 (± 9)
FEV_1 response to Salbutamol (% predicted)	36 (24 - 48) *	18 (12 - 24)	
Duration of asthma	29 (21-37)	36 (25 - 48)	
Inhaled steroids (mg/d)	1.5 (1.2 - 1.8)	0.8 (0.2 - 1.2)	
Years on systemic steroids	6.8 (3.9 - 9.7)		
Maintenance Prednisone (mg/d)	20 (15 - 30)		
Atopy (positive/negative) #	16/3	8/2	2/9

Results are expressed as the mean with range or SD (±) in parenthesis
BD, Bronchodilator
*P <.05 and ≈ P <.01 compared with ICS group
† P <.05 and § P <.001 compared with healthy subjects
Ventolin dose of 400µg
Expressed as dose equivalent to fluticasone propionate
#Atopy is defined as positive skin prick test response to at least 3 common allergens

Falcon and washed with 50ml HBSS (Hank's Balanced Salt Solution) in a centrifuge at 1600 rpm, 4°C for 10 minutes. Most of the HBSS was discarded, topped up with fresh HBSS and 2 further washes were performed. After the final wash, the cells were resuspended in 3ml RPMI 1640. (RPMI- Roswell Park Memorial Institute medium-1640 is a standard medium for mononuclear cell studies)

Ten ul of this monocyte fluid was added to 90ul of a Kimura cell stain and counted in a Neubauer counting chamber. Taking the dilution factor into account, the total number of mononuclear cells can be calculated. This was approximately 100-150 million cells per subject.

Ten ul of monocyte fluid was also mixed with Trypan Blue and loaded into the Neubauer counting chamber; the cytoplasm of dead or damaged cells turns blue. Cell viability was consistently greater than 97%.

Preparation of Tritiated Dexamethasone

[^{3}H] dexamethasone 86.0 Ci/mmol (Amersham, Buckingshamshire) came preconstituted. The calculation of the required concentration was as follows:

86.0 Ci	= 1mmol	(Ci = Curie)
1 Ci	= 1/86 mmol	
1μCi	= 1/86 nmol	and because 1μl = 1μCi
250 μl	= 250 x 1/86 nmol	
	= 2.907 nmol	
1ml	= 2.907 x 4 nmol	
	= 11.63 nmol and	
1000ml	= 11.63 μmol	
	= 11.63 x10^{-6} M	
	= 1.163 x10^{-5} M	

Thus tritiated dexamethasone was a 1.163 x10^{-5} M solution.

Therefore to bring to a 10^{-6} M solution, a 10 fold dilution was needed and therefore the dilution factor was 11.63. Next, to constitute a desired concentration one divides by the dilution factor; therefore, for 100µl stock solution, 8.6 µl [^{3}H] dexamethasone, + 91.4µl RPMI yielded a 10^{-6} M solution (100/11.63 = 8.6).

Similarly 40µl [^{3}H] dex 10^{-6} M solution + 360ul RPMI yielded
400µl [^{3}H] dex 10^{-7} M = 100 nM and taking 200µl of this + 200ul RPMI yielded 400µl [^{3}H] dex $5x10^{-8}$ M = 50 nM and sequentially half diluting resulted in: 2.5×10^{-8} M = 25 nM

1.25×10^{-8} M = 12.5 nM

0.625×10^{-8} M = 6.25nM

0.312×10^{-8} M = 3.12 nM.

Finally, note that when an equal volume of cells was added, all the above concentrations were halved. After I mastered this, I could vary the concentrations by a third or quarter to derive different binding isotherms.

Principle of the dexamethasone binding assay.

Dexamethasone is preferred to cortisol in binding experiments because it binds more tightly to GR, dissociates more slowly and it is less susceptible to metabolic inactivation.

Receptor assays have the same kinetics as enzyme assays. The problem with GR in the assay is that there is a large amount of low affinity non-specific (or non-saturable) binding that is almost unlimited. This can be overcome by using in the assay, a series of results that are due to the ligand competing with itself i.e. non-radioactive labelled competing with radio-active labelled ligand. The non- labelled agent is put in the system in more than a 100 fold excess so that the labelling only occurs at the non-saturable sites.

By subtracting the non-specific binding, the specific binding can be determined. Also by utilising precise molar concentrations and radioactivity, the number of receptor sites can be computed.

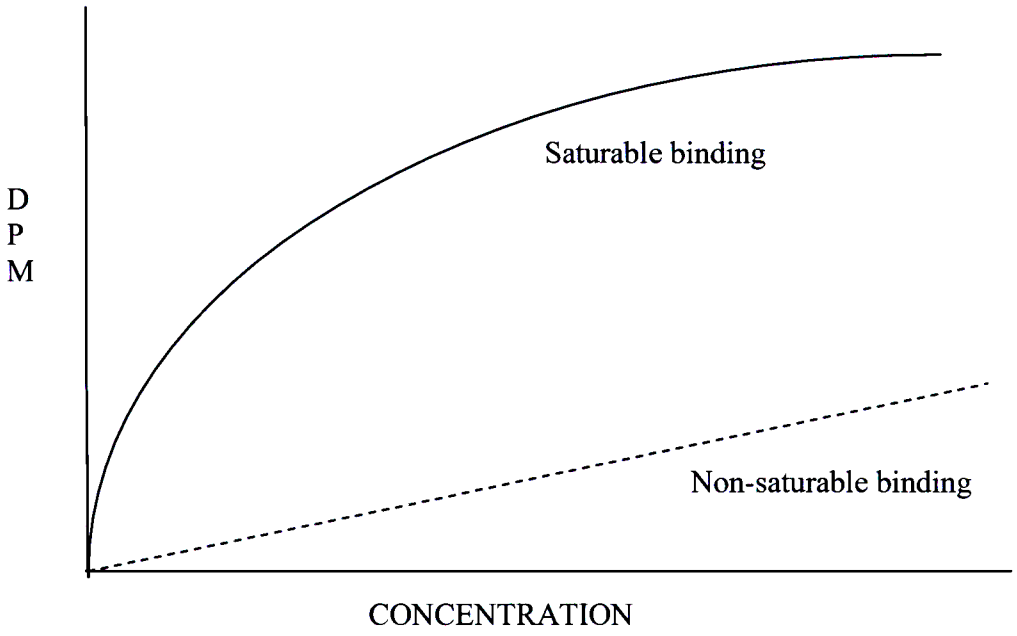


Fig 6: Graphical representation of the principle of radioligand binding studies showing saturable and non-saturable binding isotherms.
DPM = disintegrations per minute.

Tritiated dexamethasone binding assay.

GR binding characteristics of PBMCs were performed immediately after fractionation in an adaptation of the method of Crabtree [312] and exactly as described by Kam and colleagues [222]. 1.5 x 10^6 cells in 25 µl fresh RPMI were incubated with increasing concentrations of 25 µl [^{3}H] dexamethasone 86.0 Ci/mmol (Amersham, Buckingshamshire) from 0.312 to 100nM in eppendorfs for 1 hour in a shaking water bath at 37°C. Non-specific binding was measured by incubating 1.5 x 10^6 cells with 100nM and 33.3nM [^{3}H] dexamethasone with 1000 fold excess unlabelled dexamethasone each. All experiments were performed in duplicate. After incubation the tubes were centrifuged and 20 µl of supernatant aspirated for measurement of free [^{3}H] dexamethasone. The rest of the supernatant was discarded.

Nuclear Receptor Radioactivity Measurement:

Hypotonic lysis of cells in 1 set of eppendorfs was performed with 1.2 ml of 10mM Na_2 Mo O_4 and 1.5mM $MgCl_2$ (designed to stabilize the corticosteroid-nuclear complex) on ice for 30minutes. All tubes were then centrifuged for 5 minutes at 12000 rpm at 4C. The supernatant was discarded, the nuclear pellet dried and the tip of the eppendorf cut and placed in a β vial in a spectrometer.

Cytoplasmic Receptor Radioactivity Measurement:

Hypotonic lysis of the other set of PBMC's were recovered by vigorous mixing with 100 µl of 1.5 mM $MgCl_2$ containing dextran coated charcoal (this had been pre-prepared with 10ml $MgCl_2$ plus 0.1g charcoal and 0.01g dextran m.w. 60 000 to 90 000) and placed on ice for 30 minutes. Thereafter all eppendorfs were centrifuged for 5 minutes at 12 000 rpm at 4C. 100 µl of supernatant was carefully aspirated without disturbing the charcoal pellet (which can cause major contamination as this adsorbs the free steroid whilst the cytoplasmic complexes remain in the supernatant) and placed in a β vial with Bioflur for liquid scintillation counting. Specific binding for each concentration was calculated after correcting for non- specific binding.

Binding isotherms were analyzed with the PRISM curve-fitting program (GraphPad Software, San Diego, Calif).

Trial of high dose prednisone.

A random selection of the cohort was chosen to assess the effect, if any, of a short course of high dose prednisone, on lung function and glucocorticoid receptors

Seven SS patients had a course of 40 mg of oral prednisone, and 9 steroid-dependent patients supplemented their daily dose of oral prednisone to a total of 40 mg/d – both for 7 days. FEV_1 was measured before and after this trial of prednisolone, and blood was taken for GR binding experiments as well.

Data Analysis.

Data are expressed as means ± SEM of independent observations. The results obtained before or after drug treatment were compared by means of ANOVA. Median effective concentration calculations were performed with the GraphPad Prism program.

RESULTS

GR ligand binding of PBMCs.

There was no significant difference in the cell profile of the PBMCs isolated from any subject group.

Tables 3-5 show the distribution of affinity and total GR numbers per mononuclear cell in the individual patient and normal subjects.

Table 3: Glucocorticoid Receptor Number per cell and Affinity in the Oral Steroid Dependant Group

Name	Nuclear		Cytosolic		TOTAL
	Kd	Receptors	Kd	Receptors	
AC	76.68	8062	2.09	681	8743
MM	31.00	7030	3.30	824	7854
BB	55.23	9597	6.48	602	10199
AG	32.37	2401	4.70	710	3111
LH	67.92	3098	2.23	306	3404
RB	29.75	1502	3.97	473	1975
SL	30.20	4630	5.57	1132	5762
JR	20.29	2269	4.28	666	2935
TD	68.74	1935	5.42	393	2328
JM	36.78	1515	1.21	516	2031
GA	32.53	3623	4.02	832	4455
JS	62.97	7242	1.01	315	7557
AF	25.81	5242	5.00	362	5604
DH	16.52	4088	7.67	927	5015
TT	23.63	5089	6.25	543	5632
RR	47.98	6005	8.16	510	6676
JB	48.34	6448	4.95	672	7120
KP	32.63	2702	7.18	778	3480
JW	46.72	4738	4.82	684	5422
MEAN	41.37	4590	4.65	628	5224
SD	17.83	2351	2.05	217	2375

Kd, Nuc & Cyt: $p < 0.0001$
Receptors, Nuc & Cyt: $p < 0.001$

Table 4: Glucocorticoid Receptor Number per cell and Affinity in the Steroid Sensitive Asthmatic Group

Patient	Nuclear		Cytosolic		TOTAL
	Kd(nM)	Receptors	Kd(nM)	Receptors	
LW	23.95	2039	4.56	344	2383
TT	22.84	1603	6.23	537	2140
JJ	27.54	2715	2.71	289	3004
AF	25.51	1885	5.26	181	2066
SM	22.16	1798	4.25	363	2161
SS	28.20	2672	4.27	458	3130
JGB	27.22	4250	9.40	759	5009
ST	21.13	4374	6.85	798	5172
UBS	27.20	3640	3.97	484	4124
WH	27.86	5020	2.86	682	5702
MEAN	25.36	3000	5.04	490	3489
SD	2.63	1233	2.01	206	1400

Kd, Nuc & Cyt: $p < 0.001$
Receptors, Nuc& Cyt: $p<0.001$

Table 5: Glucocorticoid Receptor Number per cell and Affinity in Normal Subjects

Name	Nuclear		Cytosolic		TOTAL
	Kd(nM)	Receptors	Kd(nM)	Receptors	
EI	7.74	2248	3.22	1075	3323
OL	4.15	1743	4.25	450	2193
TH	11.62	2379	4.94	745	3124
TW	15.12	4382	2.00	469	4851
YN	13.80	4450	4.92	786	5236
HK	10.69	3390	2.56	464	3854
PP	6.77	4032	1.66	321	4353
HP	5.32	3537	4.28	510	4047
KJS	7.13	2080	2.85	279	2359
LD	9.65	4866	4.06	851	5717
RN	8.81	1965	1.53	293	2258
MEAN	9.40	3270	3.48	603	3873
SD	4.01	1033	1.30	246	983

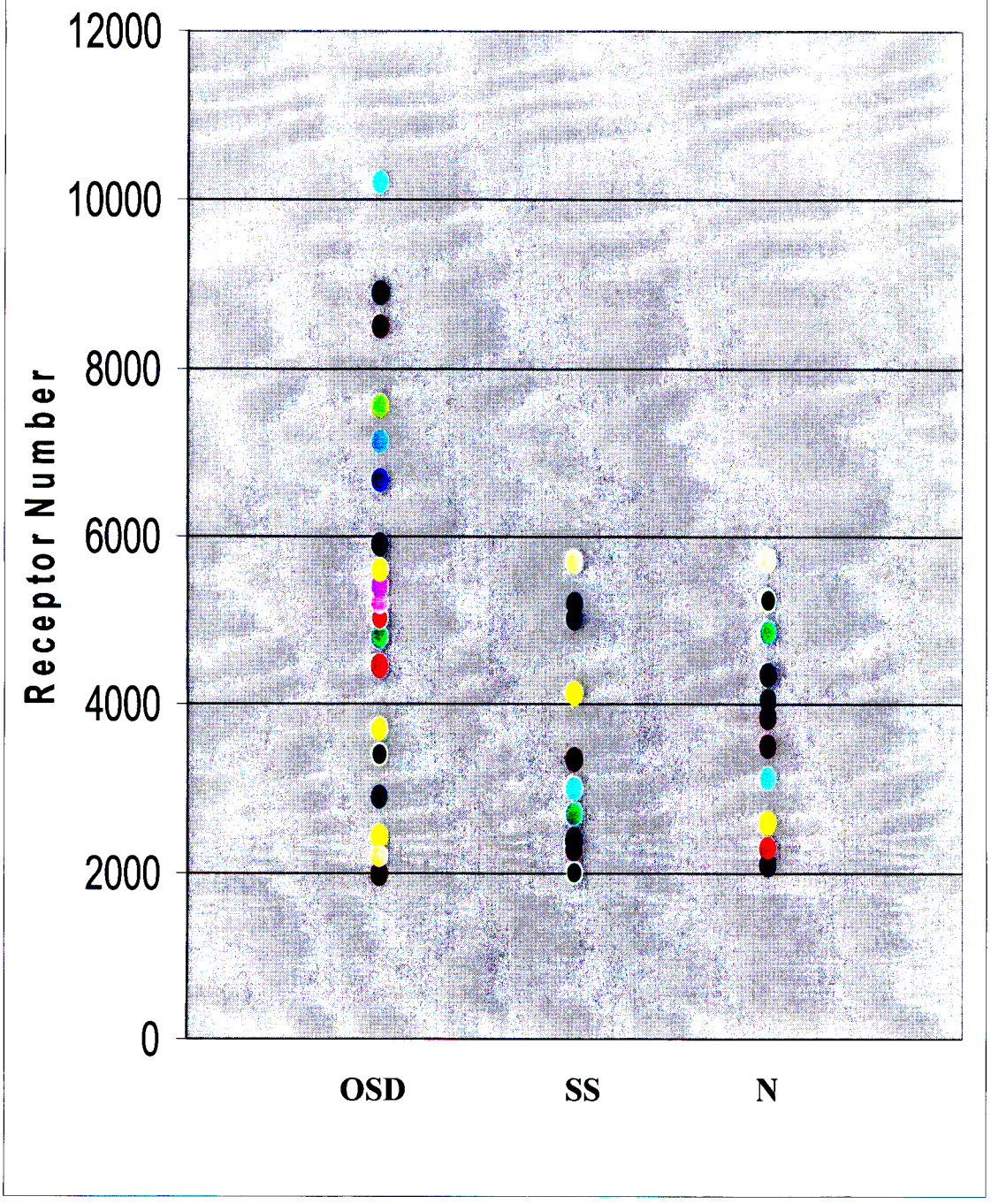
Kd, Nuc & Cyt: $p<0.01$
Receptors, Nuc & Cyt: $p<0.01$

Glucocorticoid Receptor Numbers.

Fig 7a illustrates these parameters in comparative form. Although the OSD group had a tendency to higher GR numbers, there was no statistically significant difference in nuclear receptor numbers between subject groups (steroid-dependent group, 5224 ± 2375/cell; ICS group, 3489 ± 1400/cell; healthy subjects, 3873 ± 983/cell;). The number of receptors was higher in the nucleus than in the cytoplasm (up to 10 fold higher than and as seen by Spahn as well [219]).

There were no statistically significant differences between the groups for the number of cytoplasmic receptors.

Fig 7a: Total Glucocorticosteroid Receptor Numbers in the Individual Patients in the Different Categories



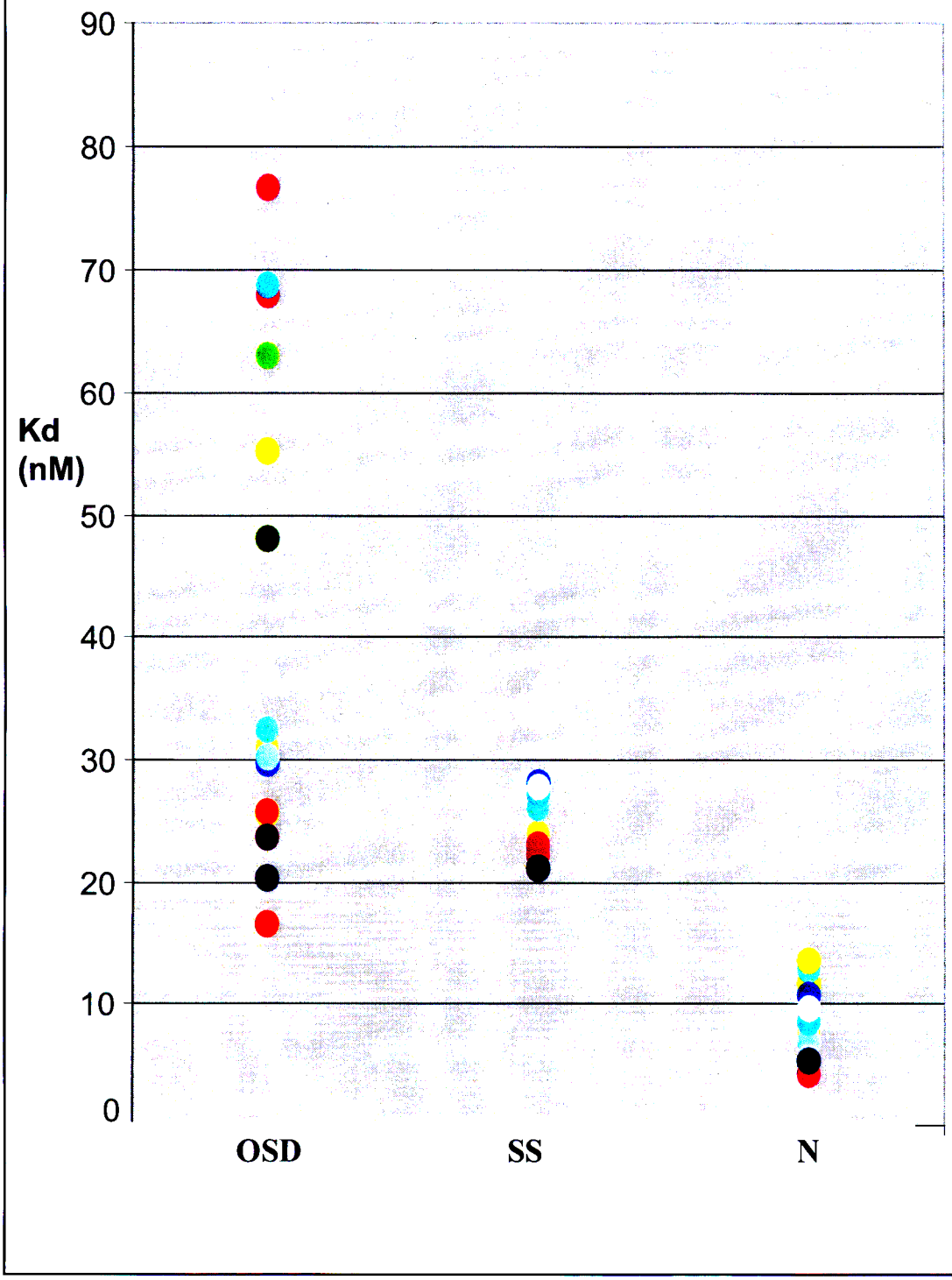
GR Affinity.

The individual ligand binding affinities of all the subjects is illustrated in Tables 3-5 and compared in Fig 7b and 7c.

The ability of dexamethasone to bind to GR was decreased in the nucleus in subjects with oral steroid-dependent asthma. Steroid-dependent subjects (Kd, 41.37 ± 17.83 nmol/L-NB: the higher the Kd, the lower the affinity) had a greater Kd compared with those with ICS controlled asthma symptoms (Kd, 25.36 ± 2.63 nmol/L; $P < .05$) or healthy subjects (Kd, 9.40 ± 4.01 nmol/L; $P < .001$). In addition, there was a significant difference in Kd between the ICS group and the healthy group ($P < .05$);

GR affinity in the cytoplasm was high in all three groups and no statistically significant differences were detected amongst any of the groups.

Fig 7b: Individual GR Affinity in the Three Subject Groups



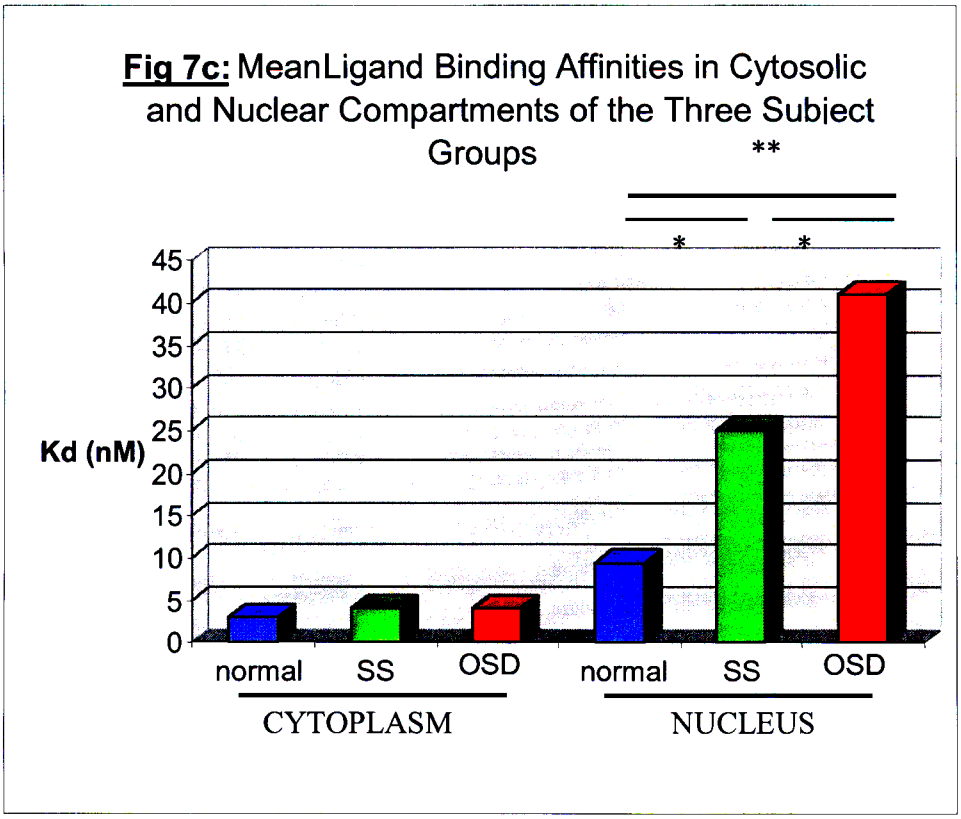


Fig 7c: GR binding parameters of PBMCs isolated from healthy subjects and patients with asthma. Ligand-binding affinity *(Kd)* in cytosolic and nuclear compartments was measured in 11 healthy subjects, 19 oral steroid–dependent *(OSD)* asthmatic patients, and 10 asthmatic patients whose symptoms were controlled with ICSs *(SS)*.

In the nuclear compartment, Steroid-dependent subjects (Kd, 41.37 ± 17.83 nmol/L) had a greater Kd compared with those with ICS controlled asthma symptoms (Kd, 25.36 ± 2.63 nmol/L;) or healthy subjects (Kd, 9.40 ± 4.01 nmol/L).

* $p < 0.01$ ** $p < 0.001$

Statistically, the differences in Kd between the nuclear & cytoplasmic compartments ranged from p= 0.001 to 0.0001 between the groups.

Response to Oral Prednisolone.

The response to an oral steroid burst in the asthmatics and changes in the glucocorticosteroid receptor status is shown in Tables 6 a,b,c and 7a,b and Fig 8.

