The Properties of Circulating Fibrocytes in Severe Asthma

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Chun-Yu Lo

To my beloved wife Eileen

Abstract

Inflammation associated with asthma mainly affects large airways and is accompanied by extensive structural changes, termed airway remodelling. 5-10% of patients with asthma suffer from severe or refractory asthma which is difficult to control despite receiving high doses of inhaled and sometimes oral corticosteroids (CS). These patients show more prominent characteristics of airway remodelling, specifically sub-epithelial fibrosis and airway smooth muscle (ASM) thickening.

My project focuses on one of the cells implicated in airway remodelling, circulating fibrocytes, which are bone marrow-derived peripheral blood mesenchymal progenitors expressing both leukocyte markers, such as CD45, and mesenchymal proteins including collagen I (Col I). Fibrocytes migrate to the sites of disease under the guidance of chemokine receptors such as CC chemokine receptor type 7 (CCR7), and differentiate into α–smooth muscle actin (α-SMA)–expressing myofibroblasts, a process that is facilitated by a variety of pro-inflammatory cytokines and growth factors. Myofibroblasts can promote subepithelial fibrosis as well as contribute to ASM thickening. Indeed, the number of fibrocytes in peripheral blood is correlated with the decline rate of forced expiratory volume in 1s (FEV_1) in patients with chronic obstructive asthma. Most importantly, there is increased recruitment of fibrocytes to

the airway wall of patients with severe asthma. However, the mechanisms driving the accumulation of fibrocytes in the airways of these patients are currently unclear.

I hypothesised that in severe asthma there are increased numbers of circulating fibrocytes that have an increased capacity to differentiate into myofibroblasts and have differential responses to pro-inflammatory mediators and asthma therapeutic agents compared to non-severe asthma.

Fibrocytes were isolated from the non-adherent non-T (NANT) cell fraction of peripheral blood mononuclear cells (PBMC) of healthy subjects and patients with non-severe or severe asthma. The number of fibrocytes (Col I+/CD45+ cells) and differentiating fibrocytes (α-SMA+ cells), as well as the expression of CCR7 and glucocorticoid receptor (GR) in fibrocytes were determined by flow cytometry. Apoptosis was determined by Annexin V/propidium iodide staining. Messenger ribonucleic acid (mRNA) expression was quantified by reverse transcription quantitative polymerase chain reaction (RT-qPCR). Fibrocytes were also isolated from the adherent fraction of PBMC.

Severe asthmatic patients had a higher number of circulating fibrocytes with a greater capacity to differentiate into myofibroblasts in culture compared to healthy subjects and patients with non-severe asthma.

Severe asthmatic fibrocytes did not have a heightened responsiveness to either interleukin (IL)-4, IL-13, nerve growth factor or brain-derived neurotrophic factor.

Dexamethasone induced apoptosis in NANT cells, including fibrocytes and differentiating fibrocytes, from healthy subjects and patients with non-severe asthma, but not in the cells from patients with severe asthma. Dexamethasone also reduced CCR7 expression in fibrocytes from patients with non-severe asthma but not in those from patients with severe asthma. The relative CS insensitivity in severe asthmatic fibrocytes may be related to the lower expression of the GR or the heightened c-Jun N-terminal kinase activity.

Salmeterol xinafoate, a long-acting β_2-adrenoceptor agonist (LABA), reduced the number, myofibroblastic differentiation and CCR7 expression of fibrocytes from healthy subjects and patients with non-severe asthma. Salmeterol did not improve the suppressive effect of dexamethasone, although it was not detrimental to dexamethasone's effect either. In contrast, tiotropium bromide, a long-acting muscarinic antagonist (LAMA), did reduce the number of fibrocytes and differentiating fibrocytes from patients with severe asthma. Increasing intracellular 3',5'-cyclic adenosine monophosphate (cAMP), the downstream signalling molecule of β_2-adrenoceptor and muscarinic M_2 receptor, by phosphodiesterase type IV

inhibitor (rolipram) and cAMP analogue (8-bromoadenosine-3',5'-cyclic monophosphate) could reduce fibrocytes from patients with severe asthma.

Patients with severe asthma have elevated numbers of circulating fibrocytes showing enhanced myofibroblastic differentiation and are less responsive to the suppressive effect of CS and LABA, but can be inhibited by LAMA. This study provides insight into a novel target for the treatment of airway remodelling in severe asthma.

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Table of Contents

Abstract....4

Acknowledgements....8

List of Figures....15

List of Tables....20

Abbreviations....21

Chapter 1: Background....25

1.1 Definition and characteristics of asthma....25

1.1.1 Definition of severe asthma....27

1.1.2 Airway inflammation in asthma....29

1.1.3 Airway remodelling in asthma....32

1.2 Fibrocytes....40

1.2.1 Mesenchymal and haematopoietic markers expressed by fibrocytes...41

1.2.2 Fibrocytes in asthma....46

1.3 Current asthma therapies....50

1.3.1 Corticosteroids....51

1.3.2 Bronchodilators....64

Rationale....77

Hypothesis....77

Aims....77

Chapter 2: Materials and Methods....80

2.1 Materials....80

2.1.1 Peripheral blood mononuclear cell and non-adherent non-T cell isolation 80

2.1.2 Cell culture 80

2.1.3 Antibodies and reagents for flow cytometry 83

2.1.4 Cell apoptosis assay for flow cytometry 84

2.1.5 Cell proliferation assay for flow cytometry 85

2.1.6 Total RNA extraction, cDNA preparation by reverse-transcriptase polymerase chain reaction and determination of mRNA expression by real-time quantitative polymerase chain reaction 85

2.2 Methods 88

2.2.1 Recruitment of healthy subjects and patients with non-severe or severe asthma 88

2.2.2 Non-adherent non-T cell isolation and culture 91

2.2.3 Adherent peripheral blood mononuclear cell isolation and culture 96

2.2.4 Flow cytometry and staining 97

2.2.5 Determination of mRNA expression 109

2.2.6 Statistical analysis 112

Chapter 3: Proliferation, myofibroblastic differentiation and CC chemokine receptor 7 expression of fibrocytes from severe asthma 113

3.1 Background 113

3.2 Results 117

3.2.1 Isolation of fibrocytes from non-adherent non-T cells 117

3.2.2 Determination the number of fibrocytes and differentiating fibrocytes in cultured non-adherent non-T cells in the presence or absence of foetal bovine serum 119

3.2.3 Comparison of the number of circulating fibrocytes in non-adherent non-T cells from healthy subjects and patients with non-severe or severe asthma..125

3.2.4 Isolation of fibrocytes from the adherent fraction of peripheral blood mononuclear cells..134

3.2.5 Comparison of the number of cultured fibrocytes derived from adherent peripheral blood mononuclear cells from healthy subjects and patients with non-severe or severe asthma..143

3.3 Discussion..146

Chapter 4: Effect of pro-fibrotic mediators on fibrocytes 159

4.1 Background..159

4.2 Results 162

4.2.1 Effect of T_H2 cytokines on the number and differentiation of fibrocytes in non-adherent non-T cells from healthy subjects and patients with non-severe or severe asthma 162

4.2.2 Effect of neurotrophins on the number and differentiation of fibrocytes in non-adherent non-T cells from healthy subjects and patients with non-severe or severe asthma 168

4.2.3 The effect T_H2 cytokines and neurotrophins on non-adherent non-T cell death, differentiation and collagen I+/CD45+ fibrocyte proliferation 173

4.2.4 Effect of T_H2 cytokines and neurotrophins on fibrocytes derived from adherent peripheral blood mononuclear cells..177

4.3 Discussion..181

Chapter 5: Do fibrocytes from patients with severe asthma display corticosteroid insensitivity? 188

5.1 Background 188

5.2 Results 191

5.2.1 Effect of systemic corticosteroids on circulating fibrocytes from patients with severe asthma 191

5.2.2 Effect of dexamethasone on the number of fibrocytes and differentiating fibrocytes within non-adherent non-T cells from healthy subjects and patients with non-severe or severe asthma 193

5.2.3 Effect of dexamethasone on the number of fibrocytes and differentiating fibrocytes within adherent fraction of peripheral blood mononuclear cells from healthy subjects and patients with non-severe or severe asthma 195

5.2.4. Effect of dexamethasone on non-adherent non-T cell apoptosis 198

5.2.5 Effect of dexamethasone on the gene expression of fibrocyte markers 201

5.2.6 Effect of dexamethasone on the expression of pro-apoptotic and anti-inflammatory genes 201

5.2.7 Role of the glucocorticoid receptor in corticosteroid action 205

5.2.8 The expression of glucocorticoid receptor in fibrocytes isolated from healthy subjects and patients with non-severe or severe asthma 209

5.2.9 Effect of T_H2 cytokines, corticosteroids, β_2-adrenoceptor agonist and antioxidant on glucocorticoid receptor expression 211

5.2.10 Effect of dexamethasone on CC chemokine receptor 7 expression in fibrocytes isolated from healthy subjects and patients with non-severe or severe asthma 214

5.2.11 Role of kinases on fibrocytes and their corticosteroid responsiveness 216

5.3 Discussion..224

Chapter 6: The effect of bronchodilators on fibrocyte function 240

6.1 Background..240

6.2 Results 243

6.2.1 Effect of β_2-adrenoceptor agonist on fibrocytes.................................243

6.2.2 Effect of muscarinic antagonist on fibrocytes...................................253

6.2.3 The role of 3',5'-cyclic adenosine monophosphate on fibrocytes derived from non-adherent non-T cells..262

6.3 Discussion..265

Chapter 7: Conclusion...276

7.1 Findings of study and contribution to the field..276

7.2 Advantages and Limitations of study and future work................................282

Reference List 289

List of Figures

Figure 1.1: The homing and differentiation of fibrocytes in asthmatic airways……..50

Figure 1.2: Anti-inflammatory actions of corticosteroids…….……………………...57

Figure 1.3: Possible molecular mechanisms of corticosteroid resistance……………63

Figure 1.4: Possible mechanisms involving β_2-adrenoceptor and muscarinic M_2 receptor-mediated cell proliferation and collagen synthesis…………………………65

Figure 1.5: Interaction of β2-adrenoceptor agonists with corticosteroid effects……..71

Figure 2.1: Isolation of non-adherent non-T cells from peripheral blood……………94

Figure 2.2: BDTM CompBeads staining by the fluorochrome-conjugated antibodies…………………………………………………………………………....99

Figure 2.3: Detection of fibrocytes and differentiating fibrocytes………………….103

Figure 2.4: Cell apoptosis assay…………………………………………………….105

Figure 2.5: Cell proliferation assay…………………………………………………108

Figure 3.1: Comparison of the efficiency of T cell depletion methods……………..118

Figure 3.2: The expression of collagen I and CD45 on α–smooth muscle actin+ cells…………………………………………………………………………….... 120

Figure 3.3: Number of fibrocytes and differentiating fibrocytes in non-adherent non-T cells cultured under foetal bovine serum-free conditions…………………………..121

Figure 3.4: Number of fibrocytes and differentiating fibrocytes in non-adherent non-T cells cultured under foetal bovine-containing conditions…………………………...123

Figure 3.5: Percentage of proliferating fibrocytes and collagen I, CD45 and α-smooth muscle actin messenger ribonucleic acid expression in non-adherent non-T cells cultured in foetal bovine serum-containing medium……………………………..125

Figure 3.6: Number of circulating fibrocytes in freshly isolated non-adherent non-T cells from healthy subjects and patients with non-severe or severe asthma………..128

Figure 3.7: Relationship between circulating fibrocytes and lung function………..129

Figure 3.8: Number of fibrocytes and differentiating fibrocytes in cultured non-adherent non-T cells from healthy subjects and patients with non-severe or severe asthma……………………………………………………………………………132

Figure 3.9: CC chemokine receptor 7 expression in non-adherent non-T cells-derived fibrocytes from healthy subjects and patients with non-severe or severe asthma…..134

Figure 3.10: The number, myofibroblastic differentiation and CC chemokine receptor 7 expression of fibrocytes derived from adherent peripheral blood mononuclear cells……………………………………………………………………………… 137

Figure 3.11: Comparison of fibrocytes derived from adherent peripheral blood mononuclear cells in the presence or absence of foetal bovine serum……………...139

Figure 3.12: The effect of fibronectin on fibrocytes derived from adherent peripheral blood mononuclear cells……………………………………………………………142

Figure 3.13: Comparison of adherent peripheral blood mononuclear cell-derived fibrocytes from healthy subjects and patients with non-severe and severe asthma...144

Figure 3.14: CC chemokine receptor 7 expression in fibrocytes from healthy subjects and patients with non-severe or severe asthma……………………………………..145

Figure 4.1: The effect of interleukin-4 on the number of fibrocytes and differentiating fibrocytes derived from non-adherent non-T cells…………………………………164

Figure 4.2: Effect of interleukin-4 on the number of fibrocytes and differentiating fibrocytes derived from non-adherent non-T cells under foetal bovine serum-free condition……………………………………………………………………………165

Figure 4.3: The effect of interleukin-4 on CC chemokine receptor 7 expression in fibrocytes derived from non-adherent non-T cells…………………………………166

Figure 4.4: The effect of interleukin-13 on the number of fibrocyte and differentiating fibrocytes derived from non-adherent non-T cells…………………………………168

Figure 4.5: The effect of interleukin-13 on CC chemokine receptor 7 expression in fibrocytes derived from non-adherent non-T cells…………………………………169

Figure 4.6: The effect of nerve growth factor on the number of fibrocyte and differentiating fibrocytes derived from non-adherent non-T cells………………….172

Figure 4.7: The effect of nerve growth factor on CC chemokine receptor 7 expression in fibrocytes derived from non-adherent non-T cells……………………………….173

Figure 4.8: The effect of brain-derived neurotrophic factor on the number of fibrocyte and differentiating fibrocytes derived from non-adherent non-T cells……………..174

Figure 4.9: The effect of brain-derived neurotrophic factor on CC chemokine receptor 7 expression in fibrocytes derived from non-adherent non-T cells…………………175

Figure 4.10: The effect of interleukin-4, interleukin-13 and nerve growth factor on apoptosis and α–smooth muscle actin expression in non-adherent non-T cells and proliferation of fibrocytes…………………………………………………………...177

Figure 4.11: The effect of T_H2 cytokines and neurotrophins on fibrocytes derived from adherent peripheral blood mononuclear cells (stimulated on day 3 after removal of adherent cells)………………...………………………………………………179

Figure 4.12: Effect of T_H2 cytokines and neurotrophins on fibrocytes derived from adherent peripheral blood mononuclear cells (stimulated on day 0 in the presence of adherent cells)…………………….…………………..……………………………180

Figure 5.1: Effect of systemic corticosteroids on circulating fibrocytes from patients with severe asthma……………………………………………………………….192

Figure 5.2: Effect of dexamethasone on the number of fibrocytes derived from non-adherent non-T cells…………………………………………………………195

Figure 5.3: Effect of dexamethasone on the number of fibrocytes derived from adherent peripheral blood mononuclear cells………………………………………197

Figure 5.4: Effect of dexamethasone on non-adherent non-T cell apoptosis in healthy subjects and patients with non-severe or severe asthma…………………………....200

Figure 5.5: Expression of fibrocyte markers in dexamethasone-treated non-adherent non-T cells from healthy subjects and patients with severe asthma………………...203

Figure 5.6: Expression of corticosteroid-inducible genes in dexamethasone-treated non-adherent non-T cells from healthy subjects and patients with severe asthma…204

Figure 5.7: Effect of glucocorticoid receptor antagonism on dexamethasone-induced reduction in the number of fibrocytes derived from non-adherent non-T cells…….206

Figure 5.8: Effect of glucocorticoid receptor antagonism on dexamethasone-induced reduction in the number of fibrocytes derived from adherent peripheral blood mononuclear cells…………………………………………………………………...208

Figure 5.9: Glucocorticoid receptor expression in fibrocytes from healthy subjects and patients with non-severe or severe asthma……………………………………….210

Figure 5.10: Effect of TH2 cytokines, asthma medications and anti-oxidant on the expression of glucocorticoid receptor in fibrocytes……………………………...213

Figure 5.11: Effect of dexamethasone on CC chemokine receptor 7 expression in fibrocytes from patients with non-severe or severe asthma………………………...215

Figure 5.12: Effect of p38 mitogen-activated protein kinase inhibitor on corticosteroid sensitivity of fibrocytes in non-adherent non-T cells from patients with asthma…218

Figure 5.13: Effect of c-jun N-terminal kinase inhibitor on corticosteroid sensitivity of fibrocytes in non-adherent non-T cells from patients with asthma…………………220

Figure 5.14: Effect of extracellular signal-regulated kinase inhibitor on corticosteroid sensitivity of fibrocytes in non-adherent non-T cells from patients with asthma…..222

Figure 5.15: Effect of phosphoinositide 3-kinases inhibitor on corticosteroid sensitivity of fibrocytes in non-adherent non-T cells from patients with asthma…..224

Figure 5.16: Corticosteroid-induced apoptosis……………………………………..228

Figure 6.1: Effect of salmeterol on fibrocytes derived from non-adherent non-T cells……………………………………………………………………………….244

Figure 6.2: Effect of salmeterol ± dexamethasone on fibrocytes derived from non-adherent non-T cells…………………………………………………………247

Figure 6.3: Effect of salmeterol ± dexamethasone on non-adherent non-T cell apoptosis in healthy subjects and patients with non-severe or severe asthma……...249

Figure 6.4: Effect of salmeterol ± dexamethasone on fibrocytes derived from adherent peripheral blood mononuclear cells……………………………………………...252

Figure 6.5: Effect of acetylcholine on fibrocytes derived from non-adherent non-T cells……………………………………………………………………………….254

Figure 6.6: Effect of salmeterol on fibrocytes derived from non-adherent non-T cells…………………………………………………………………………….....257

Figure 6.7: Effect of tiotropium on non-adherent non-T cell apoptosis in healthy subjects and patients with non-severe or severe asthma…………………………....258

Figure 6.8: Effect of tiotropium on fibrocytes derived from adherent peripheral blood mononuclear cells…………………………………………………………………...261

Figure 6.9: The effect of rolipram and 8-bromoadenosine 3',5'-cyclic monophosphate on fibrocytes derived from non-adherent non-T cells…………………………….....264

Figure 6.10: Possible effect of β_2-adrenoceptor agonist, muscarinic antagonist, phosphodiesterase inhibitor and cyclic adenosine monophosphate analogue on fibrocyte function…………………………………………………………………...271

List of Tables

Table 1.1: Refractory asthma: workshop consensus for typical clinical features…....28

Table 1.2: Molecular mechanisms of steroid resistance in patients with asthma or chronic obstructive pulmonary disease……………………………………………62

Table 2.1 Inhibitors, drugs and mediators used in the project……………………….82

Table 2.2: Wavelength of maximal excitation and emission absorption (Ex-max and Em-max) and final amount of antibodies applied for flow cytometry……………….84

Table 2.3 Primers used for reverse transcription quantitative polymerase chain reaction……………………………………………………………………………87

Table 2.4: Clinical characteristics of studied subjects……………………………….90

Abbreviations

8-Br-cAMP	8-bromoadenosine 3’,5’-cyclic monophosphate
AHR	Airway hyperresponsiveness
α-SMA	α-smooth muscle actin
APC	Allophycocyanin
ASM	Airway smooth muscle
ASMC	Airway smooth muscle cell(s)
ATS	American Thoracic Society
BDNF	Brain-derived neurotrophic factor
β_2-AR	β_2-adrenoceptor
Bim_{EL}	B-cell lymphoma 2 interacting mediator of cell death-extra long
BSA	Bovine serum albumin
cAMP	3’,5’-cyclic adenosine monophosphate
CCL	CC chemokine ligand
CCR	CC chemokine receptor
cDNA	Complementary deoxyribonucleic acid
Col	Collagen
COPD	Chronic obstructive pulmonary disease
CS	Corticosteroids
CXCL	CXC chemokine ligand

CXCR	CXC chemokine receptor
Dex	Dexamethasone
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EdU	5-ethynyl-2'-deoxyuridine
EGFR	Epidermal growth factor receptor
Epac	Exchange protein directly activated by cAMP
ERK	Extracellular-signal-regulated kinases
ERS	European Respiratory Society
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
FEV1	Forced expiratory volume in 1 s
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
FVC	Forced vital capacity
GILZ	Glucocorticoid-induced leucine zipper
GINA	Global Initiative for Asthma
GM-CSF	Granulocyte-macrophage colony stimulating factor
GR	Glucocorticoid receptor

ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
Jak	Janus kinase
JNK	c-Jun N-terminal kinases
LABA	Long-acting β_2-adrenoceptor agonist
LAMA	Long-acting muscarinic antagonist
LSP-1	leukocyte specific protein-1
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
NANT	Non-adherent non-T
NGF	Nerve growth factor
NT	Neurotrophin
p38 MAPK	p38 mitogen-activated protein kinase
PBMC	Peripheral blood mononuclear cell(s)
PC_{20}	Methacholine provocative concentration causing a 20% fall in FEV_1
PDE	Phosphodiesterase
PE	r-Phycoerythrin

PEF	Peak expiratory flow
PI3K	Phosphatidylinositol-3-kinase
PKA	Protein kinase A
RNA	Ribonucleic acid
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SABA	Short-acting β_2-adrenoceptor agonist
SAMA	Short-acting muscarinic antagonist
Sal	Salmeterol xinafoate
SEM	Standard error of the mean
SSC	Side scatter
STAT6	Signal transducer and activator of transcription, interleukin-4 induced
TGF-β	Transforming growth factor-β
T_H	CD4+ T-helper lymphocyte type
Tio	Tiotropium bromide
TNF-α	Tumour necrosis factor-α
Trk	Tropomyosin-receptor-kinase
Ut	Untreated
VEGF	Vascular endothelial growth factor
Veh	Vehicle

Chapter 1: Background

1.1 Definition and characteristics of asthma

Asthma affects 300 million people globally (Masoli, Fabian et al. 2004). The rate of asthma has increased in recent decades, paralleled by similar increases in atopic sensitization and other allergic diseases such as eczema and rhinitis (1998). Worldwide, asthma accounts for around 1% of all disability-adjusted life years lost, which is similar to that for diabetes, cirrhosis of liver, or schizophrenia (Bousquet, Bousquet et al. 2005). It is estimated that asthma accounts for about 1 in every 250 deaths worldwide (Masoli, Fabian et al. 2004).

Asthma is defined according to its clinical, physiological and pathological features by the Global Initiative for Asthma (GINA) (Bateman, Hurd et al. 2008) as a chronic inflammatory airway disorder associated with reversible airflow obstruction and airway hyperresponsiveness (AHR), manifested by recurrent episodes of wheezing, breathlessness, chest tightness and nocturnal coughing. Many cells and cellular elements are implicated in the chronic inflammation of airways. The clinical diagnosis of asthma relies on medical history of characteristic symptoms and predisposing factors, and wheezing based on physical auscultation. Lung function data provide assessment of airflow limitation and help to confirm the diagnosis of

asthma. Airflow limitation is defined as a ratio of forced expiratory volume in 1 s (FEV_1) to forced vital capacity (FVC) (FEV_1/FVC) below the normal value of 0.75–0.80 in adults. According to the American Thoracic Society (ATS) guidelines (Crapo, Casaburi et al. 2000), diagnosis of asthma can be made in patients showing a reversibility of ≥ 12% and ≥ 200 mL in FEV_1 value, and an improvement of peak expiratory flow (PEF) of 60 L/min or ≥ 20% in response to bronchodilator, or diurnal variation in PEF of > 20%. Airway hyperresponsiveness (AHR) to methacholine indicated by a methacholine provocative concentration causing a 20% fall in FEV_1 (PC_{20}) < 1mg/mL and exercise-induced bronchoconstriction (a 10% decrease in FEV_1 in response to exercise) also help to establish the diagnosis of asthma

For most conditions categorised as mild to moderate disease, symptoms are reversible and can be controlled with inhaled corticosteroids (CS) and long-acting β_2 adrenoceptor-agonists (LABAs). Approximately 5-10% of patients suffer from severe asthma which is difficult to control using the current therapies (Aburuz, Heaney et al. 2007). As these patients suffer from greater lung function impairment, longer disease duration, more daily symptoms and sinopulmonary infections, they consume more than half of the medical resources in asthmatics in terms of both time and money

(Smith, Malone et al. 1997, Serra-Batlles, Plaza et al. 1998, Wenzel, Busse et al. 2007).

1.1.1 Definition of severe asthma

Severe asthma is also known as difficult asthma as defined by the European Respiratory Society (ERS) (Chung, Godard et al. 1999), or "refractory asthma", a term coined by the ATS (2000). In accordance with the updated "International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma (Table 1.1)" (Chung, Wenzel et al. 2014), patients with severe asthma (age ≥ 6 years) require treatment with high dose inhaled CS (≥2000 μg/day beclomethasone dipropionate or equivalent dose using a dry powder inhaler or hydrofluoroalkane metered-dose inhaler or other inhaled CS in equivalent dosage) plus a second controller such as LABA or leukotriene modifier/theophylline and/or systemic CS for ≥ 50% of the previous year. In this study, I recruited patients with severe asthma by the definition of the ATS guidelines for refractory asthma in 2000 (Table 1.1) (2000): medically adherent patients with at least one of two major criteria for CS usage and at least two minor criteria of ongoing asthma are regarded as having refractory asthma while other conditions have been excluded and exacerbating factors have been well-treated.

Table 1.1: Refractory asthma: workshop consensus for typical clinical features†*

Major Characteristics		
In order to achieve control to a level of mild–moderate persistent asthma: 1. Treatment with continuous or near continuous (≥ 50% of year) oral corticosteroids 2. Requirement for treatment with high-dose inhaled corticosteroids:		
Drug	Dose (μg/d)	Dose (puffs/d)
a. Beclomethasone dipropionate	> 1,260	> 40 puffs (42 μg/inhalation) > 20 puffs (84 μg/inhalation)
b. Budesonide	> 1,200	> 6 puffs
c. Flunisolide	> 2,000	> 8 puffs
d. Fluticasone propionate	> 880	> 8 puffs (110 μg), > 4 puffs (220 μg)
e. Triamcinolone acetonide	> 2,000	> 20 puffs
Minor Characteristics		
1. Requirement for daily treatment with a controller medication in addition to inhaled corticosteroids, e.g., long-acting β-agonist, theophylline, or leukotriene antagonist 2. Asthma symptoms requiring short-acting β-agonist use on a daily or near daily basis 3. Persistent airway obstruction (FEV_1 < 80% predicted; diurnal PEF variability 20%) 4. One or more urgent care visits for asthma per year 5. Three or more oral steroid "bursts" per year 6. Prompt deterioration with ≤ 25% reduction in oral or inhaled corticosteroid dose 7. Near fatal asthma event in the past		

* Requires that other conditions have been excluded, exacerbating factors treated, and patient felt to be generally adherent.

†Definition of refractory asthma requires one or both major criteria and two minor criteria.

1.1.2 Airway inflammation in asthma

Bronchial biopsies from asthmatic patients usually display the infiltration of activated mast cells, eosinophils and activated T cells (Bhavsar, Hew et al. 2008). The main pathogenic factor of (atopic) asthma is exposure to inhaled allergens triggering a CD4+ T-helper lymphocyte type-2 (T_H2 cells) –type immune response which drives the eosinophilic inflammation (Busse and Lemanske 2001). The asthmatic inflammatory response mainly affects the larger airways (Wenzel 2006).

Most asthmatic patients are atopic (extrinsic asthma), and have a type I hypersensitivity to allergens (Wenzel 2006). Inhaled allergens crosslink immunoglobulin (Ig) E molecules on sensitized mast cells, which are recruited and maintained by stem-cell factor (SCF) and nerve growth factor (NGF) generated by epithelial cells, leading to the release of bronchoconstrictor mediators such as histamine, prostaglandin D_2 and cysteinyl leukotrienes (Kanbe, Kurosawa et al. 2000, Galli, Kalesnikoff et al. 2005, Reber, Da Silva et al. 2006). Although no culprit allergen can be identified, local synthesis of IgE is presented in the airways of non-atopic (intrinsic) asthmatic patients (Ying, Humbert et al. 2001). The infiltration of mast cells into the airway smooth muscle (ASM) is associated with AHR in asthma (Brightling, Bradding et al. 2002). Myeloid dendritic cells, which are conditioned by

thymic stromal lymphopoietin generated by mast cells and epithelial cells, release CC chemokine ligand (CCL) 17/thymus and activation regulated chemokine (TARC) and CCL22/macrophage-derived chemokine (MDC) to chemoattract and present processed peptides from inhaled allergens to CC chemokine receptor (CCR) 4-expressing T_H2 cells (Hammad and Lambrecht 2006).

CD4+ T-helper lymphocytes (T_H cells) dominate the immune response of asthma through the production of cytokines. The polarization to T_H1 cells or T_H2 cells depends on cytokine environment: T_H1 cells are triggered by interleukin (IL)-12 and IL-2, whilst T_H2 cells are triggered by IL-4. Asthma is often described as being characterised by a T_H2/T_H1 imbalance that favours T_H2 cells (Mosmann, Cherwinski et al. 1986). Infiltration of activated T_H2 cells into the asthmatic airway induces IgE synthesis, ASM hyperplasia and mucus production through IL-4 and IL-13, airway eosinophilia through IL-5 and mast cell proliferation through IL-9 (Barnes 2001). Therefore T_H2 cells are associated with disease severity (Humbert, Corrigan et al. 1997, Larche, Robinson et al. 2003, Adcock, Caramori et al. 2008). Asthmatic patients have a defect in regulatory T (T_{reg}) cells which may favour further T_H2 cell proliferation (Ling, Smith et al. 2004, Larche 2007). T_H1 cytokine interferon (IFN)-γ is up-regulated in the airway in severe asthma (Shannon, Ernst et al. 2008), which

may be associated with accumulation of T_H2 cells and exaggerated eosinophilia (Randolph, Carruthers et al. 1999, Randolph, Stephens et al. 1999).