Table 6a: Glucorticoid receptor affinity and numbers in the nuclear and cytoplasmic compartments before and after the prednisone burst

ORAL STEROID DEPENDENT GROUP

		Nuclear		Cytoplasm		Total
		Kd	Receptors	Kd	Receptors	
BB	pre	55.23	9597	6.48	602	10199
	post	45.37	4724	9.52	410	5134
JS	pre	62.97	7242	1.01	315	7557
	post	52.68	6289	5.79	707	6996
JR	pre	20.29	2269	4.28	666	2935
	post	16.36	1813	5.29	159	1972
LH	pre	67.92	3098	2.23	306	3404
	post	32.27	2968	1.15	196	3164
GA	pre	32.53	3623	4.02	832	4455
	post	25.30	2957	3.24	684	3641
MM	pre	31.00	7030	3.3	1648	8678
	post	46.84	3470	5.41	282	3752
DH	pre	16.52	4088	7.67	927	5015
	post	15.83	2290	4.56	674	2964
TD	pre	68.74	1935	5.42	393	2328
	post	49.23	1712	4.13	361	2073
JB	pre	48.34	6448	4.95	672	7120
	post	40.47	5865	3.33	458	5323

Although most subjects demonstrated a decrease in GR Kd and numbers, this did not reach statistical significance.

Table 6b: Glucorticoid receptor affinity and numbers in the nuclear and cytoplasmic compartments before and after the prednisone burst

STEROID SENSITIVE GROUP

		Nuclear		Cytoplasm		Total
		Kd (nM)	Receptors	Kd(nM)	Receptors	
SS	pre	28.2	2672	4.27	458	3130
	post	26.9	2412	3.5	477	2889
TT	pre	23.00	1603	6.00	537	2140
	post	19.61	1094	4.89	762	1856
LW	pre	24.00	2039	5.00	344	2383
	post	5.59	1480	1.10	648	2128
ST	pre	27.10	4374	6.85	798	5172
	post	23.97	3257	1.56	174	3431
JGB	pre	27.20	4250	9.40	759	5009
	post	15.14	3432	1.80	287	3719
UBS	pre	27.20	3640	3.97	484	4124
	post	27.6	3740	4.86	478	4218
WH	pre	27.90	5020	2.86	682	5702
	post	26.49	3482	4.42	323	3805

Again, although most subjects demonstrated a decrease in GR Kd and numbers, this reached statistical significance in the steroid sensitive group for receptor number only.

Table 7: Lung Function, depicted as FEV1% predicted before and after the prednisone course

ORAL STEROID DEPENDENT GROUP			STEROID SENSITIVE GROUP		
		%			%
BB	pre	72.5	ST	pre	71.3
	post	81.5		post	74.2
JS	pre	32.5	JGB	pre	61.9
	post	65.1		post	49.3
JR	pre	35.7	JJ	pre	65.4
	post	45.5		post	60.2
LH	pre	76.6	TT	pre	72.5
	post	101.3		post	60.4
GA	pre	60.3	LW	pre	75.1
	post	62.1		post	90.3
MM	pre	45.2	UBS	pre	80.2
	post	43.3		post	120.2
DH	pre	57.1	SS	pre	83.4
	post	86.2		post	80.1
TD	pre	67.5			
	post	64.9			
JB	pre	53.4			
	post	53.4			

Although many subjects had an improvement in spirometric indices, this was not statistically significant.

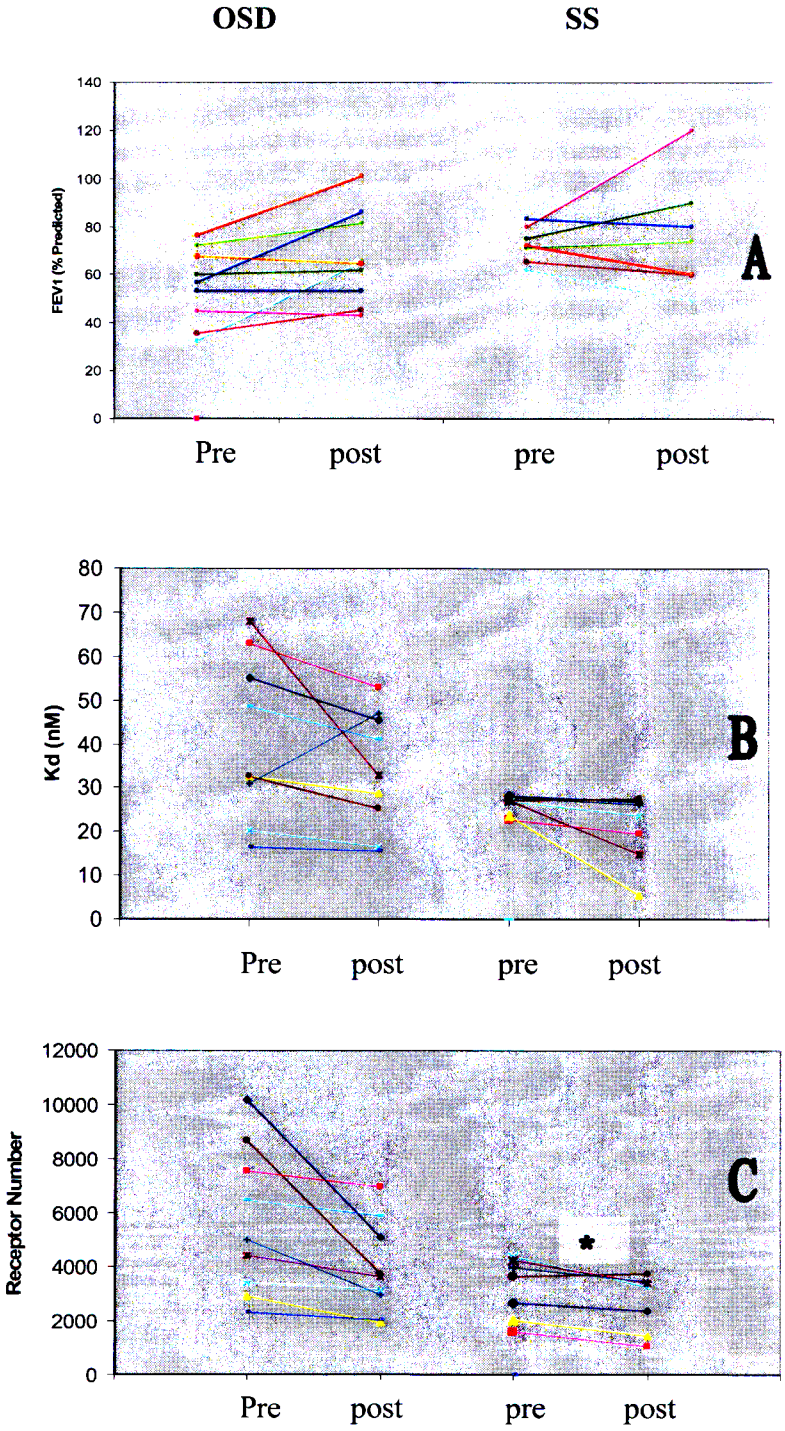


Fig 8: The effect of prednisolone (40 mg/d for 7 days) on percent predicted FEV1, GR affinity and receptor number in 9 oral-steroid-dependent and 7 SS subjects is shown. Data are expressed as individual data points (*p < .05).

Seven patients in the SS group had a baseline percent predicted FEV1 of 72 .8 ± 7.6 %, and (although increased) this was not significantly changed by a week of 40 mg of oral prednisolone (76.4 ± 23.7 % of predicted value). Nine steroid-dependent patients had their oral prednisolone increased to 40 mg for 1 week. These patients demonstrated a mean improvement of 20% ± 0.4% in FEV1 after a 1-week course of 40 mg of prednisolone (55.6 ± 15.5% of predicted value improved to 67.8 ± 7.6 %; Fig 7A). Treatment of ICS and steroid-dependent asthmatic subjects with prednisolone (40 mg/d) for 1 week did not significantly affect the Kd of ligand binding or receptor number (although most decreased, Fig 7 B, C). The altered ability of steroid-dependent and steroid-sensitive asthmatic subjects to respond to steroids may therefore relate to their reduced nuclear GR affinity for ligand. Because prednisolone did not normalise this altered affinity, this further suggests that reduced glucocorticoid responsiveness in steroid-dependent subjects is not caused by prednisolone-induced downregulation of GR expression.

CHAPTER 4: Characterisation of an in-vitro "Steroid Resistance Model" in Mononuclear Cells

Having established that oral steroid dependent asthmatics had an alteration in GR affinity, we were keen to explore the functional consequences of this altered affinity. However, we were aware that mononuclear cells incubated in medium alone in vitro for 48 hours and longer exhibit a normalisation of GR affinity. Thus it would be difficult to understand the ramifications of altered affinity if this was normalising during incubation in vitro. The first step was to confirm that GR affinity returned to normal in vitro.

4.1: Effect of incubating PBMC's with diminished GR affinity in medium alone for 48 hours.

METHODS

Mononuclear cells were isolated as described previously. In preparation for stage 2 of the study, cells were incubated in RPMI alone for 48 hours. Thereafter cells were harvested and GR affinity and numbers were determined as described in the preceding chapter and compared to results immediately prior to incubation.

RESULTS

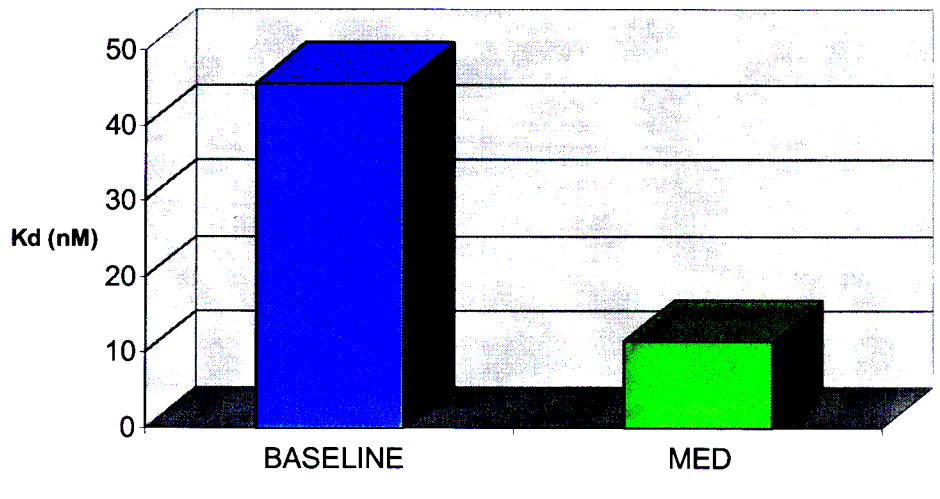
GR characteristics are depicted in Table 8 and 9 and Fig 9.

There were highly significant changes in Nuclear GR affinity in cells incubated in medium with GR affinity consistently normalising (from 45.60 ± 14.75 to 11.43 ± 2.06). This was typically associated with a decrease in the mean number of receptor sites (by approximately two thirds) as a reciprocal relationship exists between affinity and receptor numbers.

Table 8: Glucocorticoid Receptor Number and Affinity in the Nuclear compartment in PBMC's in the oral steroid dependent asthmatic subjects at baseline and after 48 hours incubation in medium alone.

Name	Nuclear: baseline		Nuclear: post 48 hr incubation in medium	
	Kd (nM)	Receptors	Kd (nM)	Receptors
MM1	31.00	7030	11.50	2374
BB1	55.23	9597	12.87	871
AG1	32.37	2401	10.97	1541
LH1	67.92	3098	10.52	1228
JW1	46.72	4738	7.15	677
KP	32.63	2702	13.23	910
TD1	68.74	1935	13.89	1191
MM2	46.84	3470	11.80	2784
BB2	45.37	4724	13.06	1322
AG2	29.13	2689	9.27	1347
MEAN	45.60	4238	11.43	1425
SD	14.75	2411	2.06	667
Kd: p< 0.00001 Receptors: p=0.002				

Fig 9: Normalisation of GR affinity (mean) in the nuclear compartment after 48 hours incubation of PBMC's in medium alone.



Cytoplasmic GR affinity (Table 9) also improved, however, receptor numbers did not change significantly. In view of the major mechanism of action of corticosteroids being exerted in the nucleus, I concentrated mainly on the nuclear component of GR characteristics in further experiments.

Table 9: Glucocorticoid Receptor Number and Affinity in the cytoplasmic compartment in PBMC's in the asthmatic subjects at baseline and after 48 hours incubation in medium alone

Name	**Cytosolic: baseline**		**Cytosolic: post 48hr incubation in medium**	
	Kd (nM)	**Receptors**	**Kd (nM)**	**Receptors**
MM1	3.30	1648	1.20	738
BB1	6.48	602	1.10	225
JW1	4.82	684	1.69	704
LH	2.23	306	2.39	348
TD1	5.42	393	6.21	484
MM2	5.41	282	5.60	274
BB2	9.52	410	2.29	380
GA2	3.24	684	4.45	640
MEAN	5.05	626	3.12	474
SD	2.29	443	2.02	199
Kd: p=0.05 **Receptors:p=0.39**				

4.2: Establishing an in-vitro "Steroid Resistance Model" with mononuclear cells.

Having documented that PBMC's develop normal GR affinity after incubation in vitro, we then attempted to decrease GR affinity by co-incubating cells with IL-2 and IL-4 in combination (and later IL-13). These were based on the experiments of Sher et al [216] after the prior detection of exclusively increased mRNA for these cytokines in BAL lymphocytes from steroid resistant asthmatics.

Methods:

Experiments with Cells Incubated with Cytokines

Cells were fractionated as described previously and then, at a concentration of 1 x 10^6/mL in RPMI with HEPES (20 mmol/L) supplemented with 10% FCS, penicillin, streptomycin, and amphotericin B were cultured for 48 hours at 37°C in medium alone and in the presence of IL-2 (50 IU/mL; R&D Systems, Abingdon, United Kingdom) with or without IL-4 (50 IU/mL, R&D Systems) and IL-13 (50 IU/mL, R&D Systems) alone. Thereafter, cells were harvested and washed in fresh RPMI after which binding experiments were performed as described previously.

RESULTS

IL-2&IL-4 or IL-13 Alone Alter GR Ligand Binding In-vitro.

The results of these experiments are depicted in Table 10 and Fig 9.

Incubation of cells with Il-2,-4 in combination led to a significant increase in GR Kd compared to that of cells in medium alone; 42.16 ± 7.0 vs 11.06 ± 2.05, $p < 0.001$. This was accompanied by a significant increase in the number of receptors; usually doubling in magnitude.

Table 10: Glucocorticoid Receptor Number and Affinity in the Nuclear compartment in PBMC's in the asthmatic subjects and normal subjects (N) after 48 hours incubation in medium alone and with IL-2 and IL-4.

Name	Nuclear: medium		Nuclear: post 48 hr IL-2,-4 incubation	
	Kd (nM)	Receptors	Kd (nM)	Receptors
MM1	11.50	2374	34.20	8442
BB1	12.87	871	50.24	4801
AG1	10.97	1541	35.01	1932
LH1	10.52	1228	38.38	2179
JW1	7.15	677	48.30	3293
KP	13.23	910	43.06	2877
TD1	13.89	1191	36.64	2835
MM2	11.80	2784	37.58	3137
BB2	13.06	1322	59.03	4480
AG2	9.27	1347	44.79	4318
N1	8.04	2422	40.23	7216
N2	9.86	2276	41.43	6860
N3	11.64	2610	39.16	7257
MEAN	11.06	1658	42.16	4587
SD	2.05	731	7.00	2176
Kd: p<0.0001				
Receptors: p=0.0001				

Treatment of PBMC's from oral steroid dependent asthmatics with IL-2,-4 (who had a high Kd initially), would maintain their high Kd whilst PBMC's from steroid sensitive asthmatics would also develop a higher Kd of approximately 40 nmol/l (compare Tables 3 and 4 to Table 10).

To summarise – no matter the source of PBMC's (any asthma severity or normal subjects- GR nuclear affinity would always decrease with co-incubation of IL-2,-4 and normalize (increase) in medium alone.

Fig 10: Establishment of the Steroid Resistance Model: Nuclear GR Affinity is Reduced (High Kd) in PBMC's Exposed to IL-2, -4 Compared to Medium

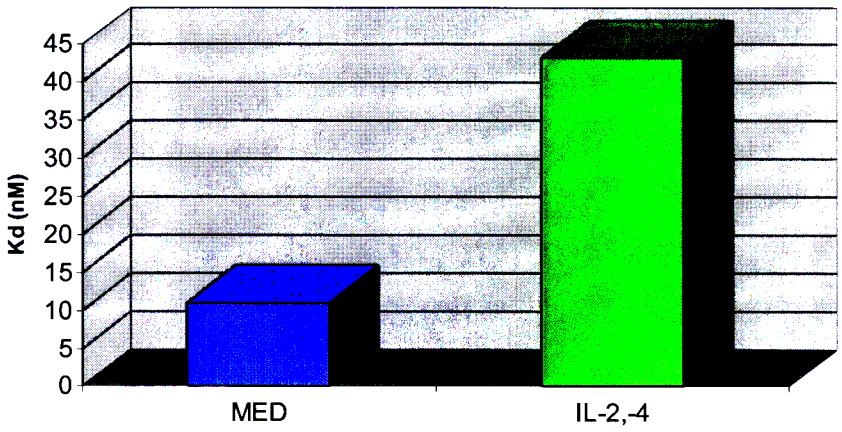


Fig 10: Confirmation of in-vitro steroid resistance as originally described by Sher et al [216]. Incubation of PBMC's in medium alone increases GR affinity (Low Kd) but co-incubation with IL-2 and IL-4 decreases GR affinity (high Kd- values similar to the oral steroid dependant group; $p < 0.0001$).

Fig 11: The Steroid Resistance Model - Increase in GR Number in Cells Incubated with IL-2, -4 Compared to Medium Alone

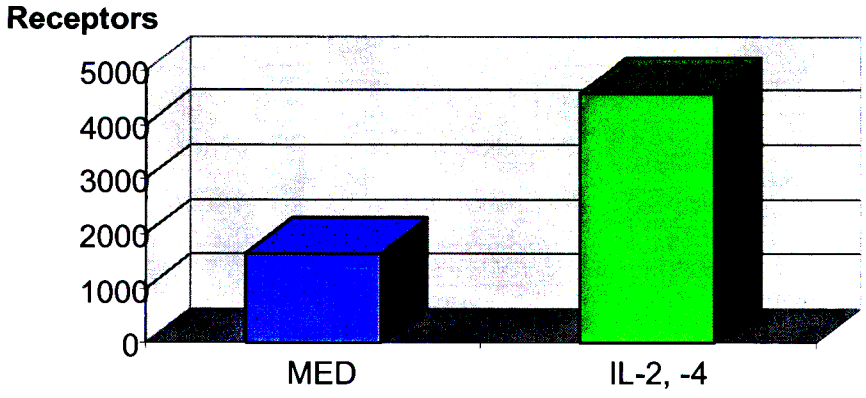


Table 11and Fig 12 illustrate an example of a patient with a poor affinity at baseline (high Kd- pre-incubation) and following culture in media alone for 48 hours resulted in a reversion of the altered nuclear Kd back to levels seen in healthy subjects. This effect was prevented by treatment of cells with IL-2 and IL-4 but not by treatment with either cytokine alone.

Table 11: Example of High Kd in OSD subject (JW) at baseline and normalisation in medium, IL-2 and IL-4 alone but preservation with this cytokine combination.

		Kd (nM)		
BASELINE	MEDIUM	IL-2 alone	IL-4 alone	IL2,-4
46.7	7.15	9	8.4	48.3

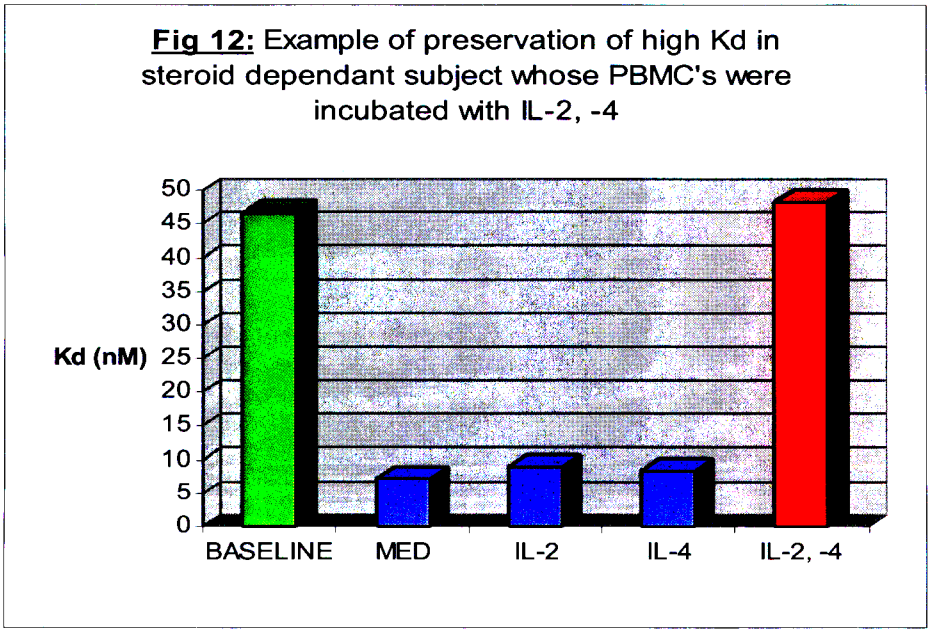


Fig 12: High GR Kd in PBMC's at baseline, ex vivo. After 48 hrs incubation in medium, IL-2, IL-4 alone GR affinity normalised. High Kd was maintained only with IL-2, -4 in combination.

I

IL-13 (50 IU/mL) alone also reduced GR ligand-binding affinity at 48 hours (12.39 ± 2.82 vs 38.91 ± 3.13 nmol/L, $p < .05$: Fig 13).

Table12: Effect on nuclear GR Kd and number in PBMC's incubated in medium with and without Interleukin 13 for 48 hours.

	Medium			IL-13	
	Kd (nM)	Receptors		Kd (nM)	Receptors
Subject1	9.47	1172		42.37	4271
Subject2	12.61	1357		36.26	3821
Subject3	15.1	933		38.1	4131
MEAN	12.39	1154		38.91	4074
SD	2.82	213		3.13	230

Kd, med & IL-13: p=0,006
Receptors, p=0.006

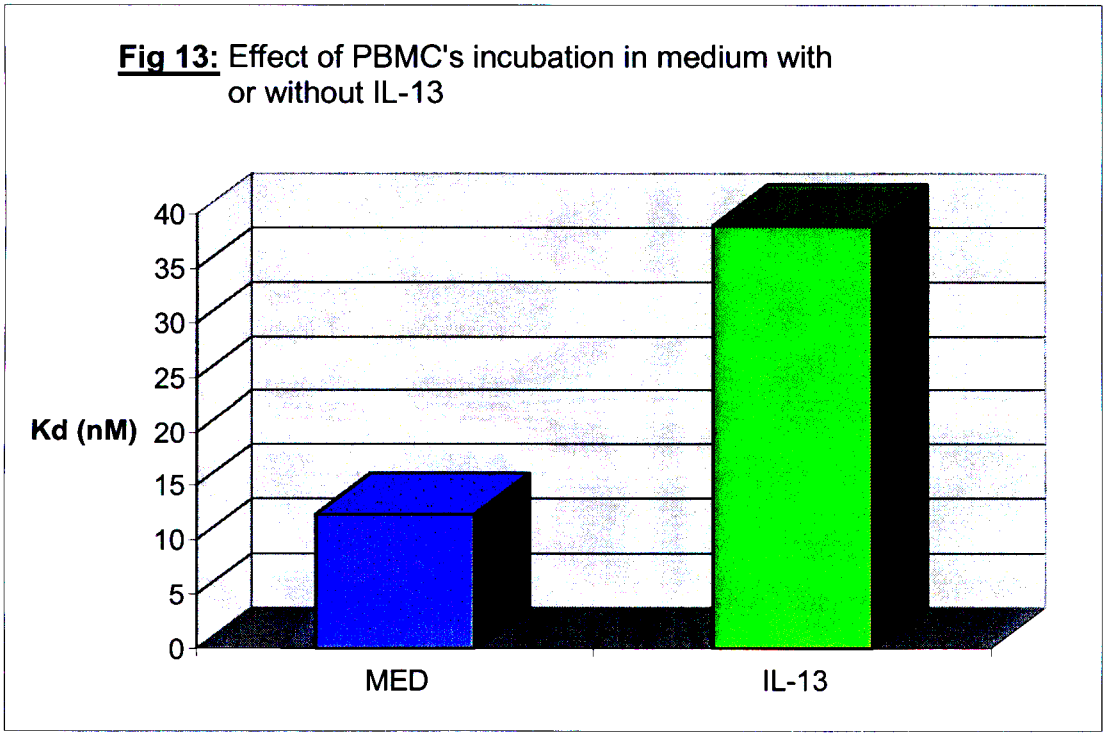


Fig 13: Confirmation that IL-13 alone also decreases GR affinity (high Kd)

4.3: Functional Consquences of Altered GR Affinity.

Having ensured that we could maintain a decreased GR binding capacity in vitro, we could now determine if there were any functional intracellular sequelae. We chose to examine the cytokine output following stimulation to ascertain if mononuclear cells are involved in the perpetuation of a pro-inflammatory state in asthma. In this regard, we chose interleukin 10 as the anti-inflammatory cytokine and GM-CSF as the protagonist of inflammation. We also co-incubated cells with dexamethasone to detect steroid resistance in vitro: i.e.-was there a difference in the dexamethasone suppressive effects in cytokine production with induction of altered GR affinity.

Methods:

GM-CSF and IL-10 Release: Experimental procedures.

PBMCs were fractionated as described previously.

Cells, (1x 10^6/mL) were incubated with medium alone or IL- 2 and IL-4 (50 IU/mL) for 48 hours in the presence of 1% FCS and thereafter stimulated with LPS (10 ng/mL; Sigma, Poole, United Kingdom) or phorbol 12-myristate 13-acetate (PMA)& Phytohemagglutinin (PHA) for 20 hours at 37°C. Plates were centrifuged, supernatants were collected, and GM-CSF and IL-10 were measured with a sandwich ELISA. GM-CSF and IL-10 concentrations in the culture supernatant were measured by using a specific ELISA calibrated with human recombinant GM-CSF (0-200 pg/mL, R&D Systems) or IL-10 (0-200 International Units/mL, R&D Systems-see next page).The concentration-dependent effects of steroids on cytokine release were also studied by adding 10^{-6}M and 10^{-7}M dexamethasone to the non-control plates for IL-10 measurement. (NB In simultaneous experiments the alteration in GR affinity was confirmed in cells incubated with IL-2,-4 as described in section 4:2).

Enzyme-linked Immunosorbent Assay for IL-10 and GM-CSF.

These cytokines were assayed using a quantitive sandwich enzyme immunoassay technique. A commercially available kit for IL-10 was used (Quantikine: R & D systems, Abingdon, Oxon, UK). Monoclonal anti IL-10 was coated onto a microtitre plate, to which standards and samples were added. An enzyme linked polyclonal antibody specific for IL-10 was added to the wells to sandwich immobilized IL-10.

Addition of a stabilized chromogen and hydrogen peroxide led to a colour development proportional to the quantity of IL-10. Samples were assayed by measurement of optical density using a spectrophotometer set to 450 nm, the lower limit of detection being 10 IU/ml.

The GM-CSF assay utilised round bottom plates that had been coated overnight with rat anti-human GM-CSF monoclonal antibody (50 µg of 2 µg /ml) at 4 ° C. After washing with phosphate buffered saline (PBS) / PBS / 10 % fetal call serum (200 µg; 2h). GM-CSF samples and standards were added to the plate overnight at 4 ° C and washed with PBS/Tween. Thereafter a biotinylated secondary anti GM-CSF antibody (100µl of 2 µg /ml in PBS /10% fetal calf serum) and 1:400 avidin peroxidose solution were sequentially added. After washing GM-CSF was measured colourmetrically at 405nm and quantified by interpolation from a standard curve. The lower limit of detection was 16 pg/ ml.

RESULTS

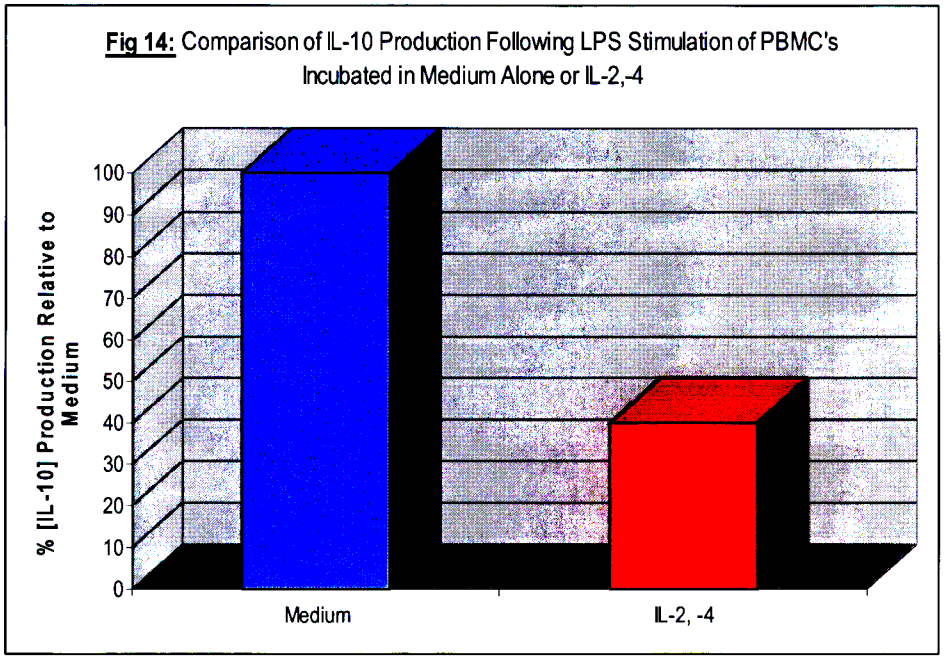
Effect of IL-2/IL-4 on GM-CSF and IL-10 Release.

The results are tabulated in Tables 13-15.

The most striking effect was the reduction in IL-10 output in cells rendered resistant in vitro; IL-10 production as measured in the supernatants was a mean of only 40% in cells treated with IL-2,-4 compared to cells in medium alone with presumed normal GR affinity; p=0.08 (Fig 14).

Table 13: Absolute Interleukin-10 production (IU/ml) by mononuclear cells at 60 hrs incubation in medium with and without IL-2,-4 following LPS stimulation.

Patient	Medium	IL-2,-4	Percentage production relative to medium
A	624	289	46
B	468	95	20
C	233	137	59
D	168	43	26
E	183	106	58
F	100	15	15
G	64	11	17
H	110	90	81
Mean	262	99	40
SD	78	36	24
p=0.02			



Effect of Dexamethasone on IL-10 Release in cells co-incubated with IL-2,-4 or medium alone.

The suppressive effects of dexamethasone were studied on the stimulated IL-10 production and are presented in Table 14.

Table 14: Absolute Interleukin-10 production at 60 hrs incubation in medium with and without IL-2,-4 following LPS stimulation and the effect of varying concentrations of dexamethasone

Patient	**Incubation in Medium**				
	Post-stimulation	**+Dex 10^{-6}M**	**%inhib**	**Dex 10^{-7}M**	**%inhib**
A	624	no dose response measured			
B	468	118	75	234	50
C	233	112	52	140	40
D	168	65	61	124	26
E	183	77	58	113	38
F	100	34	66	57	43
G	64	18	72	23	64
H	110	55	50	62	44
MEAN	262		62.00		44
SD	78		9.54		12
Patient	**Incubation with Interleukin - 2,-4**				
	Post-stimulation	**+Dex 10^{-6}M**	**%inhib**	**Dex 10^{-7}M**	**%inhib**
A	289	no dose response measured			
B	95	49	48	57	40
C	137	93	32	98	28
D	43	28	35	34	20
E	106	67	27	84	21
F	15	below detection			
G	11	below detection			
H	90	82	9	87	3
MEAN	99		30.2		22.4
SD	36		14.17		13.46
			p=0.001		p=0.015

Dexamethasone (10^{-6}M and 10^{-7}M) inhibition of IL-10 production was approximately double of the amount in medium compared to IL-2,-4 treated cells where the inhibition was 10-20%; p= 0.001 & p=0.015 respectively, i.e. steroid resistance to the effects of IL-2,-4 was demonstrated in vitro.
(Fig 15)

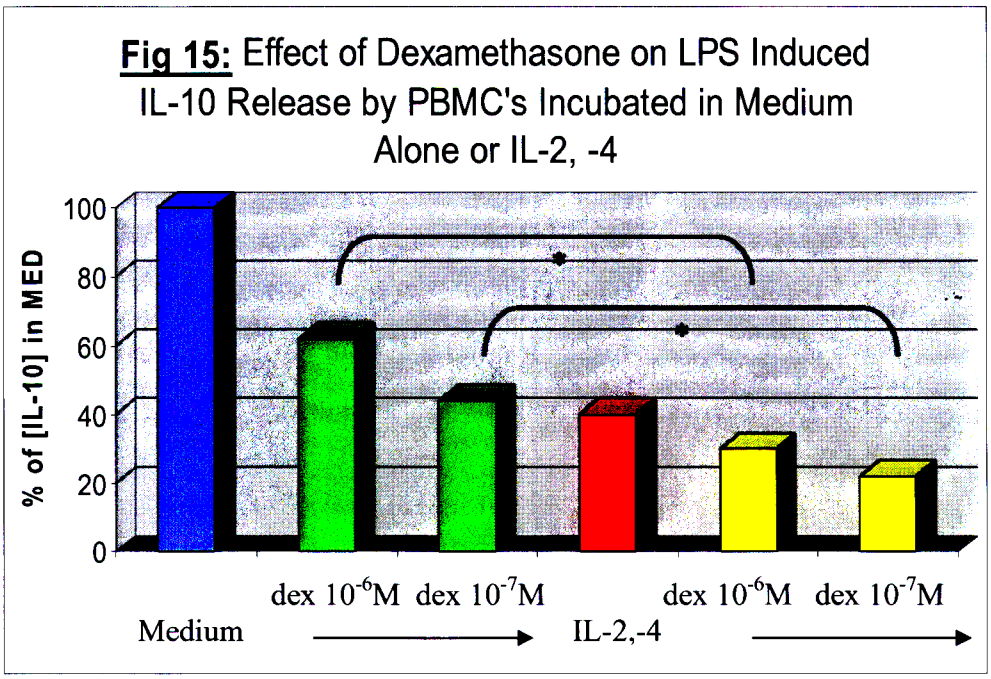


Fig 15: Results of experiments to demonstrate steroid resistance in vitro. The inhibitory effect of dexamethasone 10^{-6} M and 10^{-7} M on IL-10 release is minor compared to the dexamethasone effect on the cells incubated in medium alone, * $p<0.015$.

As regards GM-CSF, the absolute values following PMA, PHA co-stimulation are shown in Table 15 and Fig 16. PMA & PHA were chosen to give a stronger stimulus as LPS stimulation only gave low level production of GM-CSF (data not shown).

Table 15: Absolute levels of GM-CSF (pg/ml) following stimulation with PMA&PHA after PBMC incubation for 48 hours in medium alone or with IL-2,-4

	MED	**IL-2,-4**
EXPERIMENT 1	152	250
EXPERIMENT 2	90	226
EXPERIMENT 3	110	212
EXPERIMENT 4	114	186
MEAN	117	219
SD	26	27
$p<0.01$		

Following PMA & PHA stimulation, there was an approximately 2 fold increase in the amount of GM-CSF protein in the supernatant of combined IL-2/IL-4 treated cells compared to medium alone; $p<0.01$ (Fig 16).

Fig 16: Mean GM-CSF Production After PMA,PHA Stimulation After GR Affinity had Normalised or Decreased with Co-incubation with IL-2, -4

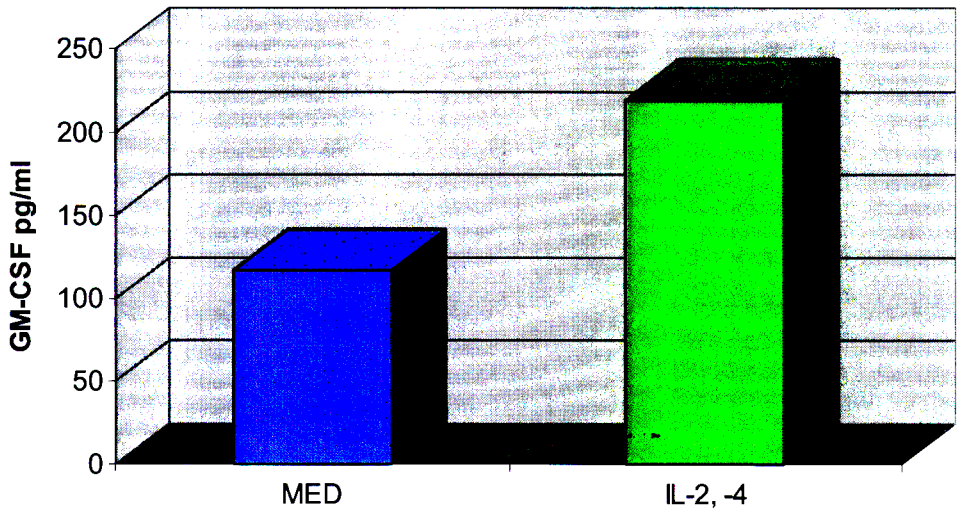


Fig 16: PBMC's were incubated in medium alone and IL-2, -4 for 48 hrs. Thereafter they were stimulated with PMA, PHA and GM-CSF measured by ELISA in the supernatant. Levels in the IL-2, -4 supernatants were almost twice the amount in media alone, $p < 0.01$.

These data on cytokine release suggest that when there is altered affinity of the glucocorticoid receptor, the output of higher GM-CSF and low IL-10 production might amplify inflammation.

CHAPTER 5: Signal-transduction Mechanisms and Functional Sequelae of Altered Affinity of the Glucocorticosteroid Receptor in-vitro.

Having established a "steroid-resistant model" we were then keen to explore how IL-2,-4 mediated altered GR affinity. There was absolutely no literature in this respect. We identified major signal transduction pathways that might react with the steroid receptor and associated transcription factors that could induce altered affinity. These were protein kinase C, Inositol 3-P kinase the Mitogen Activated Protein Kinase system and interleukin-10 and theophylline associated pathways. Specific inhibitors of the first three pathways existed and we postulated that by their co-incubation in our model, we could define possible interactions.

5.1: Experiments with Cells Incubated with Cytokines and Inhibitors of Signal Transduction.

METHODS:

PBMC's were isolated as described previously.

Cells, at a concentration of 1 x 10^6/mL in RPMI with HEPES (20 mmol/L) supplemented with 10% FCS, penicillin, streptomycin, and amphotericin B were cultured for 48 hours at 37°C in medium alone and in the presence of IL-2 (50 IU/mL; R&D Systems, Abingdon, United Kingdom) and IL-4 (50 IU/mL, R&D Systems) or IL-13 (50 IU/mL, R&D Systems). The effects of the signal-transduction pathway inhibitors wortmannin (5 nmol/L), PD098059 (10 μmol/L), Ro318220 (10 nmol/L), SB203580 (1,3,10 μmol/L – the lower concentrations to exclude possible non-specific MAPK inhibition) and theophylline; all from Calbiochem, Nottingham, United Kingdom, and IL-10 (3 ng/mL, R&D systems) on IL-2– and IL-4-stimulated modulation of GR binding characteristics were investigated by co-incubation with IL-2,-4. (**NB-** Leung's original description of in vitro steroid resistance was with IL-2,-4 and these were used in most experiments). Thereafter, cells were

harvested and washed in fresh RPMI before binding experiments were performed.

RESULTS:

The results of GR affinity and receptor numbers are shown in Table 16a, b & 17, and Figures 17 & 18.

Inhibition of the ERK MAPK pathway by PD098059 (10 μmol/L) had no effect on the altered receptor affinity induced by IL-2,-4. Inhibition of phosphoinositol 3 hydroxy kinase by wortmannin (5 nmol/L) or treatment with Ro318220 (10 nmol/L), a specific protein kinase C inhibitor and IL-10 (10 ng/mL) also failed to modulate the effect of IL-2 and IL- 4 on receptor affinity, and theophylline similarly had no effect on affinity.

Receptor numbers were largely unchanged as well.

Table 16 (a): Results of GR binding experiments of PBMC's incubated in medium with and without IL-2,-4 and inhibitors of signal transduction: Wortmannin, IL-10 and Theophylline

	Kd (nM)			**Receptors**		
			Wortmannin			Wortmannin
	med	IL-2,-4	+ IL-2,-4	med	IL-2,-4	+ IL-2,-4
Subject1	7.15	48.30	43.09	677	2797	3293
Subject2	10.52	38.38	42.47	1228	2179	2412
Subject3	9.27	44.79	38.12	1347	4318	3996
MEAN	8.98	43.82	41.23	1084	3098	3234
SD	1.70	5.03	2.71	357	1101	794
Kd, med & IL-2,-4: p= 0.001				R, med & IL-2,-4: p=0.036		
Kd, med & IL-2,-4+W: p= 0.002				R, med & IL-2,-4+W: p=0.028		
			IL-10			IL-10
	med	IL-2,-4	+ IL-2,-4	med	IL-2,-4	+ IL-2,-4
Subject1	11.50	43.48	38.79	3459	5537	4982
Subject2	11.40	41.41	37.95	1598	6860	5208
Subject3	10.72	42.19	39.90	2999	6453	5747
MEAN	11.21	42.36	38.88	2685	6283	5312
SD	0.42	1.05	0.98	969	678	393
Kd, med & IL-2, -4: p= 0.0000				R, med & IL-2,-4: p= 0.017		
Kd, med & IL-2, -4 +IL-10 :p= 0.0000				R, med& IL-2,-4+IL-10:p= 0.05		
			Theophylline			Theophylline
	med	IL-2,-4	+ IL-2,-4	med	IL-2,-4	+ IL-2,-4
Subject1	9.27	45.12	42.38	2036	4061	4283
Subject2	10.82	41.81	44.26	1872	5272	4981
Subject3	10.24	40.10	38.94	1784	3982	4121
MEAN	10.11	42.34	41.86	1897	4438	4462
SD	0.78	2.55	2.70	128	723	457
Kd, med & IL-2, -4: p= 0.0001				R, med & IL-2,-4: p= 0.004		
Kd, med & IL-2, -4 +T :p= 0.0001				R, med & IL-2,-4+T:p= 0.003		

R= Receptors W= Wortmannin T= Theophylline

Table 16(b): Results of GR binding experiments of PBMC's incubated in medium with and without IL-2,-4 and inhibitors of signal transduction : PD98039 and Ro318220

Kd(nM)				**Receptors**		
			PD98039			PD98039
	med	IL-2,-4	+ IL-2,-4	med	IL-2,-4	+ IL-2,-4
Subject1	9.47	44.21	41.10	1360	3921	3511
Subject2	9.14	39.27	43.21	1428	4021	4416
Subject3	11.50	34.20	29.87	1255	2038	2014
MEAN	10.04	39.23	38.06	1348	3327	3314
SD	1.28	5.01	7.17	87	1113	1217
Kd, med & IL-2, -4: p= 0.005				R, med & IL-2,-4: p= 0.02		
Kd, med & IL-2, -4 +PD: p= 0.005				R, med & IL-2,-4+PD: p= 0.02		
			Ro318220			Ro318220
	med	IL-2,-4	+ IL-2,-4	med	IL-2,-4	+ IL-2,-4
Subject1	10.72	42.19	37.61	2212	6453	2999
Subject2	14.10	37.58	42.24	1790	3137	2784
Subject3	16.15	34.83	39.12	2071	3761	4009
MEAN	13.66	38.20	39.66	2024	4450	3264
SD	2.74	3.72	2.36	215	1762	654
Kd, med & IL-2,-4: p = 0.003				R, med & IL-2,-4: NS		
Kd, med & IL-2,-4 +Ro: p = 0.002				R, med & IL-2,-4+Ro: NS		

R=Receptors

Fig 17: Nuclear GR Affinity in Medium With and Without Cytokines and Inhibitors of Signal Transduction

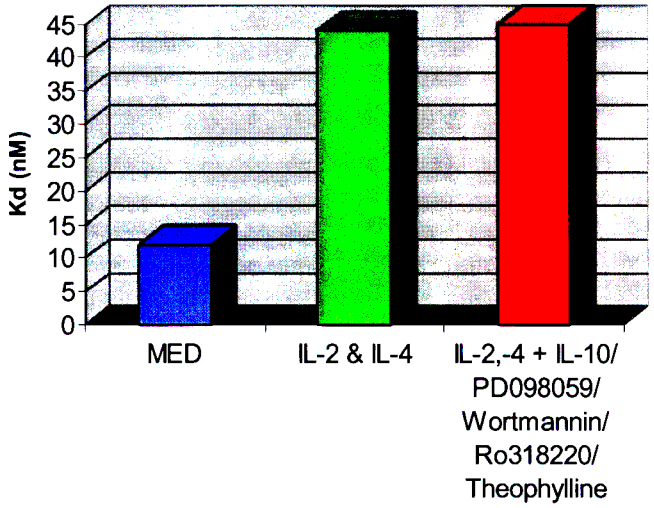


Fig 17: Effect of 48 hours incubation of PBMC's with medium alone, IL-2/IL-4 (both at 50 IU/mL combined), IL-2/IL-4 and Wortmannin (5 nmol/L), IL-10 (3 ng/mL), Ro318220 (10 nmol/L), PD098059 (10 μmol/L) or theophylline on GR ligand-binding affinity (Kd).

GR affinity normalizes in medium but increases significantly with IL-2, -4 and remains unaltered with these inhibitors of signal transduction.
There was no statistically significant difference in Kd between cells incubated with IL-2,-4 and those cells co-incubated with these signal transduction inhibitors

p38 MAPK Regulates IL-2/IL-4 Alteration of GR Ligand-binding Affinity.

In contradistinction, inhibition of the p38 MAPK pathway with SB203580 (10 μmol/L) completely prevented the IL-2/IL-4–mediated reduced receptor affinity in PBMC's; $p<0.001$ (Table 17, Fig 18a). These experiments were repeated with SB203580 at 1 and 3 μmol/L and similar effects were noted thus excluding non-specific effects of the higher concentration.

IL-2 and IL-4 co-treatment also restored the reduced levels of receptors per cell in PBMCs from steroid-dependent subjects seen after 48 hours' incubation in medium alone (Fig 18b- this did not reach statistical significance because of the high receptor numbers expressed by the patient in experiment 1).

Table 17: Results of GR binding experiments of PBMC's incubated in medium with and without IL-2,-4 and SB 203580

	Kd nM)			**Receptors**		
	med	IL-2,-4	IL-2,-4+ SB203580	med	IL-2,-4	IL-2,-4+ SB203580
Expt1	6.31	39.16	10.91	6399	11132	8402
Expt2	7.24	49.14	9.58	982	2209	906
Expt3	11.4	41.42	9.63	1598	6860	1265
Expt4	9.23	38.43	11.2	2048	4275	2205
Expt5	8.92	45.22	8.02	2786	4492	2296
Expt6	10.46	42.64	12.17	3275	5421	2996
MEAN	8.93	42.67	10.25	2848	5731	3012
SD	1.911	4.006	1.472	1923	3052	2746

Kd, IL-2,-4 & IL-2,-4 + SB203580: $p<0.001$
Kd, med & IL-2,-4 + SB203580: NS

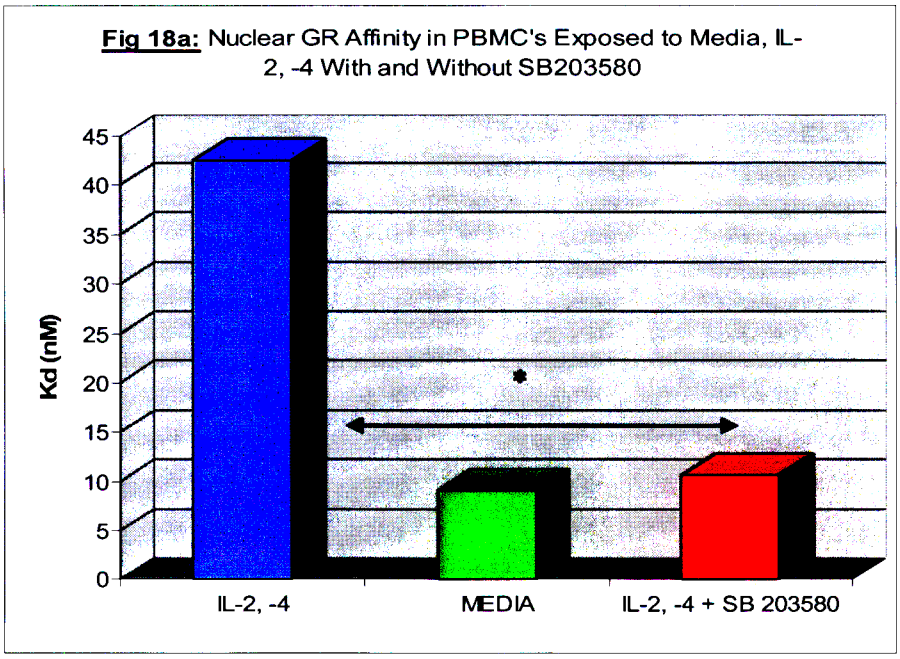


Fig 18a: Nuclear GR affinity (mean) of PBMC's, co-incubated with IL-2, -4, is reduced but increases during incubation with medium or IL-2 or IL-4 alone. Cells co-incubated with IL-2, -4 + SB 203580 increase GR affinity (low Kd); * $p<0.001$.

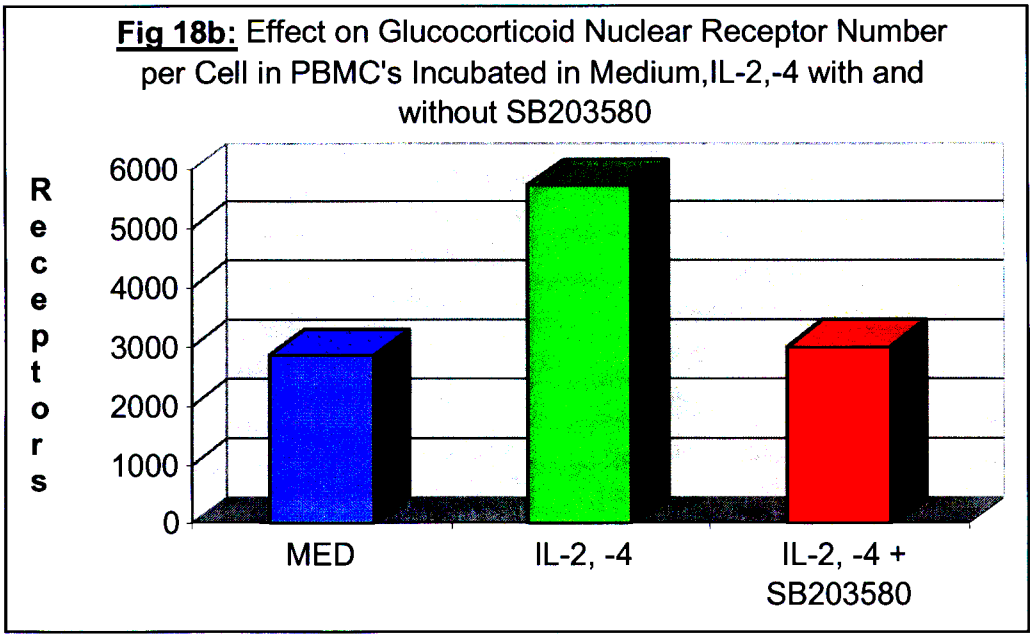


Fig 18b: When affinity increases, receptor number decreases. Here cells incubated in medium alone expressed decreased GR numbers. With co-incubation with IL-2, -4 and poor affinity (high Kd; **fig 18a**) receptor number increase. This effect on receptor number is also blocked by SB 203580.