Eosinophils accumulate at sites of allergic inflammation in response to CCL11/eotaxin-1, nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) expressed by epithelial cells (Lamkhioued, Renzi et al. 1997, Hahn, Islamian et al. 2006), IL-5 generated by T_H2 cells and mast cells, cysteinyl leukotrienes produced by eosinophils (Laitinen, Laitinen et al. 1993, Bjermer, Bisgaard et al. 2003). In severe asthma, exaggerated eosinophilia is correlated with higher level of IL-13 (Saha, Berry et al. 2008), IL-5 (Dente, Carnevali et al. 2006, Nair, Pizzichini et al. 2009) and interferon-γ (Randolph, Carruthers et al. 1999, Randolph, Stephens et al. 1999). Eosinphils release mediators, including major basic protein, cysteinyl leukotrienes, transforming growth factor (TGF)-β, reactive oxygen species (ROS) and cytokines (Weller 1997, Gleich 2000). Eosinophils promote subepithelial fibrosis through the release of TGF-β (Minshall, Leung et al. 1997), but reducing eosinophils by IL-5 specific blocking antibodies does not prevent AHR or asthma symptoms (Leckie, ten Brinke et al. 2000). Intriguingly, although cysteinyl leukotrienes-induced bronchial eosinophilia is insensitive to CS therapy (Laitinen, Laitinen et al. 1993,

Bjermer, Bisgaard et al. 2003), the presence of eosinophil is a good marker of CS responsiveness (Green, Brightling et al. 2002).

In more severe disease, IL-8 released by the bronchial epithelium and ASM cells (ASMC) recruits and promotes maturation of neutrophils (Hamilton, Torres-Lozano et al. 2003, Kikuchi, Kikuchi et al. 2006, Traves and Donnelly 2008, Al-Ramli, Prefontaine et al. 2009, Nakagome and Nagata 2011), subsequently augmenting the trans-basement membrane migration of eosinophils (Kikuchi, Kikuchi et al. 2006), thus amplifying the inflammatory response in asthma. IFN-γ is up-regulated in the airway in severe asthma (Shannon, Ernst et al. 2008), which may be associated with accumulation of T_H2 cells and exaggerated eosinophilia (Randolph, Carruthers et al. 1999, Randolph, Stephens et al. 1999). Increased number of CD8+ T lymphocytes (van Rensen, Sont et al. 2005) and higher level of oxidative stress (Chung and Marwick 2010) have also been observed in severe asthma.

1.1.3 Airway remodelling in asthma

The asthmatic inflammatory response is accompanied by extensive structural changes in the airways, such as epithelial detachment, heightened vascularity, goblet cell and submucosal gland proliferation, increased bronchial smooth muscle mass and subepithelial fibrosis, termed airway remodelling (Davies, Wicks et al. 2003, Hirota

and Martin 2013). The thickness of sub-basement membrane (Chetta, Foresi et al. 1996) and ASM (Pang and Knox 2000) is inversely correlated with post-bronchodilator FEV_1 and PC_{20}, suggesting that these changes are directly linked to airflow obstruction and AHR.

Different cells, both structural and inflammatory, have been implicated in the development of airway remodelling in asthma. Subepithelial fibrosis is a result of increased accumulation of extracellular matrix (ECM) proteins such as collagen (Col) I, Col III, and Col V, fibronectin and tenascin in the lamina reticularis (Roche, Beasley et al. 1989). These ECM proteins are released mainly by myofibroblasts, fibroblasts and ASMC in response to pro-fibrotic mediators such as transforming growth factor-β_1 (TGF-β_1) and connective tissue growth factor (Roche, Beasley et al. 1989, Jeffery, Godfrey et al. 1992, Vignola, Chanez et al. 1997, Elias, Zhu et al. 1999). On the other hand, increased ASMC layer thickness is thought be a result of ASMC hyperplasia and hypertrophy. In addition, heightened migration of ASMC (Vignola, Chanez et al. 1997) and myofibroblasts (Chen and Khalil 2006) towards the epithelium in response to epithelium-derived mediators could also contribute to this effect.

Myofibroblasts are morphologically and biochemically midway between fibroblasts and smooth muscle cells, and are responsible for tissue repair after injury (Gabbiani 1992). They express contractile α–smooth muscle actin (α-SMA) and ECM proteins including Col I (Tomasek, Gabbiani et al. 2002). In asthma there is an increase in the numbers of myofibroblasts in the subepithelial area, which directly correlates with the degree of subepithelial fibrosis (Brewster, Howarth et al. 1990). Although a large population of resident tissue myofibroblasts are mainly found under the basement membrane and in the submucosa throughout the lungs (Chen and Khalil 2006), myofibroblast numbers may increase due to the differentiation /dedifferentiation of myocytes (Wenzel and Balzar 2006), epithelial cells (epithelial-mesenchymal transition) and fibroblasts (Koumas, Smith et al. 2003). Fibroblasts do not have organised contractile elements (e.g. α-SMA), although they can undergo slow contraction due to cytoskeletal rearrangement (Singh and Hall 2008). Most fibroblasts in adult lungs exist in adventitia of vascular structures and airways, but fibroblasts can also differentiate from epithelial cells (epithelial-mesenchymal transition) or from bone-marrow derived circulating progenitor cells (fibrocytes) (Phan 2008).

Cytokines and growth factors released from structural and inflammatory cells are pivotal in airway remodelling. Collagen deposition and ASM thickening are regulated by growth factors including neurotrophins (Kilic, Sonar et al. 2011), TGF-β_1 (Roth, Johnson et al. 2004), epidermal growth factor (Stewart, Fernandes et al. 1995) and T_H2 cytokines (Barnes 2001, Hashimoto, Gon et al. 2001). Vascular endothelial growth factor (VEGF), TGF-β_1 and T_H2 cytokines are responsible for angiogenesis, epithelial cell shedding and mucus hyperplasia in airway remodelling, respectively (Undevia, Dorscheid et al. 2004, Siddiqui, Sutcliffe et al. 2007). These mediators function in an autocrine or a paracrine manner, to modulate airway remodelling (Vignola, Chiappara et al. 1997). The role of T_H2 cytokines and neurotrophins will be discussed in more detail below.

1.1.3.1 T_H2 cytokines: interleukin-4 and interleukin-13

IL-4 (also known as B cell stimulatory factor-1, 14.9kDa) and IL-13 (12.5kDa) are closely related T_H2 cytokines sharing many biological and immunoregulatory functions (Chomarat and Banchereau 1998).

IL-4 and IL-13 are released by both inflammatory (i.e. T_H2 cells, nuocytes, T_H9 cells, eosinophils, basophils, mast cells, etc.) and structural cells (i.e. smooth muscle cells) (Oliphant, Barlow et al. 2011). The genes for IL-3, IL-4, IL-5, IL-9, IL-13, and

granulocyte-macrophage colony-stimulating factor (GM-CSF), clustered on human chromosome 5q, are transcribed in cell type-specific manner upon stimulation (Koyano-Nakagawa and Arai 1996). The receptors for IL-4 and IL-13 share at least one common chain (IL-4R_{α}). Both cytokines act through a heterodimeric IL-4Rα/IL-13$R\alpha_1$ receptor (Gibejova, Mrazek et al. 2003). However, IL-4 also works through an IL-4Rα/IL-4Rγ receptor whilst IL-13 also signals through IL-4Rα/IL-13$R\alpha_2$ receptor (Chiaramonte, Mentink-Kane et al. 2003). IL-13$R\alpha_2$ is expressed intracellularly, on the cell surface, and as a soluble molecule and regulates the bioavailability of both IL-13 and IL-4. These receptors transduce the signals through janus kinases (JAK) and signal transducer and activator of transcription 6, interleukin-4 induced (STAT6), leading to transcription of IL-4 and IL-13 responsive genes (Hershey 2003).

IL-4 and IL-13 are involved in airway remodelling in asthma. Both IL-4 and IL-13 are involved in goblet cell hyperplasia and ASMC proliferation in the airways (Barnes 2001). In fibroblasts, IL-4 and IL-13 induce proliferation (Lewis, Sutherland et al. 2003), myofibroblastic differentiation (Hashimoto, Gon et al. 2001), production of CCL11/eotaxin (Richter, Puddicombe et al. 2001), and facilitate monocyte-to-fibrocyte differentiation (Shao, Suresh et al. 2008). Given the important

roles of IL-4 and IL-13 in the pathophysiology of allergic diseases and asthma, several approaches to inhibit these cytokines and their receptors are now being tested in clinical trials (Steinke 2004, Hacha, Tomlinson et al. 2012).

1.1.3.2 Neurotrophins: nerve growth factor and brain-derived neurotrophic factor

Neurotrophins are a family of proteins originally identified as growth factors which promote development and survival of the vertebrae nerve system (Lewin and Barde 1996) but have also been identified as mediators of inflammatory signals on a variety of non-neuronal tissues. Four neurotrophins, NGF, BDNF, neurotrophin 3 (NT3) and neurotrophin 4/5 (NT 4/5), have been identified in mammals, as well as additional factors such as neurotrophin 6 (NT 6) and neurotrophin 7 (NT 7) in other species.

Neurotrophins are synthesized as precursors (27 kDa) and cleaved into pro-neurotrophins (pro-NTs) that are further processed to generate mature neurotrophins (13-15 kDa) (Lessmann, Gottmann et al. 2003, Lessmann and Brigadski 2009). The mature form of BDNF, NT4 and NT3 have approximately 50% amino acid similarity to NGF(McDonald and Chao 1995).

Neurotrophins bind to cell surface receptors: the low-affinity ‘pan-neurotrophin’ p75 neurotrophin receptor (p75NTR), a member of tumour necrosis factor

receptor/Fas/CD40 superfamily, and the high-affinity and more specific tropomyosin-related kinase (Trk) receptors. Trk receptors consist of TrkA which binds to NGF, TrkB which binds to BDNF and NT4 and TrkC which binds to NT3 (Lu, Pang et al. 2005), initiating receptor auto-phosphorylation and activating intracellular signalling cascades, such as phospholipase C, phosphatidylinositol 3 kinase (PI3K) and mitogen-activated protein kinases (MAPKs). In the respiratory organs, both structural cells (nasal, bronchial and pulmonary epithelial cells, ASMC, nerves, fibroblasts and pulmonary endothelial cells) and inflammatory cells (T and B lymphocytes, eosinophils, mast cells, monocytes and macrophages) express NGF and BDNF and their receptors(Prakash, Thompson et al. 2010).

The expression of neurotrophins is increased in asthma. NGF and BDNF levels are increased in the circulation (Bonini, Lambiase et al. 1996, Lommatzsch, Schloetcke et al. 2005) and bronchoalveolar lavage fluid from allergen-challenged patients with mild allergic asthma (Virchow, Julius et al. 1998). Neurotrophins are also increased in mouse models of allergic asthma (Braun, Lommatzsch et al. 1999) and in nasal secretion of allergen-challenged allergic rhinitis patients(Raap and Braunstahl 2010).

At the cellular level, the intracellular Ca^{2+}, contractility and proliferation of human ASM is enhanced by BDNF, particularly in the presence of tumour necrosis factor (TNF)-α (Prakash, Thompson et al. 2009). NGF increases the migration and myofibroblastic differentiation of pulmonary fibroblasts MCR-5 (Micera, Vigneti et al. 2001) whereas BDNF promotes the proliferation of human ASMC (Aravamudan, Thompson et al. 2012). However, the role of neurotrophins in the development of airway remodelling, particularly in severe asthma, is currently unknown.

1.1.3.4 Airway remodelling in severe asthma

The airways of patients with severe asthma show more prominent subepithelial fibrosis and ASM thickening compared to the airways of non-severe asthmatic patients, suggesting that there is more profound airway remodelling in severe asthma (Levi-Montalcini 1998). Although current asthma treatments can reduce airway inflammation, they are less effective in attenuating airway remodelling and more specifically reducing ASM mass (Bourke, Li et al. 2011). Thus, a better understanding of the molecular and cellular mechanisms promoting airway remodelling in severe asthma may lead to the development of more effective treatments.

1.2 Fibrocytes

Fibrocytes, first described in 1994, are bone-marrow derived circulating progenitor cells that express haematopoietic markers, such as common leukocyte marker CD45, stem cell marker CD34, leukocyte specific protein-1 (LSP-1), monocyte markers CD11 and CD14, and ECM proteins, including Col I, Col III, Col V, and vimentin (Bucala, Spiegel et al. 1994, Pilling, Fan et al. 2009). Fibrocytes have the ability to differentiate into other mesenchymal cells, such as myofibroblasts or adipocytes depending on the local microenvironment, manifested by the expression of α-SMA and adipocyte lipid-binding protein respectively (Hong, Belperio et al. 2007).

Fibrocytes contribute to inflammation, angiogenesis and fibrosis by releasing mediators such as TNF-α, IL-6, IL-8, GM-CSF, VEGF, matrix metalloproteinase (MMP)-9 and TGF-β_1 (Hartlapp, Abe et al. 2001). Fibrocytes also express chemokine receptors, such as CCR3, CCR5, CCR7 and CXC chemokine receptor (CXCR) 4 which control migration and recruitment of fibrocytes to injured tissue (Phillips, Burdick et al. 2004, Sakai, Wada et al. 2006, Isgro, Bianchetti et al. 2013), intercellular adhesion molecule-1 (ICAM-1) to recruit inflammatory cells (Chesney, Bacher et al. 1997) and proteins which are important for host defence including

CD16/CD32, CD163 and for antigen presentation such as major histocompatibility complex class II (Chesney, Bacher et al. 1997).

1.2.1 Mesenchymal and haematopoietic markers expressed by fibrocytes

Fibrocytes are functionally and physiologically between leukocytes and fibroblasts. The identification of fibrocytes usually involves co-detection of at least one hematopoietic marker such as CD45 and CD34 plus a mesenchymal protein, such as Col I. Thus, fibrocytes are mostly defined as Col I+/CD45 cells (Moeller, Gilpin et al. 2009), Col I+/CD34+ cells (Chesney, Bacher et al. 1997) or Col I+/CD34+/CD45+ cells (Wang, Huang et al. 2008). Some group defined fibrocytes as Col I+/CXCR4+ cells, Col I+/CD45+/CXCR4+ cells (Garcia-de-Alba, Becerril et al. 2010) or simply Col I+ cells (Yang, Scott et al. 2002). It has been suggested that a combination of CD45RO (a low molecular weight isoform of CD45, which is still detectable after long term culture), 25F9 (a mature macrophage marker, expressed in macrophages and fibrocytes but not in monocytes), calcium binding S100A8/A9 complex (myeloid related protein 8/14; calprotectin), but not PM-2K (a marker only on mature macrophages but not on fibrocytes) is more selective for human fibrocytes (Pilling, Fan et al. 2009). While fibrocytes are myofibroblastically differentiating, they

progressively lose CD34 and gain α-SMA (Schmidt, Sun et al. 2003). In my project I defined fibrocytes as Col I+/CD45+ cells and differentiating fibrocytes as α–SMA+ cells.

1.2.1.1 Collagen I

Col I is the most abundant ECM protein amongst 29 types of collagen identified in the literature, which forms large, eosinophilic fibres known as collagen fibers (Jensen and Host 1997, Rossert, Terraz et al. 2000). It is present in most connective tissues.

Col I molecules consist of two α_1 and one α_2 chain, which are encoded by *COL1A1* gene on chromosome 17 and *COL1A2* gene on chromosome 7, respectively (Rossert, Terraz et al. 2000). The two genes produce pro-α_1(I) chain and pro-α_2(I) chain in a 2:1 ratio, and undergo extensive post-translational modification before assembling in a triple helix (Prockop, Kivirikko et al. 1979). These pro-collagen molecules are processed by enzymes outside the cell and arrange themselves into long, thin fibrils that cross-link to one another in the spaces around cells. The cross-links result in the formation of very strong mature Col I fibers (Cutroneo 2003).

A variety of soluble molecules modulate Col I synthesis (Rossert, Terraz et al. 2000). IL-1, IL-4, TGF-β, insulin-like growth fator-1 IGF-1, endothelin-1, or lipid

peroxidation products can stimulate Col I production by fibroblasts. TNF-α, IFN-γ, IL-10, prostaglandin E2 and CS inhibit Col I production by fibroblasts.

1.2.1.2 CD45

CD45, also known as leukocyte common antigen or protein tyrosine phosphatase receptor type C (PTPRC) encoded by the *PTPRC* gene, is a type I transmembrane enzyme expressed specially in haematopoietic cells regulating cell development, activation, senescence and apoptosis. CD45 is a member of the protein tyrosine phosphatase (PTP) family. Various isoforms of CD45 exist: CD45RA, CD45RB, CD45RC, CD45RAB, CD45RAC, CD45RBC, CD45RO and CD45RABC (Trowbridge and Thomas 1994).

As CD45 is exclusively expressed on differentiated haematopoietic cells except plasma cells and platelets, clinically it is a marker used to identify cells of haematopoietic origin. For example, distinguish haematopoietic stem cells from mesenchymal stem cells (Calloni, Cordero et al. 2013), and haematopoietic malignancies (lymphoma) from solid tumours (carcinoma) (Bobrow, Richards et al. 1993).

1.2.1.3 CD34

CD34, a single-pass transmembrane sialomucin encoding on human *CD34* gene, is a marker for early haematopoietic cells and vascular-associated tissue (Nielsen and McNagny 2009). CD34 is widely used for the purification for haematopoietic stem cells and progenitor cells, including circulating fibrocytes (Bellini, Marini et al. 2012). CD34 is sometimes used as one of the criteria for fibrocyte definition in line with a mesenchymal marker such as Col I (Chesney, Bacher et al. 1997, Wang, Huang et al. 2008). Some argue that CD34 may not be useful to distinguish fibrocytes from macrophages and dendritic cells, since these cell types also express weak Col I and CD34 under certain circumstances (Pilling, Fan et al. 2009). Besides, cultured fibrocytes usually lose CD34 during differentiation (Schmidt, Sun et al. 2003). CD34 is only expressed by 18% of cultured fibrocytes 1 week after isolation, whilst CD45 is still expressed by 85% of cultured fibrocytes (Phillips, Burdick et al. 2004). Therefore, in this project I chose CD45, instead of CD34, as a haematopoietic marker for fibrocyte identification.

1.2.1.4 α-smooth muscle actin

α-SMA, also known as aortic smooth muscle actin or α-actin-2 encoded by the *ACTA2* gene located on 10q22-q24, is a protein contributing to cell-generated

mechanical tension. α-SMA incorporates into stress fibers and focal adhesions, provides mechanosensory functions and contractile forces which may contribute to scar formation and fibrocontractive diseases (Wang, Zohar et al. 2006).

α-SMA comprises a very large proportion of total cell protein in smooth muscle cells and is a defining feature of myofibroblastic differentiation. Human α-SMA is a tissue-specific isoform in actin family, along with another three tissue-specific isoforms: α-cardiac, α-skeletal and γ–smooth muscle actin, and two cytoplasmic actin: γ and β non-muscle actins (Khaitlina 2001). At the early stages of the healing process, proto-myofibroblasts develop stress fibres containing cytoplasmic β-actin and γ-actin but lacking α-SMA expression (Hinz, Phan et al. 2007). However, 14-18% of total cell protein in differentiated myofibroblasts is α–SMA (Arora and McCulloch 1994).

Generation of α-SMA+ myofibroblasts is induced by mechanical stress, mediator signalling and specialized matrix proteins, such as ED-A fibronectin variant (Hinz, Phan et al. 2007). Mechanical forces stimulate α–SMA gene transcription through Rho/Rho kinase signalling (Zhao, Laschinger et al. 2007). Prolonged culture, exposure to TGF-β, platelet-derived growth factor (PDGF), angiotensin II, IL-1, IL-6, TNF-α and ROS can up-regulate α–SMA expression (Sappino, Schurch et al. 1990, Elger, Drenckhahn et al. 1993, Jarnagin, Rockey et al. 1994, Schmitt-Graff,

Desmouliere et al. 1994, Masamune, Watanabe et al. 2009, Barnes and Gorin 2011). In fibrocytes, α-SMA expression can be induced through activating mothers against decapentaplegic homolog (Smad) 2/3 and stress-activated protein kinases (SAPK)/c-Jun N-terminal kinases (JNK) MAPK pathway, and SAPK/JNK signalling acts in a positive feedback loop to modulate Smad2/3 (Hong, Belperio et al. 2007). IL-4 and IL-13 also increased α–SMA expression (Bellini, Marini et al. 2012).

1.2.2 Fibrocytes in asthma

Fibrocytes are thought to be involved in asthma pathogenesis by contributing to airway inflammation and remodelling. Circulating fibrocytes are increased in asthmatics with chronic airflow obstruction, and their number is correlated with the slope of the yearly decline in FEV_1 (Wang, Huang et al. 2008, Murray, Chen et al. 2011). Moreover, fibrocytes increasingly migrate to the ASM compartment of asthmatic airways possibly due to the release of platelet-derived growth factor (PDGF) by ASM cells (Saunders, Siddiqui et al. 2009). Fibrocyte numbers were also found to be increased in bronchial biopsies of mild asthmatic patients with fibroblasts in their bronchoalveolar lavage fluids (Nihlberg, Larsen et al. 2006). Furthermore, exposure of patients with chronic allergic asthma to allergen increases the numbers of fibrocytes in bronchial mucosa and their differentiation into myofibroblasts (Schmidt,

Sun et al. 2003). Interestingly, fibrocytes isolated from asthmatic patients with chronic airflow obstruction showed greater capacity to differentiate into myofibroblasts in response to serum, compared to non-obstructed asthmatic patients and non-asthmatic subjects, as a result of increased TGF-β_1 release (Wang, Huang et al. 2008). The behaviour of fibrocytes is also regulated by a number of mediators involved in asthmatic inflammation (see 1.2.2.1-3). Therefore, the increased recruitment of fibrocytes to the ASM compartment and their differentiation into myofibroblasts may contribute to the enhanced ASM thickness and subepithelial fibrosis observed in asthma.

1.2.2.1 Effect of T_H1, T_H2 and T_H17 cytokines on fibrocytes

The differentiation of fibrocytes into myofibroblasts is promoted by T_H2 cytokines IL-13 and IL-4, whilst it is inhibited by the T_H1 cytokines IFN-γ and IL-12 (Shao, Suresh et al. 2008, Weng, Chen et al. 2013). Asthmatic fibrocytes produce high level of collagenous (Collagen I, Collagen III, Collagen V) and non-collagenous (Hyaluronan, tenascin-C) matrix components and pro-inflammatory cytokines (IL-6, IL-11 and leukaemia inhibitory factor) upon IL-4 and IL-13 stimulation, and proliferate, express α-SMA and release CXCL1/growth-regulated oncogene α

(GROα), CXCL8/IL-8 and TNF-α in the presence of IL-17A (Bellini, Marini et al. 2012).

1.2.2.2 Effect of chemokines and their receptors (e.g. CC chemokine receptor 7) on fibrocytes

The migration of fibrocytes is guided by chemokine gradients (Figure 1.1). Fibrocytes have been shown to express CXCR4, CCR3, CCR5 and CCR7. In a mouse model of severe asthma, there are higher levels of CCL5/regulated on activation, normal T cell expressed and secreted (RANTES), CCL11/eotaxin and CCL24/eotaxin-2 in sputum fluid phase and increased expression of CCR3 and CCR5 on their fibrocytes, which may be associated with the recruitment of fibrocytes (Isgro, Bianchetti et al. 2013). The migration of circulating Col I+/CD45+/CXCR4+ fibrocytes in response to CXCL12/stromal cell-derived factor 1 (SDF-1) is associated with increased collagen deposition in the lung in a murine model of pulmonary fibrosis (Phillips, Burdick et al. 2004).

CCR7, also known as CD197, is a seven-transmembrane-spanning G-protein-coupled receptor found in inflammatory cells (fibrocytes, thymocytes, T cells, B cells, dendritic cells), structural cells (lymph node, endothelial cells, smooth muscle cells and fibroblasts) and various types of tumours (Birkenbach, Josefsen et al.

1993). The expression of CCR7 is up-regulated by IL-4 in CD8+ T lymphocytes (Seneviratne, Black et al. 2007). CCL19/macrophage inflammatory protein-3-β (MIP-3-β) and CCL21/secondary lymphoid-tissue chemokine (SLC) are the sole ligands for CCR7 (Forster, Davalos-Misslitz et al. 2008). CCL19/MIP-3-β, also known as EBI1 ligand chemokine, is highly expressed by mast cells and vessels in asthma of all severities and ASM in severe disease. Mast cells and ASMC-derived CCL19/MIP-3-β mediates ASMC migration and repair (Kaur, Saunders et al. 2006). CCL21/SLC is secreted by endothelial cells and lymph nodes and also present in lungs and tracheas (Gunn, Tangemann et al. 1998). Both CCL19/MIP-3-β and CCL21/SLC act on T cells and dendritic cells and CCL21/SLC also works on B lymphocytes and natural killer cells (Palmqvist, Wardlaw et al. 2007). Although the role of CCL19/MIP-3-β in the recruitment of fibrocytes to the ASM compartment is controversial (Saunders, Siddiqui et al. 2009), CCL21/SLC-CCR7 signalling of fibrocytes plays a key role in a murine model of renal fibrosis (Sakai, Wada et al. 2006).

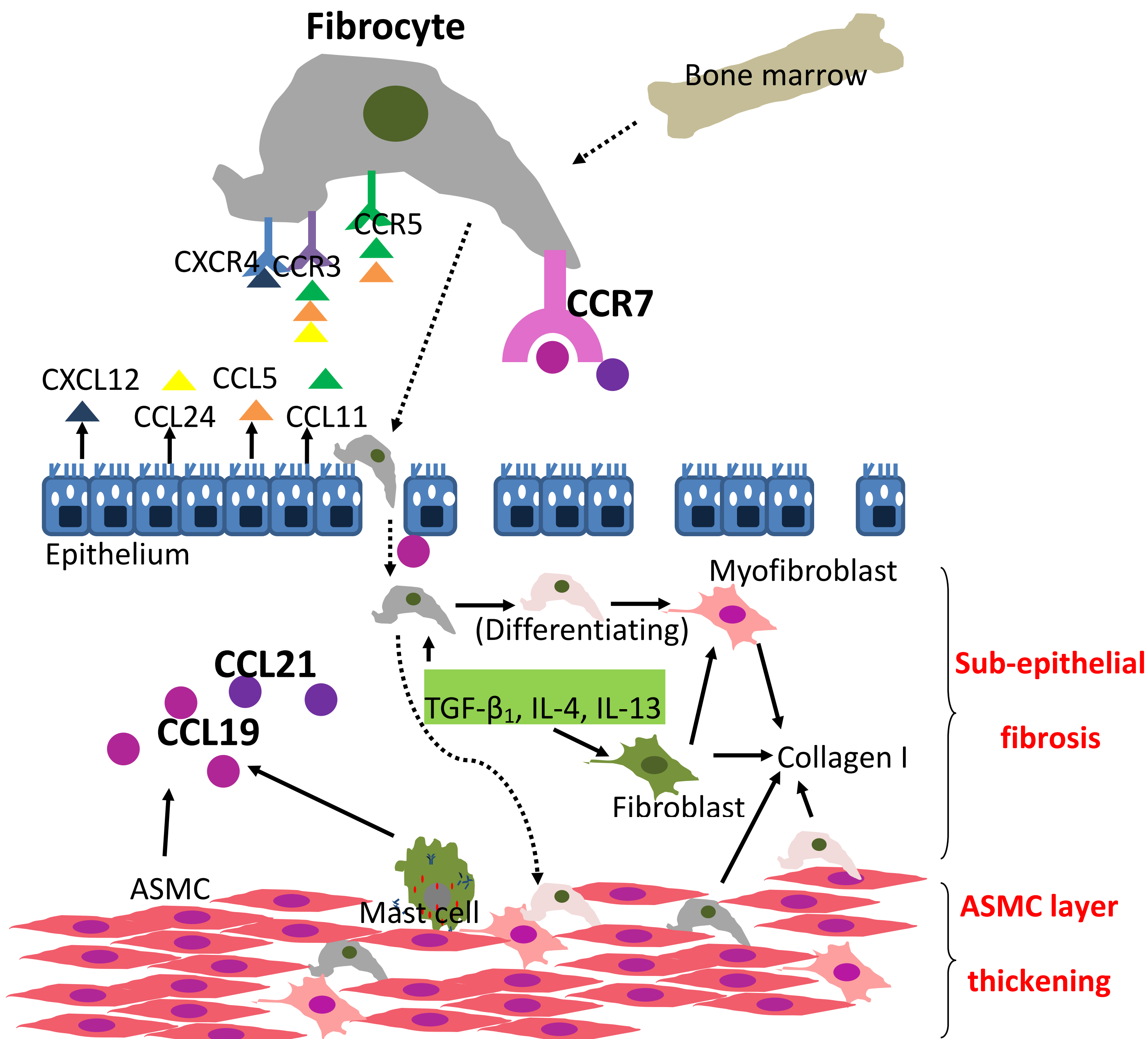


Figure 1.1: The homing and differentiation of fibrocytes in asthmatic airways. Bone marrow-derived circulating fibrocytes express chemokine receptors (CCR7, CXCR4, CCR3, CCR5) and migrate into airways under the guidance of their ligands (CCL19/MIP-3-β, CCL21/SLC; CXCL12/SDF-1; CCL5/RANTES, CCL11/eotaxin, CCL24/eotaxin-2; CCL5/RANTES, CCL11/eotaxin respectively). In the presence of IL-4, IL-13 and TGF-β_1, fibrocytes produce extracellular matrix proteins including collagen I and undergo myofibroblastic differentiation, together with resident fibroblasts, myofibroblasts and airway smooth muscle cells, leading to sub-epithelial fibrosis and airway smooth muscle cell layer thickening. CCR: CC chemokine receptor; CXCR: CXC chemokine receptor CCL: CC chemokine ligand; CXCL: CXC chemokine ligand; IL: interleukin; TGF-β_1: transforming growth factor-β_1; α-SMA: α-smooth muscle actin; ASMC: airway smooth muscle cell.

1.3 Current asthma therapies

Pharmacotherapeutic management of asthma involves chronic management and a plan for prevention of acute exacerbations. This often includes the daily use of CS (inhaled and sometimes systemic), LABAs, leukotriene modifiers, cromones, anti-IgE therapy, methylxanthines and reliever medications, usually short-acting β_2-adrenoceptor agonists (SABAs) referred to as rapid-acting. Muscarinic antagonists are also used when required (Bateman, Hurd et al. 2008).

1.3.1 Corticosteroids

CS are currently the cornerstone of asthma treatment. CS are a class of chemicals that include steroid hormones naturally produced by adrenal cortex of vertebrates and artificially synthetic analogues of these hormones. Glucocorticoids, mineralocorticoids and androgens are steroidal hormones synthesized from cholesterol within three different cellular zones of adrenal cortex (Neelon 1977). Glucocorticoids control gluconeogenesis and the anti-inflammatory response whilst mineralocorticoids promote sodium retention and androgens regulate the male characteristics, anabolism and estrogen production (Neelon 1977). CS are widely used to treat both endocrine and non-endocrine diseases (Williams 1999). Low dose CS are

used as physiological replacement for adrenal insufficiency and congenital adrenal hyperplasia. At supra-physiological dose CS, initially introduced for the treatment of rheumatoid arthritis, are administered as anti-inflammatory agents and immunosuppressants (Hench, Kendall et al. 1949).