A similar effect was also seen with SB203580 (1μ mol/L) on IL-13–induced changes in ligand-binding affinity (38.9 ± 1.8 vs 11.4 ± 1.58 nmol/L, $p < 001$, Table 18 and Fig 19).

Table18: Effect on nuclear GR Kd and number in PBMC's incubated in medium with and without Interleukin 13 and SB203580 for 48 hours

	Medium		IL-13		IL-13+SB203580	
	Kd	Receptors	Kd	Receptors	Kd	Receptors
Subject1	9.47	1172	42.37	4271	11.21	1382
Subject2	12.61	1357	36.26	3821	9.93	989
Subject3	15.1	933	38.1	4131	13.07	1817
MEAN	12.39	1154	38.91	4074	11.40	1396
SD	212.57	212	3.13	230	1.58	414

For increase in Kd with IL-13 alone: $p<0.0001$
For increase in receptor expression with IL-13 alone: $p<0.001$.

Fig 19: Effect of PBMC's incubation in medium and IL-13 with or without 1µmol/l SB203580

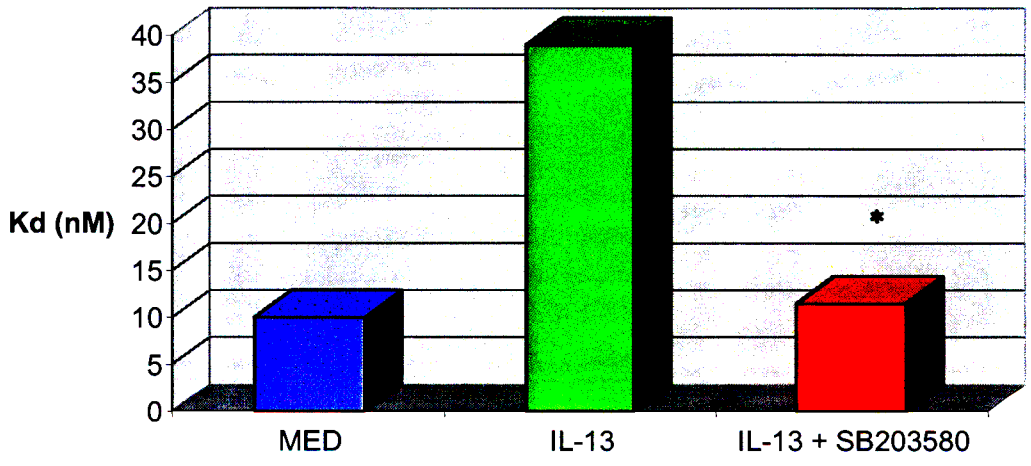


Fig 19: Confirmation that IL-13 alone also decreases GR affinity (high Kd) and normalisation with SB 203580 co-incubation, * $p<0.0001$.

5.2: Functional sequelae of restoration of GR affinity

Having identified a signal transduction inhibitor that normalised GR affinity, we proceeded to investigate if functional sequelae could also be reversed by SB 203580.

METHODs

Cells were isolated as described previously and the incubated in medium, IL-2,-4 and IL-2,-4 with SB203580. After 48 hours cells were stimulated with LPS and IL-10 measured by ELISA as described earlier.

RESULTS

IL-10 concentrations in the various experimental situations are depicted in Table 19 and Fig 20.

Co-treatment of cells with IL-2,-4 +SB203580 restored higher IL-10 production i.e. when induction of GR resistance was blocked, IL-10 production was increased.

Table 19 : Absolute Interleukin-10 production at 60 hrs incubation in medium with and without IL-2,-4 and SB203580 following LPS stimulation

Patient	Medium	IL-2,-4	Percentage production relative to medium	IL-2,4 +SB203580	% Production
D	168	43	26	142	85
E	183	106	58	194	>100
F	100	15	15	84	84
G	64	11	17	72	>100
MEAN			29		92
SD			20		9

IL-10 production with SB relative to IL-2,-4 alone, p= NS.
Decreased IL-10 production with IL-2,-4 relative to med: p= 0.003.

Fig 20: Absolute IL-10 Production Expressed as a Percentage of Medium with or without IL-2, -4 and SB203580

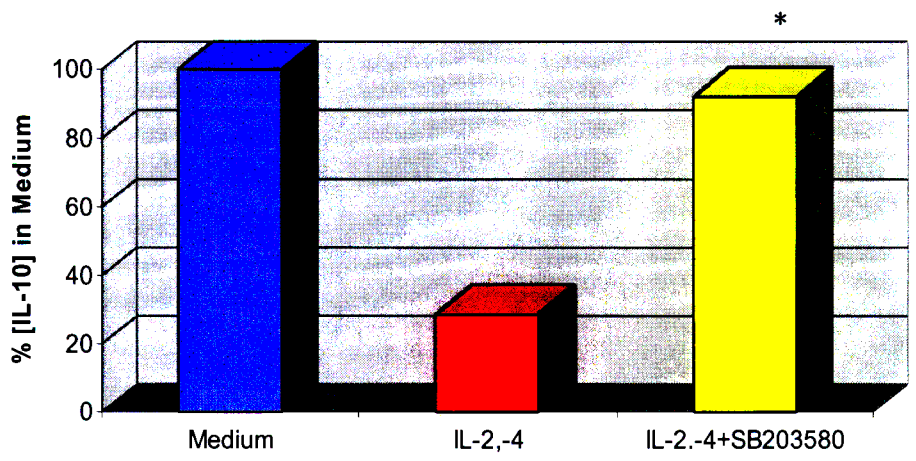


Fig 20: Results of experiments with cells incubated in medium, IL-2, -4 with and without SB 203580. Thereafter cells were stimulated with LPS. IL-10 production in medium (with normal GR affinity) was approximately 5 times more than that of IL-2, -4 cells. This effect was restored with SB 203580, *p= 0.003

5.3: Demonstration of decreased activated p38 MAPK protein

To further corroborate the effect of SB203580 on cytokine mediated altered GR affinity, we attempted to demonstrate that there was decreased intracellular activated p38 MAPK protein when mononuclear cells were incubated with IL-2,-4 & SB 203580. We chose the technique of western blotting to test this hypothesis.

Western Blot for Activated P38 MAPK.

METHODS

Cells, at a concentration of 1x 10^6 /ml in RPMI with HEPES (20 mM) supplemented with 10% FCS , penicillin, streptomycin, and amphotericin B were cultured for 48 hours in medium alone and IL-2, 50U/ml and IL-4, 50U/ml with or without SB203580. Thereafter cells were harvested, washed in fresh RPMI and lysed with 20mM Tris HCl ph7.4, 10mM EDTA 100mMNaCl 2%TritonX-100, PMSF 1mM, 1mM dithiothreitol (DTT) and the protease inhibitors aprotinin 10ug/ml and leupeptin 10ug/ml.

Protein concentration was measured by the Bradford assay and 20ug aliquots were used for Western Blotting. Proteins were added to an equal volume of 125 mM Tris-HCl, pH 6.8, 1% w/v SDS, 10% v/v glycerol, 0.1% w/v bromphenol blue, 2% v/v 2-mercaptoethanol (2× SDS loading buffer) and boiled for 5 min. Samples were separated by 10% SDS-PAGE (sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis), and the proteins were blotted onto a hybond-ECL membrane (Amersham Pharmacia Biotech). Membranes were blocked with 5% skimmed milk in Tris-buffered saline with 0.05% Tween 20, pH 7.6 overnight at 4°C and probed with Anti-ACTIVE p38 antibody (Promega) used in 1: 2000 dilutions. After washing, membranes were incubated with secondary antibody coupled to horseradish peroxidase (Amersham and Pharmacia Biotech) for 1 h at room temperature. Antibody–antigen complexes were then detected using ECL chemiluminescent detection

system according to the manufacturer's instructions (Amersham and Pharmacia Biotech). Polyvinyl chloride wrap was used to cover the blot before exposing it to acetate transparency film (Kodak, Rochester, NY). The blot was exposed for 60 s before being developed.

RESULTS

A photograph of the blot is presented below.

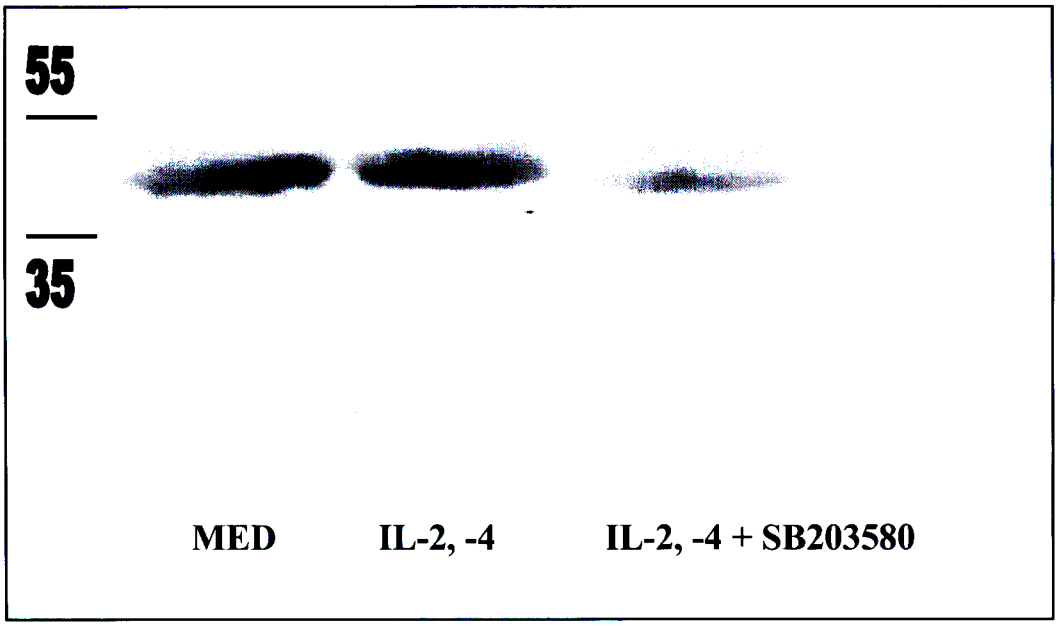


Fig 21: Western blot of activated p38 Mitogen Activated Protein Kinase (MAPK). Proteins were extracted from cells incubated in medium alone and IL-2, -4 with and without SB 203580. They were then subjected to Western blot analysis.

Molecular weight markers are indicated on the left of the above blot. There is a dense band of p38 MAPK activation with IL-2, -4 and the band density is clearly decreased with SB 203580. Non-specific induction also occurred in medium.

Thus at the final stage of the project, decreased activated p38 MAPK at the protein level was demonstrated when cells were incubated with IL-2,-4 and SB203580.

CHAPTER 6: DISCUSSION

We have focused on the glucocorticosteroid receptor in the pathogenesis of treatment resistant/ difficult asthma and used a group of oral steroid dependent asthmatics and in vitro studies to explore GR characteristics.

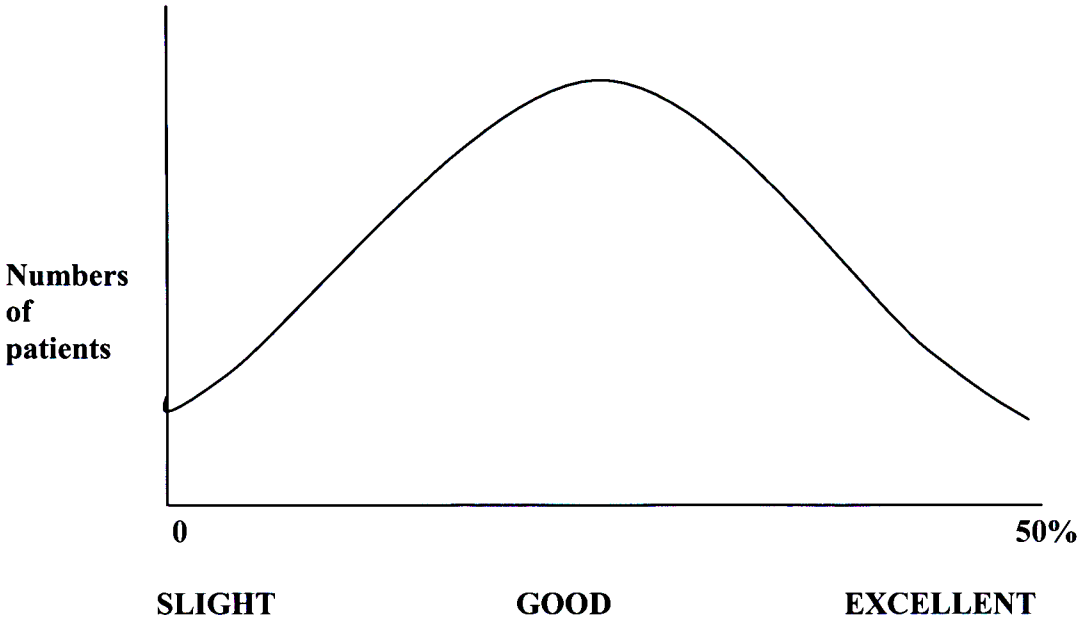
The Concept of Steroid Resistant Asthma.

The term steroid resistant asthma (SRA) is attributed to Schwartz and Carmichael [177, 178]. Thereafter a number of investigators, who focused on SRA for several years, led by Corrigan [183], Kay [184], Lee [189] and Sher [216], developed the concept further. However, the steroid receptor is crucial for a host of homeostatic processes in the body and complete steroid resistance would be incompatible with life. These subjects were in fact "partially resistant." The first problem with their definition was that it was completely arbitrary and this was perpetuated by subsequent investigators. The method of demonstrating and quantifying the bronchodilator response was not standardised. The doses of β_2 agonist varied depending on whether a metered dose inhaler or nebuliser was used and equally, steroids were given in variable doses and by different routes (see box). Clearly patients could easily fall into a category purely arbitrarily if they failed to achieve a pre-specified bronchodilator response.

	Corticosteroid
Schwartz [177]	40 mg cortisol 21- succinate
Carmichael [177, 178]	Prednisolone 20mg daily x 7 days only
Corrigan [183]	Prednisolone 20mg mgx 7 days then 40mg x 7 days
Alvarez [182]	Prednisone 45mg daily x 14 days
Lane [184]	Prednisolone 40mg daily x 14 days
Sher [216]	Prednisone 20mg twice daily x 7days

From a clinical standpoint, the concept of SRA led to much confusion. Occasionally, patients who did not even have asthma, but an asthma mimic, would be labeled SRA. This led to much deliberation in the literature and clear guidelines became available [180, 181]. In the first instance, the diagnosis of asthma must be firmly established. Secondly, the patient must be treated with an appropriate dose of ICS commensurate with the degree of severity. Finally, in the third instance, a host of remediable factors should be excluded before the patient is labeled therapy resistant or a difficult asthmatic.

Many doctors are accustomed to the bronchodilator response of β_2 agonists and do not appreciate the bronchodilator response of ICS. In general, the more severe the inflammation, the poorer the bronchodilator response to β_2 agonist- exemplified most in acute severe asthma. However, in clinical studies, improvement in airway caliber is measured by serial spirometry before a β_2 agonist is given. I reanalyzed the clinical data of Price [313] and Ostrom [314] looking at lung function improvement with ICS alone. I arbitrarily considered an improvement in FEV1 of 50% as excellent. The results are depicted in Fig 22.



MAGNITUDE OF THE FEV_1 RESPONSE TO ICS

Fig 22. The magnitude of the FEV_1 response to *ICS* follows a Gaussian distribution. This must be differentiated from the bronchodilator response to *β_2 agonists* which is dependent on the degree of inflammation and can therefore be equally variable.

Not surprisingly, consistent with most biological variables, the curve followed a Gaussian distribution [315]. Most had a good increase with ICS, some excellent and some- a poor response. It is likely that Schwartz and Carmichael and other investigators of SRA were looking at this left-end of the spectrum of steroid responders; increased or differences in inflammatory responses may be responsible for this phenomenon.

It is noteworthy that Malmstrom's analysis of the FEV1 response to the leukotriene antagonist, Montelukast, [316] compared to beclomethasone in asthma (although represented as bar graphs) also had a distribution very similar to my calculations.

The next issue is that asthma is likely to improve if a sufficient dose of steroid, whether inhaled or oral is given. In many apparent therapy resistant cases, both patient and doctor are concerned about corticosteroid side-effects and the dose chosen is a compromise that both parties are happy with (primarily from an adverse-effect point of view, although the overall cost of asthma medication is also a consideration). Even in patients that are apparently steroid sensitive, the dose may be sub-optimal if a reasonable (but incomplete) therapeutic outcome; satisfactory symptom control and lung function, is achieved. This was exemplified in my study population where although not statistically significant, many subjects in both the oral-steroid dependent and steroid sensitive groups had better lung function after a course of prednisone for just 1 week (Fig 2).

This improvement in spirometric parameters was also described by Corrigan, Lane and Leung in all their SRA cohorts as well.

When Schwartz described SRA, only inhaled beclomethasone- a relatively weak steroid- was available. Since then, more potent and lung selective steroids have become available, markedly increasing the number of patients that can achieve good asthma control [180].

The effect of using a more potent ICS was elegantly demonstrated by Nelson et al [317]. They identified a group of 111 oral steroid dependent asthmatics. In a placebo controlled manner, they then administered Fluticasone Propionate at 2 doses; 1000ug and 2000ug/ day, via MDI. The percentage of subjects that were able to *discontinue* oral steroids completely was 9%, 75% and 89% for the placebo, 1000ug and 2000ug doses respectively. The mean corresponding percentage improvement in FEV1 was 0.5%, 8% and 24% respectively with improvement in the quality of life questionnaires in the ICS groups. Not many physicians would use these doses of medication. This study illustrated that there is persistent inflammation in oral steroid dependent asthmatics and

that when one uses an ICS with a greater affinity for the steroid receptor (fluticasone propionate has approximately twice the relative receptor affinity of beclomethasone monopropionate or budesonide), a better anti-inflammatory effect is achieved. This is manifest by significant improvements in asthma control and the ability to withdraw oral corticosteroids.

The practice of not controlling asthmatics completely is more widespread than one would expect. In a recent summary of global surveys on asthma control involving approximately 11 000 patients [318], Rabe stated that only 5% of patients sampled had well controlled asthma!

Thus SRA is a misnomer and provided patients with severe asthma are properly assessed, investigated and treated, there will be very few patients with true therapy resistant asthma.

What then was the reason for the host of laboratory abnormalities seen in apparent SRA [182-189]? In large measure (one cannot exclude possible genetic and other acquired abnormalities with certainty), these reflected persistent inflammation-these were primarily the result of sub-optimal control of the asthma pathological processes. The principal reason for this assertion is that in vitro, these abnormalities were almost always reversible. As we've come to understand pathogenic mechanisms in asthma better so has come the realization that there is a process of inflammatory activation of resident lung and circulating immune cells and when these cells are removed from the body, away from excitatory stimuli that characterize asthma in vivo, they return to a non-activated state.

Reasons for Sub-optimal Response to Corticosteroids.

a. Glucocorticosteroid Receptor Number.

One of the reasons that has been proposed for a sub-optimal therapeutic response in asthma is a decrease in the number of receptors. Although there are intragenic sequences in the GR capable of down-regulation with ligand [217], most studies with SRA have not verified this [183, 184, 186]. We also could not demonstrate a decrease in receptor numbers as a cause of the poor response to CS. On the contrary, receptor numbers were preserved to high in our OSD group (although the inability to reach statistical significance might have been due to a degree of down-regulation caused by oral CS therapy). Equally compelling, is that due to the decreased affinity that we have confirmed, an increase in receptor numbers would be anticipated! Therefore, more important than the expectation of down regulation of binding sites because of treatment, is knowledge of the affinity of the receptors: a poor affinity will dictate that more receptors be expressed to compensate for the decreased binding ability as a reciprocal relationship exists between receptor number and affinity in pharmacology.

b. Alteration in GR affinity

An important factor that influences CS action is the capacity to bind to the glucocorticoid receptor. This ability is measured by the affinity/ dissociation constant-the Kd. We have demonstrated that nuclear GR affinity is altered in asthmatics and to a greater extent in oral steroid-dependent asthmatics. Thus the ability of dexamethasone to bind to GR was decreased according to the degree of asthma severity. GR affinity is therefore tiered; mild asthmatics have an approximate doubling of the Kd compared to normal, whilst severe asthmatics have double to triple the Kd value of mild asthmatics. Previous researchers of SRA did not make this observation because of smaller numbers of patients (although the trends were there) and would often group all asthmatics together.

The mean Kd in OSD asthma is approximately the same numerical value of the Kd of PBMC's rendered steroid resistant in vitro with co-incubation with cytokines. This difference was detected only in the nuclear compartment and not in the cytoplasm, possibly reflecting an effect of a nuclear protein masking the GR ligand-binding site or in an altered conformation of the activated GR.

The observation that the GR binding is only decreased in the nucleus reflects recent insights into GR activity. GR translocation to the nucleus is a highly complex process, not just DNA binding [202]. We are only just beginning to understand this. The GR-ligand complex has to be actively transported through the nuclear pore. It is possible that further chaperone molecules and other compounds – a result of diverse signal transduction activation- link with the GR to modulate this action. Within the nucleus there is a reassembly before and after gene interaction before it is actively exported out back into the cytoplasm [202].

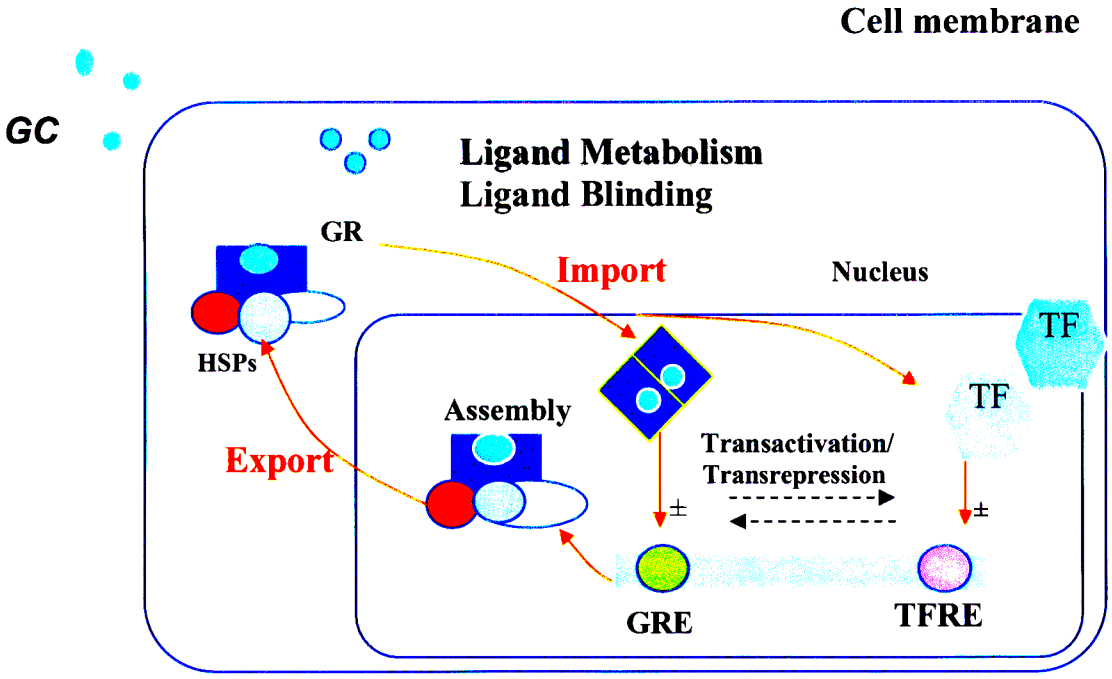


Fig 23.Contemporary View of Corticosteroid Action

Translocation to the nucleus is an active process of import through the nuclear pore. A number of signal transduction proteins are likely to modulate this process. After gene interaction the GR is reassembled before export out of the nucleus.

This altered affinity of dexamethasone for GR may reflect either an intrinsic defect in the GR within these patients or may relate to changes in the receptor induced by the increased level of inflammation in subjects with more severe asthma. The reversal of the reduced binding affinity by means of incubation with healthy media suggests that the latter is a more likely possibility. Our results confirm the differences in Kd between asthmatic and healthy subjects at baseline originally described by Leung and coworkers [216, 220, 222].