1.3.1.1 Corticosteroids and asthma treatment

CS can be administered as controller medications for persistent asthma in different ways: by inhalation, orally or parenterally (Bateman, Hurd et al. 2008). Inhaled CS, such as budesonide and fluticasone, reduce asthma symptoms (Juniper, Kline et al. 1990), improve quality of life (Juniper, Kline et al. 1990) and lung function (Juniper, Kline et al. 1990), control airway inflammation (Jeffery, Godfrey et al. 1992) and reduce frequency and severity of exacerbations (Pauwels, Lofdahl et al. 1997), asthma mortality (Suissa, Ernst et al. 2000) and AHR (2000). Low and high dose inhaled CS in adults are defined as equivalent to < 500 µg and > 1000 µg of beclomethasone dipropionate per day, respectively (Bateman, Hurd et al. 2008). Increasing to higher doses provides little further benefit (Powell and Gibson 2003). Oral CS such as prednisolone may be required for asthma exacerbations. However, high dose inhaled CS and systemic CS are associated with adverse effects including adrenal suppression, bone loss, cataract and glaucoma (Bateman, Hurd et al. 2008).

Therefore, the addition of controller medications, such as LABAs, for patients >5 years of age in step 2 of asthma control, is beneficial to achieve better control and reduce the adverse effect of CS (Bateman, Hurd et al. 2008).

CS suppress inflammatory responses by reducing inflammatory cell number and pro-inflammatory mediator release, and increasing the expression anti-inflammatory cytokines, chemokines, receptors and adhesion molecules (Chung and Barnes 1999).

The effect of CS on airway remodelling is less clear. Long-term inhaled CS treatment does not eliminate airway remodelling (van Essen-Zandvliet, Hughes et al. 1994, Ward and Walters 2005), although some reports show that inhaled CS attenuate subepithelial fibrosis and airway vascularity (Hoshino 2004). Inhaled CS have been shown to reduce reticular basement membrane layer thickness of asthmatic airways and collagen deposition (Hoshino, Nakamura et al. 1998, Hoshino, Nakamura et al. 1998), but other studies failed to demonstrate a significant effect (Jeffery, Godfrey et al. 1992). CS inhibit pro-remodelling responses such as the myofibroblastic differentiation of fibroblasts (Olivieri, Chetta et al. 1997) and the proliferation of ASMC (Chung and Barnes 1999), but some reported the anti-mitogenic effect only occurs in the presence of normal basement membrane (laminin) but not pathological

basement membrane (collagen), suggesting CS are less effective in established airway remodelling (Bonacci, Harris et al. 2003) .

1.3.1.2 Mechanism of corticosteroid action

Genomic actions of CS are mediated by nuclear translocation of glucocorticoid receptors (GR; more specially, GR-α) and subsequent regulation of gene transcription (Figure 1.2). The GR, also known as nuclear receptor subfamily 3, group C, member 1 (NR3C1), is a ligand-dependent transcription factor. GR belongs to the superfamily of steroid/thyroid/retinoid acid receptor proteins that function as ligand-dependent transcription factors (Evans 1988). The GR is composed of three major functional domains: the N-terminal domain, the central DNA-binding domain, and the ligand-binding domain (LBD). Two isoforms, GR-α and GR-β, originate from the same *NR3C1* gene on chromosome 5 (5q31-32) in human through alternative splicing of the GR primary transcript (Hollenberg, Weinberger et al. 1985, Francke and Foellmer 1989, Encio and Detera-Wadleigh 1991). The structural differences are in the LBD, where the last 50 amino acids of GR-α are replaced by a non-homologous 15-amino acid sequence in GR-β. GR-α is the predominant isoform to which cortisol and other CS bind (Hollenberg, Weinberger et al. 1985) whilst GR-β does not bind

CS. Most of the studies analysing GR expression did not distinguish between GR-α and GR-β isoforms. In my thesis, "GR" denotes total GR, unless otherwise specified.

In the inactive state, unbound cytoplasmic GR forms a heterocomplex with chaperone proteins such as heat shock protein 70 and FK506-binding protein 52 (Cadepond, Schweizer-Groyer et al. 1991, Pratt, Morishima et al. 2006). Upon CS binding, GR dissociates from chaperone proteins, homodimerises via the C-terminal LBD and translocates to the nucleus (Bledsoe, Montana et al. 2002), where GR inactivates the pro-inflammatory genes by recruitment of co-repressors and inhibition of transcription factors (trans-repression). GR suppresses gene transcription by interacting with negative glucocorticoid response element (cis-repression) on gene promoters. Post-transcriptionally, CS may reduce the mRNA stability of certain inflammatory proteins by inducing the expression of proteins which destabilise these mRNAs (Barnes 2010).

GR also acts through glucocorticoid-response element (GRE) on gene promoters to activate transcription of anti-inflammatory genes (transactivation), such as glucocorticoid-induced leucine zipper (*GILZ*; also known as TSC22 domain family protein 3) (Beaulieu and Morand 2011). Human GILZ protein is constitutively expressed in many human tissues (D'Adamio, Zollo et al. 1997, Ayroldi, Migliorati et

al. 2001) and is rapidly up-regulated by CS via direct binding of GR to GRE in the promoter of the gene encoding GILZ (Asselin-Labat, Biola-Vidamment et al. 2005).

GR also mediates some of its actions in a nongenomic manner, such as phosphorylation of PI3K and protein kinase B (Akt) which leads to activation of endothelial nitric oxide synthase and ultimate vasorelaxation (Hafezi-Moghadam, Simoncini et al. 2002, Losel and Wehling 2003).

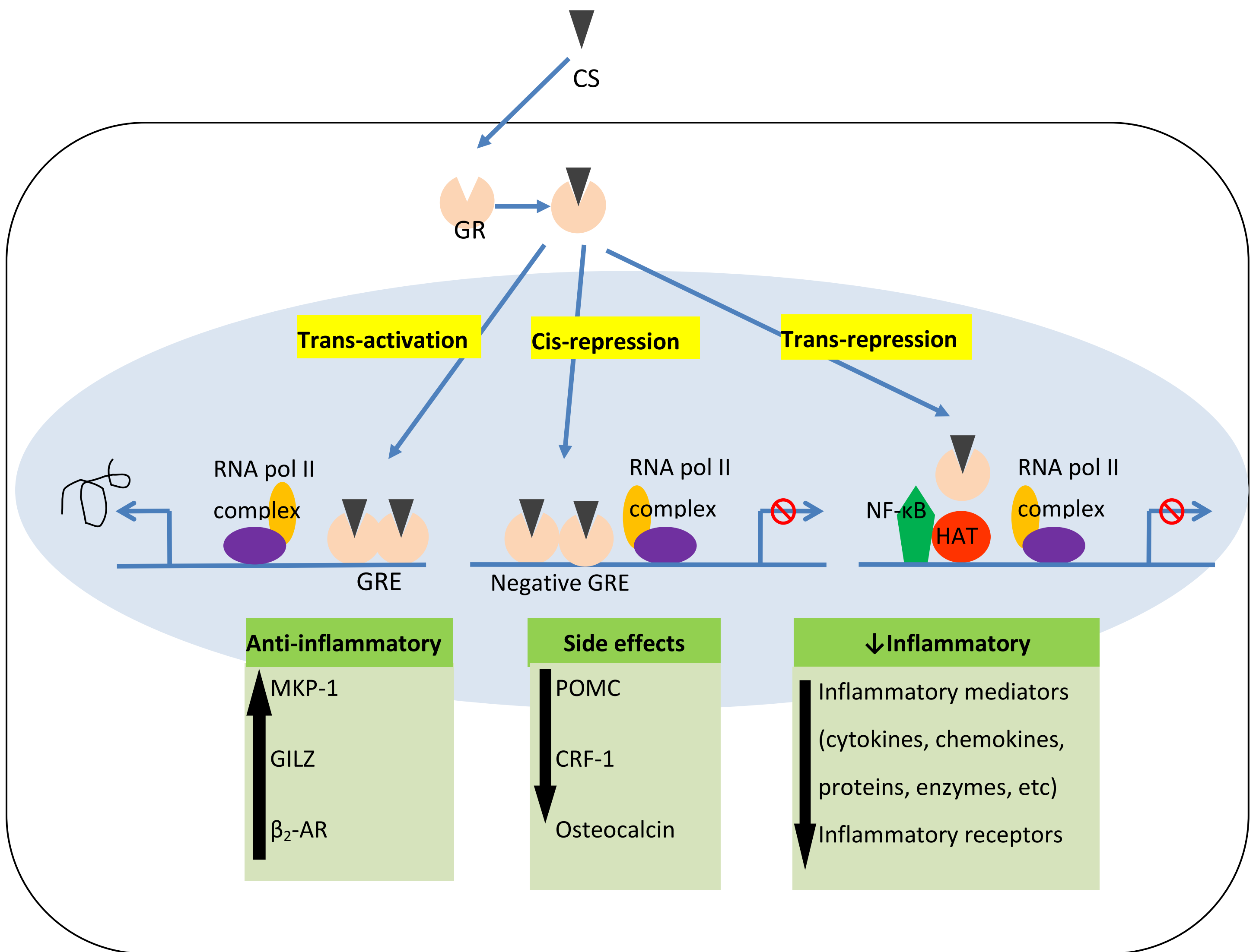


Figure 1.2: Anti-inflammatory actions of corticosteroids. CS enter the cell and bind to glucocorticoid receptors (GR) in the cytoplasm that translocate to the nucleus. Binding of the activated GR homodimer to a GRE in the promoter region of anti-inflammatory genes leads to their transactivation. GR homodimers also interact with negative GREs to suppress inflammatory genes by cis-repression. Through transrepression, the GR-CS complex interacts and inactivates proinflammatory transcription factors, such as nuclear factor-κB (NF‐κB) and activator protein-1 (AP-1), thus switching off expression of inflammatory genes. RNA pol II: RNA polymerase II. MKP-1: mitogen-activated protein kinase phosphatase-1; GILZ: glucocorticoid-induced leucine zipper; β_2-AR: β_2-adrenoceptor; POMC: proopiomelanocortin; CRF-1: corticotrophin releasing factor. Modified from Barnes PJ. J Steroid Biochem Mol Biol 2010;31:76 and Holgate ST *et al*. Nat Rev Immunol 2008;8:218.

To investigate the function of GR, dexamethasone and RU-486, the agonist and the antagonist respectively, were used in this project. Dexamethasone is a synthetic CS, which is 25 times more potent than cortisol/hydrocortisone in its glucocorticoid effect, with minimal mineralocorticoid effect (Cantrill, Waltman et al. 1975). Clinically, dexamethasone is indicated for the treatment of anaphylaxis, allergy, inflammation, emesis and brain edema in cancer patients, to promote the maturation of preterm fetal lungs in pregnant mother (Williams 1999) as well as the diagnosis of Cushing's syndrome (dexamethasone suppression test) (Crapo 1979). RU486, also known as mifepristone and Roussel-Uclaf-38486, is a synthetic steroid compound with anti-glucocorticoid and anti-progesterone effects (Spitz and Bardin 1993). Structurally, RU486 is characterized by the bulky phenyl-aminodimethyl group substituent in the 11β-position of the steroidal skeleton, which interacts with the specific region of the receptor binding pocket in LBD and induces transconformation differences in the LBD (Cadepond, Ulmann et al. 1997). Its affinity for GR in rat hepatoma tissue culture cells is three times higher than that of dexamethasone (Gagne, Pons et al. 1985). Clinically RU486 is used as GR antagonist for refractory Cushing syndrome, and as progesterone receptor antagonist for emergency contraception or medical abortion (Spitz and Bardin 1993).

1.3.1.3 Corticosteroid insensitivity in severe asthma

Patients with severe asthma are less responsive to treatment and require high dose inhaled or even systemic CS therapy (Chung, Wenzel et al. 2014). CS insensitivity has been associated with comorbidities such as obesity (Sutherland, Goleva et al. 2008), smoking (Chalmers, Macleod et al. 2002), low vitamin D levels (Gupta, Sjoukes et al. 2011) and particularly significant in the non-eosinophilic (low T_H2 inflammation) phenotype (Berry, Morgan et al. 2007). Apart from high-dose inhaled CS, one third of adult patients with severe asthma require regular oral CS to maintain some degree of asthma control (Ogirala, Aldrich et al. 1991, ten Brinke, Zwinderman et al. 2004). However, only 11% patients of childhood difficult asthma are completely CS-unresponsive to single intramuscular injection of triamcinolone (Bossley, Saglani et al. 2009).

Relative CS-insensitivity in severe asthma is also observed at the cellular and molecular levels. ASMC from patients with asthma are resistant to the anti-proliferative and anti-inflammatory effect of CS (Roth, Johnson et al. 2004, Chang, Bhavsar et al. 2012). Many *in vitro* studies focus on the impaired transrepession in severe asthma. Dexamethasone is less effective in suppressing the release of IL-1β, CXCL8/IL-8, and CCL3/macrophage inflammatory protein-1-α

(MIP-1-α) from endotoxin-stimulated peripheral blood mononuclear cells (PBMC) and alveolar macrophages, and the release of CCL11/eotaxin and CXCL8/IL-8 from TNF-α-stimulated ASMC in severe asthma (Hew, Bhavsar et al. 2006, Bhavsar, Hew et al. 2008, Chang, Bhavsar et al. 2012). Also, IL-2 combined with IL-4 reduces CS sensitivity in PBMC through down-regulating GR-α, leading to attenuated GILZ expression and consequently reduced apoptosis (Vazquez-Tello, Halwani et al. 2013).

Many mechanisms may lead to CS resistance in severe asthma. Activation of MAPK pathways (Abe, Donnelly et al. 2001), up-regulation of GR-β subunit (Roth, Johnson et al. 2004), oxidative stress and decreased histone deacetylase (HDAC) activity (Bhavsar, Hew et al. 2008) are postulated to be involved in mechanisms leading to CS insensitivity in severe asthma (Table 1.2, Figure 1.3).

1.3.1.4 Effect of corticosteroids on fibrocyte function

There is an increase in the number of infiltrating fibrocytes in the renal interstitium of patients with chronic kidney diseases, which is reduced after corticosteroid therapy with a parallel reduction in urinary CCL2/monocyte chemotactic protein (MCP-1) level (Sakai, Furuichi et al. 2010). Dexamethasone reduces the proliferation and IL-13 release in fibrocytes from healthy subjects (Hayashi, Kawakita et al. 2014). Moreover, dexamethasone reduces the expression of α-SMA and the release of IL-6 and IL-10 in

IL-17-primed fibrocytes from healthy subjects (Hayashi, Kawakita et al. 2013). Nonetheless, the effect of CS on fibrocytes from patients with asthma remains unclear whilst it is unknown whether fibrocytes isolated from patients with severe asthma show CS insensitivity.

Table 1.2: Molecular mechanisms of corticosteroid resistance in patients with asthma or chronic obstructive pulmonary disease.

Familial glucocorticoid resistance
GR modification ↑ Phosphorylation: ↓ nuclear translocation ↑ p38MAPKα caused by IL-2 plus IL-4 or IL-13 in patients with severe asthma or caused by MIF in patients with severe asthma ↑ p38MAPKγ in patients with severe asthma ↑ JNK1 caused by pro-inflammatory cytokines in patients with severe asthma ↑ ERK caused by microbial superantigens in patients with severe nonallergic asthma ↓ MKP-1 in patients with severe asthma ↓ PP2A in patients with severe asthma Nitrosylation: ↑ NO from inducible NO synthase Ubiquitination: ↑ degradation by proteasome
Increased GR-β expression
Increased proinflammatory transcription factors AP-1, JNK
Immune mechanisms ↓ T_{reg} cells (↓IL-19, ↓vitamin D_3) ↑ T_H17 (IL-17)
Defective histone acetylation ↓ Acetylation of lysine-5 on histone 4 in patients with severe asthma ↓ HDAC2 in patients with COPD and patients with severe asthma and smokers with asthma ↑ Oxidative stress ↑ PI3Kδ activation

GR: glucocorticoid receptor; MAP: mitogen-activated protein; MAPK: mitogen-activated protein kinase; MKP-1: MAPK phosphatase-1; JNK: c-jun N-terminal kinase; ERK, extracellular signal-regulated kinase; PI3K: phosphoinositide 3-kinase; PPA2: protein phosphatase 2; NO: nitric oxide; IL: interleukin; MIF, macrophage migration inhibitory factor; AP-1: activator protein-1; T_{reg}: CD4+ regulatory T cell; HDAC2: histone deacetylase 2.

Modified Barnes PJ, J Allergy Clin Immunol 2013;131:636

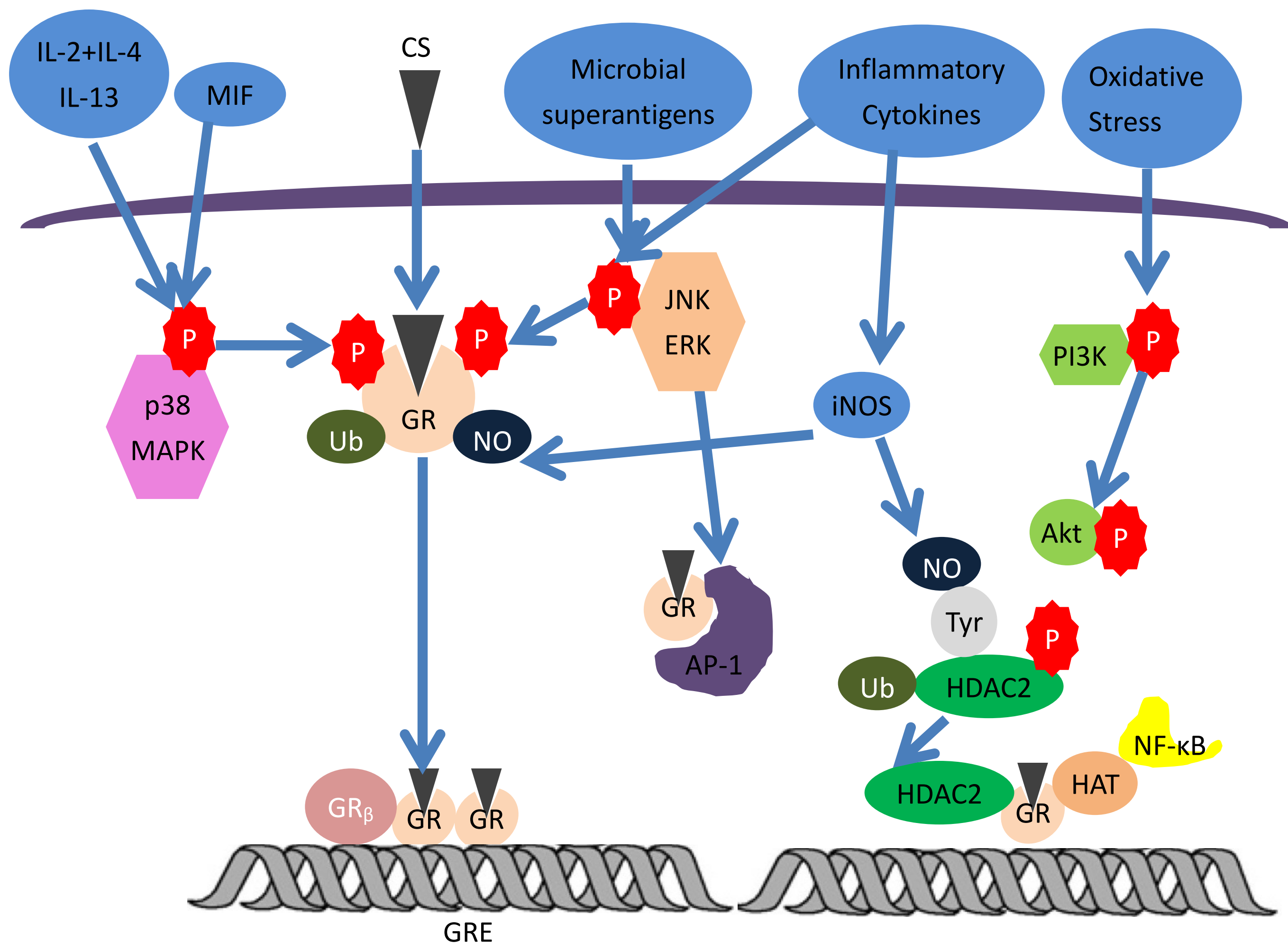


Figure 1.3: Possible molecular mechanisms of corticosteroid resistance. The phosphorylation (P) of glucocorticoid receptors (GR) leads to reduced nuclear translocation of GR. GR can be phosphorylated by p38 mitogen-activated protein kinase (p38 MAPK), c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK), which can be activated by a variety of stimuli such as interleukins (IL), macrophage migration inhibitory factor (MIF) and microbial superantigens. Nitric oxide (NO), generated by inducible nitric oxide synthase (iNOS), can nitrate tyrosine residues (Tyr) on GR and histone deacetylase 2 (HDAC2), leading to inactivation of GR and HDAC2. GR and HDAC2 can also be ubiquitinated (Ub), which results in degradation of GR and HDAC2 by the proteasome. Oxidative stress activates phosphoinositide-3-kinase (PI3K) and downstream Akt (protein kinase B) and subsequently inhibits HDAC2. Sequestration of nuclear factor κB (NF-κB) by activator protein 1 (AP-1) and increased GR-β to glucocorticoid responsive element (GRE) binding sites are also associated with corticosteroid insensitivity.

1.3.2 Bronchodilators

Bronchodilators are widely used for the treatment of asthma. Bronchodilators reduce resistance in the bronchi and bronchioles and increase airway flow to the lungs (Cazzola, Page et al. 2012). These agents could be either short-acting or long-acting: short-acting bronchodilators are rescue relievers for acute bronchospasms, while long-acting bronchodilators reduce symptoms and episodes of exacerbations (Bateman, Hurd et al. 2008). Three classes of bronchodilators are prescribed clinically: β_2-adrenoreceptor (β_2-AR) agonists, muscarinic antagonists and xanthines/phosphodiesterase (PDE) inhibitors. These compounds all involve 3',5'-cyclic adenosine monophosphate (cAMP) signalling (Figure 1.5). The effect of bronchodilators on fibrocyte function has not been reported. In this project I focus on two long-acting compounds: salmeterol xinafoate (salmeterol) and tiotropium bromide (tiotropium); PDE4 inhibitor rolipram has been used to investigate the downstream signalling of β_2-AR and muscarinic M_2 receptor.

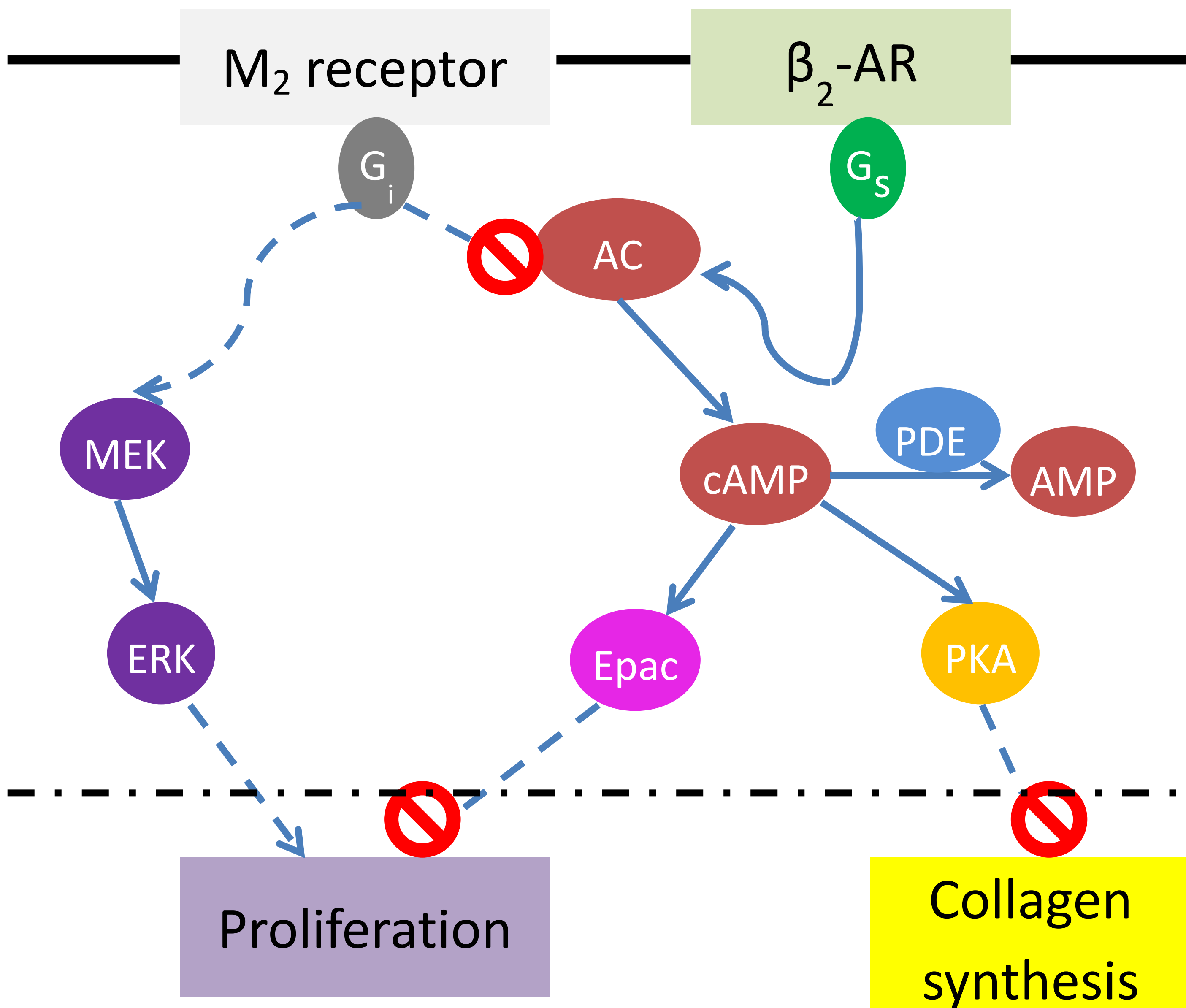


Figure 1.4: Possible mechanisms involving β_2-adrenoceptor and muscarinic M_2 receptor-mediated cell proliferation and collagen synthesis. β_2-AR and M_2 receptor coupled to adenylyl cyclase stimulatory and inhibitory, respectively. AC catalyses the conversion of adenosine triphosphate to cAMP and pyrophosphate. cAMP, which inhibits the proliferation and collagen synthesis in pulmonary fibroblasts, can be hydrolysed by PDE. M_2 receptor can also stimulate fibroblast proliferation via MEK/ERK pathway. β_2-AR: β_2-adrenoceptor. AC: adenylyl cyclase. cAMP: 3',5'-cyclic adenosine monophosphate. Epac: exchange protein activated by cAMP. MEK: MAP kinase activating enzyme. ERK: extracellular signal-regulated kinase. PDE" phosphodiesterase. Modified from Racké K *et al.* *Naunyn-Schmiedeberg's archives of pharmacology* 2008;378:193.

1.3.2.1 β_2-adrenoceptor agonists

The β_2-AR, also known as ADRB2, is a 413-amino acid long seven-transmembrane, G-protein coupled receptor (GPCR) which binds adrenaline or neurotransmitters affecting muscles or organs (Henderson, Baldwin et al. 1990). Despite the lack of direct sympathetic innervation of ASM, β-ARs are expressed throughout the lungs (Barnes 1995). β_2-AR represents 70% of pulmonary β-AR and are localised to both structural cells (ASMC, fibroblasts, epithelial cells, submucosal glandular cells, endothelial cells, type I and type II alveolar cells) and inflammatory cells (mast cells, macrophages, neutrophils, lymphocytes, eosinophils). Activation of β_2-ARs, which are linked to adenylyl cyclases by G_s proteins, increases intracellular cAMP levels. In ASMC, cAMP increases protein kinase A (PKA) activity, which phosphorylates downstream protein modulators (Giembycz and Raeburn 1991), leading to the inhibition of phosphoinositol hydrolysis, a reduction in intracellular Ca^{2+} levels, and subsequent activation of large conductance K^+ channels and relaxation of airway smooth muscle (Jones, Charette et al. 1990). Activation of β_2-AR/G_s protein/cAMP/PKA also inhibits collagen II production in chondrocytes (Mitchell, Lai et al. 2011) and proliferation of mammary epithelial MCF-10A cells (Bruzzone, Sauliere et al. 2014). Activation of β_2-AR/G_s/cAMP/exchange protein directly

activated by cAMP (Epac) inhibits the proliferation of astrocytoma cells (Mostafavi, Khaksarian et al. 2014). Switching the coupling of β_2-ARs from G_s to G_i results in desensitization due to termination of β_2-AR signalling (Johnson 2001).

Conventional SABAs, such as salbutamol (albuterol), fenoterol and terbutaline, work rapidly (within 3 to 5 min), but their effects may only last 4-6 hr. As-needed SABA is used to relieve symptoms when asthma control is lost or not achieved. All controller treatments serve to reduce the use of SABAs, including the regular use of LABAs (Bateman, Hurd et al. 2008), because high dose SABA could worsen asthma even leading to death, especially in patients who do not receive concurrent inhaled CS therapy (Taylor, Sears et al. 1996).

Inhaled LABAs such as salmeterol and formoterol, have bronchodilator action lasting up to 12 hr (Lotvall 2002). Salmeterol has a delayed onset and is a partial agonist of low intrinsic efficacy (Moore, Khan et al. 1998). Salmeterol initially partitions rapidly into the lung cells (< 1 min), with its long lipid tail anchoring on the hydrophobic exosite within the fourth domain of the β_2-AR, then slowly being released back outside the cell (25 min), where salmeterol's "albuterol head group" can specifically and repeatedly engage and disengage the active site of the β_2-AR (Johnson, Butchers et al. 1993). Salmeterol's relatively slow onset results from the

slow desorption from the cells (Johnson 2001), and its long duration thanks to its slow dissociation (Green, Spasoff et al. 1996). Its intrinsic efficacy (intrinsic ability to activate β_2-ARs independent of tissue properties) may reduce side effects and desensitization.

β_2-AR agonists also have non-bronchodilator effects. Both SABA and LABA reduce the release of histamine from mast cells (Nials, Ball et al. 1994), leading to protection against acute exacerbations of asthma (Pauwels, Lofdahl et al. 1997). LABAs inhibit plasma exudation by preventing separation of endothelial cells in postcapillary venules (Baluk and McDonald 1994, Bolton, Lefevre et al. 1997), and decrease vascular densities in the subepithelial lamina propria in asthmatic airways (Orsida, Ward et al. 2001). Although salmeterol monotherapy does not attenuate collagen deposition in airways *in vivo* (Roberts, Bradding et al. 1999, Vanacker, Palmans et al. 2002), formeterol inhibits the synthesis of collagen in pulmonary fibroblasts *in vitro* (Lamyel, Warnken-Uhlich et al. 2011). LABAs also repress the proliferation and the expression of α–SMA, adhesion molecules and GM-CSF in pulmonary fibroblasts/myofibroblasts *in vitro* (Silvestri, Fregonese et al. 2001, Spoelstra, Postma et al. 2002, Lamyel, Warnken-Uhlich et al. 2011). Therefore, β_2-AR agonists have some anti-inflammatory effects in asthma therapy.

The combination of an inhaled CS and LABA is the mainstream therapy for moderate-to-severe asthma (Chung and Adcock 2004), which is superior to high dose inhaled CS monotherapy (Woolcock, Lundback et al. 1996, Masoli, Weatherall et al. 2005) and avoids LABA monotherapy-associated death (Cockcroft and Sears 2013). An addition of salmeterol to inhaled CS therapy greatly improves the symptoms and lung function in asthmatic patients whose disease cannot be easily controlled by inhaled CS (Greening, Ind et al. 1994).

Combination therapy provides many additional or synergistic anti-inflammatory effects which cannot be achieved by either inhaled CS or LABA therapy alone. β_2-AR agonists increase the expression of tachykinin neurokinin receptor in ASMC, that may increase bronchoconstriction, but can be prevented by inhaled CS (Katsunuma, Mak et al. 1998). CS also decrease eosinophil recruitment and increased late response to allergen induced by salbutamol (Gauvreau, Jordana et al. 1997). The combination of CS and LABA inhibits release of CXCL8/IL-8 and CCL11/eotaxin release from human ASMC and GM-CSF from epithelial cells, as well as ASMC proliferation (Pang and Knox 2000, Korn, Jerre et al. 2001). Salmeterol also enhances the inhibitory activity of fluticasone propionate on ICAM-1 expression in pulmonary fibroblasts (Silvestri, Fregonese et al. 2001).

At the molecular level, CS may increase the number of β_2-ARs (Baraniuk, Ali et al. 1997) and modulate the coupling between β_2-AR and G_s protein (Mak, Nishikawa et al. 1995) (Figure 1.6). On the other hand, β_2-AR agonists also improve CS sensitivity by increasing intracellular cAMP, leading to protein kinase A (PKA)-induced GR nuclear translocation (Eickelberg, Roth et al. 1999) or attenuated GR phosphorylation (Mercado, To et al. 2011). However, some argue that high concentration of LABA activates cAMP response element-binding protein (CREB), which may interfere the anti-inflammatory effect of CS through its interaction with NF-κB and AP-1 (Adcock, Stevens et al. 1996). This detrimental effect of LABA on CS effect has been observed in rat lungs (Peters, Adcock et al. 1995), but not in human monocytes (Seldon, Stevens et al. 1998).

The effect of combination therapy on airway remodelling is less established though. In ovalbumin-challenged rats, combining salmeterol with fluticasone propionate attenuates goblet cell hyperplasia, but promotes the deposition of fibronectin and collagen in the airway wall (Vanacker, Palmans et al. 2002).

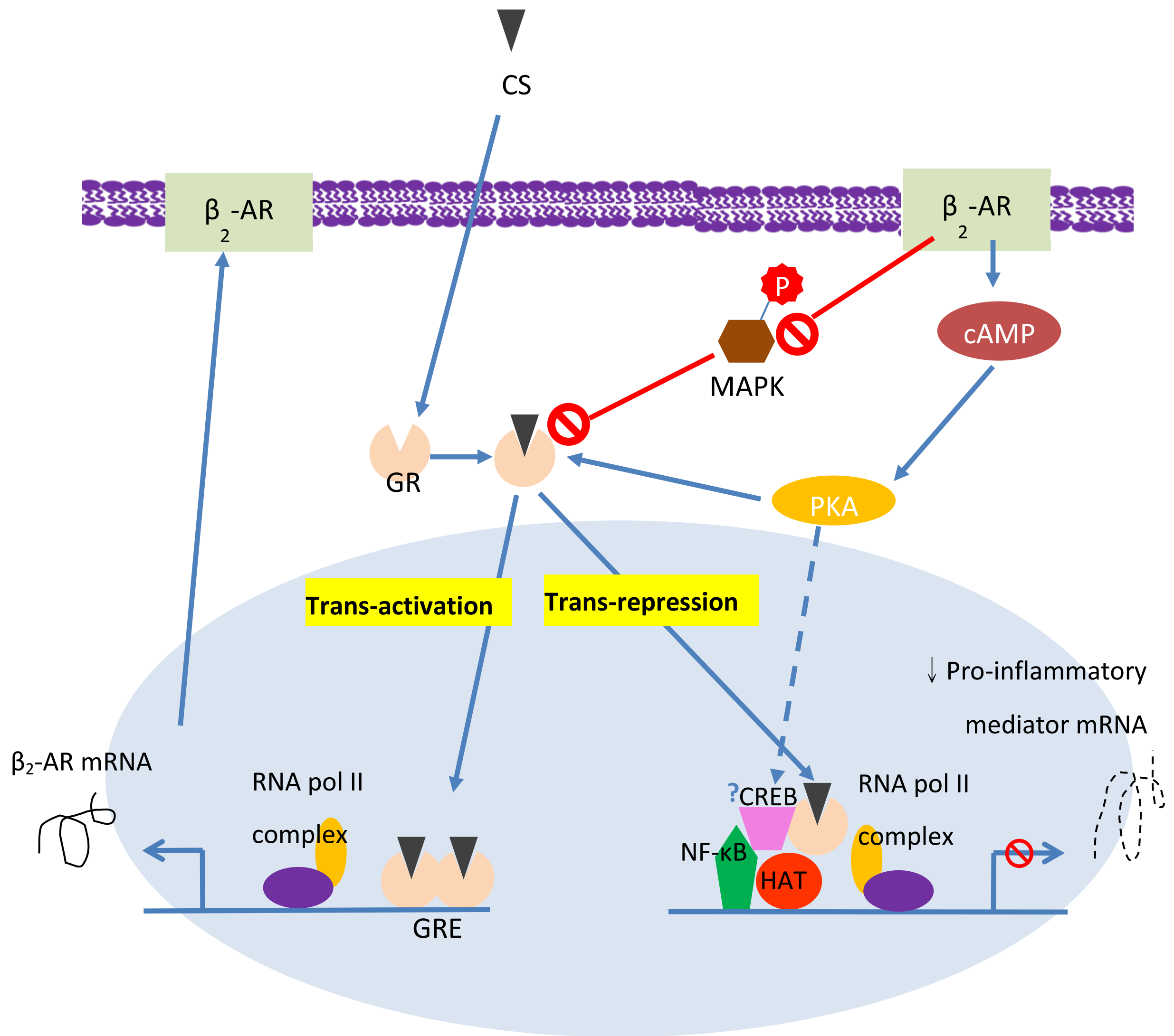


Figure 1.5: Interaction of β_2-adrenoceptor agonists with corticosteroid effects. β_2-AR agonists increase production of intracellular 3',5'-cyclic adenosine monophosphate (cAMP) and subsequent activation of downstream protein kinase A (PKA), enhancing glucocorticoid receptor (GR) nuclear translocation and DNA binding. β_2-AR agonists also interfere the activity of mitogen-activated protein kinases, which affect the phosphorylation status of GR. High concentration of β_2-AR agonists activate cAMP response element-binding protein (CREB) which may block the effect of CS through interact with other transcriptional factors, such as nuclear factor-κB (NF-κB), in some species (e.g. rat). CS bind to glucocorticoid response elements (GRE) in the promoter region of β2-AR gene, leading to increased transcription of β_2-AR. RNA pol III: ribonucleic acid polymerase type III. HAT: histone acetyltransferase.

1.3.2.2 Muscarinic antagonists

Muscarinic receptors, also known as muscarinic acetylcholine receptors (mAChRs) or M receptors, are seven-transmembrane helix G-protein coupled receptors (GPCRs) which respond to acetylcholine from the parasympathetic nervous system (Caulfield and Birdsall 1998). Muscarinic receptors can be divided into five subgroups: M_1, M_2, M_3, M_4 and M_5 (Caulfield and Birdsall 1998). Activation of G_q protein-coupled M_1, M_3 and M_5 receptors up-regulates phospholipases whereas activation of G_i protein-coupled M_2 and M_4 down-regulates intracellular cAMP (Simon, Strathmann et al. 1991).

In respiratory organs (Gosens, Zaagsma et al. 2006), acetylcholine induces bronchospasm and mucus secretion. Acetylcholine is mostly secreted by efferent vagal nerves but also by bronchial epithelial cells and inflammatory cells in a paracrine or autocrine manner (Sastry and Sadavongvivad 1978). Postsynaptic M_1 receptors, as well as nicotinic receptors, mediate neurotransmission in airway parasympathetic ganglia and presynaptic M_2 autoreceptors produce negative feedback to ganglionic transmission. The postganglionic neurons regulate secretion of mucus glands (M_3) and the contraction, synergy and cytokine release of airway smooth muscle cells (M_3, although M_2 is the predominant subtype in smooth muscle cells).

Stimulation of M_3 activates phospholipase C, resulting in activation of protein kinase C and generation of inositol 1,4,5-trisphosphate, which increases intracellular Ca^{2+} and induces ASM contraction (Billington and Penn 2002). M_2 activation inhibits adenylyl cyclase and downstream PKA and Epac (Iancu, Ramamurthy et al. 2008). In pulmonary fibroblasts, cAMP inhibits proliferation through Epac pathway and down-regulates the expression of Col I through the PKA pathway (Racke, Haag et al. 2008). Activation of M_2 and M_3 may lead to proliferation of pulmonary fibroblasts through phosphorylation of mitogen-activated protein kinase kinase/ extracellular signal-regulated kinases cascades (Racke, Haag et al. 2008). Muscarinic receptors also mediate the release of cytokines and chemokines from epitheial cells (M_1, M_2, M_3) and macrophages (M_3) (Spears, Donnelly et al. 2009).

Ipratropium bromide (ipratropium) is a topically active non-selective short-acting muscarinic antagonist (SAMA) widely used in asthmatic patients. The effect of ipratropium starts to act within 15 to 30 minutes, takes 90 minute to reach maximal bronchodilation, with a 6 hr duration (Essayan 2001). Ipratropium can be used along with SABA through nebulizers for asthma exacerbation, sometimes as a controller via metered dose inhaler to reduce daily use of β_2-AR agonist or as an alternative choice for patients with severe asthma who do not tolerate or respond to β_2-AR agonists

(Simon, Strathmann et al. 1991, Caulfield and Birdsall 1998, Chung, Wenzel et al. 2014).

Tiotropium bromide (tiotropium) is a highly potent LAMA. The binding affinities of tiotropium and the short-acting muscarinic antagonist ipratropium are similar, but tiotropium dissociates from M_1 and M_3 much more slowly compared with ipratropium (14.6 hr vs 0.11hr and 34.7 hr vs 0.26 hr respectively), whereas dissociation from M_2 is relatively similar (3.6 hr vs 0.035 hr) (Birkenbach, Josefsen et al. 1993, Disse, Speck et al. 1999). Tiotropium improves the outcome of asthma management, although it is often regarded as only an alternative to LABA. The addition of tiotropium to high dose inhaled CS in patients with moderate-to-severe asthma improves the lung function and symptoms and the effect is not inferior to the addition of the LABA salmeterol but is superior to a doubling of the dose of the inhaled CS (Palmqvist, Wardlaw et al. 2007, Peters, Kunselman et al. 2010). In patients taking high dose inhaled CS and LABA, the addition of tiotropium improves FEV_1, reduces as-needed use of SABA and the risk of severe exacerbation (Rot and von Andrian 2004, Palmqvist, Wardlaw et al. 2007).

Tiotropium reduces airway inflammation (Meurs, Dekkers et al. 2013). In chronic obstructive pulmonary disease (COPD) patients, tiotropium induces apoptosis

of CD8+ T lymphocytes and reduces apoptosis of CD4+ T lymphocytes (Profita, Riccobono et al. 2012). In addition, it reduces the chemotactic activity of macrophages and neutrophils (Buhling, Lieder et al. 2007, Vacca, Randerath et al. 2011). Tiotropium attenuates the increase in the mass and contractility of ASM, mucus hypertrophy and eosinophil infiltration in ovalbumin-challenge guinea pigs (Gosens, Bos et al. 2005).

Fibrotic alteration is a key characteristic of airway remodelling caused by pulmonary fibroblasts/myofibroblasts, which are potential targets for tiotropium therapy. In fibroblasts, tiotropium abolishes proliferation and collagen synthesis induced by muscarinic signalling (Racke, Haag et al. 2008). Intriguingly, although tiotropium has a kinetic selectivity for M_1 and M_3 receptors over M_2 receptors, it still inhibits the behaviours of pulmonary fibroblasts through an M_2-predominant effect (Barnes 2000).

The effect of LAMAs on fibrocyte function is currently unknown.

1.3.2.3 Phosphodiesterase inhibitors

Cyclic nucleotide PDEs catalyse the hydrolysis of the phosphodiester bond in the second messenger molecules cAMP and cyclic guanosine monophosphate (cGMP) (Essayan 2001). Inhibition of PDEs accumulates the intracellular concentrations of

these cyclic nucleotides, resulting in activation of PKA and protein kinase G (PKG) (Maurice, Ke et al. 2014). For example, theophylline is a non-selective PDE inhibitor with relative weak bronchodilatory and anti-inflammatory effect (Barnes 2013).

Selective PDE4 inhibitors show therapeutic potential in asthma, COPD and allergic rhinitis (Dyke and Montana 2002). Rolipram is a first generation selective PDE4 inhibitor, reversing AHR and bronchoconstriction in guinea pigs chronically exposed to endotoxin, a model for severe asthma and COPD (Toward and Broadley 2001). However, the therapeutic utility of rolipram is restricted due to dose limiting side-effects such as nausea and emesis (Hebenstreit, Fellerer et al. 1989). Currently, RPL554, a dual PDE3/4 inhibitor, and CHF 6001, an inhaled PDE4 inhibitor, are under clinical development for the treatment of asthma (Matera, Page et al. 2014).

Rationale

Patients with severe asthma show exaggerated airway remodelling despite being on high-dose inhaled, and often systemic CS treatment. The airways of patients with severe asthma also show increased fibrocyte recruitment to the lamina propria and ASM compartments, suggesting that fibrocytes may contribute to the development of remodelling in severe asthma (Saunders, Siddiqui et al. 2009). However, it is currently unknown whether the increased presence of fibrocytes in severe asthmatic airways is a result of aberrant proliferation, differentiation and/or migration, or resistance to anti-asthma treatments.

Hypothesis

I hypothesise that:

In severe asthma there are increased numbers of circulating fibrocytes that have an increased capacity to differentiate into myofibroblasts and have differential responses to pro-inflammatory mediators and asthma therapeutic agents compared to non-severe asthma, thus contributing to sub-epithelial fibrosis and ASM layer thickening.

Aims

In order to address this hypothesis these specific aims were set:

1. Compare the number of fibrocytes and differentiating fibrocytes, as well as CC chemokine receptor 7 (CCR7) expression in fibrocytes, isolated from the peripheral blood of healthy subjects and patients with non-severe or severe asthma, both immediately after isolation and following culture.

2. Compare the effect of pro-inflammatory T_H2 cytokines (interleukin-4 and interleukin-13) and neurotrophins (nerve growth factor and brain-derived neurotrophic factor) on the number of fibrocytes and differentiating fibrocytes as well as on CCR7 expression in fibrocytes from healthy subjects and patients with non-severe or severe asthma.

3. Compare the effect of corticosteroids (dexamethasone) on the number of fibrocytes and differentiating fibrocytes, as well as the expression of CCR7 in fibrocytes from all groups. Also, study the mechanisms through which corticosteroids mediate their effects on fibrocyte function and compare the expression of the glucocorticoid receptor between the fibrocytes of the three groups.

4. Compare the effect of β_2-adrenergic receptor agonists (salmeterol xinafoate) and muscarinic antagonists (tiotropium bromide) on the number of fibrocytes and differentiating fibrocytes as well as on CCR7 expression in fibrocytes from healthy subjects and patients with non-severe or severe asthma.

5. Compare the responses of fibrocytes isolated from the adherent and non-adherent fraction of peripheral blood mononuclear cells.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Peripheral blood mononuclear cell and non-adherent non-T cell isolation

Phosphate-buffered saline (PBS), pure water and ethylenediaminetetraacetic acid (EDTA) were from Sigma-Aldrich (Poole, UK). Ficoll-Paque^{TM} PLUS was from GE HealthCare (Uppsala, Sweden). Anti-coagulant acid-citrate dextrose (ACD, di-Sodium hydrogen citrate) was from Merck KGaA (Darmstadt, Germany). Sheep red blood cells (RBC) 100% washed pooled cells was from Rockland (Gilbertsville, Pennsylvania, USA). CD3 MicroBead kit, LD column and VarioMACS™ Separator were from Miltenyi Biotec (Auburn, California, USA). Trypan Blue Stain and PBS (10X) were from Invitrogen –Life Technologies (Paisley, UK).

2.1.2 Cell culture

Iscove's Modified Dulbecco's Medium (IMDM), Dulbecco's Modified Eagle Medium (DMEM), cell culture grade bovine serum albumin 35% (BSA), foetal bovine serum (FBS), non-essential amino-acid solution and L-glutamine were from Sigma-Aldrich. 6-well tissue culture plates (growth surface area 9.6 cm^2/well) were from Corning

(Corning, New York, USA). Human fibronectin was from BD Biosciences (San Jose, California, USA). The details for different cell treatments used in this thesis are shown in Table 2.1.

Table 2.1 Inhibitors, drugs and mediators used in the project

Treatment	Source	Stock solution
Recombinant human IL-4	R&D Systems (Abingdon, UK)	100 μg/mL in sterile PBS containing 0.1% BSA
Recombinant human IL-13	R&D Systems	50 μg/mL in sterile PBS containing 0.1% BSA
Recombinant human β-NGF	R&D Systems	100 μg/mL in sterile PBS containing 0.1% BSA
Recombinant human BDNF	R&D Systems	25 μg/mL in sterile PBS containing 0.1% BSA
Dexamethasone	Sigma-Aldrich	10^{-3} M in cell-culture grade H_2O
RU486	Abcam (Cambridge, UK)	0.1 M in DMSO
N-acetyl cysteine	Sigma-Aldrich	0.613 M in cell-culture grade H_2O
SB202190	Sigma-Aldrich	10^{-2} M in DMSO
SP600125	Sigma-Aldrich	10^{-2} M in DMSO
U0126	EMD Millipore	0.5 M in DMSO
LY294002	Cayman Chemical (Ann Arbor,, Michigan, USA)	10^{-2} M in DMSO
Salmeterol xinafoate	Sigma-Aldrich	10^{-2} M in DMSO
Acetylcholine chloride	Sigma-Aldrich	0.1 M in cell-culture grade H_2O
Tiotropium bromide	Biorbyt Ltd (Cambridge, UK)	0.2 M in DMSO
Rolipram	Sigma-Aldrich	2.65×10^{-2} M in DMSO
8-Br-cAMP	Abcam	0.1 M in cell-culture grade H_2O

IL-4: interleukin-4; IL-13: interleukin-13; NGF: nerve growth factor; BDNF: brain-derived neurotrophic factor; 8-Br-cAMP: 8-Bromoadenosine 3',5'-cyclic monophosphate

2.1.3 Antibodies and reagents for flow cytometry

BD falcon round bottom polystyrene tube, BD FACSCanto™ II device and BD FACSDiva software were from BD Biosciences. For detection of cell surface markers, antibodies conjugated to different fluorochromes were used either alone or in combination. The spectral characteristics and working dilutions of the antibodies used are listed in Table 2.2. Fluorochrome-labelled isotype controls were used as negative controls. BD™ CompBeads (Anti-Mouse Ig, κ/Negative Control Compensation Particles Set) were from BD Biosciences.

Table 2.2: Wavelength of maximal excitation and emission absorption (Ex-max and Em-max) and final amount of antibodies applied for flow cytometry

Fluorochrome-labelled antibodies	Company	Ex-max (nm)	Em-max (nm)	Final amount (µg)
Unlabelled-mouse $IgG_{1\,\kappa}$ anti-human Col I	Millipore	N/A	N/A	0.5
Unlabelled-mouse $IgG_{1\kappa}$ anti-human isotype control	DAKO	N/A	N/A	0.5
FITC-rabbit F(ab')2 anti-mouse immunoglobulins	DAKO	494	520	2.75
FITC-mouse $IgG_{1\,\kappa}$ anti-human Col I	Millipore	488	518	0.5
FITC-mouse $IgG_{1\kappa}$ anti-human isotype control	Millipore	488	518	0.5
APC- mouse $IgG_{1\kappa}$ anti-human CD45	BD	650	660	0.015
APC-mouse $IgG_{1\kappa}$ anti-human isotype control	BD	650	660	0.015
PE-mouse IgG_{2A} anti-human α-SMA	R&D	496	578	0.125
PE-mouse IgG_{2A} anti-human isotype control	R&D	496	578	0.125
PE-mouse IgG_{1} anti-human CD3	BD	496	578	0.0625
PE-mouse IgG_{1} anti-human isotype control	BD	496	578	0.0625
Unlabelled-rabbit IgG anti-human glucocorticoid receptor	Abcam	N/A	N/A	0.228
Unlabelled-rabbit IgG anti-human isotype control	Abcam	N/A	N/A	0.228
PE-goat IgG anti-rabbit polyclonal (secondary)	Abcam	488	575	0.3
PE-mouse IgG_{2A} anti-human CCR7	BD	496	578	0.125

Ex-max and Em-max: maximal excitation and emission absorption; FITC: fluorescein isothiocyanate; PE: R-phycoerythrin; APC: allophycocyanin; Col I: collagen I; α-SMA: α-smooth muscle actin; GR: glucocorticoid receptor; CCR7: CC chemokine receptor 7

2.1.4 Cell apoptosis assay for flow cytometry

Fluorescein isothiocyanate (FITC) Annexin V/Dead Cell Apoptosis Kit with FITC annexin V and propidium iodide was from Invitrogen –Life Technologies.

2.1.5 Cell proliferation assay for flow cytometry

Click-iT® 5-ethynyl-2'-deoxyuridine (EdU) Flow Cytometry Assay Kit with Pacific Blue™ azide was from Invitrogen –Life Technologies.

2.1.6 Total RNA extraction, cDNA preparation by reverse-transcriptase polymerase chain reaction and determination of mRNA expression by real- time quantitative polymerase chain reaction

The RNeasy Mini Kit, QIAshredder spin columns, DNAse enzyme and SYBR Green PCR Master Mix Reagent were from Qiagen (Crawley, UK). β-mercaptoethanol was from Sigma-Aldrich. The NanoDrop 1000 spectrophotometer was from Thermo Scientific (Epsom, UK). The deoxynucleotides (dNTPs) were from Bioline (London, UK). Avian myeloblastosis virus (AMV) reverse transcriptase reaction buffer and enzyme, random primers, recombinant RNasin ribonuclease inhibitor and nuclease-free water were from Promega (Southampton, U.K). The G-storm thermal cycler GS1 was from G-storm (Somerton, UK). The Rotor Gene 3000 real-time cycler, capillary tubes, and the Rotor Gene 6000 Series software (version 1.7) were from Corbett Research (Sydney, Australia).

Primers for real-time quantitative polymerase chain reaction (real-time PCR) are listed in Table 2.3. Commercial primers for α-smooth muscle actin (α-SMA; Hs_ACTA2_va.1_SG; QT02407307), CD45 (Hs_PTPRC_5_SG; QT01869931) and glucocorticoid-induced leucine zipper (GILZ; TSC22D3_vb.1_SG; QT00999747) were from Qiagen. Customised collagen I (Col I) and 18S primers were produced by Eurofins MVG Operon (Ebersberg, Germany). Customised B-cell lymphoma 2 interacting mediator of cell death-extra long (Bim_{EL}) primer was produced by Sigma-Genosys (Suffolk, UK). Col I primer was designed according to sequences published in the literature (Aoudjehane, Pissaia et al. 2008). Primers for Bim_{EL} and 18S were designed according to their published sequences using the GenScript online primer design software. Primer specificity was measured by using the online sequence analysis software BLAST (www.ncbi.nlm.nih.gov/BLAST/).

Table 2.3 Primers used for real-time PCR

Gene	Primer Sequences	Source
18S	Forward: 5'-CTT AGA GGG ACA AGT GGC G-3' Reverse: 5'-AGC CTG AGC CAG TCA GTG TA-3'	Eurofins MVG Operon
Col I	Forward: 5'-CCT CAA GGG CTC CAA CGA G-3' Reverse: 5'-TCA ATC ACT GTC TTG CCC CA-3'	Eurofins MVG Operon
CD45	QT01869931 (Hs_PTPRC_5_SG)	Qiagen
α-SMA	QT02407307 (Hs_ACTA2_va.1_SG)	Qiagen
Bim_{EL}	Forward: 5'-CGA TCC TCC AGT GGG TAT TT-3' Reverse: 5'-ACT CTT GGG CGA TCC ATA TC-3'	Sigma-Genosys
GILZ	QT00999747 (TSC22D3_vb.1_SG)	Qiagen

2.2 Methods

2.2.1 Recruitment of healthy subjects and patients with non-severe or severe asthma

Healthy subjects and patients with non-severe or severe asthma, aged between 20 and 65, were recruited (Table 2.4). All subjects were non-smokers or ex-smokers with < 5 pack-year smoking history (quit > than 3 years ago) without significant co-morbidities e.g. ischaemic heart disease, diabetes, malignancy. Subjects with forced expiratory volume in 1 s (FEV_1) reversibility ≥ 12% or provocative concentration of methacholine causing a 20% fall in FEV_1 (PC_{20}) < 8 mg/mL were diagnosed as asthmatic patients. Severe asthma was defined according to the American Thoracic Society guidelines for refractory asthma (Chapter 1.1), with the presence of at least one of two major criteria for corticosteroid (CS) usage (inhaled beclomethasone >1260 μg/day or equivalent), and at least two minor criteria (2000). Asthmatic patients who did not meet the criteria were included as patients with non-severe asthma. This study has been approved by the Royal Brompton & Harefield NHS Trust/National Heart & Lung Institute Ethics Committee (London - Chelsea REC 08/H0708/109). Informed consent was given.

Compared to healthy subjects and patients with non-severe asthma, the patients with severe asthma used higher doses of inhaled CS, and about two third of them were on oral prednisolone treatment. Generally, these patients were more senior, predominantly female and had worse lung function.

Table 2.4: Clinical characteristics of studied subjects

	Healthy	Non-severe Asthma	Severe Asthma
Number	18	19	44
Age, years	40.2 ± 2.3	44.6 ± 3.7	49.2 ± 2.4
Gender, Female/Male	8/10	10/8	31/13
Duration of asthma, years	N/A	29.0 ± 11.5	28.8 ± 15.7*
Smoking status	None	None	None
Inhaled corticosteroid dose, µg BDP equivalent	0	342.1 ± 108.5	2095 ± 118.4**##
Atopy	10 (55.6 %)	11 (57.9 %)	27 (61.4 %)
Receiving oral corticosteroids	0 (0 %)	0 (0 %)	29 (66.0 %)
Pre-bronchodilator FEV_1 (L)	3.5 ± 0.2	2.8 ± 0.2**	1.9 ± 0.1***#
Pre-bronchodilator FEV_1 of predicted value (%)	97.9 ± 2.9	87.1 ± 3.1**	67.9 ± 3.5***##
FEV_1/FVC (%)	87.4 ± 1.6	73.4 ± 1.8***	69.4 ± 1.9***

Data are expressed as mean ± SEM. BDP, beclomethasone dipropionate (Beclomethasone 1µg = Fluticasone 0.5µg = Budesonide 0.640µg); FEV_1, forced expiratory volume in 1s; FVC, forced vital capacity.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to healthy subjects.

# $p < 0.05$, ## $p < 0.01$ compared to patients with non-severe asthma.

2.2.2 Non-adherent non-T cell isolation and culture

Non-adherent non-T (NANT) cells were isolated from peripheral blood as previously described in the literature (Wang, Huang et al. 2008, Wang, Huang et al. 2012, Weng, Chen et al. 2013) with modification in the method of T cell depletion. Peripheral blood mononuclear cells (PBMC) were separated from whole blood using Ficoll-Paque™ PLUS density gradient centrifugation. Briefly, whole blood containing acid citrate dextrose (ACD) was diluted with equal volume of PBS. 10 mL of diluted cell suspension was carefully layered over 3 mL of Ficoll-Paque™ PLUS in a 15 mL conical tube and centrifuged at 300×g for 30 min, at 20 °C. PBMC were harvested from the interface, carefully transferred to a new 50 mL conical tube, mixed with PBS, and centrifuged at 300×g for 12 min, at 20 °C. RBC lysis was carried out by brief hypotonic shock using 1.8 mL of pure water to re-suspend the cell pellet for 30 s, and stopped by adding 200 μL of 10× concentrated PBS. The PBMC were then diluted with IMDM supplemented with 20% FBS to a density of 4×10^6 PBMC/mL, and cells were seeded in 6-well tissue culture plates (4×10^6 PBMC/well) and incubated at 37°C for 90 min to allow attachment of monocytes. The non-adherent cell fraction was then obtained and centrifuged at 300×g for 12 min.

Non-adherent cells were then depleted of T cells by treating with sheep RBC, or anti-CD3-labelled magnetic beads. The two methods are described below.

Treatment with sheep RBC: This method is based on the ability of T cells to bind to sheep RBC, forming E-rosette structures which can be then removed by Ficoll-Paque density gradient centrifugation (Wang, Huang et al. 2008). Briefly, non-adherent PBMC were re-suspended in 2 mL of IMDM and mixed with 100% sheep RBCs for 1 hr at 4°C. The E-rosette–forming cells were separated by Ficoll-Paque centrifugation as described above. The NANT cell fraction was harvested from the interface. This method was only used in chapter 3.2.1 for protocol optimisation.