The magnitude of the decreased GR affinity seen in our oral-steroid dependent subjects was almost identical to that reported by Corrigan and Sher as well [183, 216]. The normalisation of GR affinity when we incubated cells in medium alone was the experience of these investigators as well.

GR affinity is dynamic and can change diurnally and over time and appears to be proportional to the degree of inflammation. Thus when inflammatory stimuli and pathological features of asthma increase at night [224], GR affinity decreases, and may account for a decreased therapeutic response of CS at night i.e. from decreased binding capacity to steroid receptors. This may be one of the reasons for the characteristic nocturnal symptomatology of asthma. This has also translated into clinical practice in that, when nocturnal symptoms are particularly troublesome, an increased dose of CS is administered in the evening with a better treatment outcome! It is likely that these higher doses are able to overcome the decreased GR affinity with improved GR mediated effects.

Another report that illustrates the dynamic nature of GR affinity was serial studies of GR affinity in patients with allergic asthma. With the onset of the allergy season, serial GR Kd measurements performed in these patients demonstrated a sequential decline in affinity [223]. Intriguingly, symptoms *only* appeared *after* the GR affinity had already declined. It is tempting to postulate that endogenous anti-inflammatory mechanisms fail when this affinity alters and inflammation escalates, precipitating symptoms.

To illustrate the importance of inflammation influencing GR affinity, the following study is salutary. Nimmagadda et al studied 13 steroid dependent asthmatics [226]. GR affinity was studied serially over 1 year whist the patients received Fluticasone Propionate 1000- 2000 μg/day. The patients gradually improved, oral steroid requirements dropped substantially and ECP levels declined. GR Kd which was a mean of 42.5nM at the outset improved to

19.5nM at 6 weeks and was maintained for the rest of the year. (Interestingly, the initial Kd is about the same as my oral steroid group and the improved value similar to my SS group). Thus to a certain extent GR affinity is auto-regulated. As a more potent CS is used which can bind with greater avidity, so inflammation is brought under control, and these decreased inflammatory stimuli improve the affinity of GR!

In another report Spahn et al [219], studied a group of asthmatics with severe symptoms, decreased FEV1 with marked diurnal variation requiring prednisone therapy. Median FEV1 improved by 25% following treatment. The GR binding affinity was a median of 29nM and decreased significantly after the prednisone course. Serum ECP and sIL-2R (soluble IL-2 receptor which is only up-regulated with immune activation) that were elevated before treatment also decreased significantly following clinical improvement. These observations again support abnormal GR binding during periods of uncontrolled asthma during which there is heightened inflammation and that this is reversible with high dose corticosteroid therapy.

Establishing the "Steroid Resistance Model"

One of the difficulties in studying GR characteristics of immune cells ex vivo is that outside the body and presumably away from inciting/ inflammatory stimuli the GR affinity rapidly normalizes (as seen consistently in over 200 binding experiments done by me).

It was therefore fortuitous that we were aware of the in vitro experiments of Leung et al, who after initially demonstrating increased mRNA for IL-2,-4, and -13 in BALF cells of SRA [220,221] used these cytokines to manipulate GR affinity in vitro [222]. We confirmed that diminished GR affinity could be induced in vitro by incubating PBMCs with IL-2/IL-4 in combination only or IL-13 alone i.e. that we could render mononuclear cells "glucocorticoid resistant".

Since their first description was with IL-2,-4 we used this as a "Steroid Resistance model", and could now examine mechanisms of resistance and the functional consequences thereof.

Functional sequelae of steroid resistance in vitro

There is a paucity of data on the functional sequelae of this steroid resistance model. Leung's group was able to show that IL-2,-4 resistant cells secreted more IL-6 and expressed more GR β [222, 204]. They also demonstrated that PBMC's with diminished GR binding affinity were less sensitive to the suppressive effects of CS on lymphocyte proliferation [222]. These cells were co-incubated with a mitogen (PMA/ionomycin) and increasing concentrations of methylprednisolone for a further 72 hrs. Thereafter cells were pulse labelled with ^{3}H- thymidine and counted. There was a greater proliferative response in the control cells than GR resistant cells.

They also addressed the issue of the clinical relevance of a two to three fold decrease in GR binding affinity. They argued that since physiological levels of cortisol can regulate IgE dependent allergic inflammation, and since diminished GR binding renders calls less susceptible to the inhibitory effect of PMA/ionomycin proliferation, that it is possible that the magnitude of decreased GR affinity seen could impair the ability of endogenous steroids to suppress ongoing airway inflammation [222]. This same degree of decrease GR affinity was seen in their subsequent experiments with concomitant diminished GR mediated effects.

We were able to demonstrate that ccombined treatment of cells with IL-2 and IL-4 (inducing altered GR affinity of the same magnitude as Leung et al) resulted in increased expression of the proinflammatory cytokine GM-CSF and reduced expression of the anti-inflammatory cytokine IL-10. In addition, we showed that the ability of dexamethasone to inhibit LPS- or PMA/PHA–stimulated IL-10 production in PBMCs pretreated with IL-2 and IL-4 was

impaired i.e. steroid resistance in vitro. Utilizing this model as well, Larsson observed that the inhibitory effect of budesonide on GM-CSF was also attenuated [319].

This suggests that the balance of cytokine production in a situation of diminished GR affinity favours a pro-inflammatory state that may contribute to asthma severity.

The Role of GM-CSF in Asthma

Asthma is characterized by up-regulation of GM-CSF. There is an increased expression in bronchial epithelium and airway T lymphocytes and eosinophils [132].

Increased circulating levels of GM-CSF are seen in chronic and acute asthma [141, 140].

It has a permissive effect on eosinophil function and amplifies inflammation [137, 304]. A phenotypically high GM-CSF producing asthmatic sub-group was noted to have more difficult asthma [303]. Our data on elevated GM-CSF as a function of induced resistance is consistent with this body of literature. Again, when GR affinity is decreased, this is accompanied by elevated GM-CSF which exacerbates asthma control. By contrast, when clinical asthma improves with corticosteroids or concomitant LABA therapy, this is paralleled by decreased airway elaboration of GM-CSF [305, 311].

The Role of IL-10 in Asthma

Interleukin-10 is a major regulator of inflammation. The data on defective IL-10 expression and production in asthma is remarkably consistent. Our finding of diminished IL-10 production when mononuclear cells are rendered resistant in vitro is concordant with

- the significant reduction of IL-10 m RNA and protein expression in alveolar macrophages of asthmatics compared to non-asthmatics [298]
- a similar in vitro study performed by Hawrylowicz et al where T lymphocytes rendered resistant had diminished IL-10 production [320]

Of the many beneficial molecular effect of corticosteroids in ameliorating inflammation was the restoration of reduced IL-10 release from macrophages from asthmatics following ICS therapy [295] with a concomitant reduction in MIP-1α, GM-CSF and IFN-γ. In vitro, CS drive T cell differentiation towards a phenotype with greater IL-10 and a less pro-inflammatory cytokine profile [321].

The delicate balance of inflammatory control was illustrated in a study where despite similar doses of corticosteroids, an unstable asthma group showed a decrease in IL-10 producing T cells in peripheral blood compared to stable subjects [322].

It is interesting to note that one of the mechanisms whereby theophylline might exert its beneficial effect is by increasing IL-10 secretion [323]. Furthermore during specific immunotherapy, Th cells secrete more IL-10, which may contribute to controlling allergic phenomena.

Although most of the data favour both inflammation and the association with diminished GR affinity decreasing IL-10 production by immune cells, there may be IL-10 genetic polymorphisms predisposing to uncontrolled asthma [101, 102].

Thus in asthma the decrease in IL-10 may favour a pro-inflammatory state rendering the patient difficult or therapy resistant.

Signal Transduction Pathways that are not Involved in Decreasing GR Affinity.

Despite the fact that several intracellular signalling pathways related to second messengers, phosphorylation compounds and transcription factors are transduced through the ERK MAPK pathway, inhibition by PD098059 had no effect on the altered receptor affinity induced by IL-2,-4. Similarly, blocking phosphoinositol 3 hydroxy kinase by wortmannin or treatment with Ro318220, a specific protein kinase C inhibitor, theophylline and IL-10 all failed to modulate the effect of IL-2 and IL- 4 on receptor affinity.

Concerns have been raised about the single mediator/receptor antagonism/ enhancement strategy that has been the subject of intense research [324]. Studies with novel agents such as monoclonal antibodies against IL-5 and recombinant IL-12- both directed at eosinophils, have essentially failed [325, 326]. In the former, eosinophils disappeared from the circulation and the airways with virtually no change in the asthma phenotype; physiological parameters such as airway hyper responsiveness, the late asthmatic reaction, lung function and symptoms remained static. Recombinant IL-12 was associated with poor clinical outcomes for asthma and significant toxicity [326].

Antagonising TNF-α is slightly more promising.

Howarth et al, studied the role of TNF alpha in corticosteroid dependent asthma [327]. Bronchoalveolar lavage was performed in control subjects and mild and severe asthmatics. The asthmatics were also subjected to endobronchial biopsies. The severe asthmatics had increased levels of TNF

alpha in BALF with accompanying increased TNF alpha gene expression and immunoreactive cells in bronchial tissue. Amongst this severe group, 17 subjects were then given etanercept (Enbrel(R)), the soluble TNF alpha receptor-IgG1Fc fusion protein, for 3 months in an open-label study. Asthma symptom scores, lung function and BHR all improved.

A definitive placebo-controlled study with etanercept 25mg twice weekly over 10weeks was then performed [328]. In this study, patients with refractory asthma had increased expression of membrane-bound TNF-alpha, TNF-alpha receptor 1, and TNF-alpha-converting enzyme by peripheral-blood monocytes. A lower level of expression of these markers was noted in mild asthmatics which was identical to normal controls. A significant improvement was noted in BHR, quality of life scores and post-bronchodilator (but not pre-bronchodilator) FEV1.

However, there were no significant differences between groups in single-flow nitric oxide concentration, calculated alveolar nitric oxide concentration, sputum total or differential cell counts, or sputum eosinophilic cationic protein, interleukin-8, or cysteinyl leukotriene concentrations. These suggest that TNF-α blockers have modest effects and not a broad range of anti-inflammatory effects and cannot be used without corticosteroids.

Combined IL-2,-4 or IL-13 Induced Diminished Glucocorticoid Receptor Affinity Normalises with the p38 MAPK Inhibitor, SB203580, In-vitro.

SB203580, the specific p38 MAPK inhibitor consistently ameliorated both the diminished affinity and number of receptor sites induced by IL-2 and IL-4 or IL-13 alone in our experiments. Furthermore we were able to demonstrate decreased activated p38 MAPK protein quantatively by Western Blot in cells treated with IL-2,-4 and SB203580.

IL-13 has similar effects to IL-4 and the evidence from cDNA cloning of the IL-13 receptor suggests that the IL-4R α chain is shared by the IL-13R. We were able to show that IL-13 could also induce altered GR affinity in vitro and that this could be reversed with SB203580. As IL-4 is required in the steroid resistance model, it is therefore conceivable that IL-13 could alter GR affinity through the IL-4 Receptor through mechanisms that are not elucidated presently.

SB203580 is a specific pyrinidyl imidazole inhibitor of both α and β isoforms of p38 and p38-2 MAP kinases that prevent activation of its downstream effector MAPK-activating protein kinase 2. This MAPK signalling cassette is highly conserved in many species. P38MAPK responds primarily to stressful and inflammatory stimuli e.g. tumour necrosis factor- alpha, IL-1 and lipopolysaccharide. Potential cellular targets of MAPK include PLA_2 and p90 s6 kinase.

Activation of the MAPK system results in pro-inflammatory cytokine and chemokine production. These result in cellular recruitment and perpetuation of the inflammatory process. By way of example, p38 MAPK regulates IL-5 synthesis and SB 203580 was able to inhibit eosinophil recruitment by human T cells [329].

Intracellular MAPK cascades modulate signalling and nuclear transcription via e.g. NF-κB. These processes are known to interact with GR mediated responses as well. P38MAPK has been shown to be essential for the mitogenic response of IL-2 [267] and its inhibition predictably normalised affinity as IL-4 needs the co-operation of IL-2 to decrease GR affinity.

However, the precise mechanism whereby GR affinity normalises is not known. GR is a phosphoprotein that contains consequences for numerous kinases such as protein kinase A, casein kinaseII, protein kinase C, cyclin

dependent kinases, glycogen synthase kinase3 and MAPK [330]. MAPK are involved in transcriptional and post -transcriptional regulation of pro-inflammatory cytokine expression. By way of example, many of the mRNA's coding for genes involved in inflammation are unstable and are stabilised by p38MAPK signalling [331]. The latter generates proteins that bind to AU rich sequences in the 3' untranslated region of inflammatory mediator genes stabilising the mRNA. The consequence is that mRNA levels can be rapidly adjusted and the amplitude of the inflammatory response can be magnified. These effects are precisely opposite to those observed with glucocorticoids.

There are various domains in the GR through which subsequent interaction may occur with MAPK. These activation pathways can interact with GR, modifying each others actions and occurring at the level of the transcription factors themselves [332]. Eevidence suggests that GR can inhibit the activity of both ERK and JNK enzymes, thus limiting the inflammatory response [333-336].

Rogatsky et al, have, in turn, shown reciprocal inhibition of rat GR reporter gene activity by JNKs by means of a direct phosphorylation of serine 246 through the consensus nonpolar-X-Ser/Thr-Pro motif, whereas ERK can inhibit GR action by means of an indirect effect, possibly through phosphorylation of a cofactor [337]. Our data cannot determine whether this is a direct or indirect effect of p38 MAPK or whether GR phosphorylation alters ligand-binding affinity directly, but our data in Fig 18a &18b clearly show that SB203580 can modify ligand binding parameters. Previous data have shown that reversible serine-threonine phosphorylation of GR may influence the binding of the hormone to the cytoplasmic GR complex, GR nuclear translocation, GR binding to glucocorticoid response elements, and formation of an activated transcriptional complex [338].

Our data suggest that IL-2/IL-4 may alter the functional activity of GR through phosphorylation, by means of p38 MAPK within the nucleus. The resultant effect is to change GR binding characteristics and probably also its ability to interact with components of the transcription apparatus. This may result from either a change in GR conformation caused by association of distinct cofactors or partial blocking of the ligand-binding domain caused by association of the GR with nuclear transcriptional modulating proteins. Similar results have been seen after nitric oxide treatment of GR, whereby nitrosylation of GR at an hsp90 interaction site modified ligand binding [339].

The GR phosphorylation sites and the sequences immediately surrounding it are highly conserved, suggesting functional conservation for transcriptional antagonism between the GR and p38 MAPK. Leucine-rich sequences may be involved in nuclear export [340], or this region may form a surface that interacts with factors involved in transcriptional regulation [341]. Interactions between the rat GR and the transcriptional complex involve several domains within the N-terminal region of GR, including a leucine-rich domain surrounding the p38 MAPK site [341], may alter or disrupt the protein-protein interactions regulating GR action.

It is possible that p38 MAPK induces GR phosphorylation thus providing a novel mechanism for downregulation of GR activity in T cells in response to proinflammatory stimuli, leading to relative glucocorticoid resistance or oral-steroid dependence. Enhanced activation of this pathway as a result of stimulation by inflammatory mediators, such as IL-2 and IL-4, which are elevated at the site of inflammation in corticosteroid-resistant asthma, may modify GR directly, leading to recruitment of distinct GR-associated proteins and resulting in reduced glucocorticoid function.

Another possibility is altered receptor affinity due to chaperone proteins during the nuclear importation of GR. An end substrate of p38 MAPK activation is a different heat shock protein: HSP27 [342]. This might complex with the GR-CS complex after activation.

Targeted treatment with low doses of p38 MAPK inhibitors may reverse glucocorticoid resistance and improve the anti-inflammatory effects of glucocorticoids in these glucocorticoid-dependent or partially glucocorticoid resistant patients with asthma, who are usually unresponsive to other currently available therapies. Both agents have a broad range of anti-inflammatory effects that are likely to be synergistic in attenuating inflammation. Caution would have to be exercised to ensure no beneficial MAPK pathways are inadvertently blocked.

Conclusion.

Steroid Resistant Asthma –SRA-is a misnomer. The glucocorticoid receptor is essential for life and true steroid resistance would be fatal. The inflammatory nature of asthma is extremely complex and thus far the single mediator amelioration has met with little success; glucocorticoids will remain the mainstay of treatment for many years to come. There is however, a spectrum of responses to ICS with therapy resistant asthma at one end of the spectrum. These patients have often been erroneously labelled as steroid resistant/ oral steroid dependent; most of them will respond to higher doses of high affinity ICS such as fluticasone propionate or ciclesonide [317, 343]. It is also possible that other mechanisms are driving the inflammation and some patients may well respond to anti-TNF agents or anti IgE therapy [344], by-passing the steroid un-responsive pathways.

The vast majority of apparently steroid resistant subjects described previously and our experience has revealed that there is persistent inflammation that can be detected in the airways and by activation markers of inflammatory cells in the peripheral blood. Genetic factors may play a part; polymorphisms of mediators or receptor isoforms that cannot transduce an anti-inflammatory signal effectively or are antagonised. Examples include the DPP10 gene linked to difficult asthma [170] and the high GM-CSF expressors in children [303].

However, most patients have an altered GR affinity that is acquired due to inflammation; in vitro GR affinity normalises away from the inciting stimuli of the disease. It is likely that there is a spectrum of altered GR affinity as well; with mild asthma exhibiting a slight alteration in GR binding- that is still capable of effecting most corticosteroid action whilst severe asthma is accompanied by poor GR binding and consequent limited control of inflammation.

Thus GR affinity is an index of asthma control as well. The greater the inflammation, the poorer the affinity and as inflammation resolves GR affinity improves [219, 226].

GR affinity is dynamic- it changes diurnally and over time, dependent on exogenous aeroallergens [224, 223] and the degree of inflammation. The data on GR binding capacity, including ours, suggest that with increasing inflammatory stimuli, GR affinity decreases, and endogenous and therapeutic doses of corticosteroids become inadequate to control inflammation. This explains the clinical observation of increased symptoms and the need for supplementing ICS.

Inflammation should not be seen as one dimensional; that it is present or not. Rather there are grades-exemplified by GR-β. GR-β expression is a function of inflammation. Normal subjects have low grade production, it is increased in mild asthma, even more is produced in severe asthma and the highest levels occur in fatal asthma [210, 206, 205]. Being unable to bind CS, the resultant anti-inflammatory effect is increasingly inadequate with greater GR-β expression- worse still; it inhibits the functional GR-α.

Perhaps inflammation is different in different categories of asthma; there may be negligible TNF-α activation in mild asthma but this is a feature of severe asthma [327, 328].

Apart from GR and its variants and diverse types of cytokine and other mediators there may also be a discrete cell predominance in distinct types of asthma e.g. neutrophil dominant asthma [31-33].

Whatever the reason, genetic or non-genetic inflammatory activation, inflammation dictates GR affinity and this very affinity dictates GR function and the ability to bring inflammation under control.

We have used a steroid resistance model to demonstrate that p38 MAPK is one pathway for altered GR affinity due to IL-2,-4. The magnitude of that alteration is the same as the GR Kd in oral steroid dependent subjects. When cells are rendered resistant in vitro, the output of inflammatory cytokines favours a pro-inflammatory state: high GM-CSF and low IL-10 production- by extrapolation, we believe that this is what happens in refractory asthma as well. p38 MAPK inhibitors have the potential to improve GR affinity and enhance corticosteroid activity in difficult asthma.

Epilogue

The Royal Brompton has a proud tradition of respiratory research excellence. When one looks at the unimpressive façade, it's hard to comprehend; however, its people that make up an institution.

I was trained by fellow scientists. The research fellows I trained in turn in the steroid resistance model went on to demonstrate that p38 MAPK indeed phosphorylates GR. This and the principal findings of my work were published in The Journal of Allergy and Clinical Immunology [345] prompting an editorial by Chrousos- one of the foremost researchers into the molecular activity of the glucocorticoid receptor [202].

There are over eight p38 MAPK inhibitors in development currently for a variety of inflammatory disorders including obstructive airways disease [346].

My research was awarded a prize by the Allergy and Clinical Immunology Assembly of the European Respiratory Society at their annual ERS Conference for innovative research in asthma.

REFERENCES.

1. Elias JA, Lee CG, Zheng T, Ma B, Homer RJ, Zhu Z. New insights into the pathogenesis of asthma. J Clin Invest 2003; 111: 291-297.

2. Lange P, Parner J, Vestbo J. A 15-year follow-up study of ventilatory function in adults with asthma. N Engl J Med 1998; 339: 1194-1200.

3. Dijkstra A, Vonk JM, Jongepier H, Koppelman GH, Schouten JP, ten Hacken NHT, Timens W, Postma DS. Lung function decline in asthma: association with inhaled corticosteroids, smoking and sex. Thorax 2006; 61: 105-110.

4. Barnes PJ, Adcock IA. How do corticosteroids work in asthma? Ann Intern Med 2003; 139: 359-370

5. Lilly CM. Diversity of asthma: Evolving concepts of pathophysiology and lessons from genetics. J Allergy Clin Immunol 2005; 1115: S526-S531.

6. Burrows B, Martinez FD, Halonen M, Barbee RA, Cline MG. Association of asthma with serum IgE levels and skin-test reactivity to allergens. N Engl J Med 1989; 320: 271-277.

7. Sears MR, Burrows B, Flannery EM, Herbison GP, Hewitt CJ, Holdaway MD. Relationship between airway responsiveness and serum Ig E in children with asthma and in apparently normal children. N Engl J Med 1991; 325: 1067- 1071.

8. Busse WW, Lemanske RF. Asthma. N Engl J Med 2001; 344: 350- 362.

9. Bousquet J, Jeffery P, Busse WW, Johnson M, Vignola AM. Asthma: from bronchoconstriction to airway inflammation and remodeling. Am J Respir Crit Care Med 2000; 161: 1720-1745.

10. Rothenberg ME. Eosinophilia. N Engl J Med 1998; 338: 1592- 1600.

11. Kirby JG, Hargreave RE, Gleich GJ, O'Byrne PM. Bronchoalveolar cell profiles of asthmatic and non asthmatic subjects. Am Rev Respir Dis 1987; 136: 379-383.

12. Corrigan CJ, Hartnell A, Kay AB. T lymphocyte activation in acute severe asthma. Lancet 1988; 1: 1129-1132.

13. Wilson JW, Djukanovic R, Howart PH, Holgate ST. Lymphocyte activation in bronchoalveolar lavage and peripheral blood in atopic asthma. Am Rev Respir Dis 1992; 45: 958-960.

14. Rothenberg ME, Owen WF, Silberstein DS, Woods J, Soberman RJ, Austen KF, Stevens RL. Human eosinophils have prolonged survival, enhanced functional properties, and become hypodense when exposed to human interleukin 3. J Clin Invest 1988; 81: 1986- 1992.

15. Lopez AF, Williamson DJ, Gamble JR. Recombinant human interleukin 5 is a selective activator of human eosinophil function. J Clin Invest 1986; 78: 1220-1228.

16. Robinson DS, Hamid Q, Ying S, Tsicopoulos A, Barkans J, Bentley AM, Corrigan C, Durham SR, Kay AB. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. N Engl J Med 1992; 326: 298-304.

17. Kelly EA, Rodriguez RR, Busse WW, Jarjour NN. The effect of segmental bronchoprovocation with allergen on airway lymphocyte function. Am J Respir Crit Care Med 1997; 156: 1421- 1428.

18. Robinson DS, Hamid Q, Bentley A, Ying S, Kay B, Durham SR. Activation of CD4 + T cells, increased Th2-type cytokine mRNA expression, and eosinophil recruitment in bronchoalveolar lavage after allergen inhalation challenge in patients with atopic asthma. J Allergy Clin. Immunol 1993; 92: 313- 924

19. Bousquet J, Chanez P, Lacoste JY, Barneon G, Ghavanian N, Enander I, Venge P, Ahlstedt S, Simone-Lafonteine J, Godard P. Eosinophilic inflammation in asthma. N Engl Med 1990 ; 323 : 1033- 1039.

20. Flavahan NA, Slifman NR, Gleich GJ, Vanouette PM. Human eosinophil major basic protein causes hyperreactivity of respiratory smooth muscle: role of the epithelium. Am Rev Respir Dis 1988; 136: 685-688.

21. Leckie MJ, ten Brinke A, Khan J, Diamant Z, O'Connor BJ, Walls CM, Mathur AK, Cowley KC, Chung KF, Djukanovic R, Hansel TT, Holgate ST, Sterk PJ, Barnes PJ. Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. Lancet 2000; 356: 2144-2148.

22. Gosset P, Tillie-Leblond I, Oudin S, Parmentier O, Wallaert B, Joseph M, Tonel AB. Production of chemokines and proinflammatory and anti-inflammatory cytokines by human alveolar macrophages activated by Ig E receptors. J Aller Clin Immunol 1999; 103: 289-297.

23. Mautino G, Oliver N, Chanez P, Bousquet J, Capony F. Increased release of matrix metalloproteinase-9 in bronchoalveolar lavage fluid and by alveolar macrophages of asthmatics. Am J Respir Cell Mol Biol 1997; 17: 583- 591.

24. Balbo P, Silvestri M, Rossi GA, Crimi E, Burastero SE. Differential role of CD 80 and CD86 on alveolar macrophages in the presentation of allergen to T lymphocytes in asthma. Clin Exp Allergy 2001; 31: 625-636.