Antibody-labelled magnetic beads: This method involves labelling T cells using anti-CD3 antibodies conjugated to magnetic beads, and then removing them by placing in a magnetic field according to the manufacturer's instructions. Briefly, 1×10^7 non-adherent cells were re-suspended in 80 µL of PBS containing 0.5% BSA and 2 mM EDTA. The cells were then mixed with 20 µL of CD3 MicroBeads and incubated for 15 min at 4°C. Finally, cells were washed, re-suspended in 500 µL of PBS and applied onto a pre-rinsed LD column in the magnetic field of a MACS

Separator. T cells were retained in the column whilst the NANT cells were collected in the flow-through fraction.

Following extraction, viable NANT cell number was determined by Trypan Blue staining and haemocytometer counting, and cells were prepared for flow cytometry or culture. The NANT cell isolation procedure is illustrated in Figure 2.1.

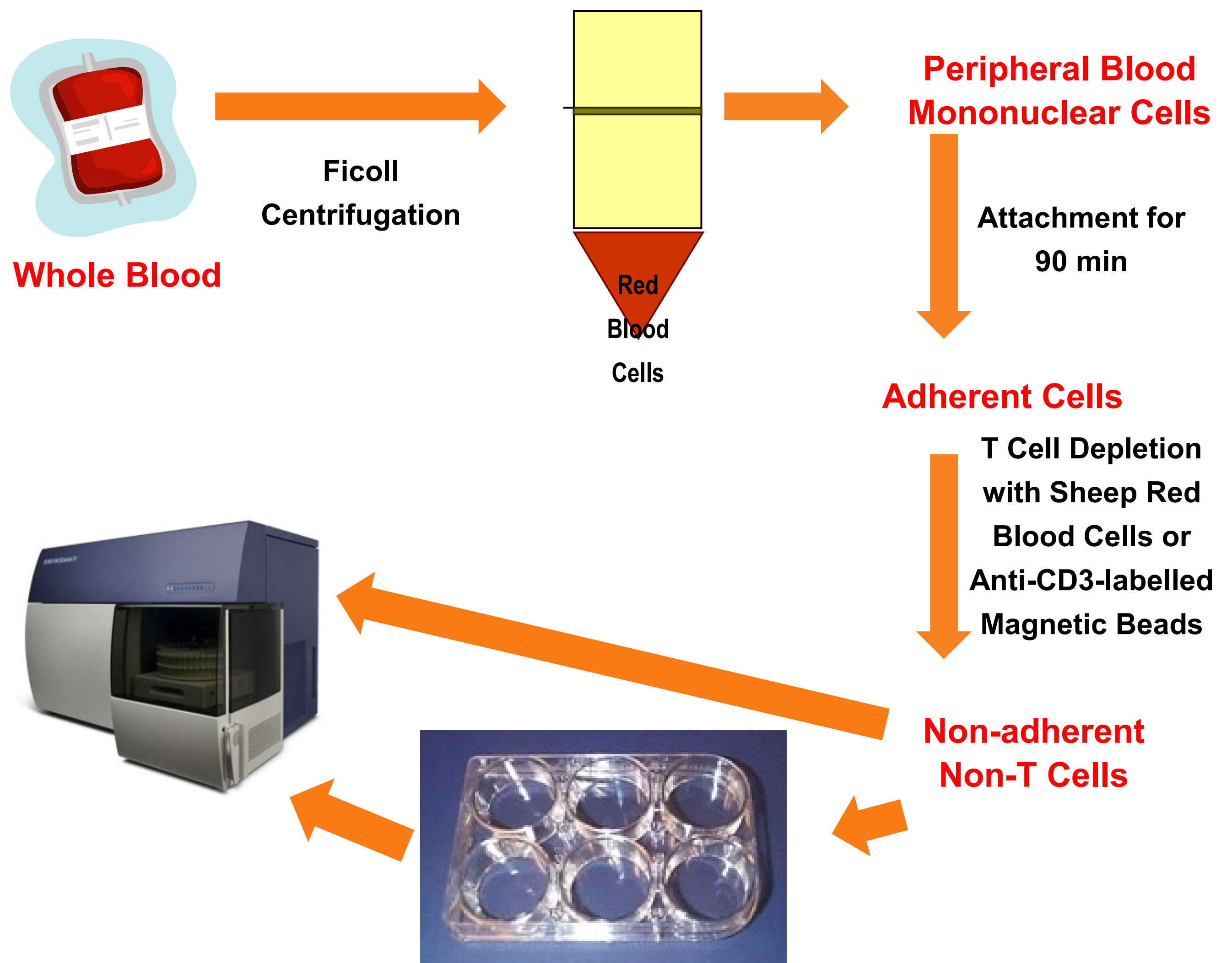


Figure 2.1: Isolation of non-adherent non-T cells from peripheral blood. PBMC were isolated from whole blood by Ficoll-Paque centrifugation. PBMC were seeded in 6-well tissue culture plates and incubated for 90 min to allow attachment of monocytes. Non-adherent cells were collected, and T cells were depleted by anti-CD3-labelled magnetic beads or sheep RBC. NANT cells were then prepared for flow cytometry or culture.

To culture the fibrocytes derived from NANT cells, $1x10^6$ freshly isolated NANT cells were re-suspended in 2 mL of FBS-containing medium (IMDM supplemented with 30% FBS and 1% BSA) (Wang, Huang et al. 2008, Wang, Huang et al. 2012, Weng, Chen et al. 2013) or FBS-free medium (IMDM supplemented with 2mM non-essential amino acids and 0.1% BSA; only used in Chapter 3.2.2 and Chapter 4.2.1). Cells were then seeded in 6-well tissue culture plates (10^6 cells/well) and incubated in a humidified incubator at 37 °C, 5% CO_2 for the required time periods in the presence or absence of the treatments (IL-4. IL-13, NGF, BDNF, dexamethasone, RU486, N-acetylcysteine, SB202190, SP600125, U0126, LY294002, salmeterol, N-acetylcysteine, tiotropium, rolipram, 8-Br-cAMP). The medium was not changed throughout the experiment, to avoid removal of un-differentiated fibrocytes which were found in suspension (Wang, Huang et al. 2008, Wang, Huang et al. 2012, Weng, Chen et al. 2013). Adherent cells were recovered by scrapping, and collected together with non-adherent cells. Viable NANT cell number was determined by Trypan Blue staining and haemocytometer counting, and cells were prepared for flow cytometry.

2.2.3 Adherent peripheral blood mononuclear cell isolation and culture

PBMC were isolated from whole blood containing acid citrate dextrose (ACD) using density gradient centrifugation with Ficoll-Paque™ PLUS as described above (Chapter 2.2.2). PBMC (4×10^6 cells/well) re-suspended in 1 mL of FBS-containing medium (DMEM supplemented with 10% FBS and L-glutamate 2 mM) (Abe, Donnelly et al. 2001, Yang, Scott et al. 2002) were seeded in 6-well tissue culture plates pre-coated with fibronectin (25 μg/mL or 2.6 μg/cm^2). Non-adherent cells with old media were removed by a single gentle aspiration and 2 mL of new media were added on day 3 with or without treatment (dexamethasone, RU486, salmeterol, tiotropium, IL-4, IL-13, NGF, BDNF). Adherent cells were harvested by medium flushing and scraping on day 6. Viable adherent cell were determined by Trypan Blue staining and haemocytometer counting. Fibrocytes (Col I+/CD45+ cells) and differentiating fibrocytes (α-SMA+ cells) were determined by flow cytometry.

For condition optimisation, FBS-free medium (DMEM supplemented with 0.1% BSA, 1X non-essential amino acids, L-glutamate 2 mM; Chapter 3.2.4.2 and Chapter 4.2.4), different amount of fibronectin (0-10 μg/mL or 0-1.0 μg/cm^2; Chapter 3.2.4.3)

and length of culture (3-14 day, Chapter 3.2.4.1) and treatment (treated cells on day 0 without changing medium on day 3, Chapter 4.2.4) have also been tried.

2.2.4 Flow cytometry and staining

Cells stained with antibodies or dyes were prepared in BD falcon round bottom polystyrene tube. Flow cytometry was performed using a BD FACSCanto™ II device and analysis was carried out using the BD FACSDiva software.

2.2.4.1 Colour compensation

In order to avoid spectral overlap and thus false positive events in the flow cytometric analysis, colour compensation was carried out. CompBeads are polystyrene beads coupled to an anti-Ig κ light chain antibody allowing the binding of the fluorochrome-conjugated antibodies. Moreover, beads without anti-Ig κ light chain antibody were used as negative control.

Staining of the BD™ CompBeads was carried out according to the manufacturer's instructions. Briefly, 60 μL of BD™ CompBeads Negative Control and BD™ CompBeads were added to 100 μL of staining buffer (PBS containing 0.1% BSA) and incubated with the fluorochrome-labelled antibodies at the working dilutions (Table 2.2), for 30 min at room temperature. The beads were then washed

using PBS, spun at 200×g for 10 min, re-suspended in PBS and transferred to polystyrene tubes for flow cytometry. In Figure 2.2, I demonstrated how to carry out compensation to avoid spill over between fluorescence channels when performing three colour staining using mouse-anti-human anti-Col I/FITC-anti-mouse, R-phycoerythrin (PE)-anti-CC chemokine receptor 7 (CCR7) and allophycocyanin (APC)-anti-CD45 antibodies. The singlet bead population was selected based on forward scatter (FSC) and side scatter (SSC) characteristics (gate P1; Figure 2.2A) and the mean fluorescence intensity for each antibody was determined for the positive (gate P2) and negative (gate P3) populations. All antibodies, at the dilutions used, gave a good resolution between negative and positive populations (Figure 2.2B-D). Compensation was automatically calculated by the FACSDiva software.

For multicolour staining involving non-mouse antibodies (i.e. glucocorticoid receptor; GR) or dyes (i.e. cell proliferation assay and cell apoptosis assay), fluorescence compensation for spectral overlap was done manually.

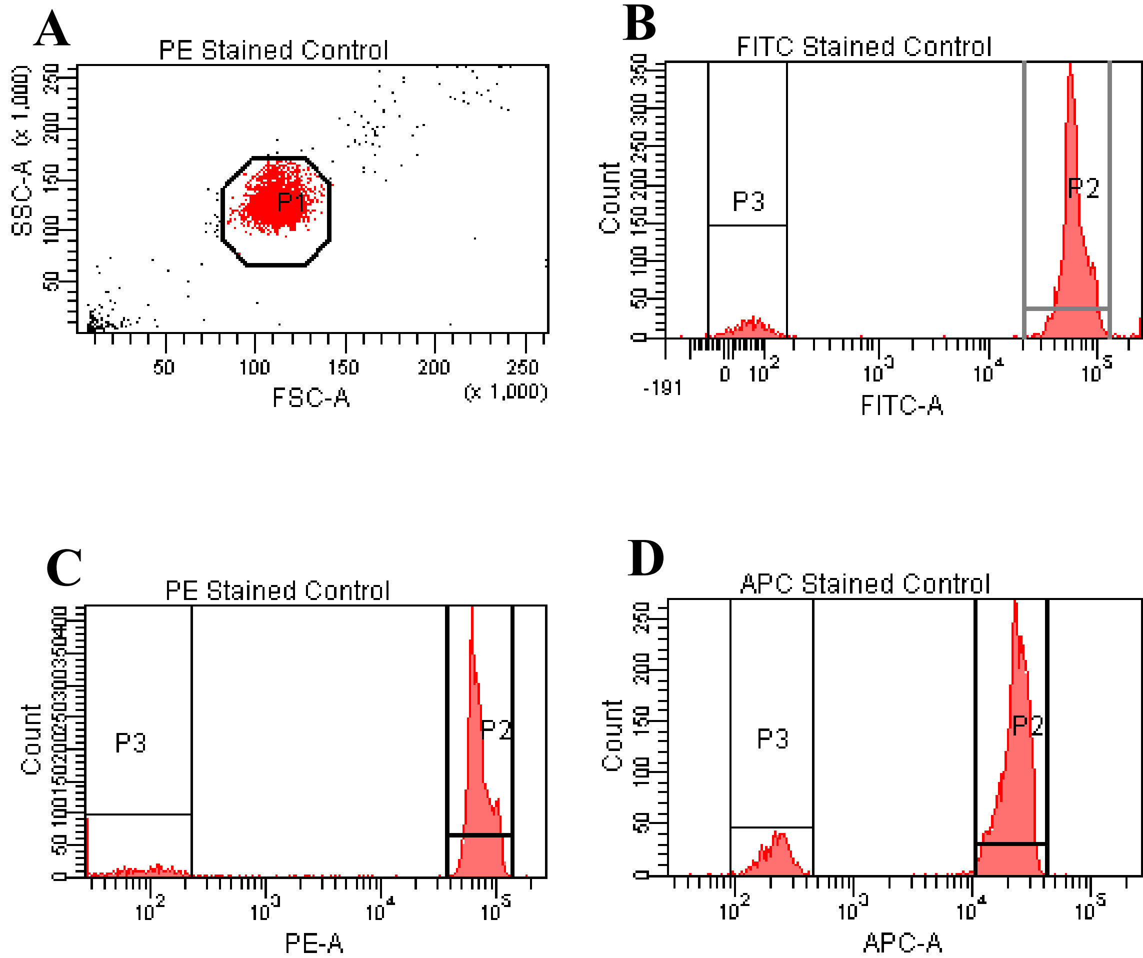


Figure 2.2: BD™ CompBeads staining by the fluorochrome-conjugated antibodies. BD™ CompBeads and BD™ CompBeads Negative Control were stained using anti-Col I/FITC-anti-mouse, PE-anti-CCR7 and APC-anti-CD45 antibodies. Beads were selected based on their FSC and SSC characteristics (**A**;P1), and the mean fluorescence intensity for each antibody was determined for the positive (P2) and negative (P3) populations (**B-D**).

2.2.4.2 Detection of fibrocytes (Collagen I+/CD45+ cells) and their CC chemokine receptor 7 or glucocorticoid receptor expression

Two types of anti-Col I antibodies have been used in this project because commercial flurochrome (FITC)-directly labelled mouse anti-human antibody was only available after 2013. For Col I+/CD45+ double staining or Col I+/CD45+/CCR7 triple staining, Col I was stained with unlabelled primary mouse anti-human Col I antibody (and isotype control antibody) and FITC-conjugated secondary rabbit anti-mouse antibody. For GR detection and cell proliferation assay, FITC-labelled mouse anti-human anti-Col I antibody (and its isotype control antibody) was used.

For Col I+/CD45+ or Col I+/CD45+/CCR7+ staining (Wang, Huang et al. 2008, Moeller, Gilpin et al. 2009, Wang, Huang et al. 2012, Weng, Chen et al. 2013), 5×10^5 NANT cells or adherent PBMC were permeabilised with 120 µL of 1X BD FACS™ Permeabilizing Solution 2 for 15 min, then washed with PBS (1 mL, 2700 rpm, 7 min, 4°C) and incubated with primary anti-Col I antibody (or isotope control antibody) for 30 min, followed by PBS washing (1mL, 2700 rpm, 7 min, 4°C) and incubation with FITC-conjugated secondary antibody for 30 min. After washing with PBS (1 mL, 2700 rpm, 7 min, 4 °C), cells were incubated with the APC-conjugated anti-CD45 antibody (or its isotype control antibody), with or without PE-conjugated anti-CCR7

antibody (or its isotype control antibody) for 30 min. Cells were washed with PBS (1 mL, 2700 rpm, 7 min, 4°C) and re-suspended in 500 μL of PBS for flow cytometric analysis (Figure 2.3B). Fibrocyte number was determined as: % Col I+/CD45+ cells × NANT cell number.

For detection of GR in fibrocytes, NANT cells were incubated with APC-conjugated anti-CD45 antibody (or its isotype control antibody) for 30 min, washed with PBS (1 mL, 2700 rpm, 7 min, 4 °C), permeabilised with 120 μL of 1X BD FACS™ Permeabilizing Solution 2 for 15 min, then washed with PBS (1 mL, 2700 rpm, 7 min, 4°C) and incubated with FITC-conjugated anti-Col I antibody and primary rabbit-anti-human unconjugated anti-GR antibody (or their isotype control antibodies) for 30 min, followed by PBS washing (1 mL, 2700 rpm, 7 min, 4°C) and incubation with PE-conjugated goat-anti-rabbit antibody for 30 min. Cells were washed with PBS (1 mL, 2700 rpm, 7 min, 4 °C) and re-suspended in 500 μL of PBS for flow cytometric analysis.

2.2.4.3 Detection of fibrocytes undergoing myofibroblastic differentiation (α-smooth muscle actin+ cells)

To detect the differentiating fibrocytes, 2×10^5 cells were permeabilised with 120 μL of 1X BD FACS™ Permeabilizing Solution 2, followed by PBS washing (1 mL, 2700

rpm, 7 min, 4°C) and staining with PE-conjugated anti-α-smooth muscle actin (α-SMA) antibodies (or its isotype control antibody) for 30 min. Cells were washed with PBS (1 mL, 2700 rpm, 7 min, 4 °C) and re-suspended in 500 μL of PBS for flow cytometric analysis (Figure 2.3C). Differentiating fibrocyte number were determined as: % α-SMA+ cells × NANT cell number.

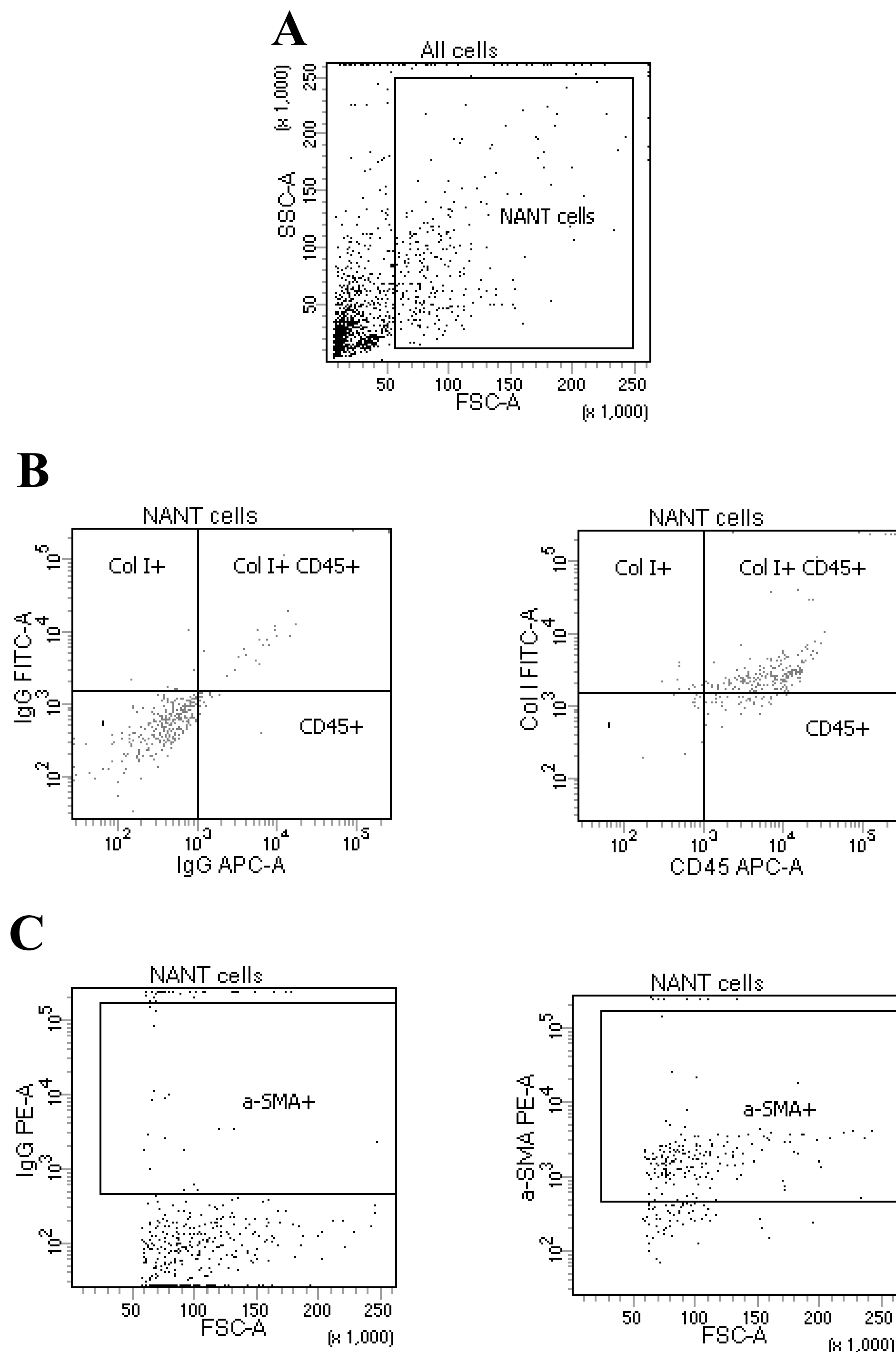


Figure 2.3: Detection of fibrocytes and differentiating fibrocytes. Representative flow cytometry scatter plots from one experiment are shown. NANT cells from one healthy subject were cultured for 7 days in FBS-containing medium. (**A**) NANT cells were gated on forward and side scatter plots (Wang, Huang et al. 2008). (**B**) NANT cells were stained with primary Col I antibody/FITC-conjugated secondary and APC-conjugated anti-CD45 antibody (or isotype control antibodies). Col I+/CD45+ cells were identified as fibrocytes. (**C**) NANT cells were stained with PE-conjugated α–SMA antibody (or isotype control antibody). α-SMA+ cells were identified as differentiating fibrocytes.

2.2.4.4 Cell apoptosis assay

Apoptosis in NANT cells were evaluated by FITC Annexin V/Dead cell apoptosis kit according to manufacturer's instruction. In apoptosis, phosphatidylserine is translocated from the inner cytoplasmic surface of the cell membrane to the outer leaflet where it is recognised by annexin V. Propidium iodide binds to nucleic acids and is only permeating the cell membranes of dead cells. Briefly, $1x10^5$ NANT cells were harvested, washed in cold PBS (1 mL, 2700 rpm, 7 min, 4°C) and pelleted. An unstained negative control was used. Cells were re-suspended in 1X annexin-binding buffer (10^5 cells in 100 μL) in the presence of FITC-annexin V (5 μL) and propidium iodide (0.1 μg) for 15 min. The live (annexin V^-/propidium $iodide^-$), early apoptotic (annexin V^+/propidium $iodide^-$) and late apoptotic (annexin V^+/propidium $iodide^+$) NANT cells were then determined using the FITC channel and PE channels of the flow cytometer.

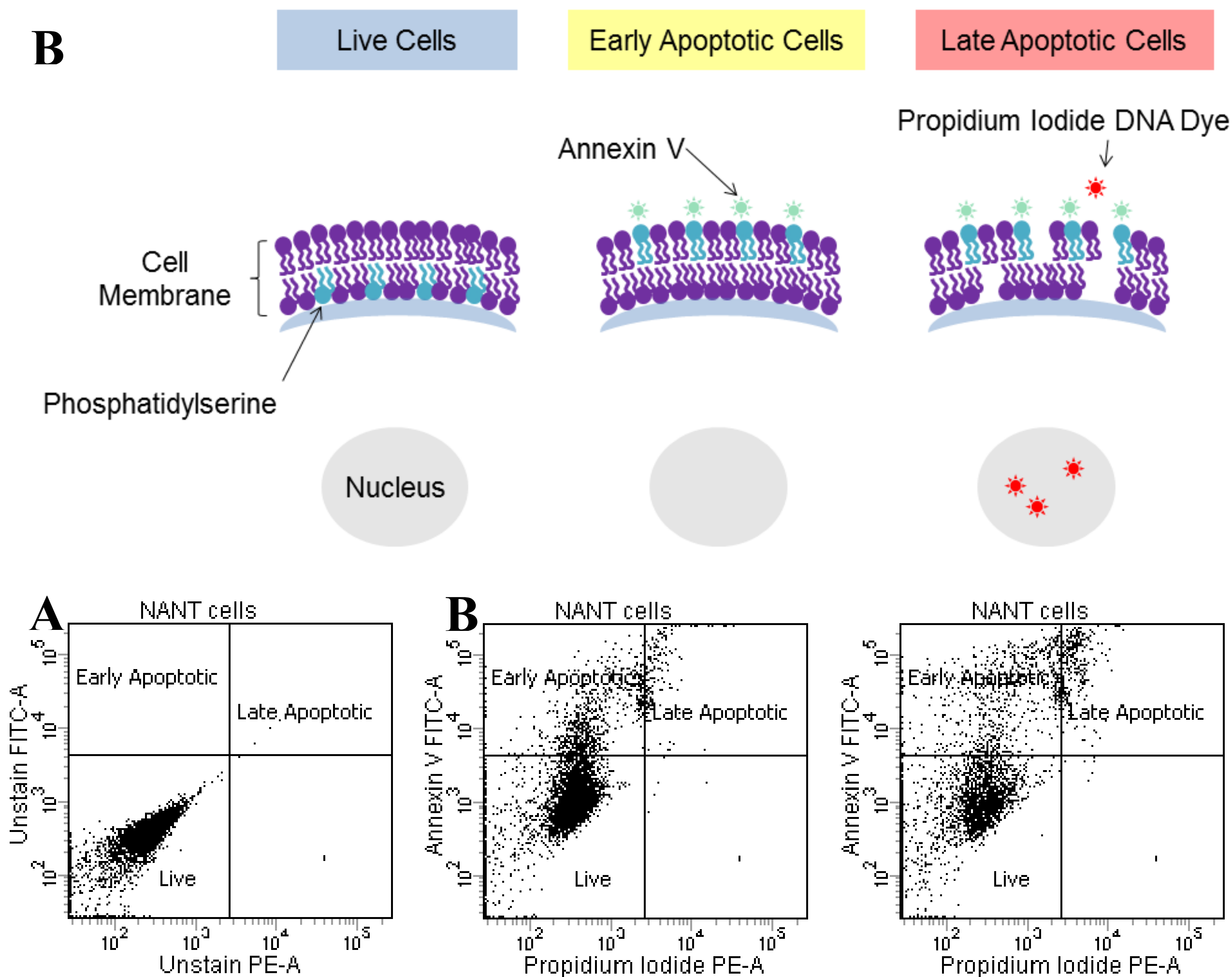


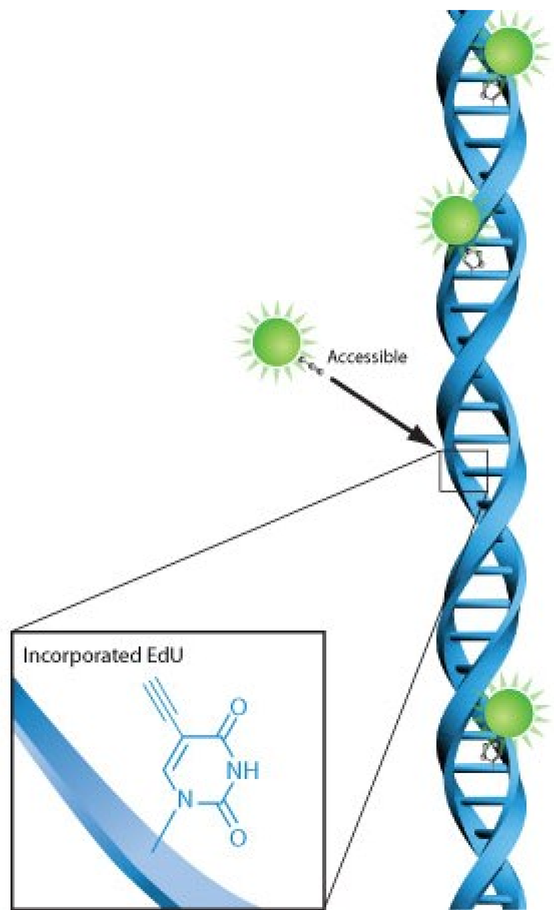
Figure 2.4: Cell apoptosis assay. (**A**) Phosphatidylserine is translocated from inner cytoplasmic surface of apoptotic cell membrane to the outer leaflet for recognition by annexin V. Propidium iodide binds to nuclear acid and is only permeant in the late apoptotic cells. An unstained sample is required to set negative thresholds (**B**). Early apoptosis (annexin V^+/propidium iodide$^-$) and late apoptosis (annexin V^+/propidium iodide$^+$) was determined in freshly isolated NANT cells (**C**) and 3-day cultured NANT cells (**D**) from a healthy subject using flow cytometry.

2.2.4.5 Cell proliferation assay

The Click-iT® EdU Flow Cytometry Assay Kit is an alternative to the bromodeoxyuridine (BrdU) assay which detects the percentage of S-phase cells in the population. EdU is a nucleoside analogue of thymidine with alkyne in its ethynyl moiety. EdU can be determined by a click reaction, a copper catalyzed covalent reaction between an alkyne and an azide, which is coupled to Pacific Blue™ in this kit (Figure 2.5 A). As a result, the EdU incorporated in synthesizing DNA during cell proliferation can be detected by flow cytometry.

The identification of proliferating fibrocytes derived from NANT cells was achieved by staining CD45, Col I and EdU according to the manufacturer's instructions. Briefly, $1x10^6$ NANT cells were incubated with EdU (10 μM) in FBS-containing medium for 1 hr. Cells were then harvested, washed with PBS (1mL, 2700 rpm, 7 min, 4°C), pelleted then stained with APC-conjugated mouse anti-human CD45 (or its isotype control antibody) for 30 min. Cells were then washed with PBS (1 mL, 2700 rpm, 7 min, 4°C), pelleted and fixed with 100 μL of Click-iT® fixative (4% paraformaldehyde) for 15 min at room temperature. Cell were then washed (1 mL, 2700 rpm, 7 min, 4°C), pelleted again and permeabilised with 100 μL of 1X Click-iT® saponin-based permeabilization and washing reagent for 15 min, then

mixed with 0.5 mL of Click-iT® reaction cocktail containing Copper (II) sulphate, Pacific Blue™ dye azide and reaction buffer additive for 30 min at room temperature. Cell were then washed (1 mL, 2700 rpm, 7 min, 4°C), pelleted, permeabilised with 120 μL of 1X BD FACS Permeabilizing Solution 2, washed (1 mL, 2700 rpm, 7 min, 4°C), and stained with FITC-conjugated mouse anti-human Col I antibody (or isotype control antibody). The proportion of Col I+/CD45+/EdU+ cells within Col I+/CD45+ cells (% Col I+/CD45+/EdU+ cells) was determined by flow cytometry (Figure 2.5B-D).