25. Lambrecht BN, Salomon B, Klatzmann D, Pauwels RA. Dendritic cells are required for the development of chronic eosinophilic airway inflammation in response to inhaled antigen in sensitized mice. J Immunol 1998; 160: 4090-4097.

26. Nocker RE, Out TA, Weller FR, Mul EP, Jansen HM, van der Zee JS. Influx of neutrophils into the airway lumen at 4h after segmental allergen challenge in asthma. Int Arch Allergy Immunol 1999; 119: 45-53.

27. Teran LM, Carroll MP, Frew AJ, Redington AE, Davies DE, Lindley I, Howarth PH, Church MK, Holgate ST. Leukocyte recruitment after local endobronchial allergen challenge in asthma. Relationship to procedure and to airway interleukin -8 release. Am J Respir Crit Care Med 1996; 154: 469- 476.

28. Woodruff PG, Fahy JV. A role for neutrophils in asthma? Am J Med 2002; 112: 498-500.

29. Kraft M, Cassell GH, Henson JE, Watson H, Williamson J, Marmion BP, Gaydos CA, Martin RJ. Detection of Mycoplasma pneumoniae in the airways of adults with chronic asthma. Am J Respir Crit Care Med 1998; 158: 998- 1001.

30. Sur S, Crotty TB, Kephart GM, Hyma BA, Colby TV, Reed CE, Hunt LW, Gleich GJ. Sudden-onset fatal asthma: a distinct entity with few eosinophils and relatively more neutrophils in the airway submucosa? Am R Resp Dis 1993; 148: 713-719.

31. Wenzel SE, Szefler SJ, Leung DYM, Sloan SS, Rex MD, Martin RJ. Bronchoscopic evaluation of severe asthma: Persistent inflammation associated with high dose glucocorticoids. Am J Respir Crit Care Med 1997; 156: 737-743.

32. Tonnel AB, Gosset P, Tillie-Leblond I. Characteristics of the Inflammatory Response in Bronchial Lavage Fluids from Patients with Status asthmaticus. Int Arch Allergy Immunol 2001; 124: 267-271.

33. Carroll NG, Mutavdzic S, James AL. Increased mast cells and neutrophils in submucosal mucous glands and mucus plugging in patients with asthma. Thorax 2002; 57: 677 – 682.

34. March CJ, Mosley B, Larsen A, Cerretti DP, Braedt G, Price V, Gillis S, Henney CS, Kronheim SR, Grabstein K. Cloning, sequence and expression of two distinct interleukin-1 complementary DNAs. Nature 1985; 315: 641-7.

35. Kern JA, Lamb RJ, Reed JC, Daniele RP, Nowell PC. Dexamethasone inhibition of interleukin 1 beta production by human monocytes: posttranscriptional mechanisms. J Clin Invest 1988; 81: 237-44.

36. McKean DJ, Podzorski RP, Bell MP, Nilson AE, Huntoon CJ, Slack J, Dower SK, Sims J.Murine T helper cell-2 lymphocytes express type I and type II IL-1 receptors, but only the type I receptor mediates costimulatory activity. J Immunol 1993; 151: 3500-3510.

37. Colotta F, Dower SK, Sims JE, Mantovani A. The type II `decoy` receptor: a novel regulatory pathway for interleukin 1. Immunol Today 1994; 15: 562-566.

38. Cao Z, Xiong J, Takeuchi M, Kurama T, Goeddel DV .TRAF6 is a signal transducer for interleukin-1. Nature 1996; 383: 443-446.

39. Yamamoto KK, Gonzalez GA, Biggs WH 3rd, Montminy MR. Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. Nature 1988; 334: 494-498.

40. Kaur P, Saklatvala J. Interleukin 1 and tumour necrosis factor increase phosporylation of fibroblast proteins. FEBS Lett 1988; 241: 6-10.

41. Lipsky PE, Thompson PA, Rosenwasser LJ, Dinarello CA. The role of interleukin 1 in human B cell activation: inhibition of B cell proliferation and the generation of immunoglobulin-secreting cells by an antibody against human leukocytic pyrogen. J Immunol 1983; 130: 2708-2714.

42. Godding V, Stark JM, Sedgwick JB, Busse WW. Adhesion of activated eosinophils to respiratory epithelial cells is enhanced by tumor necrosis factor-alpha and interleukin-1 beta. Am J Resp Cell Mol Biol 1995; 13: 555- 562.

43. Sousa AR, Lane SJ, Nakhosteen JA, Lee TH, Poston RN. Expression of interleukin –1 beta (IL- 1 beta) and interleukin –1 receptor antagonist (IL-1 ra) on asthmatic bronchial epithelium. Am J Respir Crit Care Med 1996; 154: 1061-1066.

44. Sousa AR, Trigg CJ, Lane SJ, Hawksworth R, Nakhosteen JA, Poston RN, Lee TH. Effect if inhaled glucocorticoids on IL-1 beta and IL-1 receptor antagonist (IL-1 ra) expression in asthmatic bronchial epithelium. Thorax 1997; 52: 407-410.

45. Morgan DA, Ruscetti FW, Gallo R. Selective in vitro growth of T lymphocytes from normal human bone marrow. Science 1976; 193: 1007-1008.

46. Levi Schaffer F, Barkans J, Newman TM, Ying S, Wakelin M, Hohenstein R, Barak V, Lacy P, Kay AB, Moqbel R. Identification of interleukin -2 in human peripheral blood eosinophils. Immunology 1996; 87: 155-161.

47. Aoki Y, Qiu D, Uyei A, Kao PN. Human airway epithelial cell express interleukin -2 in vitro. Am J Physiol 1997; 272: L276-L286.

48. Taniguchi T, Minami Y. The IL-2/IL-2 receptor system: a current overview. Cell 1993; 73: 5-8.

49. Hatakeyama M, Mori H, Doi T, Taniguchi T. A restricted cytoplasmic region of IL-2 receptor beta chain is essential for growth signal transduction but not for ligand binding and internalization. Cell 1989; 59: 837-845.

50. Nakamura Y, Ozaki T, Kamei T, Kawaji K, Banno K, Miki S, Fujisawa K, Yasuoka S, Ogura T. Increased granulocyte/macrophage colony-stimulating factor production by mononuclear cells from peripheral blood of patients with bronchial asthma. Am Rev Respir Dis 1993; 147: 87-91.

51. Renzi PM, Sapeinza S, Waserman S, Du T, Olivenstein R, Wang NS, Martin JG. Effect of interleukin-2 on the airway response to antigen in the rat. Am Rev Respir Dis 1992; 146: 163-169.

52. Elwood W, Lotvall JO, Barnes PJ, Chung KF. Effect of dexamethasone and cyclosporine A on allergen-induced airway hyper responsiveness and inflammatory cell response in sensitized Brown –Norway rats. Am Rev Respir Dis 1992; 145: 1289-1294.

53. Alexander AG, Barnes NC, Kay AB. Cyclosporin A in corticosteroid-dependent chronic severe asthma. Lancet 1992; 339: 324-327.

54. Fung MC, Hapel AJ, Ymer S, Cohen DR, Johnson RM, Campbell HD, Young IG. Molecular cloning of cDNA for murine interleukin-3. Nature 1984; 307: 233-237.

55. Arai KI, Lee F, Miyajima A. Cytokines; co-ordinators of immune and inflammatory responses. Annu Rev Biochem 1990; 59: 783-836.

56. Hayashida K, Kitamura T, Gorman DM, Arai K, Yokota T, Miyajima A. Molecular cloning of a second subunit of the receptor for human granulocyte-macrophage colony-stimulating factor (GM-CSF): reconstitution of a high –affinity GM-CSF receptor. Proc Natl Acad Sci USA 1990; 87: 9655-9659.

57. Sornsen P, Mui AL, Krystal G. Interleukin-3 stimulates the tyrosine phosphorylation of the 140-kilodalto interleukin-3 receptor. J Biol Chem 1989; 264: 19253-19258.

58. Isfort R, Huhn RD, Frackelton AR, Ihle JN. Stimulation factor – dependent myeloid cell lines with interleukin induces tyrosine phosphorylation of several cellular substrates. J Biol Chem 1988; 263: 19203-19209.

59. Sun Q, Woodcock JM, Rapoport A, Stomski FC, Korpelainen EI, Bagley CJ, Goodall GJ, Smith WB, Gamble JR, Vadas MA, Lopez AF. Monoclonal anti body 7G3 recognizes the N-terminal domain of the human interleukin-3 (IL-3) receptor alpha- chain and functions a specific IL-3 receptor antagonist. Blood 1996; 87: 83-92.

60. Robinson DS, Hamid Q, Bentley AM, Durham SR, Kay AB. Activation of CD4+ T cells and increased IL-4, IL-5 and GM-CSF mRNA positive cells in bronchoalveolar lavage fluid (BAL) 24 hours after allergen inhalation challenge of atopic asthmatic patients. J Allergy Clin Immunol 1993; 92: 313-324.

61. Blotta MH, Marshall ID, DeKruyff RH, Umetsu DT. Cross-linking of the CD40 ligand on human CD4+ lymphocytes generates a co stimulatory signal that up-regulates IL-4 synthesis. J Immunol 1996; 156: 133-140.

62. Blotta MH, DeKruyff RH, Umetsu DT. Corticosteroids inhibit IL-12 production in human monocytes and enhance their capacity to induce IL-4 synthesis in CD4+ lymphocytes. J Immunol 1997; 158: 5589-5595.

63. Idzerda RL, March CJ, Mosley B, Lyman SD, Vanden Bos T, Gimpel SD, Din WS, Grabstein KH, Widmer MB, Park LS et al. Human interleukin-4 receptor confers biological responsiveness and defines a novel receptor superfamily. J Exp Med 1990; 171: 861-873.

64. Kotsimbos TC, Ghaffar O, Minshall EM, Humbert M, Durham SR, Pfister R, Menz G, Kay AB, Hamid QA. Expression of the IL-4 receptor alpha – subunit is increased in bronchial biopsy specimens from atopic and nonatopic asthmatic subjects. J Allergy Clin Immunol 1998; 102: 859-666.

65. Imani F, Rager KJ, Catipovic B, Marsh DG. Interleukin-4 (IL-4) induces phosphatidylinositol 3-kinase (p85) dephosphorylation. Implications for the role of SHP-1 in the IL-4 induced signals in human B cells. J Biol Chem 1997; 272: 7927-7931.

66. Swain SL, Weinberg AD, English M, Huston G. IL-4 directs the development of Th2 - like helper effectors. J Immunol 1990; 145: 3796-3806

67. Le Gros G, Ben-Sasson SZ, Seder R, Finkelman FD, Paul WE. Generation of interleukin 4 (IL-4) producing cells in vivo and in vitro: IL-2 and IL-4 are required for in vitro generation of IL-4- producing cells. J Exp Med 1990; 172: 921-929.

68. Brusselle G, Kips J, Joos G, Bluethmann H, Pauwels R. Allergen-induced airway inflammation and bronchial responsiveness in wild-type and interleukin-4-deficient mice. Am J Respir Cell Mol Biol 1995; 12: 254-259.

69. Coyle AJ, Gros G, Bertrand C, Tsuyuki S, Heusser CH, Kopf M, Anderson GP. Interleukin-4 is required for the induction of lung Th2 mucosal immunity. Am J Respir Cell Mol Biol 1995; 13: 54-59.

70. Gavett SH, O'Hearn DJ, Karp CL, Patel EA, Schofield BH, Finkelman FD, Wills-Karp M. Interleukin-4 receptor blockade prevents airway responses induced by antigen challenge in mice. Am J Physiol 1997; 272: L253-L261.

71. Shi HZ, Deng JM, Xu H, Zong ZX, Xiao CQ, Liu ZM, Qin SM, Jiang HX, Liu GN, Chen YQ. Effect of inhaled interleukin-4 on airway hyper reactivity in asthmatics. Am J Respir Crit Care Med 1998; 157: 1818-1821.

72. Sanderson, CJ, Campbell HD, Young IG. Molecular and cellular biology of eosinophil differentiation factor (interleukin-5) and its effects on human and mouse B cells. Immunol Rev 1988; 102: 29-34.

73. Takatsu K, Takaki S, Hitoshi Y. Interleukin-5 and its receptor system: implication in the immune system and inflammation. Adv Immunol 1994; 57: 145-149.

74. Hamid Q, Azzawi M, Ying S, Moqbel R, Wardlaw AJ, Corrigan CJ, Bradley B,. Durham SR, Collins JV, Jeffrey PK. Expression of mRNA for interleukin-5 in mucosal bronchial biopsies from asthma. J Clin. Invest 87: 1991; 1541-1549.

75. Robinson DS, Ying S, Bently AM, Meng Q, North J, Durham SR, Kay AB, Hamid Q. Relationships among numbers of bronchoalveolar lavage cells expressing messenger ribonucleic acid for cytokines, asthma symptoms, and airway methacholine responsiveness in atopic asthma. J Allergy Clin Immunol 1993; 92: 397-405.

76. Humbert M, Corrigan CJ, Kimmitt P, Till SJ, Kay AB, Durham SR. Relationship between IL-4 and IL-5 mRNA expression and disease severity in atopic asthma. Am J Respir Crit Care Med 1997; 156: 704-711.

77. Corrigan CJ, Haczku A, Gemou-Engesaeth V, Doi S, Kikuchi Y, Takatsu K, Durham SR, Kay AB. CD-4 T-lymphocyte activation in asthma is accompanied by increased serum concentrations of interleukin-5: effect of glucocorticoid therapy. Am Rev Respir Dis 1993; 147: 540-547.

78. Gulbenkian AR, Egan RW, Fernandez X, Jones H, Kreutner W, Kung T, Payvandi F, Sullivan L, Zurcher JA, Watnick AS. Interleukin -5 modulates eosinophil accumulation in allergic guinea pig lung. Am Rev Respir Dis 1992; 146: 263- 266.

79. Mauser PJ, Pitman A, Witt A, Fernandez X, Zurcher J, Kung T, Jones H, Watnick AS, Egan RW, Kreutner W, Adams GK. Inhibitory effect of the TRFK-5 anti IL-5 antibody in a guinea pig model of asthma. Am Rev Respir Dis 1993; 148: 1623-1627.

80. Renauld JC, Groethals A, Houssiau F, Merz H, Van Roost E, Van Snick J. Human P40/IL-9. Expression in activated CD4+ T cells, genomic organization and comparison with the mouse gene. J Immunol 1990; 144: 4235-4241.

81. Houssiau FA, Renauld JC, Stevens M, Lehmann F, Lethe B, Coulie PG, Van Snick J. Human T cell lines and clones respond to IL-9. J Immunol 1993; 150: 2634-2640.

82. Dugas B, Renauld JC, Pene J, Bonnefoy JY, Pete-Frere C, Braquet P, Bousquet J, Van Snick J, Mencia-Huerta JM. Interleukin-9 potentiates the interleukin-4 induced immoglobulin (IgG, IgM and IgE) production by normal human B lymphocytes. Eur J Immunol 1993; 23: 1687-1692.

83. Hultner L, Druez C, Moeller J, Uyttenhove C, Schmitt E, Rude E, Dormer P, Van Snick J. Mast cell growth-enhancing activity (MEA) is structurally related and functionally identical to the novel mouse T cell growth factor P40/TCGFIII (interleukin 9). Eur J Immunol 1990; 20: 1413-1416.

84. Temann UA, Geba GP, Rankin JA, et al. Expression of interleukin 9 in the lungs transgenic mice causes airway inflammation, mast cell hyperplasia and bronchial hyper responsiveness. J Exp Med 1998; 188: 1307-1320.

85. Fiorentino DF, Bond MW, Mosmann TR. Two types of mouse helper T cells. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. J Exp Med 1989; 170: 2081-2089.

86. Enk AH, Katz SI. Identification and induction of keratinocyte-derived IL-10. J Immunol 1992; 149: 92-95.

87. Spits H, de Waal Malefyt R. Functional characterization of human IL-10. Int Arch Allergy Appl Immunol 1992; 99: 9-15.

88. Berkman N, John M, Roesems G, Jose PJ, Barnes PJ, Chung KF. Inhibition of macrophage inflammatory protein-1α by interleukin-10: differential sensitivities in human blood monocytes and alveolar macrophage. J Immunol 1995; 155: 4412-4418.

89. Liu Y, Wei SH, Ho AS, de Waal Malefyt, Moore KW. Expression cloning and characterization of a human IL-10 receptor. J Immunol 1994; 152: 1821-1829.

90. Wang P, Wu P, Siegel MI, Egan RW, Billah MM. Interleukin (IL) -10 inhibits nuclear factor kappa B (NF kappa B) activation in human monocytes. IL-10 and IL-4 suppress cytokine synthesis by different mechanisms. J Biol Chem 1995; 270: 9558-9563.

91. Fiorentino DF, Zlotnik A, Mossmann TR, Howard M, O'Garra A. IL-10 inhibits cytokine production by activated macrophages. J Immunol 1991; 147: 3815-3822.

92. de Waal Malefyt R, Abrams J, Bennett B, Figdor CG, de Vries JE. Interleukin 10 (IL-10) inhibits synthesis by human monocytes: an auto regulatory role of IL-10 produced by monocytes. J Exp Med 1991; 179: 1209-1220.

93. Seitz M, Loetscher P, Dewald B, Towbin H, Galatti H, Baggiolini M. Interleukin-10 differentially regulates cytokine inhibitor and chemokine release from blood mononuclear cells and fibroblasts. Eur J Immunol 1995; 25: 1129-1132.

94. de Waal Malefyt R, Yssel H, Roncarolo MG, Spits H, de Vries JE. Inteleukin-10. Curr Opin Immunol 1992; 4: 314-320.

95. de Waal Malefyt M, Haanen J, Spits H Roncarolo MG, te Velde A, Figdor C, Johnson K, Kastelein R, Tssel H, de Vries JE . Interleukin 10 (IL -10)and viral IL-10 strongly reduce antigen –specific human T cell proliferation by diminishing the antigen –presenting capacity of monocytes via down regulation of class II major histocompatibility complex expression. J Exp Med 1991; 174: 915-924.

96. Cunha FQ, Moncada S, Liew FY. Interleukin-10 (IL-10) inhibits the induction of nitric oxide synthase by interferon gamma in murine macrophages. Biochem Biophys Res Commun 1992; 182: 1155-1159.

97. John M, Hirst SJ, Jose PJ, Robichaud A, Berkman N, Witt C, Twort CH, Barnes PJ, Chung KF. Human airway smooth muscle cells express and release RANTES in response to Th-1 cytokines: regulation by Th-2 cytokines and corticosteroids. J Immunol 1997; 158: 1841-1847.

98. John M, Au BT, Jose PJ, Lim S, Saunders M, Barnes PJ, Mitchell JA, Belvisi MG, Chung KF. Expression and release of interleukin-8 by human airway smooth muscle cells: inhibition by Th-2 cytokines and corticosteroids. Am J Respir Cell Mol Biol 1998; 18: 84-90.

99. Moore KW, O'Garra A, de Waal Malefyt R, Vieira P, Mosmanm TR. Interleukin -10. Annu Rev Immunol 1993; 11: 165-90.

100. Schandene L, Alonso Vega C Willems F, Gerard C, Delvaux A, Velu T, Devos R, de Boer M, Goldman M. B7/CD28- dependant IL-5 production by human resting T cells is inhibited by IL-10. J Immunol 1994; 152: 4368-74.

101. Rosenwasser LJ, Borish L. Genetics of atopy and asthma: the rationale behind promoter -based candidate gene studies (IL-4 and IL-10). Am J Respir Crit Care Med 1997; 156: S152-155.

102. Lim S, Crawley E, Woo P, Barnes PJ. Haplotype associate with low interleukin -10 production in patients with severe asthma. Lancet 1998; 352: 113.

103. Chung F. Anti-inflammatory cytokines in asthma and allergy: interleukin-10, interleukin-12, interferon gamma. Mediators Inflamm 2001; 10: 51-59.

104. Gately MK, Renzetti LM, Magram J, Stern AS, Adorini L, Gubler U. The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. Annu Rev Immunol 1998; 16: 495–521.

105. Gavett SH, O'Hearn DJ, Li X, Huang SK, Finkelman FD, Wills-Karp M. Interleukin-12 inhibits antigen- induced airway hyper-responsiveness, inflammation and Th2 cytokine expression in mice. J Exp Med 1995 ; 182 : 1527- 1536.

106. van der Pouw Kraan TC, Boeije LC, de Groot ER, Stapel SO, Snijders A, Kapsenberg ML. Reduced production of I-12 and IL-12 dependent IFN- gamma release in patients with allergic asthma. J Immunol 1997; 158 : 5560-5565

107. Minty A, Chalon O, Derocq J-M, Dumont X, Guillemot JC, Kaghad M, Labit C, Leplatois P, Liauzun P, Miloux B et al. Interleukin -13 is a new human lymphokine regulating inflammatory and immune responses. Nature 1993; 362 : 248-250.

108. Doucet C, Brouty-Boye D, Pottin-Clemenceau C, Jasmin C, Canonica GW, Azzarone B. IL-4 and IL-13 specifically increase adhesion molecule and inflammatory cytokine expression in human lung fibroblasts. Int Immunol 1998; 10: 1421- 1433.

109. de Waal Malefyt R, Figdor CG, Huijbens R, Mohan -Peterson S, Bennett B, Culpepper J, Dang W, Zurawski G, de Vries JE. Effects of IL-13 on phenotype, cytokine production, and cytotoxic function of human monocytes. J Immunol 1993; 151: 6370-6381.

110. Berkman N, Roesems G, Jose PJ, Barnes PJ, Chung KF. Interleukin -13 inhibits expression of macrophage-inflammatory protein-l α from human blood monocytes and alveolar macrophages. Am J Respir Cell Mol Biol 1995; 15: 382-389.

111. John M, Hirst SJ, Jose PJ, Barnes PJ, Chung KF. Human airway smooth muscles express and release RANTES in response to Th-1 cytokines: regulation by Th-2 cytokines and corticosteroids. J Immunol1997; 158: 1841-1847.

112. John M, Au BT, Jose PJ, Lim S, Saunders M, Barnes PJ, Mitchell JA, Belvisi MG, Chung KF. Expression and release of interleukin-8 by human airway smooth muscle cells: inhibition by Th-2 cytokines and corticosteroids. Am J Respir Cell Mol Biol 1998; 18: 84-90.

113. Punnonen J, Aversa G, Cocks BG, McKenzie AN, Menon S, Zurawski G, de Waal Malefyt R, de Vries JE. Interleukin 13 induces interleukin-4-independent IgG4 and IgE synthesis and CD23 expression by human B cells. Proc Natl Acad Sci USA 1993; 90: 3730-3734.

114. Naseer T, Minshall EM, Leung DY, Laberge S, Ernst P, Martin RJ, Hamid Q. Expression of IL-12 and IL-13 mRNA in asthma and their modulation in response to steroid therapy. Am J Respir Crit Care Med 1997; 155: 845-851.

115. Grabstein KH, Eisenman J, Shanebeck K, Rauch C, Srinivasen S, Fung V, Beers C, Richardson J, Schoenborn MA, Ahdieh A et al. Cloning of a T cell growth factor that interacts with the beta chain of the interleukin-2 receptor. Science 1994; 264: 965-968.

116. Badolato R, Ponzi AN, Millesimo M, Notarangelo LD, Musso T. Interleukin -15 (IL-15) induces IL-8 and monocyte chemotactic protein 1 production in human monocytes. Blood 1997; 90: 2804-2809.

117. Rumsaeng V, Cruikshank WW, Foster B, Prussin C, Kirshenbaum AS, Davis TA, Kornfeld H, Center DM, Metcalfe DD. Human mast cells produce the CD4+ T lymphocyte chemoattractant factor, IL-16. J Immunol 1997; 159: 2904-2910.

118. Cruikshank WW, Long A, Torpy RE, Kornfeld H, Carroll MP, Teran L, Holgate ST, Center DM. Early identification of IL-16 (lymphocyte chemoattractant factor) and macrophage inflammatory protein 1 α (MIP-1 α) in bronchoalveolar lavage fluid of antigen-challenged asthma. Am J Respir Cell Mol Biol 1995; 13: 738-747.

119. Fossiez F, Djossou O, Chomarat P, et al. T cell interleukin -17 induces stromal cells to produce proinflammatory and hematopoietic cytokines (see comments) J Exp Med 1996; 183: 2593-2603.

120. Ushio S, Namba M, Okura T, Hattori K, Nukada Y, Akita K, Tanabe F, Konishi K, Micallef M, Fujii M, Torigoe K, Tanimoto T, Fukuda S, Ikeda M, Okamura H, Kurimoto M. Cloning of the cDNA for human IFN gamma-inducing factor, expression in *Escherichia coli* and studies on the biologic activities of the protein. J Immunol 1996; 156: 4274-4279.

121. Xu D, Chan WL, Leung BP, Hunter D, Schulz K, Carter RW, McInnes IB, Robinson JH, Liew FY. Selective expression and functions of interleukin 18 receptor on T helper (Th) type 1 but not Th2 cells. J Exp Med 1998; 188: 1485-1492

122. Matsumoto S, Tsuji-Takayama K, Aizawa Y, Koide K, Takeuchi M, Ohta T, Kurimoto M. Interleukin-18 activates NF-kappaB in murine T helper type 1 cells. Biochem Boiphys Res Commun 1997; 234: 454-457.

123. Oppman B, Leswley R, Blom B, Timans JC, Xu Y, Hunte B, Vega F, Yu N, Wang J, Singh K, Zonin F, Vaisberg E, Churokova T, Liu M, Gorman D, Wagner J, Zurawski S, Liu Y, Abrams JS, Moore KW, Rennick D, de Waal-Malefyt R, Hannum C, Bazan JF, Kastelein RA. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. Immunity 2000; 13: 715–725.