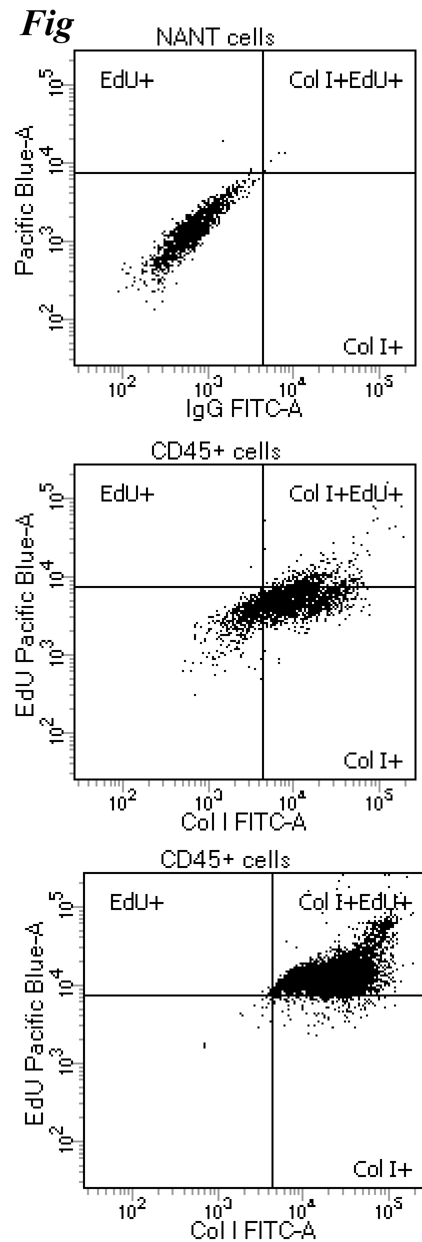


Figure 2.5: Cell proliferation assay. NANT cells were incubated with EdU (10μM) for 1 hour prior to staining with APC-conjugated anti-CD45 antibody, Click-iT® EdU Flow Cytometry Assay Kit (coupled to Pacific Blue™ dye) and FITC-conjugated anti-Col I antibody. A click reaction (copper catalyzed reaction) occurs between the azide coupled to Pacific Blue™ dye and alkyne on EdU incorporated into synthesizing DNA in proliferating cells (**A**; modified from http://jablonskidiagram.com/illust.html). NANT cells stained with isotype control antibodies were used as a negative control (**B**). Proliferating fibrocytes (Col I+/CD45+/EdU+ cells) were identified by flow cytometry immediately after isol\ated from peripheral blood (**C**) or after 7 days in culture (**D**).

2.2.5 Determination of messenger ribonucleic acid expression

2.2.5.1 Ribonucleic acid extraction

NANT cells were cultured in 6-well tissue culture plates at a density of $1x10^6$ cells/well in FBS-containing medium. After treatment as indicated, cells were recovered by aspiration, flushing and scraping, followed by washing with PBS (1 mL, 2700 rpm, 7 min, 4°C) and centrifugation. Cells were then lysed by resuspending in 350 µL of RLT buffer containing 1% of β-mercaptoethanol for 5 min at room temperature. The lysates were stored at -80 °C until RNA was extracted.

Total RNA was isolated from NANT cells by using the RNeasy Mini Kit according to the protocol recommended by the manufacturer. Briefly, cell lysates were homogenised by centrifugation through QIAshredder spin columns at 12,000 rpm for 2 min, mixed with an equal volume (350 µL) of 70% ethanol, transferred to RNeasy Mini spin column and centrifuged at 10,000 rpm for 15 s. The RNA retained on the silica-based membrane was then washed using 350 µL of buffer RW1 to remove contaminants, incubated with a DNase enzyme for 15 min at room temperature to remove DNA contamination. The columns were finally washed once with 350µL buffer RW1 and twice with 500 µL buffer RPE. The extracted RNA product was finally eluted from the column by applying 30 µL of H_2O and

centrifuging at 10,000 rpm for 1 min, and quantified using a NANODrop 1000 spectrophotometer. A ratio of absorbance at 260 nm over the absorbance at 280 nm (A260/A280) between 1.8 and 2.1 was indicative of highly purified RNA. The concentration of RNA was calculated using the following equation:

RNA concentration (µg/µL)= (A_{260} × 40 µg/mL × dilution factor)/1000

2.2.5.2 Reverse transcriptase PCR

To denature the RNA strands, approximately 0.25-0.5 µg of diluted RNA product in a final volume of 10 µL was incubated for 5 min at 70 °C on the heating block of a G-storm thermal cycler. The denatured RNA was then mixed with 10 µL of reverse-transcription reaction mix which contained dNTPs (2 mM), random primers (25ng/mL), recombinant RNasin ribonuclease inhibitor (40 U), AMV reverse transcriptase (10U) and 1X AMV reverse transcriptase reaction buffer. The mixture was returned to G-storm thermal cycler, incubated at 42 °C for 60 min followed by incubation at 90 °C for 4 min to inactivate the reverse transcriptase enzyme. The final complementary DNA (cDNA) product was then diluted in 20 µL of nuclease-free H_2O.

2.2.5.3 Real-time PCR

2.5 µL of cDNA was added to capillary tubes containing 7.5 µL of reaction mixture, which consisted of SYBR Green PCR master Mix Reagent (containing HotStarTaq DNA polymerase, dNTPs and SYBR Green I dye) and gene specific primers. The PCR reaction was carried out in the Roter Gene 3000 real-time cycler, where the cycling conditions were 15 min at 95 °C (enzyme activation) followed by 35-60 cycles of 20 s at 94 °C (denaturation step), 20 s at either 60 °C (18S and Bim) or 55 °C (Col I, α-SMA, CD45, GILZ) (annealing step) and 20 s at 72 °C (extension step).

During the extension step, DNA-bound SYBR Green was excited at 470 nm and fluorescence emission was detected at 510 nm. The data were analysed by Rotor-Gene 6000 Series Software (version 1.7). mRNA expression was quantified and normalised to 18S ribosomal RNA expression by the ΔΔCt method using the equation $2^{-\Delta\Delta Ct}$. ΔCt for each sample is the difference between the threshold cycle of the target gene and the threshold cycle of 18S [ΔCt = Ct (target) - Ct (18S)]. ΔΔCt is the difference between the average ΔCt of a treated sample and the average ΔCt of the untreated control [Average ΔCt (treatment) - Average ΔCt (untreated)]. Melting curve analysis was carried out to ensure the presence of one specific PCR product.

2.2.6 Statistical analysis

Data are presented as median with interquartile range. Statistical analysis was carried out using the GraphPad Prism v.5 software package (GraphPad Prism Software Inc, San Diego, CA, USA). The Wilcoxon matched pairs test is used to compare two related samples. For ≥ two treatments, results were analysed using the Friedman test for intra-group comparisons, followed by Dunn's post-hoc test to determine differences between the control group and each treatment group. The differences between two disease groups were determined by Mann-Whitney test. The differences amongst ≥ three disease groups were determined by Kruskal-Wallis test, followed by Dunn's post-hoc test. Correlations between parameters were determined by Spearman's rank correlation. For comparisons, a p value < 0.05 was considered as statistically significant.

Chapter 3: Proliferation, myofibroblastic differentiation and CC chemokine receptor 7 expression of fibrocytes from severe asthma

3.1 Background

Airway remodelling is a key factor of asthma pathogenesis and involves epithelial detachment, goblet cell and submucosal gland proliferation, increased airway smooth muscle (ASM) mass and subepithelial fibrosis (Davies, Wicks et al. 2003). ASM layer thickening and subepithelial fibrosis in asthma develop, at least partly, as a result of airway smooth muscle cell (ASMC) hyperplasia and hypertrophy (Woodruff, Dolganov et al. 2004, James, Elliot et al. 2012), and an increased presence of myofibroblasts (Brewster, Howarth et al. 1990) in the airway wall. The airways of patients with severe asthma show more prominent subepithelial fibrosis and ASM thickening compared to the airways of patients with non-severe asthma. This suggests that these patients show more profound airway remodelling despite taking high doses of inhaled, and often systemic corticosteroids (CS) (Levi-Montalcini 1998, Benayoun, Druilhe et al. 2003, Bumbacea, Campbell et al. 2004). A better understanding of the mechanisms driving airway remodelling in severe asthma is thus crucial in order to improve current asthma treatments.

Fibrocytes are thought to play a key role in the development of asthmatic airway remodelling, as they migrate from the circulation to the lung in response to inflammation, and home to the airway wall where they differentiate into myofibroblasts (Gomperts and Strieter 2007). The CC chemokine receptor (CCR) 7 is an important mediator of fibrocyte migration (Gomperts and Strieter 2007). CC chemokine ligand (CCL) 19/macrophage inflammatory protein-3-β (MIP-3-β), a ligand of CCR7, is increased in the airways of patients with asthma (Kaur, Saunders et al. 2006). Moreover, increased recruitment of fibrocytes to the ASM compartment has been shown in patients with asthma, whilst the number of fibrocytes in the bronchial biopsies of mild asthmatic patients correlates with the extent of subepithelial fibrosis (Nihlberg, Larsen et al. 2006, Saunders, Siddiqui et al. 2009). Intriguingly, fibrocytes isolated from patients with asthma, demonstrating chronic airflow obstruction, showed a greater capacity to differentiate into myofibroblasts *in vitro,* in response to treatment with the patient's own serum (Wang, Huang et al. 2008). Therefore, the increased recruitment of fibrocytes to the ASM compartment and their differentiation into myofibroblasts may contribute to asthmatic airway remodelling and airflow obstruction.

A recent study by Saunders *et al.* demonstrates increased localisation of fibrocytes in the ASM compartment and the lamina propria of patients with severe refractory asthma, suggesting an important role for these cells in the development of airway remodelling in these patients (Saunders, Siddiqui et al. 2009). However, the mechanism leading to the accumulation of fibrocytes in the airways of these patients is unclear and, therefore, needs further investigation.

The isolation of fibrocytes from peripheral blood is extensively described in the literature. There are three main methods. The first and most widely used method entails generation of fibrocytes by culture of the adherent fraction of peripheral blood mononuclear cells (PBMC) for a period of 5 days to 6 weeks (Bucala, Spiegel et al. 1994). The second method involves the differentiation of fibrocytes from cultured $CD14^{+}$ monocytes (Yang, Scott et al. 2002). Growth of fibrocytes using the above two methods has been reported both under foetal bovine serum (FBS)-free and FBS-dependent conditions. The above two methods primarily examine fibrocytes derived from the one of a number of cell types that can differentiate from monocyte fraction in PBMC, including dendritic cells, macrophages and osteoclasts (Geissmann, Auffray et al. 2008), and the CD14+ monocyte method avoids the interference of T cells before the initial replacement of media. The third method involves the culture of

the non-adherent fraction of PBMC, after depletion of T cells, in the presence of serum for 7 days (Wang, Huang et al. 2008).

In this chapter, I describe the optimisation of the isolation and culture of fibrocytes from the non-adherent non-T (NANT) cell fraction of PBMC and compared the number of circulating fibrocytes (collagen I (Col I)+/CD45+ cells) and differentiating fibrocytes (α–smooth muscle actin (SMA)+ cells) as well as the expression of CCR7 in fibrocytes from healthy subjects and patients with non-severe or severe asthma. In addition, I optimised the isolation of fibrocytes from the adherent fraction of PBMC from a small number of patients in order to compare the responses of fibrocytes isolated from the different PBMC fractions.

3.2 Results

3.2.1 Isolation of fibrocytes from non-adherent non-T cells

3.2.1.1 Optimisation of method for T cell depletion.

In order to determine the most efficient method to deplete T cells from the NANT cell fraction, I compared two widely used methods. The first method involved incubation of cells with sheep red blood cells (RBC), leading to the formation of E-rosette complexes with T cells which were then removed by Ficoll centrifugation (Wang, Huang et al. 2008). The second method involved the use of anti-CD3 antibodies conjugated to magnetic beads (CD3 MicroBeads). In order to compare the efficiency of the two methods, I determined the total number of NANT cells and the percentage of CD3-positive cells by flow cytometry, after T cell depletion. Before T cell depletion, the percentage of CD3+ T cells in non-adherent PBMC was 75.5% (Figure 3.1B). The percentage of residual CD3+ cells in NANT cells was 6.6% after using the sheep RBC method (Figure 3.1C) and 3.1% after using the CD3 MicroBeads method (Figure 3.1D). The CD3 MicroBeads method was more reproducible and resulted in less NANT cell loss compared to the sheep RBC method (data not shown). Therefore, the CD3 MicroBeads method was used in all subsequent experiments.

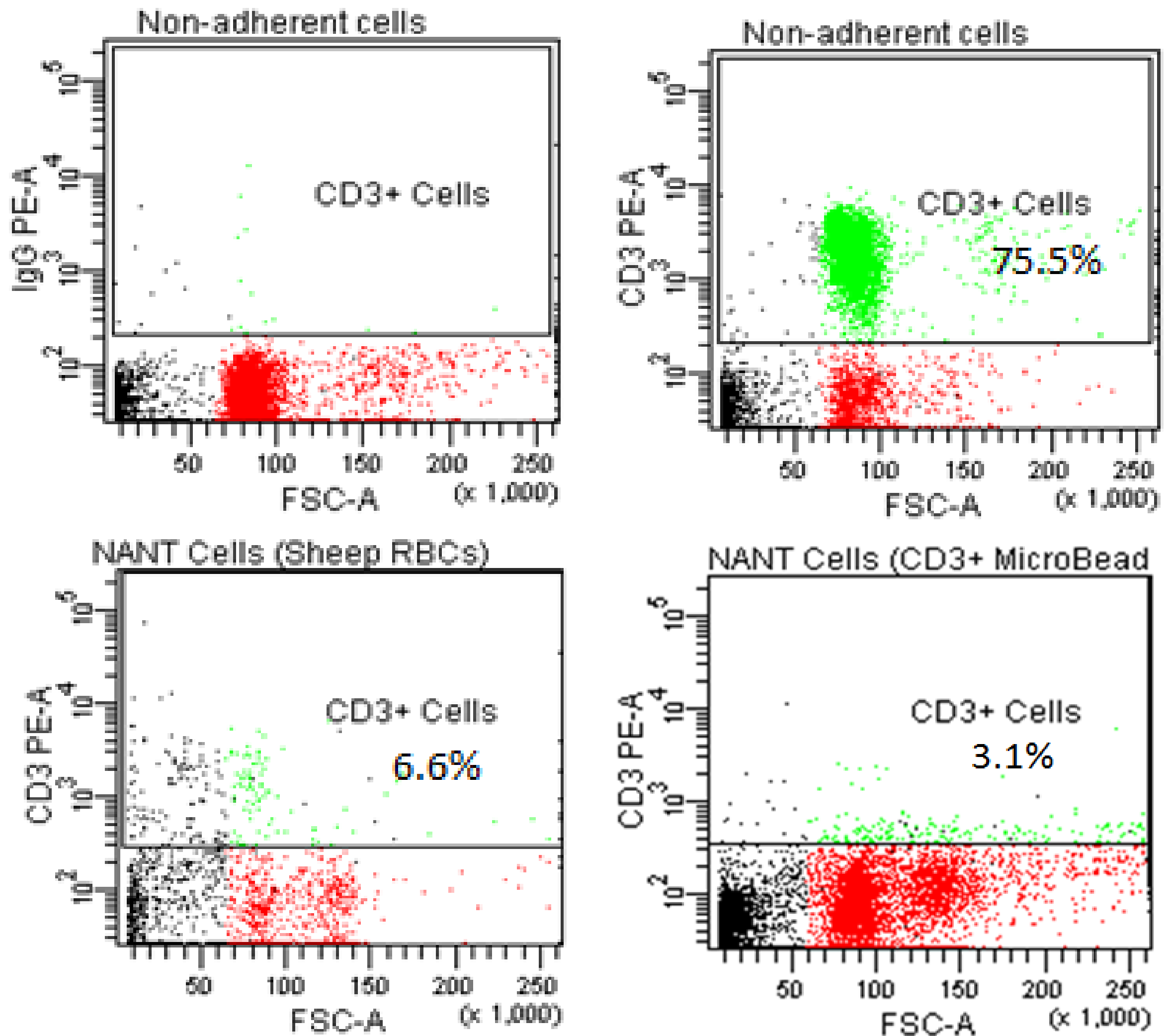


Figure 3.1: Comparison of the efficiency of T cell depletion methods. The non-adherent fraction of PBMC was depleted of T cells using sheep RBC or CD3 MicroBeads. CD3-positive cells were identified by staining with a phycoerythrin (PE)-conjugated anti-CD3 antibody followed by flow cytometric analysis. (**A**) The negative threshold for CD3 expression was set by incubating cells with a PE-labelled isotype control. (**B**-**D**) The CD3-positive population was identified in all viable cells before T cell depletion (B) and following T cell depletion using sheep RBC (**C**) and CD3 MicroBeads (**D**). Data are representative of one experiment.

3.2.2 Determination the number of fibrocytes and differentiating fibrocytes in cultured non-adherent non-T cells in the presence or absence of foetal bovine serum

Fibrocytes are known to differentiate from PBMC in culture both under FBS-dependent and FBS-free conditions. The resulting fibrocytes, however, show distinct phenotypes (Curnow, Fairclough et al. 2010). I therefore placed NANT cells (10^6 cells/well) for 3, 7 or 14 days in culture, and then determined the number of fibrocytes by staining for Col I and CD45 expression and differentiating fibrocytes by staining for α-SMA. I employed single α-SMA staining to identify differentiating fibrocytes, having previously determined that the majority of cells in the NANT cell population expressing α-SMA are fibrocytes. This was done by identifying the percentage of CD45+/Col I+/α-SMA+ cells in the NANT cells of a healthy subject after 3 days in culture (Figure 3.2). Fully differentiated fibrocytes were identified by light microscopy, as adherent, "spindle-shaped" cells (Figure 3.3E).

Under FBS-free conditions the percentage of fibrocytes and differentiating fibrocytes in cultured NANT cells increased in a time-dependent manner reaching ~20% after 7 days in culture (Figure 3.3A and C). However, the absolute numbers of fibrocytes and differentiating fibrocytes did not change significantly after culture

(Figure 3.3B and D). Furthermore, very few spindled-shaped cells were observed suggesting that fibrocytes may not fully differentiate into myofibroblasts under these conditions (Figure 3.3E).

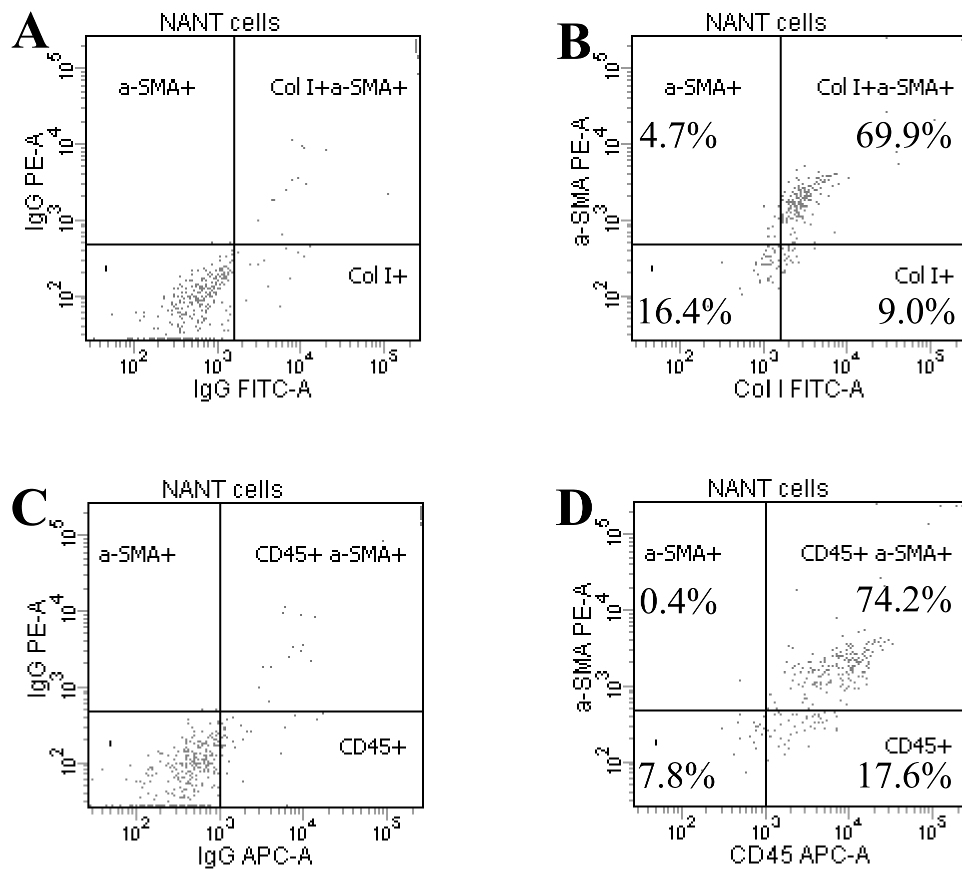


Figure 3.2: The expression of collagen I and CD45 on α–smooth muscle actin+ cells. NANT cells from one healthy subject were harvested after 3 days in culture and stained with antibodies for Col I, α-SMA and CD45 or their respective IgG isotype controls (**A** and **C**). The percentage of Col I+/α-SMA+ (**B**) and CD45+/ α-SMA+ cells (**D**) was determined by flow cytometry.

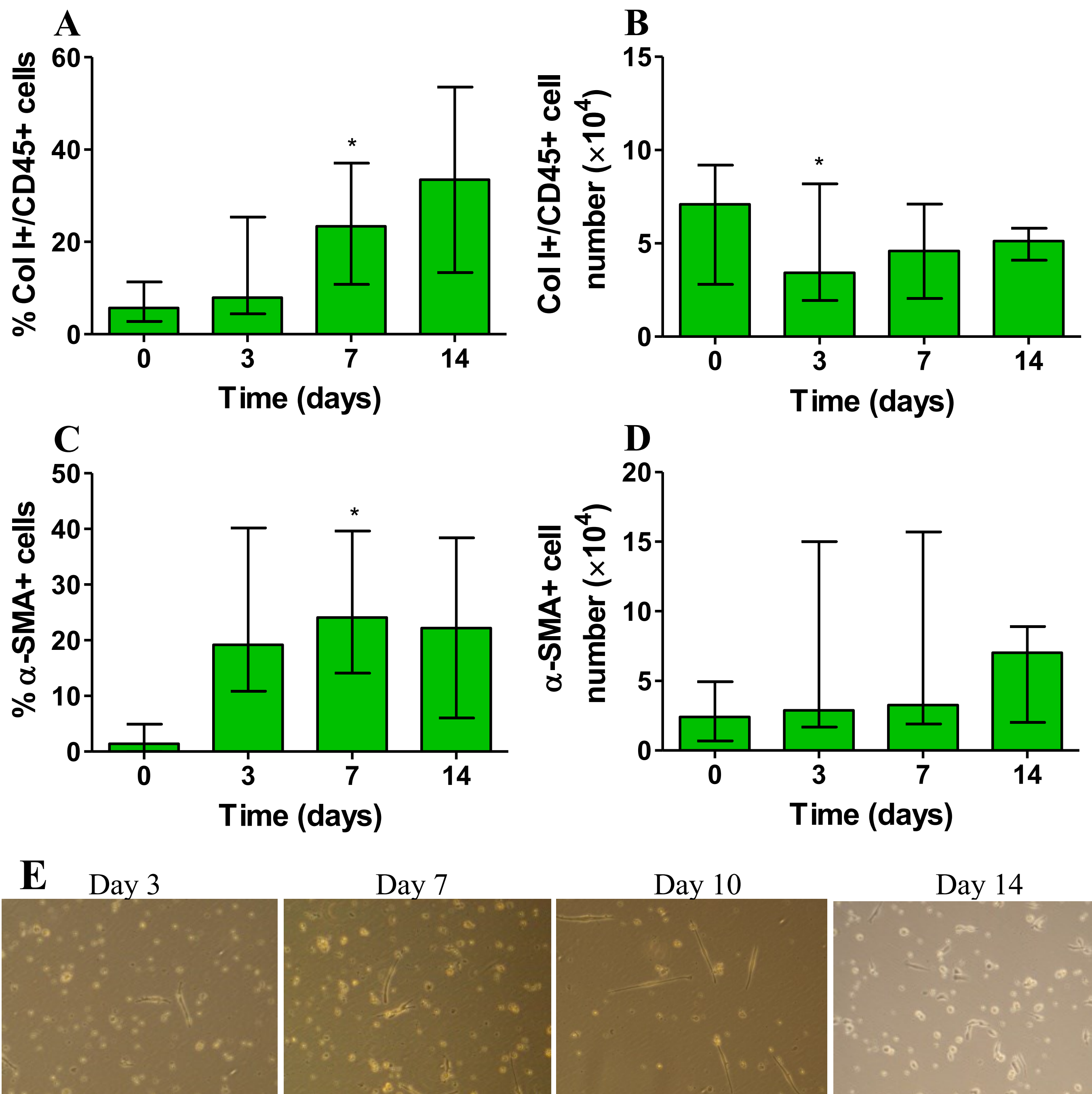


Figure 3.3: Number of fibrocytes and differentiating fibrocytes in non-adherent non-T cells cultured under foetal bovine serum-free conditions. NANT cells isolated from healthy subjects were placed in culture for a 14-day period in FBS-free medium. (**A-D**) Cultured NANT cells were then harvested and counted at 3, 7 and 14 days. The cells were then analysed by flow cytometry. Fibrocytes were identified by staining for Col I and CD45 (**A, B**). Fibrocyte differentiation was determined by staining for α-SMA (**C, D**). Bars represent median with interquartile range of 4-5 healthy subjects under FBS-free conditions. The percentage and number of cells at different time points were compared by Friedman test, followed by Dunn's post-hoc test. * $p < 0.05$ compared to day 0. Spindle-shaped cells were identified by light microscopy and photographs were taken after 3, 7, 10 and 14 days. Photographs are representative of one experiment (**E**).

Under FBS-containing conditions, the percentage of fibrocytes and differentiating fibrocytes in NANT cells of healthy subjects increased in a time-dependent manner reaching ~50% of the total cell population after 7 days in culture and declining 14 days post-culture (Figure 3.4A and C). As a result, the absolute number of fibrocytes and differentiating fibrocytes also increased and peaked 3 days post-culture (Figure 3.4 B and D). The increase in differentiating fibrocytes was accompanied by the appearance of adherent "spindle-shaped" cells after 7 days, peaking after 10 days in culture, suggesting that fibrocytes can fully differentiate into myofibroblast-like cells in the presence of FBS (Figure 3.4E). These data suggest that in the presence of FBS, cultured fibrocytes show prolonged survival and the ability to differentiate more readily into myofibroblasts. Thus, all subsequent experiments were carried out in the presence of FBS.

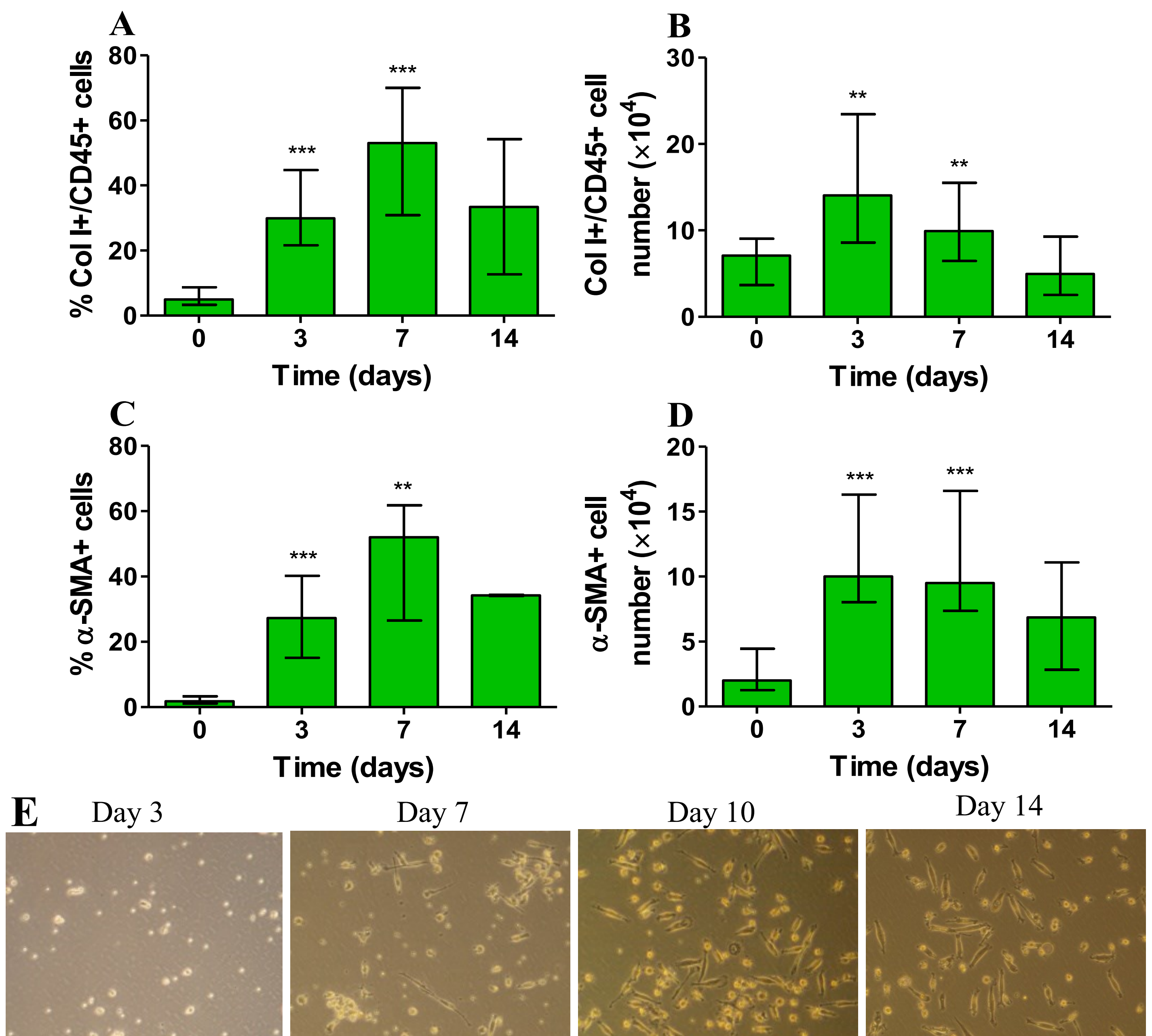


Figure 3.4: Number of fibrocytes and differentiating fibrocytes in non-adherent non-T cells cultured under foetal bovine-containing conditions. NANT cells isolated from healthy subjects were placed in culture for a 14 day period in FBS-containing medium. (**A-D**) Cultured NANT cells were then harvested and counted at 3, 7 and 14 days. The cells were then analysed by flow cytometry. Fibrocytes were identified by staining for CD45 and collagen I (**A, B**). Fibrocyte differentiation was determined by staining for α-SMA (**C, D**). Bars represent median with interquartile range of 4-17 healthy subjects under FBS-free conditions. The percentage and number of cells at different time points were compared by Friedman test, followed by Dunn's post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to day 0. Spindle-shaped cells were identified by light microscopy and photographs were taken after 3, 7, 10 and 14 days. Photographs are representative of one experiment (**E**).