124. Hurst SD, Muchamuel T, Gorman DM, Gilbert JM, Clifford T, Kwan S. New IL-17 family members promote Th1 or Th2 responses in the lung: in vivo function of the novel cytokine IL-25. J Immunol 2002; 169: 443-453.

125. Ikeda K, Nakajima H, Suzuki K, Kagami SI, Hirose K, Suto A, Saito Y, Iwamoto I. Mast cells produce interleukin-25 upon Fc{epsilon}RI-mediated activation. Blood 2003; 101: 3594- 3596.

126. Gearing AJ, Beckett P, Christodoulou M, Churchill M, Clements J, Davidson AH, Drummond AH, Galloway WA, Gilbert R, Gordon JL et al. Processing of tumour necrosis factor-alpha precursor by metallaproteinases. Nature 1994; 370: 555-557.

127. Eder J. Tumor necrosis factor alpha and interleukin 1 signalling: do MAPKK kinases connect it all? Trends Pharmacol Sci 1997; 18: 319-22.

128. Ohkawara Y, Yamauchi K, Tanno Y. et al. Human lung mast cells and pulmonary macrophages produce tumor necrosis factor-α in sensitized lung tissue after IgE receptor triggering. Am J Respir Cell Mol Biol 1992; 7: 385- 392.

129. Yates DH, Barnes PJ, Thomas PS. Tumor necrosis factor-α alters human bronchial reactivity and induces inflammatory cell influx. Am Rev Respir Dis 1993; 147: A1011.

130. Shah A, Church MK, Holgate ST. Tumor necrosis factor alpha: a potential mediator of asthma. Clin Exp Allergy 1995; 25: 1038-1044.

131. Hayashida K, Kitamura T, Gorman DM, Arai K, Yokota T, Miyajima A. Molecular cloning of a second subunit of the receptor for human granulocyte-macrophage colony-stimulating factor (GM-CSF); reconstitution of a high affinity GM-CSF receptor. Proc Natl Acad Sci USA 1990; 87: 9655-9699.

132. Bussolino F, Wang JM, Defilippi P. Granulocyte- and granulocyte-macrophage-colony stimulating factor induce human endothelial cells to migrate and proliferate. Nature 1989; 337: 471-473.

133. Kitamura T, Sato N, Arai K, Miyajima A. Expression cloning of the human IL-3 receptor cDNA reveals a shared subunit for the human IL-3 and GM-CSF receptors. Cell 1991; 66: 1165-74.

134. Martinez-Moczygemba M, Huston DP. Biology of common beta receptor-signalling cytokines: IL-3, IL-5 and GM-CSF. J Allergy Clin Immunol 2003; 112: 653-655.

135. Kotsimbos AT, Humbert M, Minshall E. Upregulation of alpha GM-CSF-receptor in non-atopic asthma but not in atopic asthma. J Allergy Clin Immunol 1997; 99: 666-672.

136. Hallsworth MP, Giembycz MA, Barnes PJ. Cyclic AMP-elevating agents prolong or inhibit eosinophil survival depending on prior exposure to GM-CSF. Br J Pharmacol 1996; 117: 79-86.

137. Sousa AR, Poston RN, Lane SJ, Nakhosteen JA, Lee TH. Detection of GM-CSF in asthmatic bronchial epithelium and decrease by inhaled corticosteroids. Am Rev Respir Dis 1993; 147: 1557-1561

138. Broide DH, Lotz M, Cuomo AJ, Coburn DA, Federman EC, Wasserman SI. Cytokines in symptomatic asthmatic airways. J Allergy Clin Immunol 1992; 89: 958-967.

139. Broide DH, Firestein GS. Endobronchial allergen challenge: demonstration of cellular source of granulocyte macrophage colony-stimulating factor by in situ hybridization. J Clin Invest 1991; 88: 1048-53.

140. Brown PA, Crompton GK, Greening AP. Proinflammatory cytokines in acute asthma. Lancet 1991; 338: 590-593.

141. Nakamura Y, Ozaki T, Kamei T, Kawaji K, Banno K, Miki S, Fujisawa K, Yasuoka S, Ogura T. Increased granulocyte-macrophage colony-stimulating factor production by mononuclear cells from peripheral blood of patients with bronchial asthma. Am Rev Respir Dis 1993; 147: 87-91.

142. Xing Z, Ohkawara Y, Jordana M, Graham. F, Gauldie J. Transfer of granulocyte-macrophage colony –stimulating factor gene to rat lung induces eosinophilia, monocytosis, and fibrotic reactions. J Clin Invest 1996; 97: 1102-1110.

143. Romagnini S. Regulation and deregulation of human IgE synthesis. Immunol today 1990; 11: 316-321.

144. Lack G, Bradley KL, Hamelmann E, Renz H, Loader J, Leung DY, Larsen G, Gelfand EW. Nebulised IFN-gamma inhibits the development of secondary allergic responses in mice. J Immunol 1996; 157: 1432-1439.

145. Lack G, Nelson HS, Amran D, Oshiba A, Jung T, Bradlry KL, Giclas PC, Gelfanf EW. Rush immunotherapy results in antigen-specific alterations in lymphocyte function and interferon- gamma production in CD4+ T cells. J Allergy Clin Immunol 1997; 99: 530-538.

146. Bentley AM, Hamid Q, Robinson DS, Shotman E, Meng Q, Assoufi B, Kay AB, Durham SR. Prednisolone treatment in asthma. Reduction in the numbers of eosinophils, T cells, tryptase-only positive mast cells, and modulation of IL-4, IL-5, and interferon-gamma cytokine gene expression within the bronchial mucosa. Am J Respir Crit Care Med 1996; 153: 551-556.

147. Gifford GE, Lohmann-Matthess ML. Gamma interferon priming of mouse and human macrophages for induction of tumour necrosis factor production by bacterial lipopolysaccharide. J Natl Cancer Inst 1987; 78: 121-124.

148. Look DC, Rapp R, Keller BT, Holtzman MJ. Selective induction of intracellular adhesion molecule-1 by interferon-gamma in human airway epithelial cells. Am J Physiol 1992; 263: L79-L87.

149. Sporn SA, Eierman DF, Johnson CE, Morris J, Martin G, Ladner M, Haskill S. Monocyte adherence results in selective induction of novel genes sharing homology with mediators of inflammation and tissue repair. J Immunol 1990; 144: 4434-4441.

150. Matsushima K, Larsen CG, Du Bois GC. Purification and characterisation of a novel monocyte chemotactic and activating factor produced by a human myelomonocytic cell line. J Exp Med 1989; 169: 1485- 1490.

151. Schall TJ, Jongstra J, Dyer BJ, Jorgensen J, Clayberger C, Davis MM, Krensky AM. A human T cell-specific molecule is a member of a new gene family. J Immunol 1988; 141: 1018-25.

152. Jose PJ, Griffiths-Johnson DA, Collins PD, Walsh DT, Moqbel R, Totty NF, Truong O, Hsuan JJ, Williams TJ. Eotaxin: a potent eosinophil chemoattractant cytokine detected in a guinea pig model of allergic airways inflammation. J Exp Med 1994; 179: 881-887.

153. Bonecchi R, Bianchi PP, Bordignon PP, D'Ambrosio D, Lang R, Borsattti A, Sozzani S, Allavena P, Gray PA, Mantovani A, Sinigaglia F. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. J Exp Med 1998; 187: 129-134.

154. Neote K, Digregorio D, Mak JY, Koruk R, Scahall TJ. Molecular cloning, functional expression and signalling characteristics of a C-C chemokine receptor. Cell 1993; 72: 415-425.

155. Heath H, Qin S, Rao P, Wu L, LaRosa G, Kassam N, Ponath PD, Mackay CR. Chemokine receptor usage by human eosinophils. The importance of CCR3 demonstrated using an antagonistic monoclonal antibody. J Clin Invest 1997; 99: 178-184.

156. Kuna P, Reddigari SR, Rucinski D, Oppenheim JJ, Kaplan AP. Monocyte chemotactic and activating factor is a potent histamine-releasing factor for human basophils. J Exp Med 1992; 175: 489-493.

157. Rollins BJ, Walz A, Baggiolini M. Recombinant human MCP-1/JE induces chemotaxis, calcium flux, and the respiratory burst in human monocytes. Blood 1991; 78: 1112-1116.

158. Bywater M, Rorsman F, Bongcam Rudloff E. Expression of recombinant platelet-derived growth factor A- and B-chain homodimers in rat-1 cells and human fibroblasts reveals differences in protein processing and autocrine effects. Mol Cell Biol 1998; 8: 2753-2762.

159. Raghu G, Masta S, Meyers D, et al. Collagen synthesis by normal and fibrotic human lung fibroblasts and the effect of transforming growth factor β. Am Rev Respir Dis 1989; 140: 95-100.

160. Folkman J, Klagsbrun M. Vascular physiology. A family of angiogenic peptides. Nature 1987; 329: 671-672.

161. Cohen P, Noveral JP, Bhala A, Nunn SE, Herrick DJ, Grunstein MM. Leukotriene D4 facilitates airway smooth muscle cell proliferation via modulation of the IGF axis. Am J Physiol 1995; 269: L151-L157.

162. Mosmann TR, Coffman RL. Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol 1989; 7: 145-173.

163. Castro M, Chaplin D, Walter M, Holtzman M. Could asthma be worsened by stimulating the T-helper type 1 immune response? Am J Respir Mol Cell Biol 2000; 22: 143-146.

164. Hansen G, Berry G, DeKruyff RH, Umetsu DT. Allergen-specific Th1 cells fail to counterbalance Th2 cell induced airway hyper reactivity but cause severe airway inflammation. J Clin Invest 1999; 103: 175-183.

165. Lawrence S, Beasley R, Doull I. Genetic analysis of atopy and asthma as quantitive traits and ordered polychotomies. Ann Hum Gen 1994; 58: 359-368.

166. Holgate ST. Asthma genetics: waiting to exhale. Nat Genet 1997; 15: 227-229.

167. Liggett S. Polymorphisms of the β_2 adrenergic receptor and asthma. Am J Respir Crit Care Med 1997; 156: S156-S162.

168. Moffat M, Cookson W. Tumour necrosis factor haplotypes and asthma. Hum Mol Genet 1997; 6: 551-554.

169. Van Eerdewegh P, Little RD, Dupuis J, Del Mastro RG, Falls K, Simon J, ...Holgate ST. Association of the ADAM 33 gene with asthma and bronchial hyper responsiveness. Nature 2002; 418: 426-430.

170. Allen M, Heinzmann A, Noguchi E, Abecasis G, Broxholme J, Ponting CP, et al. Positional cloning of a novel gene influencing asthma from chromosome 2q14. Nat Genet 2003; 35: 258-263.

171. Strachan DR. Hay fever, hygiene, and household size. BMJ 1989; 299: 1259-1260.

172. Ball T, Castro-Rodriguez J, Griffith K, Hilberg CJ, Martinez FD, Wright AL. Siblings, day-care attendance and the risk of asthma and wheezing during childhood. N Engl J Med 2000; 348; 543 -548.

173. Hollenberg SM, Weinberger C, Ong ES, et al. Primary structure and expression of a functional human glucocorticoid receptor cDNA. Nature 1985; 318: 635-641.

174. Barnes PJ. Therapeutic strategies for allergic diseases. Nature 1999; 402 (suppl): B31-B38.

175. Karin M. New twists in the gene regulation by glucocorticoid receptor: Is DNA binding dispensable? Cell 1998; 93: 487-490.

176. Barnes PJ. Inhaled glucocorticoids for asthma. N Engl J Med 1995; 332: 868-875.

177. Schwartz HJ, Lowell FC, Melby JC. Steroid resistance in bronchial asthma. Ann Intern Med 1968; 69: 493 - 499.

178. Carmichael J, Paterson IC, Diaz P, Crompton GE, Kay AB, Grant IWB. Corticosteroid resistance in chronic asthma. Br Med J 1981; 282: 1419 - 1422.

179. American Thoracic Society. ATS standardisation of spirometry: 1994 update. Am J Resp Crit Care Med 1994; 152: 1107-1136.

180. Chung KF for the ERS Task Force. Difficult/therapy- resistant asthma. ERJ 1999; 13: 1198-1208.

181. Woolcock AJ. Steroid resistant asthma: what is the clinical definition? Eur Respir J 1993; 6: 743-747.

182. Alvarez J, Surs W, Leung DYM, Ikle' D, Gelfand EW, Szefler SJ. Steroid-resistant asthma: Immunologic and pharmacologic features. J Allergy Clin Immunol 1992; 89: 714-721.

183. Corrigan CJ, Brown PH, Barnes NC, Szefler SJ, Tsai JJ, Frew AJ, Kay AB. Glucocorticoid resistance in chronic asthma: Glucocorticoid pharmacokinetics, glucocorticoid receptor characteristics, and inhibition of peripheral blood T cell proliferation by glucocorticoids in vivo. Am Rev Respir Dis 1991; 144: 1016-1025.

184. Kay A B, Diaz P, Carmichael J, Grant I W B. Corticosteroid - resistant chronic asthma and monocyte complement receptors .Clin Exp Immunology 1981; 44: 576-580.

185. Poznansky M C , Gordan A C H , Douglas J G ,Krajewski A S , Wyllie A H , Grant I W B . Resistance to methylprednisolone in cultures of blood mononuclear cells from glucocorticoid - resistant asthmatic patients. Clin Sci 1984; 67: 639-645.

186. Wilkinson JR, Crea AEG, Clark TJH, Lee TH. Identification and characterisation of a monocyte- derived neutrophil activating factor in corticosteroid-resistant bronchial asthma. J Clin Invest 1989; 84: 1930-1941.

187. Vecchiarelli A , Siracusa A , Ceaci E , Puliti M , Abbriti G . Effect of corticosteroid treatment on interleukin - 1 and tumour neurosis factor secretion of monocytes from subject with asthma. Clin Exp Allergy 1992; 22: 365-370.

188. Corrigan CJ, Brown PH, Barnes NC. Glucocorticoid resistance in chronic asthma. Am Rev Respir Dis 1991; 144: 1026-1032.

189. Lane SJ, Sousa AR, Poston RN, Lee TH. In vivo cutaneous tuberculin response to prednisolone in corticosteroid resistant bronchial asthma. J Allergy Clin Immunol 1993; 91: 222A.

190. Cato AC, Nestl A, Mink S. Rapid actions of steroid receptors in cellular signalling pathways. Sci STKE 2002; 2002(138): RE9.

191. Pederson KB, Vedeckis WV. Quantification and glucocorticoid regulation of glucocorticoid receptor transcripts in two human leukaemic cell lines. Biochemistry 2003; 42: 10978-10990.

192. Kino T, Chrousos GP. Glucocorticoid and mineralocorticoid resistance/ hypersensitivity syndromes. J Endocrinol 2001; 169: 437-445.

193. Adcock IM. Molecular mechanisms of glucocorticosteroid actions. Pulm Pharmacol Ther 2000; 13: 115-26.

194. Barnes PJ, Karin M. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. N Engl J Med 1997; 336:1066-71.

195. Barnes PJ. Efficacy of inhaled corticosteroids in asthma. J Allergy Clin Immunol 1998; 102: 531-8.

196. Cosio BG, Mann B, Ito K, Jazrawi E, Barnes PJ, Chung KF, Adcock IA. Histone acetylase and deacetylase activity in alveolar macrophages and blood mononocytes in asthma. Am J Respir Crit Care Med 2004; 170: 141-147.

197. Swantek JL, Cobb MH, Geppert TD. Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) is required for lipopolysaccharide stimulation of tumor necrosis factor alpha (TNF-alpha) translation: glucocorticoids inhibit TNF-alpha translation by blocking JNK/SAPK. Mol Cell Biol 1997; 17: 6274-6282.

198. Caelles C, Gonzalez-Sancho JM, Munoz A. Nuclear hormone receptor antagonism with AP-1 by inhibition of the JNK pathway. Genes Dev 1997; 11: 3351- 3364.

199. Rider LG, Hirasawa N, Santini F, Beaven MA. Activation of the mitogen activated protein kinase cascade is suppressed by low concentrations of dexamethasone in mast cells. J Immunol 1996; 157: 2374- 2380.

200. Hirasawa N, Sato Y, Fujita Y, Mue S, Ohuchi K. Inhibition by dexamethasone of antigen-induced c-Jun N-terminal kinase activation in rat basophilic leukemia cells. J Immunol 1998; 161: 4939- 4943.

201. Galon J, Franchimont D, Hiroi N, Frey G, Boettner A, Ehrhart-Bornstein M, et al. Gene profiling reveals unknown enhancing and suppressive actions of glucocorticoids on immune cells. FASEB J 2002; 16: 61-71.

202. Kino T, Chrousos GP. Tissue-specific glucocorticoid resistance hypersensitivity syndromes: multifactorial states of clinical importance.J Allergy Clin Immunol 2002; 109: 609-613.

203. Oakley RH, Sar M, Cidlowski JA. The human glucocorticoid receptor beta-isoform: expression, biochemical properties, and putative function. J Biol Chem 1996; 271: 9550-9559.

204. Leung DYM, Hamid Q, Vottero A, Szefler SJ, Surs W, Minshall E, Chrousos GP, KLemm DJ. Association of glucocorticoid insensitivity with increased expression of glucocorticoid receptor beta. J Exp Med 1997; 186: 1567-1574.

205. Christodoulopoulos P, Leung DY, Elliott MW, Hogg JC, Muro S, Toda M, Laberge S, Hamid QA. Increased number of glucocorticoid receptor beta expressing cells in the airways in fatal asthma. J Allergy Clin Immunol 2000; 106: 479-484.

206. Hamid QA, Wenzel SE, Hauk PJ, Tsicopoulos A, Wallaert B, Lafitte JJ. Increased glucocorticoid receptor β in airway cells of glucocorticoid insensitive asthma. Am J Respir Crit Care Med 1999; 159: 1600-1604.

207. Sousa AR, Lane SJ, Cidlowski JA, Staynov DZ, Lee TH. Glucocorticoid resistance in asthma is associated with elevated in vivo expression of the glucocorticoid receptor beta-isoform. J Allergy Clin Immunol 2000; 105: 943-950.

208. Gagliardo R, Chanez P, Vignola AM, Bousquet J, Vachier I, Godard P, Bonsignore G, Demoly P. Mathieu M. Glucocorticoid receptor α and β in glucocorticoid dependent asthma. Am J Respir Crit Care Med 2000; 162: 7-13.

209. Oakley RH, Jewell CM, Yudt MR, Bofetiado DM, Cidlowski JA. The dominant negative activity of the human glucocorticoid receptor beta-isoform. J Biol Chem 1999; 274: 27857-27866.

210. Webster JC, Oakley RH, Jewell CM, Cidlowski JA. Pro-inflammatory cytokines regulate human glucocorticoid receptor gene expression and lead to the accumulation of the dominant negative β isoform: a mechanism for the generation of glucocorticoid resistance. Proc Natl Acad Sci USA 2001; 98: 6865 – 6870.

211. Gougat C, Jaffuel D, Gagliardo R, Henriquet C, Bousquet J, Demoly P, Mathieu M. Over expression of the human glucocorticoid receptor α and β isoforms inhibit AP-1 and NF-κB activities independently. J Mol Med 2002; 80: 309-318.

212. Nocturnal asthma is associated with reduced glucocorticoid receptor binding affinity and decreased steroid responsiveness at night. J Allergy Clin Immunol 1999; 103: 66-71.

213. Barnes PJ, Greening AP, Crompton GK. Glucocorticoid resistance in asthma. Am J Respir Crit Care Med 1995; 152: S125-S140.

214. Adcock IM. Steroid resistance in asthma. Molecular mechanisms. Am J Respir Crit Care Med 1996; 154: S58-S61.

215. Lane SJ, Lee TH. Glucocorticoid receptor characteristics in monocytes of patients with Corticosteroid – resistant bronchial asthma. Am Rev Respir Dis 1991; 143: 1020-1025.

216. Sher ER, Leung DY, Surs W, Kam JC, Zieg G, Kamada AK, Szefler SJ. Steroid-resistant asthma. Cellular mechanisms contributing to inadequate response to glucocorticoid therapy. J Clin Invest 1994; 93: 33-39.

217. Burnstein Kl, Jewell CM, Cidlowski JA. Human GR receptor cDNA contains sequences sufficient for receptor down-regulation. J Biol Chem 1990; 265: 7284-7291.

218. Pujols L, Mullol J, Perez M, Roca-Ferrer J, Juan M, Xaubet A, Cidlowski JA, Picado C. Expression of the human glucocorticoid receptor the α and β isoforms in human respiratory epithelial cells and their regulation by dexamethasone. Am J Respir Cell Mol Biol 2001; 24: 49-57.

219. Spahn J, Leung DYM, Surs W, Harbeck RJ, Nimmagadda S, Szefler SJ. Reduced glucocorticoid binding affinity in asthma is related to ongoing allergic inflammation. Am J Respir Crit Care Med 1995; 151: 1709-1714.

220. Leung DYM, Martin RJ, Szefler JS. The airways of steroid resistant versus steroid sensitive asthma are associated with different patterns of cytokine gene expression. J Allergy Clin Immunol 1994; 93: 209A.

221. Spahn JD, Szefler SJ, Surs W, Doherty DE, Nimmagadda Sai R, Leung DYM . A novel action of IL-13. Induction of diminished monocyte glucocorticoid receptor-binding affinity. J Immunol 1996, 157: 2654-2659.

222. Kam JC, Szefler JS, Surs W, Sher ER, Leung DYM. Combination IL-2 and IL-4 reduces glucocorticoid receptor-binding affinity and T cell response to glucocorticoids. J Immunol 1993; 151: 3460-3466.

223. Nimmagadda SR, Szefler SJ, Spahn JD, Surs W, Leung DY. Allergen exposure decreases glucocorticoid receptor binding affinity and steroid responsiveness in atopic asthmatics. Am J Respir Crit Care Med. 1997; 155: 87-93.

224. Kraft M, Vianna E , Martin RJ, Leung DYM . Nocturnal asthma is associated with reduced glucocorticoid receptor binding affinity and decreased steroid responsiveness at night. J Allergy Clin Immunol 1999; 103: 66-71.

225. Spahn JD, Leung DYM, Surs W, Harbeck RJ, Nimmagadda Sai R, Szefler SJ. Reduced glucocorticoid binding affinity in asthma is related to ongoing allergic inflammation. Am J Respir Crit Care Med 1995; 151: 1709-14.

226. Nimmagadda SR, Spahn JD, Nelson HS, Jenkins J, Szefler SJ, Leung DY. Fluticasone propionate results in improved glucocorticoid receptor binding affinity and reduced oral glucocorticoid requirements in severe asthma. Ann Allergy Asthma Immunol. 1998; 81: 35-40.

227. Barnes PJ, Adcock IM .Anti-inflammatory actions of steroids: molecular mechanisms. Trends Pharmacol Sci 1993 ; 14: 436-41.

228. Berridge MJ. Inositol triphosphate and diacylglycerol: two interacting second messengers. Annu Rev Biochem 1987; 56: 159-193

229. Condliffe AM, Cadwallader KA, Walker TR, Andrew RC, Cowburn RS, Chilvers ER. Phosphoinositide 3-kinase: a critical signalling event in pulmonary cells. Respir Res 2000; 1: 24–29.

230. Hershenson MB, Kelleher MD, Naureckas ET, Abe MK, Rubinstein VJ, Zimmermann A, Bendele AM, McNulty JA, Panettieri RA, Solway J: Hyperoxia increases airway cell S-phase traversal in immature rats in vivo. Am J Respir Cell Mol Biol 1994; 11: 296- 303.

231. Panettieri RA, Murray RK, Eszterhas AJ, Bilgen G, Martin JG: Repeated allergen inhalations induce DNA synthesis in airway smooth muscle and epithelial cell in vivo. Am J Physiol 1998; 274: L417–L424.

232. Scott PH, Belham CM, Al-Hafidh J, Chilvers ER, Peacock AJ: A regulatory role for cAMP in phosphatidylinositol 3-kinase/p70 ribosomal S6 kinase-mediated DNA synthesis in platelet-derived growth- factor-stimulated bovine airway smooth-muscle cells. Biochem J 1996; 318: 965–971.

233. Condliffe AM, Hawkins PT, Stephens LR, Haslett C, Chilvers ER: Priming of human neutrophil superoxide generation by tumour necrosis factor is signalled by enhanced phosphatidylinositol 3,4,5-trisphosphate but not inositol 1,4,5-trisphosphate accumulation. FEBS Lett 1998; 439: 147–151.

234. Palframen RT, Collins, PD, Severs, NJ, Rothery S, Williams TJ, Rankin SM: Mechanisms of acute eosinophil mobilization from bone marrow by interleukin 5: the role of specific adhesion molecules and phosphatidylinositol 3-kinase. J Exp Med 1998; 188: 1621–1632.

235. Dunzendorfer S, Meirhofer C, Wiedermann CJ: Signalling in neuropeptide- induced migration of human eosinophils. J Leukoc Biol 1998; 64: 828–834.

236. Hofmann C, Dichmann S, Zimpfer U, Czech W, Herouy Y, Wagner E, Norgauer J: Metabolism and function of 3-D-phosphorylated phosphoinositides in C5a-stimulated eosinophils. Biochem Biophys Res Commun 2000; 269: 816–821.

237. Pan ZK, Chen LY, Cochrane CG, Zuraw BL: fMet-leu-phe stimulates proinflammatory cytokine gene expression in human peripheral blood monocytes: the role of phosphatidylinositol 3-kinase. J Immunol 2000; 164: 404–411.

238. Goekjian PG, Jirousek MR. Protein kinase C inhibitors as novel anticancer drugs. Expert Opin Investig Drugs 2001; 10: 2117–2140.

239. Evans DJ, Lindsay MA, Webb BL, Kankaanranta H, Giembycz MA, O'Connor BJ, Barnes PJ. Expression and activation of protein kinase C-zeta in eosinophils after allergen challenge. Am J Physiol 1999; 277: L233–L239.

240. Vachier, P. Chanez, T. Radeau, D.C. Le, C. Leger and P. Godard, Cellular protein kinase C activity in asthma. Am J Respir Crit Care Med 1997; 155: 1211–1216.

241. Page K, Li J, Zhou L, Iasvovskaia S, Corbit KC, Soh JW, Weinstein I B, Brasier AR, Lin A, Hershenson M.B. Regulation of airway epithelial cell NF-kappa B-dependent gene expression by protein kinase C delta. J. Immunol 2003. 170, 5681–5689.

242. Whelchel A, Evans J, Posada J. Inhibition of ERK activation attenuates endothelin-stimulated airway smooth muscle cell proliferation. Am J Respir Cell Mol Biol 1997; 16: 589-596.

243. Matsubara M, Ohmori K, Hasegawa K. Histamine H1 Receptor-Stimulated Interleukin 8 and Granulocyte Macrophage Colony-Stimulating Factor Production by Bronchial Epithelial Cells Requires Extracellular Signal-Regulated Kinase Signalling via Protein Kinase C. Int Arch Allergy Immunol 2006; 139: 279- 284.

244. Lewis TS, Shapiro PS, Ahn NG, Signal Transduction through MAP kinase cascades. Adv Cancer Res 1998; 74: 49-139.

245. Brunet A, Pouysségur J. Mammalian MAP kinase modules: how to transduce specific signals. Essays Biochem 1997; 32: 1-16.

246. Robinson MJ, Cobb MH. Mitogen activated protein kinase pathways. Cur Opin Cell Biol 1997; 9: 180-6.

247. Yamaguchi K, Shirakabe K, Shibuya H et al. Identification of a member of the MAPKKK family as a potential mediator of TGF-beta signal transduction. Science 1995; 270: 2008-2011.

248. Zhang S, Han J. Sells MA et al. Rho family GTPases regulate p38 MAPK through downstream mediator PAK1. J Biol Chem 1995; 270: 23934-23936.

249. Stein B, Balwin AS, Ballard DW Jr. Greene WC, Angel P, Herrlich P. Cross coupling of the NFkB p65 and Fos/Jun transcription factors produces potentiated biological function. EMBO J 1993; 12:3879-3891.

250. Allessi DR, Cuenda A, Cohen P. Dudley DT, Saltiel AR. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. J Biol Chem 1995; 270: 27489-27494.

251. Wang YZ, Bonner JC. Mechanism of extracellular signal-regulated kinase (ERK)-1 and ERK-2 activation by vanadium pentoxide in rat pulmonary myofibroblasts. Am J Respir Cell Mol Biol 2000; 22: 590-597.

252. Laan M, Lotvall J, Chung KF, Linden A.IL-17-induced cytokine release in human bronchial epithelial cells in vitro: role of mitogen-activated protein (MAP) kinases. Br J Pharmacol. 2001; 133: 200-206.

253. Lee PJ, Zhang X, Shan P, Ma B, Lee CG, Homer RJ, Zhu Z, Rincon M, Mossman BT, Elias JA. ERK1/2 mitogen-activated protein kinase selectively mediates IL-13-induced lung inflammation and remodelling in vivo. J Clin Invest 2006; 116: 163-173.

254. Blanchet S, Ramgolam K, Baulig A, Marano F, Baeza-Squiban A. Fine particulate matter induces amphiregulin secretion by bronchial epithelial cells. Am J Respir Cell Mol Biol 2004; 30: 421-427.

255. Tachimoto H, Kikuchi M, Hudson SA, Bickel CA, Hamilton RG, Bochner BS . Eotaxin-2 Alters Eosinophil Integrin Function via Mitogen-Activated Protein Kinases. Am J Respir Cell Mol Biol. 2002; 26: 645-649.

256. Lee JC, Laydon JT, McDonnel PC et al. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. Nature 1994; 372: 739-46.

257. Tong L, Pav S, White DM, Rogers S, Crane KM, Cywin CL, Brown ML, Pargellis CA, A highly specific inhibitor of human p38 MAP kinase binds in the ATP pocket. Nat. Struct. Biol 1997; 4:311- 316.

258. Cohen P. The search for physiological substrates of MAP and SAP kinases in mammalian cells. Trends Cell Biol 1997; 7: 353-761.

259. Kyriakis JM, Avruch J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev 2001; 81, 807–869.

260. J.C. Lee, J.T. Laydon, P.C. McDonnell, T.F. Gallagher, S. Kumar, D. Green, D. McNulty, M.J. Blumenthal, J.R. Heys, S.W. Landvatter, et al., A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. Nature 1994; 372: 739-742.

261. Beyaert R, Cuenda A, Vande Berghe W et al. The p38/RK mitogen-activated protein kinase pathway regulates interleukin-6 synthesis response to tumour necrosis factor. EMBO J 1996; 15: 1914-1923.

262. Shapiro L, Dinarello CA. Osmotic regulation of cytokine synthesis in vitro. Proc Nat Acad Sci USA 1995; 92: 12230-12234.

263. Tashimoto S, Matsumoto K. Gon Y et al. p38 MAP kinase regulates TNFα, IL-1α and PAF-induced RANTES and GM-CSF production by human bronchial epithelial cells. Clin Exp Allergy 1999; 30: 48-55.

264. Campbell J, Ciesielski CJ, Hunt AE, Horwood NJ, Beech JT, Hayes LA, Denys A, Feldmann M, Brennan FM, Foxwell BMJ. A novel mechanism for TNF-a regulation by p38 MAPK: involvement of NF-κB with implications for therapy in rheumatoid arthritis. J Immunol 2004; 173: 6928– 6937.

265. Chen CC, Wang JK., p38 but not p44/42 mitogen-activated protein kinase is required for nitric oxide synthase induction mediated by lipopolysaccharide in RAW 264.7 macrophages. Mol. Pharmacol 1999; 55: 481–488.

266. Duan W, Chan JH, McKay K, Crosby JR, Choo HH, Leung BP, Karras JG, Wong WS. Inhaled p38{alpha} Mitogen-Activated Protein Kinase Antisense Oligonucleotide Attenuates Asthma in Mice. Am J Respir Crit Care Med 2005; 171: 571-578.

267. Crawley JB, Rawlinson L, Lali FV, Page TH, Saklatvala J, Foxwell BM. T cell proliferation in response to interleukins 2 and 7 requires p38MAP kinase activation. J Biol Chem1997; 272: 15023-15027.

268. Bergstrand H. Phosphodiesterase inhibition and theophylline. Eur J Respir Dis 1980; 61: 37- 44.

269. Fredholm BB, Persson CGA. Xanthine derivatives as adenosine antagonists. Eur J Pharmacol 1982; 81: 673- 6.

270. Dent G, Giembycz MA, Rabe KF, Barnes PJ. Inhibition of eosinophil cyclic nucleotide PDE activity and opsonized zymosan-stimulated respiratory burst by type IV-selective PDE inhibitors. Br J Pharmacol 1991; 103: 1339-1346.

271. Cohan VL, Showell HJ, Fisher DA, Pazoles CJ, Watson JW, Turner CR, Cheng JB. In vitro pharmacology of the novel phosphodiesterase type 4 inhibitor, CP-80633. J Pharmacol Exp Ther 1996; 278: 1356- 61.

272. Hatzelmann A, Tenor H, Schudt C. Differential effects of non-selective and selective phosphodiesterase inhibitors on human eosinophil functions. Br J Pharmacol 1995; 114: 821-31.

273. Walker BAM, Jacobson MA, Knight DA, Salvatore CA, Weir T, Zhou D, Bai TR. Adenosine A3 receptor expression and function in eosinophils. Am J Respir Cell Mol Biol 1997; 16: 531-537.

274. Ezeamuzie CI, Philips E. Adenosine A3 receptors on human eosinophils mediate inhibition of degranulation and superoxide anion release. Br J Pharmacol 1999; 127: 188-94.

275. Knight K, Zheng X, Rocchini C, Jacobson M, Bai T, Walker B. Adenosine A3 receptor stimulation inhibits migration of human eosinophils. J Leukoc Biol 1997; 62: 465-468.

276. Ezeamuzie CI. Involvement of A3 receptors in the potentiation by adenosine of the inhibitory effect of theophylline on human eosinophil degranulation: possible novel mechanism of the anti-inflammatory action of theophylline. Bio Pharmacol 61 (2001) 1551-1559.

277. Pauwels RA. New aspects of the therapeutic potential of theophylline in asthma. J Allergy Clin Immunol 1989;83:548-553.

278. Sullivan PJ, Bekir S, Jaffer Z, Page C, Jeffery P, Costello J. Anti-inflammatory effects of low-dose oral theophylline in atopic asthma. Lancet 1994; 343: 1006-1008.

279. Banner KH, Page CP. Anti-inflammatory effects of theophylline and selective phosphodiesterase inhibitors. Clin Exp Allergy 1996; 26: (Suppl 2)2-9.

280. Dutoit JI, Salome CM, Woolcock AJ. Inhaled corticosteroids reduce the severity of bronchial hyper responsiveness in asthma but oral theophylline does not. Am Rev Respir Dis 1987; 136: 1174--1178.

281. Sullivan P, Bekir S, Jaffar Z, Page C, Jeffery P, Costello J. Anti-inflammatory effects of low-dose oral theophylline in atopic asthma. Lancet 1994; 343: 1006-1008.

282. Ward AJ, McKenniff M, Evans JM, Page CP, Costello JF. Theophylline-an immunomodulatory role in asthma? Am Rev Respir Dis 1993; 147:518-23.

283. Evans DJ, Taylor DA, Zetterstrom O, Chung KF, O'Connor BJ, Barnes PJ. Comparison of low-dose inhaled budesonide plus theophylline and high-dose inhaled budesonide for moderate asthma. N Engl J Med 1997; 337: 1412-1418.

284. Ukena D, Harnest U, Sakalauskas R, Magyar P, Vetter N, Steffen H, Leichtl S, Rathgeb F, Keller A, Steinijans VW. Comparison of addition of theophylline to inhaled steroid with doubling of the dose of inhaled steroid in asthma. Eur Respir J 1997; 10: 2754-2760.

285. Spatafora M, Chiappara G, Merendino AM, D'Amico D, Bellia V, Bonsignore G. Theophylline suppresses the release of tumour necrosis factor-alpha by blood monocytes and alveolar macrophages. Eur Respir J 1994; 7: 223-228.

286. Ghezzi P, Dinarello CA. IL-1 induces IL-1. Specific inhibition of IL-1 production by IFN-gamma. J Immunol 1988; 140: 4238-4244.

287. Zocchi MR, Pardi R, Gromo G, Ferrero E, Ferrero ME, Besana C, Rugarli C. Theophylline induced non specific suppressor activity in human peripheral blood lymphocytes. J Immunopharmacol 1985;7: 217-234.

288. Bruserud O. The effect of theophyllamine on T-lymphocyte activation in vitro. Clin Immunol Immunopathol 1984; 32: 111-118.

289. Lim S, Tomita K, Carramori G, Jatakanon A, Oliver B, Keller A, Adcock I, Chung KF, Barnes PJ. Low-dose theophylline reduces eosinophilic inflammation but not exhaled nitric oxide in mild asthma. Am J Respir Crit Care Med 2001; 164: 273-276.

290. Sagara H, Makino S, Chibana N, Ota M, Holgate ST, Church MK, Fukuda T. Theophylline at Therapeutic Concentrations Inhibits NF-[kappa] B Activation in Human Lung Mast Cells. International Archives of Allergy and Immunology. 2001; 124: 371-379.

291. Barnes PJ. Cytokine-directed therapies for the treatment of chronic airway diseases. Cytokine Growth Factor Rev 2003; 14: 511-522.

292. Pretolani M, Goldman M. IL-10: a potential therapy for allergic inflammation? Immunol Today 1997; 18: 277–280.

293. Moore KW, O'Garra A, de-Waal-Malefyt R, Vieira P, Mosmann TR.Interleukin-10. Annu Rev Immunol 1993; 11: 165-190.

294. Borish L, Aarons A, Rumbyrt J, Cvietusa P, Negri J, Wenzel S. Interleukin-10 regulation in normal subjects and patients with asthma. J Allergy Clin Immunol 1996; 97: 1288-1296.

295. John M, Lim S, Seybold J, Robichaud A, O'Connor B, Barnes PJ, et al. Inhaled corticosteroids increase IL-10 but reduce MIP-1α, GM-CSF and IFN-γ release from alveolar macrophages in asthma. Am J Respir Crit Care Med 1998; 157: 256-262.

296. Turner DM, Williams DM, Sankaran D, Lazarus M, Sinnott PJ, Hutchinson IV. An investigation of polymorphism in the interleukin-10 gene promoter. Eur J Immunogenet 1997; 24: 1-8.

297. Kube D, Platzer C, von Knethen A, Straub H, Bohlen Hafner M, Tesch H. Isolation of the human interleukin 10 promoter. Characterization of the promoter activity in burkitts's lymphoma cell lines. Cytokine 1996; 7: 1-7.

298. Tomita K, Lim S, Hanazawa T, Usmani O, Stirling R, Chung KF, Barnes PJ, Adcock IM. Attenuated production of intracellular IL-10 and IL-12 in monocytes from patients with severe asthma. Clin Immunol 2002; 102: 258-266.

299. Zuany-Amorim C, Haile S, Leduc D, Dumarey C, Huerre M, Vargaftig BB, Pretolani M. Interleukin-10 inhibits antigen-induced cellular recruitment into the airways of sensitized mice. J Clin Invest 1995; 95: 2644-2651.

300. Oh JW, Seroogy CM, Meyer EH, Akbari O, Berry G, Fathman CG, et al. CD4 T-helper cells engineered to produce IL-10 prevent allergen-induced airway hyperreactivity and inflammation. J Allergy Clin Immunol 2002; 110: 460-468.

301. Akdis CA, Blesken T, Akdis M, Wuthrich B, Blaser K. Role of interleukin 10 in specific immunotherapy. J Clin Invest 1998; 102: 98-106.

302. Fedorak RN, Gangl A, Elson CO, Rutgeerts P, Schreiber S, Wild G, et al. Recombinant human interleukin 10 in the treatment of patients with mild to moderately active Crohn's disease. Gastroenterology 2000; 119: 1473-1482.

303. La Grutta, Gagliardo R, Mirabella F, Pajno GB, Bonsignore G, Bousquet J, Bellia V, Vignola AM. Clinical and biological heterogeneity in children with moderate asthma. Am J Resp Crit Care Med 2003; 167: 1490-1495.

304. Inoue H, Aizawa H, Fukuyama S, Takata S, Matsumoto K, Shigyo M, Koto H, Hara N. Effect of Inhaled Glucocorticoid on the Cellular Profile and Cytokine Levels in Induced Sputum from Asthmatic Patients. Lung 1999; 177: 53- 62.

305. Nagata M, Sedgwick JB, Kita H, Busse WW. Granulocyte macrophage colony-stimulating factor augments ICAM-1 and VCAM-1 activation of eosinophil function. Am J Respir Cell Mol Biol 1998; 19: 158-166.

306. Pauwels R, Lofdahl CG, Postma DS, Tattersfield AE, O'Byrne P, Barnes PJ, Ullman A. Effect of inhaled formoterol and budesonide on exacerbations of asthma. Formoterol and Corticosteroids Establishing Therapy (FACET) International Study Group. N Engl J Med 1997; 337: 1405-1411.

307. Shrewsbury S, Pyke S, Britton M. Meta-analysis of increased dose of inhaled steroid or addition of salmeterol in symptomatic asthma (MIASMA). BMJ 2000; 320: 1368-1373.

308. Pang L, Knox AJ. Regulation of TNFa-induced eotaxin release from cultured human airway smooth cells by β2 -agonists and corticosteroids. FASEB J 2001; 15: 261-269.

309. Eickelberg O, Roth M, Lorx R, Bruce V, Fudiger J, Johnson M, Block L H. Ligand independent activation of the glucocorticoid receptor by the β2-adrenergic receptor agonists in primary human lung fibroblasts and vascular smooth muscle cells. J Biol Chem 1999; 274: 1005-1010.

310. Roth M, Rudiger JJ, Bihl MP, et al. The β2-agonist formoterol activates the glucocorticoid receptor in vivo. Eur Respir J 2000; 16: 437S.

311. Spoelstra FM, Postma DS, Hovenga H, Noordhoek JA, Kauffman HF. Additive anti-inflammatory effect of formoterol and budesonide on human lung fibroblasts. Thorax 2002; 57: 237- 241.

312. Crabtree GR, Smith K, Munck A. Glucocorticoid receptors. In Methods of Haematology: The Leukaemic Cell. Catovsky D, Chanarin I, Beutler E, Brown EB, and Jacobs A, eds Churchill Livingstone, New York 1981; 252 - 269.

313. Price Price DB, Hernandez D, Magyar P, Fiterman J, Beeh KM, James IG, Konstsantopoulos S, Rojas R, van Noord JA, Pons M, Gilles L, Leff JA. Randomised controlled trial of montelukast plus inhaled budesonide versus double dose inhaled budesonide in adult patients with asthma. Thorax 2003; 58: 211-216

314. Ostrom NK, Decotiis BA, Lincourt WR. Comparative Efficacy and Safety of Low-dose Fluticasone Propionate and Montelukast in Children with Persistent Asthma. J Pediatr 2005; 147: 213-220.

315. Irusen EM. The corticosteroid dose-response curve in asthma and how to identify patients for adjunctive and alternative therapy. SA Fam Pract 2006; 48(2) 34-42.

316. Malmstrom K, Rodriguez- Gomez G, Guerra J. Oral montelukast, inhaled beclomethasone, and placebo for chronic asthma: a randomized controlled trial. Ann Intern Med 1999; 130: 487-495.

317. Nelson HS, Busse WW, deBoisblanc BP, Berger WE, Noonan MJ, Webb DR, Wolford JP, Mahajan PS, Hamedani AG, Shah T, Harding SM. Fluticasone propionate powder: oral corticosteroid-sparing effect and improved lung function and quality of life in patients with severe chronic asthma. J Allergy Clin Immunol 1999; 103; 267-275.

318. Rabe KF, Vermiere PA, Soriano JB, Maier WC. Clinical management of asthma in 1999: the Asthma Insights and Reality in Europe (AIRE) study. Eur Respir J 2000; 16: 802-807.

319. Larsson S, Lofdahl CG, Linden M. IL-2 and IL-4 counteract budesonide inhibition of GM-CSF and IL-10, but not of IL-8, IL-12 or TNF- production by human mononuclear blood cells. Br J Pharmacol 1999; 127: 980-7.

320. Hawrylowicz CH, Richards D, Loke TK, Corrigan C, Lee T. A defect in corticosteroid- induced IL-10 production in T lymphocytes from corticosteroid-resistant asthmatic patients. J Allergy Clin Immunol 2002; 109: 369-370.

321. Richards DF, Fernandez M, Caulfield J, Hawrylowicz CM. Glucorticoids drive human CD8+ T cell differentiation towards a phenotype with high IL-10 and reduced IL-4, IL-5 and IL- 3 production. Eur J Immunol 2000; 30: 2344-2354.

322. Matsumoto K, Inoue H, Fukuyama S, Tsuda M, Ikegami T, Kibe A, Yoshiura Y, Komori M, Hamasaki N, Aizawa H, Nakanishi Y. Decrease of Interleukin-10-Producing T Cells in the Peripheral Blood of Severe Unstable Atopic Asthmatics despite similar doses of ICS. Int Arch Allergy Immunol 2004; 134: 295-302.

323. Mascali JJ, Cvietusa P, Negri J, Borish L. Anti-inflammatory effects of theophylline: modulation of cytokine production. Ann Allergy Asthma Immunol 1996; 77: 34-38.

324. Spina D, Page CP. Asthma - a need for a rethink? Trends Pharmacol Sci. 2002; 23: 311-315.

325. Leckie MJ. Anti-interleukin-5 monoclonal antibodies: preclinical and clinical evidence in asthma models. Am J Respir Med 2003; 2: 245-259.

326. Bryan SA, Bryan SA, O'Connor BJ, Matti S, Leckie MJ, Kanabar V, Khan J, Warrington SJ, Renzetti L, Rames A, Bock JA, Boyce MJ, Hansel TT, Holgate ST, Barnes PJ. Effects of recombinant human interleukin-12 on eosinophils, airway hyper-responsiveness, and the late asthmatic response. Lancet 2000; 356: 2149-2153.

327. Howarth PH, Babu KS, Arshad HS, Lau LC, Buckley MG, McConnell W, Beckett P, Ali MA, Chauhan A, Wilson SJ, Reynolds A, Davies DE, Holgate ST. Tumour Necrosis Factor (TNF{alpha}) as a novel therapeutic target in symptomatic corticosteroid-dependent asthma. Thorax 2005; 60: 1012- 1018.

328. Berry MA, Hargadon B, Shelley M, Parker D, Shaw DE, Green RH, Bradding P, Brightling CE, Wardlaw AJ Parvord ID. Evidence of a Role of Tumor Necrosis Factor [alpha] in Refractory Asthma. . N Engl J Med 2006; 354:697-710.

329. Mori A, Kaminuma O, Miyazawa K, Ogawa K, Okudaira H, Akiyama, K.p38-Mitogen-Activated Protein Kinase regulates Human T cell IL-5 synthesis. J Immunol, 1999, 163: 4763-4771.

330. Bodwell JE, Webster JC, Jewell CM, Cidlowski JA, Hu JM, Munck A. Glucocorticoid receptor phosphorylation: overview, function and cell cycle-dependence. J Steroid Biochem Mol Biol 1998; 65: 91-99.

331. Caput D, Beutler B, Hartog K, Thayer R, Brown-Shimer S, Cerami A. Identification of a common nucleotide sequence in the 3' untranslated region of mRNA molecules specifying inflammatory mediators. Proc Natl Acad Sci USA 1986; 83: 1670-1674

332. Karin M, Hunter T. Transcriptional control by protein phosphorylation: signal transmission from the cell surface to the nucleus. Curr Biol 1995; 5: 747-757.

333. Swantek JL, Cobb MH, Geppert TD. Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) is required for lipopolysaccharide stimulation of tumor necrosis factor alpha (TNF-alpha) translation: glucocorticoids inhibit TNF-alpha translation by blocking JNK/SAPK. Mol Cell Biol 1997; 17: 6274-6282.

334. Caelles C, Gonzalez-Sancho JM, Munoz A. Nuclear hormone receptor antagonism with AP-1 by inhibition of the JNK pathway. Genes Dev 1997; 11: 3351-3364.

335. Rider LG, Hirasawa N, Santini F, Beaven MA. Activation of the mitogen activated protein kinase cascade is suppressed by low concentrations of dexamethasone in mast cells. J Immunol 1996; 157: 2374- 2380.

336. Hirasawa N, Sato Y, Fujita Y, Mue S, Ohuchi K. Inhibition by dexamethasone of antigen-induced c-Jun N-terminal kinase activation in rat basophilic leukemia cells. J Immunol 1998; 161: 4939-4943.

337. Rogatsky I, Logan SK, Garabedian MJ. Antagonism of glucocorticoid receptor transcriptional activation by the c-Jun N-terminal kinase. Proc Natl Acad Sci U SA 1998; 95: 2050-2055.

338. Bamberger CM, Schulte HM, Chrousos GP. Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids. Endocr Rev 1996; 17: 245-261.

339. Galigniana MD, Piwien-Pilipuk G, Assreuy J. Inhibition of glucocorticoid receptor binding by nitric oxide. Mol Pharmacol 1999; 55: 317- 323.

340. Lee MS, Silver PA. RNA movement between the nucleus and the cytoplasm. Curr Opin Genet Dev 1997; 7: 212-219.

341. Almlof T, Wallberg AE, Gustafsson JA, Wright AP. Role of important hydrophobic amino acids in the interaction between the glucocorticoid receptor tau 1-core activation domain and target factors. Biochemistry 1998; 37: 9586- 9594.

342. Haddad JJ, Land SC. Redox/ROS regulation of lipopolysaccharide-induced mitogen-activated protein kinase (MAPK) activation and MAPK-mediated TNF-alpha biosynthesis. Br J Pharmacol. 2002; 35: 520-536.

343. Bateman ED, Karpel J, Casale T, Wenzel S, Banerji D. Ciclesonide reduces the need for oral steroid use in adult patients with severe, persistent asthma. Chest 2006; 129: 1176-1187.

344. Chapman KR, Cartier A, Hebert J, McIvor RA, Schellenberg RR. The role of Omalizumab in the treatment of severe allergic asthma. Can Respir J 2006; 13 Suppl B: 1B-9B.

345. Irusen EM, Matthews JG, Takahashi A, Barnes P, Chung KF, Adcock IM. P38MAPkinase-induced glucocorticoid receptor phosphorylation reduces its activity: role in steroid insensitive asthma. J Allergy Clin Immunol 2002; 109: 649-657.

346. Adcock IA, Chung KF, Caramori G, Ito K. Kinase inhibitors and airway inflammation. Eur J Pharmacol 2006; 533: 118-132.