In order to determine whether the observed increase in the percentage of fibrocytes in cultured NANT cells is as a result of fibrocyte proliferation or differentiation of NANT cells into fibrocytes, I determined the rate of deoxyribonucleic acid (DNA) synthesis by measuring 5-ethynyl-2'-deoxyuridine (EdU) incorporation in fibrocytes and the expression of Col I, CD45 and α-SMA messenger ribonucleic acid (mRNA) in NANT cells after culture for different times. The proportion of proliferating fibrocytes, identified as EdU+ fibrocytes (Col I+/CD45+/EdU+ cells) increased in a time-dependent manner peaking after 96 hours in culture (Figure 3.5A), indicating that fibrocytes can proliferate in culture. On the other hand, CD45 mRNA remained unchanged whilst Col I mRNA was reduced after 12 hr in culture suggesting that there is possibly no differentiation of other NANT cells into fibrocytes (Figure 3.5B). However, the expression of α-SMA mRNA was increased after 12-48 hr in culture, confirming my previous findings, showing myofibroblastic differentiation of fibrocytes in culture (Figure 3.5B).

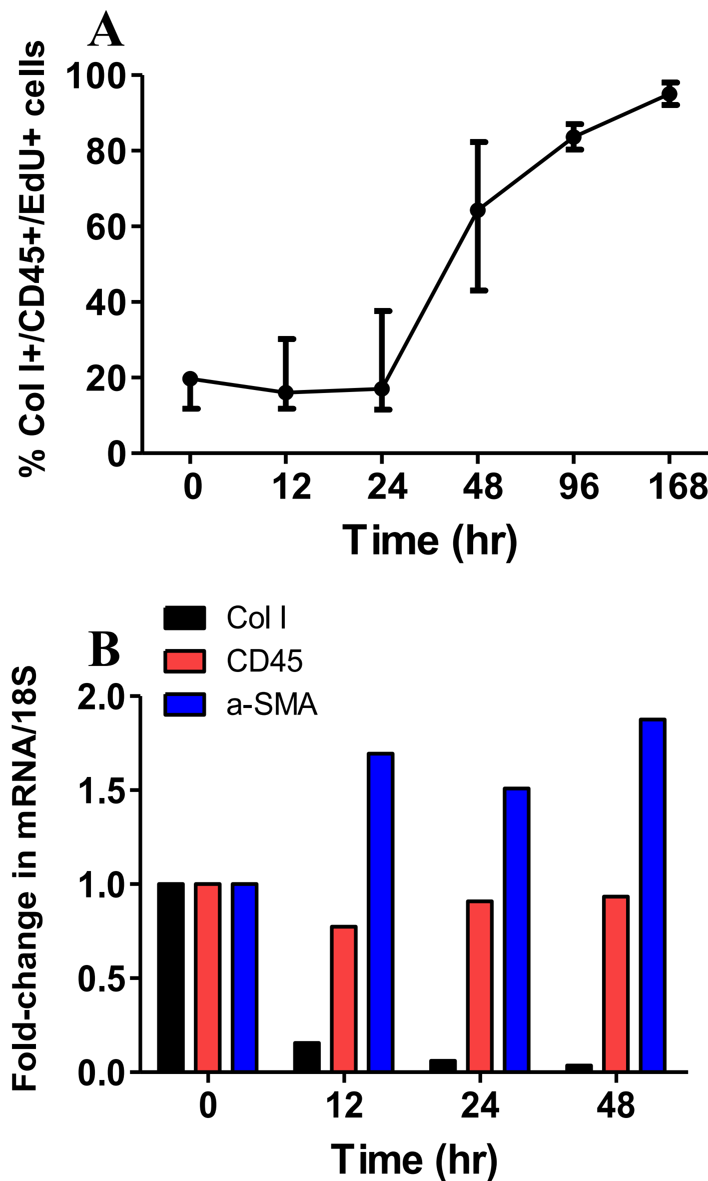


Figure 3.5: Percentage of proliferating fibrocytes and collagen I, CD45 and α-smooth muscle actin messenger ribonucleic acid expression in non-adherent non-T cells cultured in foetal bovine serum-containing medium. (**A**) NANT cells were placed in culture in the presence of FBS for 12-168 hr and then incubated with EdU for 1 hr. The percentage of EdU+ fibrocytes (Col I+/CD45+/EdU+ cells) within the NANT cell population was identified by flow cytometry. Symbols are median with bars representing interquartile range of 3 healthy subjects. (**B**) NANT cells were placed in culture in the presence of FBS for 12-48 hr and Col I, CD45 and α-SMA mRNA was determined by real-time PCR. Bars represent median of 2 healthy subjects.

3.2.3 Comparison of the number of circulating fibrocytes in non-adherent non-T cells from healthy subjects and patients with non-severe or severe asthma

Having optimised the conditions for isolation and culture of fibrocytes from NANT cells, I compared the number of fibrocytes in the NANT cells of healthy subjects and patients with non-severe or severe asthma immediately after isolation and also after culture.

3.2.3.1 Determination of circulating fibrocyte numbers in healthy subjects and patients with non-severe or severe asthma

NANT cells were isolated from the peripheral blood of healthy subjects and patients with non-severe or severe asthma, and the percentage of fibrocytes (Col I+/CD45+ cells) was determined by flow cytometry. There was no significant difference between the number of NANT cells per mL of blood from healthy subjects and patients with non-severe or severe asthma (Figure 3.6A). However, the NANT cells of patients with severe asthma contained a higher percentage of fibrocytes compared to that of healthy subjects ($p < 0.001$) and patients with non-severe asthma ($p < 0.01$; Figure 3.6B). These differences were thus reflected in the absolute number of fibrocytes per mL of blood, which was increased in patients with severe asthma compared to patients with

non-severe asthma ($p < 0.01$) and healthy subjects ($p < 0.001$), and also in patients with non-severe asthma compared to healthy subjects ($p < 0.05$; Figure 3.6C).

Furthermore, I studied the relationship between the number of fibrocytes and the lung function of the subjects in my cohort. The absolute counts of fibrocytes per mL of blood inversely correlated with the pre-bronchodilator forced expiratory volume in 1 s (FEV_1) of predicted value of the patients ($p < 0.01$, Figure 3.7A) and FEV_1/ forced vital capacity (FVC) ratio ($p < 0.01$, Figure 3.7B), suggesting that the number of circulating fibrocytes is associated with airway obstruction.

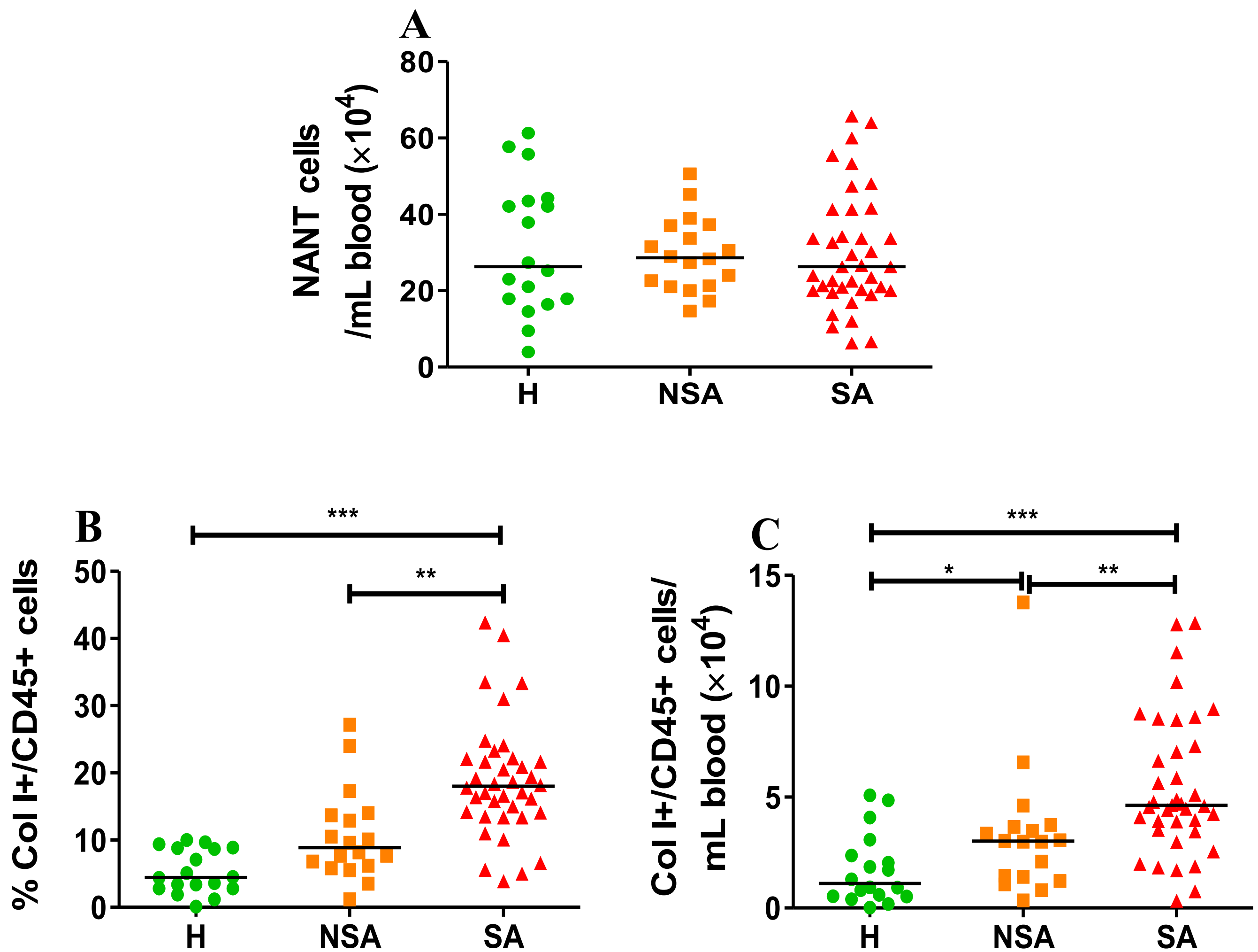


Figure 3.6: Number of circulating fibrocytes in freshly isolated non-adherent non-T cells from healthy subjects and patients with non-severe or severe asthma. NANT cells were isolated from peripheral blood of healthy subjects (H, n = 18) and patients with non-severe (NSA, n = 18) or severe asthma (SA, n = 38). The number of viable NANT cells was determined by Trypan blue staining and haemocytometer counting (**A**). The percentage of fibrocyte (Col I+/CD45+ cells) was determined by flow cytometry (**B**). The number of fibrocytes per mL of blood was deduced from the percentage of fibrocytes and the total number of NANT cells (**C**). Horizontal lines represent the median values for each group. The differences between disease groups were determined by Kruskal-Wallis test, followed by Dunn's post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

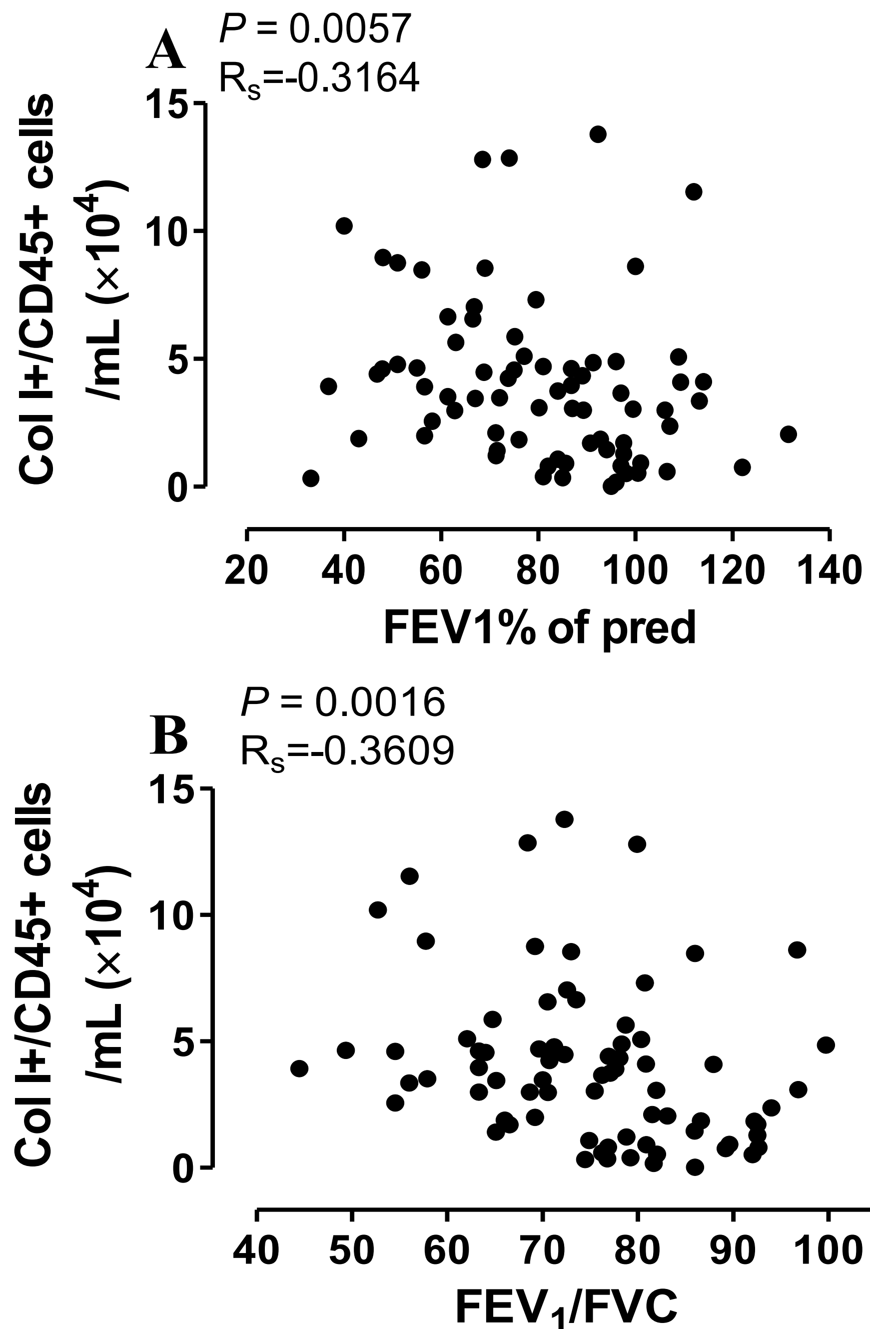


Figure 3.7: Relationship between circulating fibrocytes and lung function. NANT cells were freshly isolated from peripheral blood of healthy subjects (n = 18) and patients with non-severe (n = 18) or severe asthma (n = 38). The percentage of fibrocytes (Col I+/CD45+ cells) in NANT cells was determined by flow cytometry and the number of fibrocytes per mL of blood was deduced. The correlation of fibrocyte number with FEV_1 % predicted (**A**) and FEV_1/FVC (**B**) was determined using Spearman's rank correlation. R_s: Spearman's rank correlation.

3.2.3.2 Comparison of the number of fibrocytes and differentiating fibrocytes in non-adherent non-T cells from healthy subjects and patients with non-severe or severe asthma after culture

NANT cells from healthy subjects and patients with non-severe or severe asthma were cultured in FBS-containing culture medium. After 3, 7 and 14 days in culture, cells were collected and analysed for fibrocyte numbers by staining for Col I and CD45 expression and for myofibroblastic differentiation by staining for α-SMA.

In line with my findings in the healthy subjects, there was an increase in the number of fibrocytes in the NANT cells of patients with non-severe asthma peaking after 3 days in culture, returning back to baseline after 14 days in culture (Figure 3.8A). In contrast, although the number of fibrocytes in the NANT cells of patients with severe asthma were higher immediately after isolation (day 0) their number did not increase further after 3 days in culture, reducing after 7-14 days in culture (Figure 3.8A). There was no significant difference in the number of fibrocytes in cultured NANT cells across the three groups.

There was an increase in the number of differentiating fibrocytes in the NANT cells of patients with non-severe or severe asthma after 3 days in culture, reducing after 14 days in culture in agreement with my findings in NANT cells from healthy

subjects (Figure 3.8B). However, the number of differentiating fibrocytes in the NANT cells of patients with severe asthma after 3 days in culture was significantly higher compared to healthy subjects ($p < 0.001$) and patients with non-severe asthma ($p < 0.001$; Figure 3.8B).

Therefore, there are more differentiating fibrocytes suggesting that fibrocytes from patients with severe asthma may have a greater capacity to differentiate to myofibroblasts.

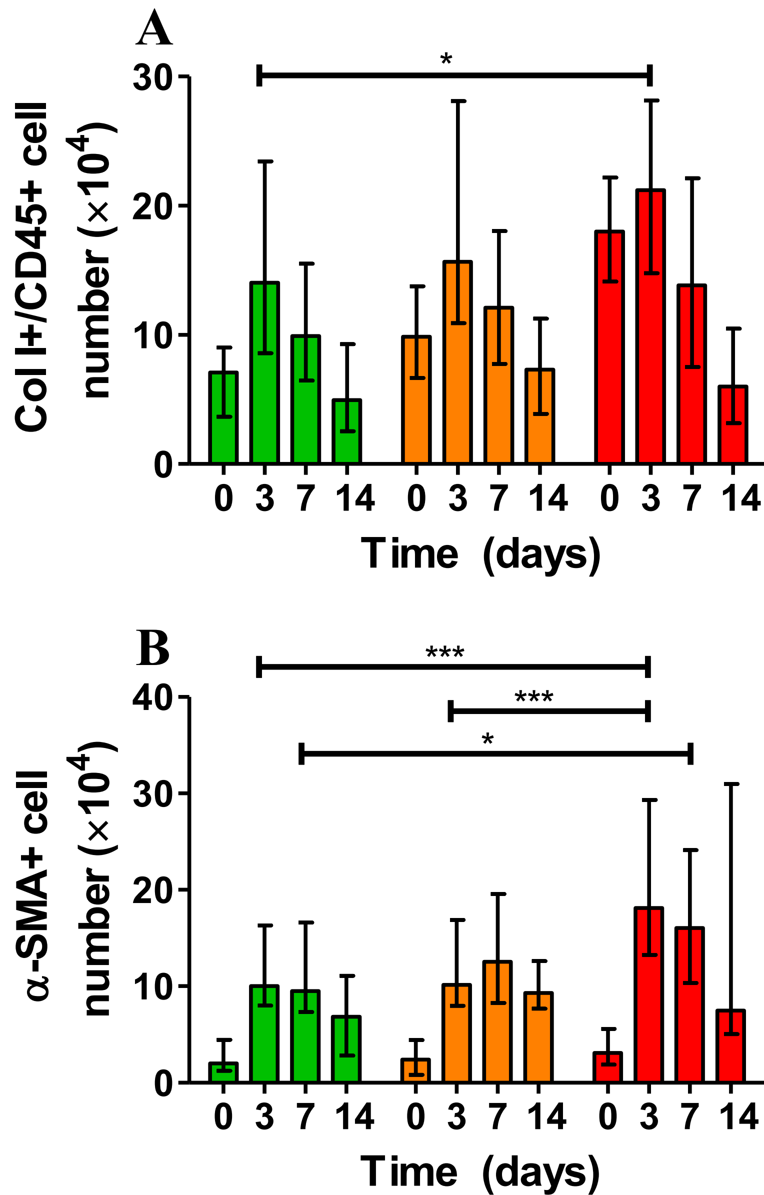


Figure 3.8: Number of fibrocytes and differentiating fibrocytes in cultured non-adherent non-T cells from healthy subjects and patients with non-severe or severe asthma. The number of fibrocytes (Col I+/CD45+ cells; **A**) and differentiated fibrocytes (α-SMA+ cells; **B**) were determined in NANT cells isolated from healthy subjects (■, n = 5-18) and patients with non-severe (■, n = 4-18) or severe asthma (■, n = 5-38) by flow cytometry, after 3-14 days in culture. Bars represent median with interquartile range under FBS-containing conditions. The numbers of cells at different time points in each disease group were compared by Friedman test, followed by Dunn's post-hoc test. The differences between disease groups were determined by Kruskal-Wallis test, followed by Dunn's post-hoc test. * $p < 0.05$, *** $p < 0.001$.

3.2.3.3 Comparison of CC chemokine receptor 7-expressing fibrocytes in non-adherent non-T cells from healthy subjects and patients with non-severe or severe asthma after culture

As CCR7 is an important mediator of fibrocyte migration to the airways (Phillips, Burdick et al. 2004, Sakai, Wada et al. 2006), I compared the expression of CCR7 in fibrocytes from healthy subjects and patients with non-severe or severe asthma. The intensity of CCR7 expression in CCR7+ fibrocytes was quantified by the CCR7 median fluorescence intensity (MFI) ratio (background fluorescence of isotype control was subtracted from the MFI of the sample). No significant difference was observed between healthy subjects and patients with non-severe or severe asthma in terms of the proportion of CCR7+ fibrocytes (% Col I+/CD45+/CCR7+ cells) and CCR7 MFI ratio immediately after isolation or after culture for 3 or 7 days (Figure 3.9A-B). Thus, it is unlikely that the increased migration of fibrocytes to the airway wall of patients with severe asthma is due to increased expression of CCR7.

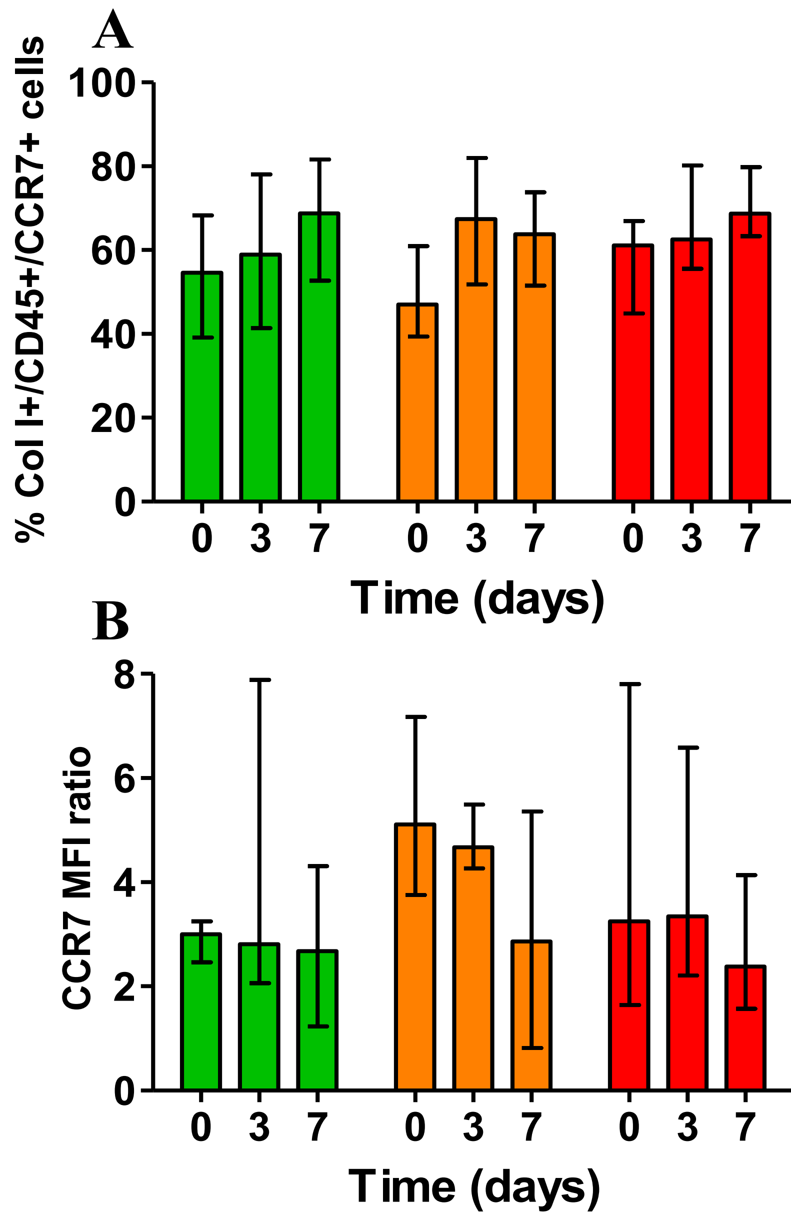


Figure 3.9: CC chemokine receptor 7 expression in non-adherent non-T cells-derived fibrocytes from healthy subjects and patients with non-severe or severe asthma. The percentage of CCR7+ fibrocytes (Col I+/CD45+/CCR7+ cells) **(A)** and the corresponding MFI ratio **(B)** were determined in NANT cells from healthy subjects (■, n = 7) and patients with non-severe (■, n = 10) or severe asthma (■, n = 12) by flow cytometry, immediately after isolation or after 3-7 days in culture. Bars represent median with interquartile range. The expression of CCR7 at different time points was compared by Friedman test. The expression of CCR7 in cells from different disease groups were determined by Kruskal-Wallis test.

3.2.4 Isolation of fibrocytes from the adherent fraction of peripheral blood mononuclear cells

To obtain a more complete picture of the responses of fibrocytes from patients with severe asthma, I also isolated fibrocytes from adherent PBMC and compared their response to fibrocytes isolated from NANT cells. There are a number of different protocols for isolating fibrocytes from adherent PBMC in the literature, differing in the length of incubation, the composition of the cell culture medium and the concentration of fibronectin used to pre-coat the cell culture plates. I therefore determined the optimum conditions for isolating fibrocytes from adherent PBMC.

3.2.4.1 Determination of optimum incubation time

PBMC (4×10^6 cells/well) from healthy subjects were seeded in 6-well tissue culture plates (growth surface area 9.6cm^2/well) pre-coated with fibronectin (25 μg/mL, or 2.6 μg/cm^2). FBS-containing medium was replaced every 3 days. The number of adherent PBMC and percentage of fibrocytes and differentiating fibrocytes was determined after 3, 6, 10 or 14 days.

There was a time-dependent increase in the percentage of fibrocytes in the adherent PBMC population which peaked after 10 days in culture, reaching 73% of

the total number of cells (day 3, $p < 0.05$; day 6-14, $p < 0.001$; Figure 3.10A). Due to the reduction in total adherent PBMC number related to media changing, the absolute number of fibrocytes (day 0, 14.1×10^4) was not significantly different after 3-6 days in culture (day 3, 14.9×10^4, $p = 0.8125$; day 6, 15.8×10^4, $p = 0.6125$), whilst it decreased after 10-14 days (day 10, 10.6×10^4, $p = 0.3125$; day 14, 7.4×10^4, $p = 0.1875$; Figure 3.10B). At the same time, there was an increase in the percentage of differentiating fibrocytes which was maximal after 10 days (67%, $p < 0.001$; Figure 3.10C). The absolute number of differentiating fibrocytes was also increased, peaking after 6-10 days in culture ($\sim 9.5 \times 10^4$, $p < 0.05$, Figure 3.10D). The increase in differentiating fibrocytes was accompanied by an accumulation of spindle-shaped, myofibroblast-like cells which also peaked after 6 days in culture (Figure 3.10G-J). In contrast, the percentage of CCR7-positive fibrocytes in the adherent PBMC did not change over time (Figure 3.10E-F).

Therefore, there is an increase in the percentage of fibrocytes and differentiating fibrocytes in the adherent fraction of PBMC after culture, which is maximal after 6 days. Thus, for all subsequent experiments the effects of the different treatment on fibrocytes in adherent PBMC were studied over a 6-day period.

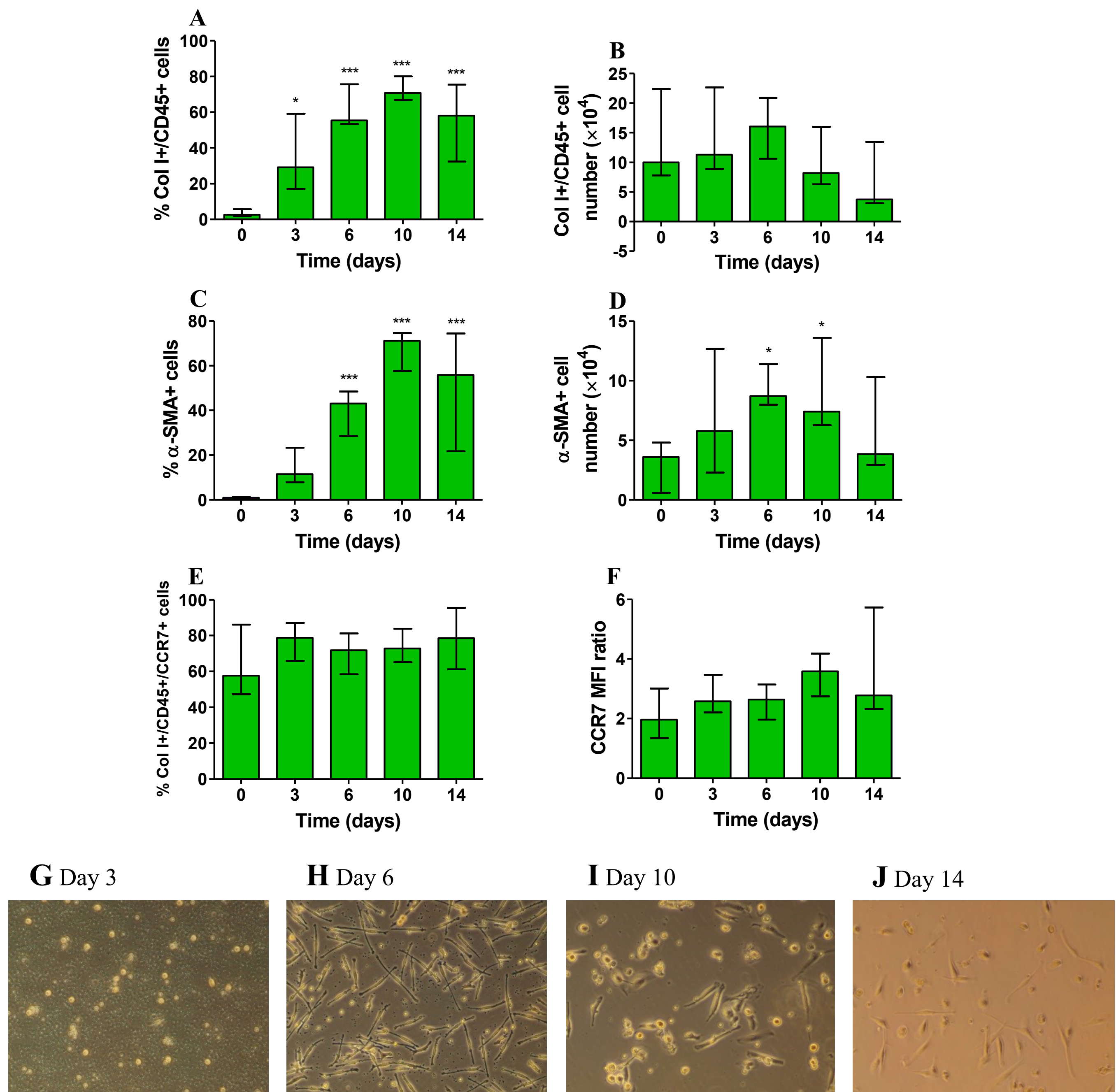


Figure 3.10: The number, myofibroblastic differentiation and CC chemokine receptor 7 expression of fibrocytes derived from adherent peripheral blood mononuclear cells. PBMC from 5 healthy subjects were seeded on plates pre-coated with fibronectin (25µg/mL). FBS-containing medium were changed every 3 days. Adherent PBMC were harvested after 3, 6, 10 and 14 days in culture. Fibrocytes (Col I+/CD45+ cells) (**A**-**B**) and differentiating fibrocytes (α-SMA+ cells) (**C**-**D**) as well as the percentage of CCR7+ fibrocytes (% Col I+/CD45+/CCR7+ cells) (**E**) and CCR7 MFI ratio (**F**) were determined by flow cytometry. Bars represent median with interquartile range. The percentage, number and CCR7 expression of cells at different time points were compared by Friedman test, followed by Dunn's post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to day 0. Spindle-shaped cells were identified by light microscopy and photographs were taken after 3, 6, 10 and 14 days (**G**-**J**). Photographs are representative of one experiment.

3.2.4.2 Determination the number of fibrocytes and differentiating fibrocytes in cultured adherent peripheral blood mononuclear cells in the presence or absence of foetal bovine serum

PBMC from healthy subjects suspended in either FBS-containing medium or FBS-free medium were seeded in 6-well tissue culture plates pre-coated with fibronectin (25 μg/mL). Non-adherent cells were removed and media were replenished on day 3. Adherent cells were harvested on day 6. Fibrocytes and differentiating fibrocytes were identified by flow cytometry.

There were more adherent PBMC ($p < 0.05$), as well as fibrocytes ($p < 0.001$) and differentiating fibrocytes ($p < 0.05$), in the presence of FBS (Figure 3.11A-C) Under microscopy, more spindle-shaped cells were observed in FBS-containing media (Figure 3.11D-E).

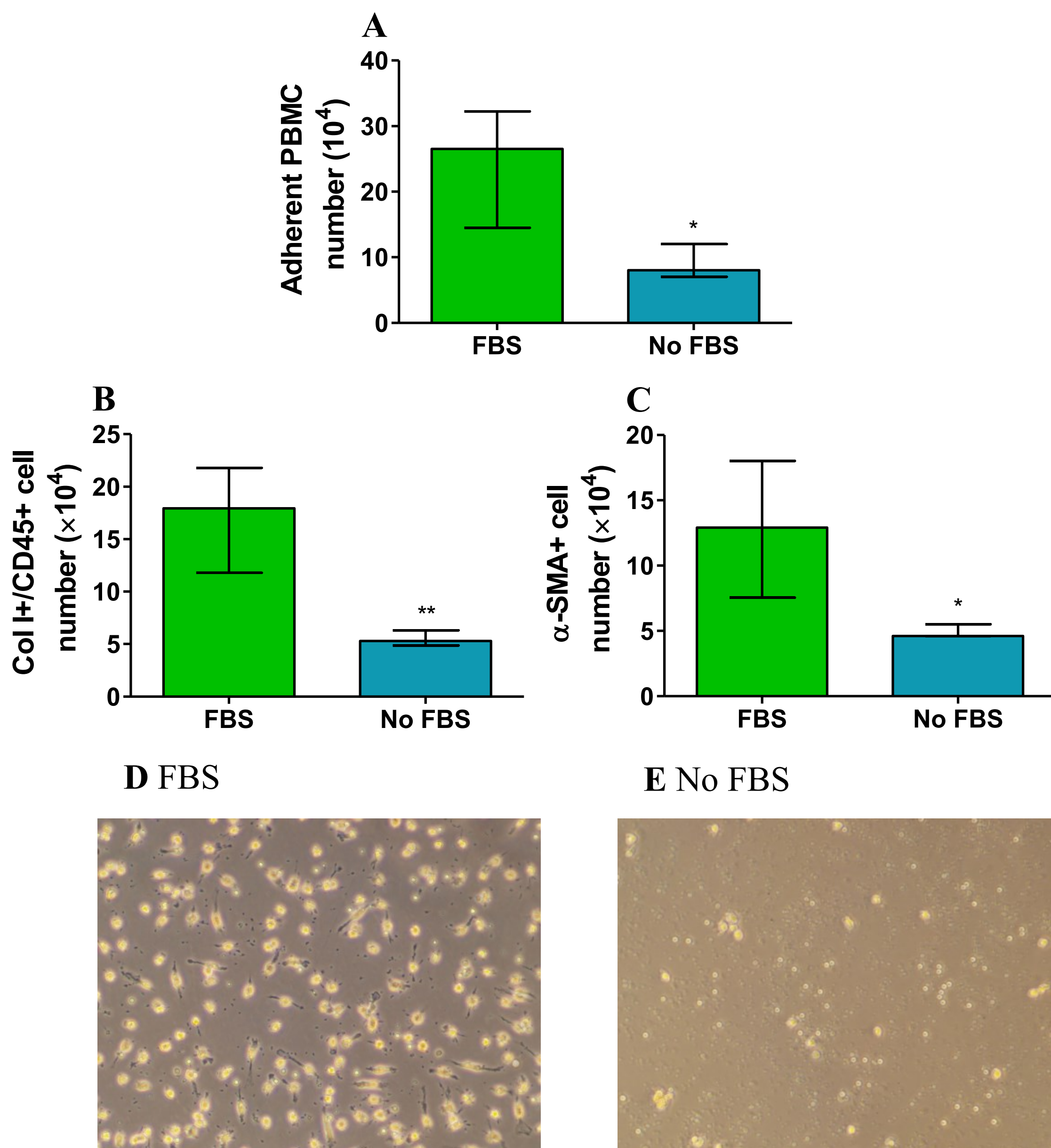


Figure 3.11: Comparison of fibrocytes derived from adherent peripheral blood mononuclear cells in the presence or absence of foetal bovine serum. PBMC from 6 healthy subjects were cultured in plates pre-coated with fibronectin (25 μg/mL) in the presence or absence of FBS. Medium was changed after 3 days in culture. Adherent cells were harvested and counted after 6 days in culture (**A**) and the number of fibrocytes (**B**; Col I+/CD45+ cells) and differentiating fibrocytes (**C**; α-SMA+ cells) within the adherent cell population was determined using flow cytometry. Bars represent median with interquartile range. The differences were determined by Wilcoxon matched pairs test. * $p < 0.05$, ** $p < 0.01$ compared to FBS-containing group. Spindle-shaped cells were identified by light microscopy and photographs were taken after 6 days (**D**-**E**). Photographs are representative of one experiment.

3.2.4.3 Determination of optimum fibronectin concentration

To determine the optimum concentration of fibronectin required, 6-well tissue culture plates were coated with different concentrations of fibronectin (0 – 25 μg/mL) for 60 minutes prior to plating the PBMC. PBMC from healthy subjects were incubated in FBS-containing medium, which was replenished on day 3. Adherent PBMC were harvested and counted on day 6. The percentage of fibrocytes and differentiating fibrocytes was determined by flow cytometry. Also, spindle-shaped, myofibroblast-like cells were identified by light microscopy.

Coating the wells with fibronectin led to an increase in the number of adherent PBMC (Figure 3.12A), but did not significantly affect the percentage of fibrocytes and differentiating fibrocytes in adherent PBMC (Figure 3.12B and D). The number of fibrocytes in the adherent PBMC was also increased by all concentrations of fibronectin (Figure 3.12C), whilst an increase in differentiating fibrocytes was more pronounced in the presence of 25 μg/mL of fibronectin (Figure 3.12E). In line with the α-SMA staining data, the number of spindle-shaped cells increased in the fibronectin-coated wells after 6 days in culture and was more pronounced (Figure 3.12F-H). Pre-coating with fibronectin led to higher number of adherent PBMC and

fibrocytes, which can more readily differentiate into myofibroblasts. Thus, in all subsequent experiments I used fibronectin at 25 μg/mL.

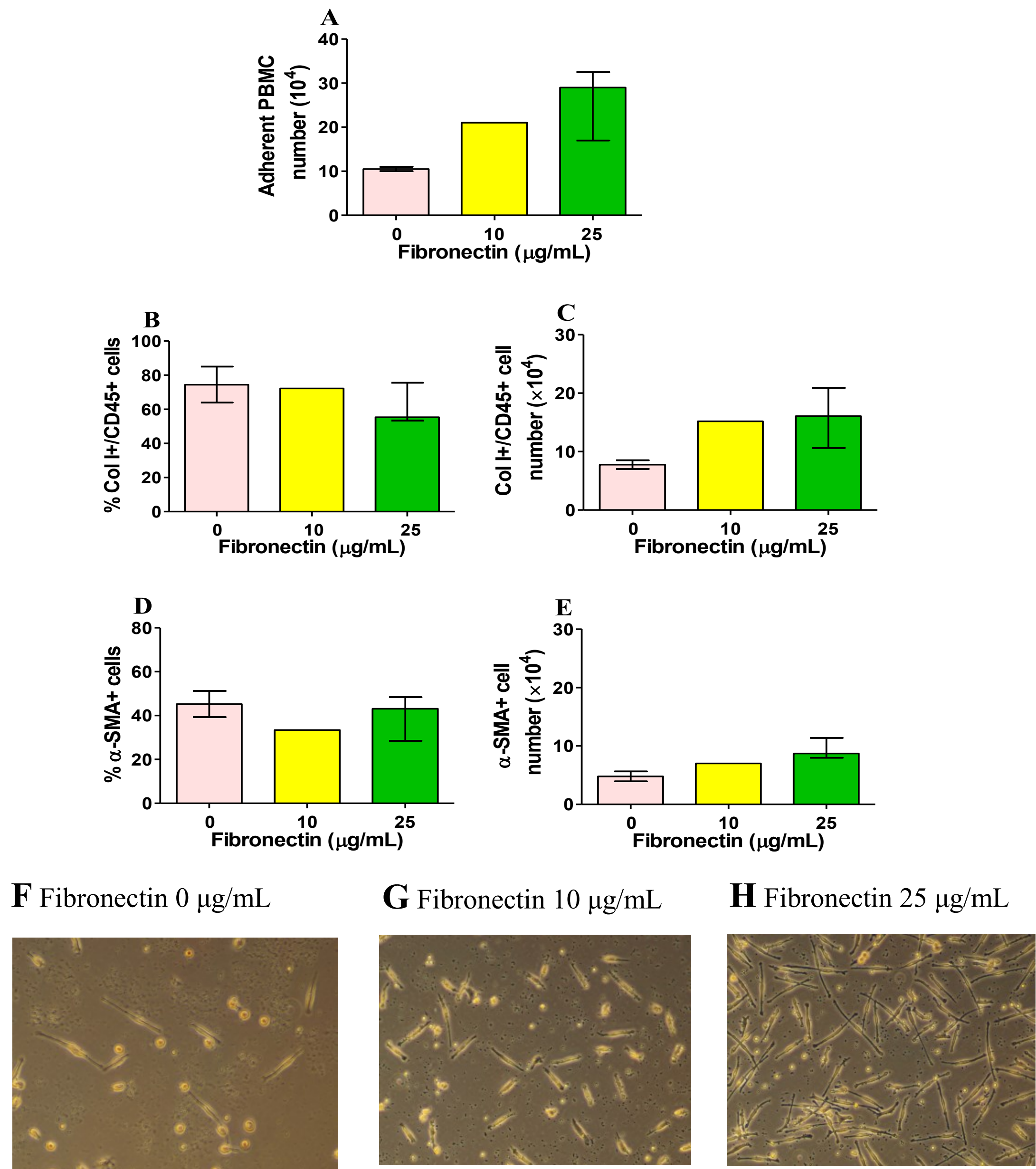


Figure 3.12: The effect of fibronectin on fibrocytes derived from adherent peripheral blood mononuclear cells. PBMC from healthy subjects were seeded in 6-well tissue culture plates pre-coated with fibronectin at the concentration of 0 μg/mL (0 μg/cm^2, n = 2), 10 μg/mL (1.0 μg/cm^2, n = 1) or 25 μg/mL (2.6 μg/cm^2, n = 5) in FBS-containing medium. Medium was changed on day 3 and adherent cells were harvested and counted (**A**) after 6 days in culture. Fibrocytes (Col I+/CD45+ cells) (**B-C**) and differentiating fibrocytes (α-SMA+ cells) (**D-E**) were also determined by flow cytometry. Spindle-shaped cells were identified by light microscopy and photographs were taken after 6 days (**F-H**). Photographs are representative of one experiment. Bars represent median with interquartile range.

3.2.5 Comparison of the number of cultured fibrocytes derived from adherent peripheral blood mononuclear cells from healthy subjects and patients with non-severe or severe asthma

PBMC from healthy subjects and patients with non-severe or severe asthma were seeded in plates pre-coated with fibronectin, in the presence of FBS. The number of adherent PBMC and percentage of fibrocytes and differentiating fibrocytes was determined after 6 days.

The percentage of fibrocytes (~70%) and differentiating fibrocytes (~65%) in cultured adherent PBMC were similar (Figure 3.13B and D). However, there was a trend towards that patients with asthma, especially non-severe asthma, having more cells adhered on the plates (Figure 3.13A), including fibrocytes and differentiating fibrocytes (Figure 3.13C and E). Morphologically, more spindle-shaped cells appeared in plates with PBMC from patients with asthma, especially non-severe asthma than from healthy subjects (Figure 3.13F-H).

Amongst these three groups, the expression of CCR7 were also similar (% Col I+/CD45+/CCR7+ cell 62.0% - 67.6%, $p = 0.85$; CCR MFI ratio 2.7-3.3, $p = 0.61$) (Figure 3.14A-B).

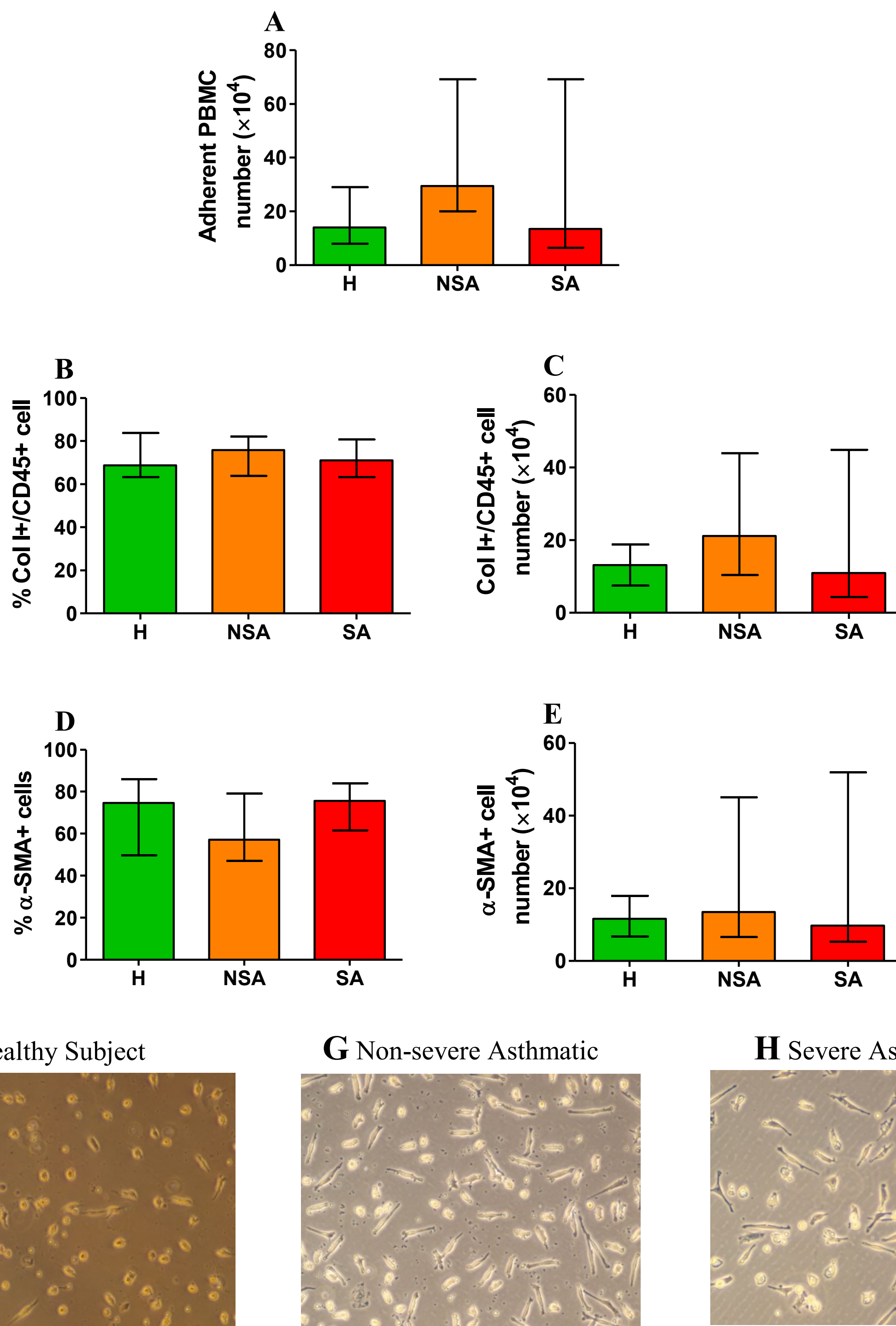


Figure 3.13: Comparison of adherent peripheral blood mononuclear cell-derived fibrocytes from healthy subjects and patients with non-severe and severe asthma. PBMC from healthy subjects (n = 9) and patients with non-severe (n = 6) or severe asthma (n = 6) were placed in plates pre-coated with fibronectin, in the presence of FBS. Adherent cells were harvested and counted **(A)** after 6 in culture. Fibrocytes (Col I+/CD45+ cells) (**B**, **C**) and differentiating fibrocytes (α-SMA+ cells) (**D**, **E**) were determined by flow cytometry. Bars represent median with interquartile range. The differences between disease groups were determined by Kruskal-Wallis test. Spindle-shaped cells were identified by light microscopy and photographs of fibrocytes from a healthy subject **(F)**, a non-severe asthmatic patient **(G)** and a severe asthmatic patient **(H)** were taken after 6 days.

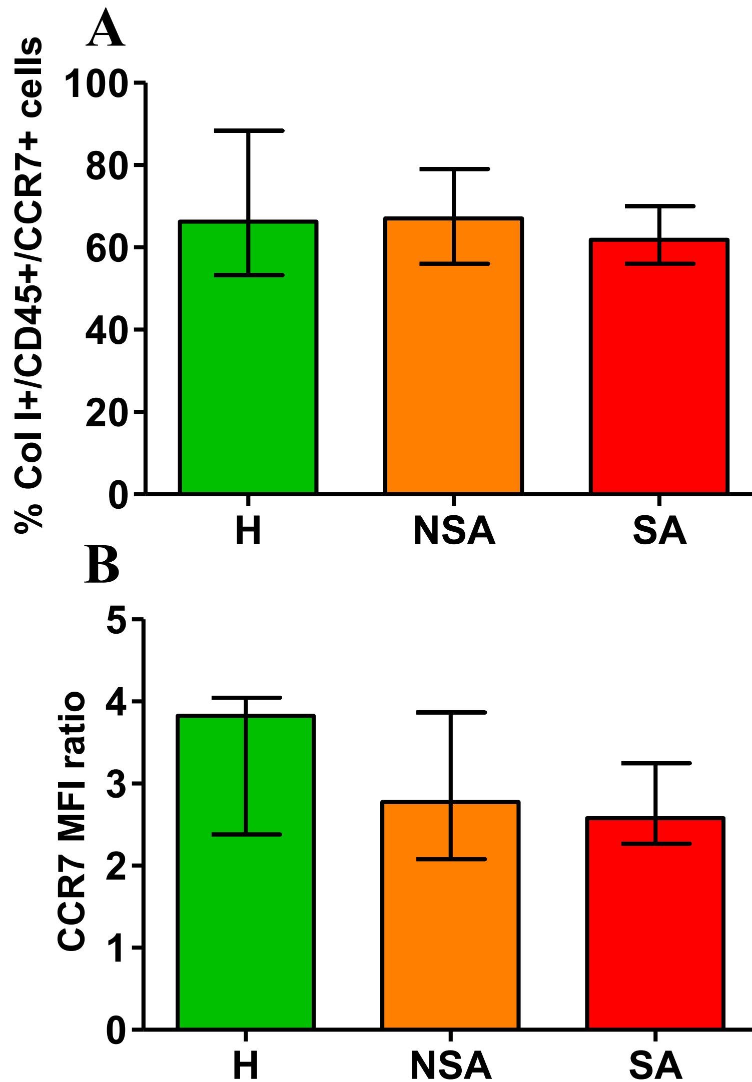


Figure 3.14: CC chemokine receptor 7 expression in fibrocytes from healthy subjects and patients with non-severe or severe asthma. PBMC isolated from healthy subjects (H, n = 9) and patients with non-severe (NSA, n = 6) or severe asthma (SA, n = 6) were placed in plates pre-coated with fibronectin, in the presence of FBS. Adherent cells were harvested after 6 days in culture. **(A)** The percentage of CCR7+ fibrocytes (Col I+/CD45+/CCR7+ cells) and **(B)** the CCR7 MFI ratio was determined by flow cytometry. Bars represent median with interquartile range. The differences between disease groups were determined by Kruskal-Wallis test.

3.3 Discussion

In this chapter I determined the optimal conditions for isolating and detecting fibrocytes from the NANT cell fraction of PBMC. I showed that, when placed in culture, fibrocytes can undergo proliferation whilst at the same time increase their α-SMA expression suggesting that they undergo myofibroblastic differentiation. This was confirmed by the appearance of spindle-shaped, myofibroblast-like cells. Patients with severe asthma were found to have a higher number of fibrocytes in their NANT cells compared to healthy subjects and patients with non-severe asthma, immediately after isolation. The number of circulating fibrocytes inversely correlated with the lung function of the subjects in my cohort. Furthermore, the fibrocytes in the NANT cells from patients with severe asthma also had a greater capacity to differentiate into myofibroblasts in culture. I also optimised the isolation of fibrocytes from adherent PBMC in order to compare the responses of the fibrocytes in the different fractions. However, I observed no differences in the number or differentiation capacity of fibrocytes, in the adherent fraction of PBMC, between healthy subjects and patients with non-severe or severe asthma. Although Saunders *et al.* reported patients with severe asthma have more fibroytes in peripheral blood and bronchial biopsies (Saunders, Siddiqui et al. 2009), currently no other group has compared the number or

myofibroblastic differentiation of cultured fibrocytes amongst healthy subjects and patients with non-severe or severe asthma.

Although there is a plethora of publications demonstrating the isolation of fibrocytes from peripheral blood, there is a disparity in the methods used. There are three methods reported in the literature. The most widely used method involves generating fibrocytes by differentiation from the adherent fraction of PBMC (Bucala, Spiegel et al. 1994) or CD14+ monocytes (Yang, Scott et al. 2002). The main part of my study was carried out on fibrocytes isolated from the NANT cell fraction of PBMC, as shown by Wang *et al.* (Wang, Huang et al. 2008). This study demonstrated that fibrocytes are more abundant in the NANT cell fraction of PBMC and that NANT cells represent more than 80% of the fibrocyte population (Wang, Huang et al. 2008). Moreover, as other progenitor cells with similar phenotype such as endothelial progenitor cells are present in the adherent fraction of PBMC, using the non-adherent fraction possibly results in less contaminating cells (Rehman, Li et al. 2003). As fibrocytes comprise just a small proportion of total circulating leukocytes, removal of T cells augments the percentage of target cells in the total cell population, improving the specificity of flow cytometry. T cells were depleted using anti-CD3

labelled-magnetic beads. This method was selected over the sheep RBCs method, used by Wang *et al.*, as it was found to be more efficient and specific.

In the literature, fibrocytes are defined and quantified by either their morphology or cell markers. Pilling *et al.* identified fibrocytes as spindle-shaped cells with oval nucleus in adherent PBMC (Shao, Suresh et al. 2008, Pilling, Vakil et al. 2009). However, the number of fibrocytes could be over-estimated since some other cells such as endothelial progenitor cells may also develop similar elongated shape (Asahara, Murohara et al. 1997). This method also neglects the floating fibrocytes which have not attached to the tissue culture plates. Pilling *et al.* found fibrocytes are the only cells which express the combination of CD45RO, 25F9, and S100A8/A9, but not PM-2K (macrophages have CD45RO, 25F9, S100A8/A9 and PM-2K) (Pilling, Fan et al. 2009), but this definition has not been widely accepted. More commonly, fibrocytes are defined by a combination of Col I+, CD45+ and/or CD34+. In studies which quantify fibrocytes count by flow cytometry, fibrocytes are identified as Col I+ cells (Yang, Scott et al. 2002), Col I+/CD34+ cells (Chesney, Bacher et al. 1997), Col I+/CD45+ cells (Moeller, Gilpin et al. 2009) or Col I+/CD34+/CD45+cells (Wang, Huang et al. 2008) in PBMC. Since fibrocytes quickly lose the CD34 expression during differentiation (Schmidt, Sun et al. 2003), inclusion of CD34 in the criteria

may under-estimate the count of differentiating/differentiated fibrocytes. CXCR4 is occasionally adopted as one of the criteria (Garcia-de-Alba, Becerril et al. 2010), but only 83% of Col I+/CD45+ fibrocytes express CXCR4 (Wang, Huang et al. 2008). Therefore, I defined fibrocytes as Col I+/CD45+ PBMC in this project.

In this project, α-SMA+ cells in NANT cells are regarded as differentiating fibrocytes. Sometimes these α–SMA+ NANT cells are regarded as myofibroblasts (Wang, Huang et al. 2008, Wang, Huang et al. 2012, Weng, Chen et al. 2013). Although bone-marrow derived smooth muscle progenitor cells also are present in PBMC (Simper, Stalboerger et al. 2002), I confirmed that most of the α-SMA+ cells in NANT cells are also positive for fibrocyte markers, i.e. Col I and CD45.

There is conflicting evidence in the literature concerning the use of FBS for fibrocyte culture. FBS-containing and FBS-free conditions may represent different phases of the inflammation and remodelling process (Curnow, Fairclough et al. 2010). Due to the change of endothelial permeability and tissue integrity, the concentration of serum-derived proteins in the diseased site is initially high but falls gradually due to the repair process. Fibrocytes cultured in FBS-containing and FBS-free conditions may activate different gene pathways in terms of inflammatory and immune processes, lipid metabolism, chemotaxis and RNA processing (Curnow, Fairclough et al. 2010).

Fibrocytes cultured in FBS-containing medium had up-regulated toll-like receptor (TLR)-4, IL-1β, chemokines CCL2/monocyte chemotactic protein 1 (MCP1), CCL3/macrophage inflammatory protein 1α (MIP-1-α), CCL7/monocyte-specific chemokine 3 (MCP3), CCL22/macrophage-derived chemokine (MDC), and the C5a complement receptor in comparison to fibrocytes cultured in FBS-free medium (Curnow, Fairclough et al. 2010). Therefore, I studied the responses of cultured fibrocytes in the presence and absence of serum. My data showed that the presence of FBS leads to an increase in the number of viable fibrocytes in both NANT cells and in adherent PBMC, accompanied by an increase in rate of DNA synthesis demonstrated by EdU proliferation assay, suggesting that fibrocytes proliferate in culture. Increased numbers of differentiating fibrocytes are also accompanied by α-SMA mRNA up-regulation, indicating cultured fibrocytes were actively transforming into myofibroblasts. In contrast, the number of fibrocytes and differentiating fibrocytes in either NANT cells or adherent PBMC was fewer in the absence of FBS, which might suggest less proliferation, less differentiation or more cell death. As a result, I observed fibrocytes in both NANT cells and adherent PBMC in FBS-containing media in the subsequent experiments.

Having identified the optimum conditions for fibrocyte isolation and culture, I compared the responses of fibrocytes from healthy subjects and patients with non-severe or severe asthma. Increased circulating fibrocytes has also been found in patients in healing/repairing process or with other fibrotic diseases, such as burn wound (Yang, Scott et al. 2002), scleroderma with interstitial lung disease (Mathai, Gulati et al. 2010) and idiopathic pulmonary fibrosis (Moeller, Gilpin et al. 2009). My data showed an increase in the number of fibrocytes in freshly isolated NANT cells from patients with severe asthma compared to healthy subjects and patients with non-severe asthma. Thus, fibrocytes appear to be more abundant in the circulation of patients with severe asthma. The number of circulating fibrocytes was inversely correlated with lung function suggesting a role of fibrocytes in the development of airway obstruction. Indeed, Wang *et al.* had reported that the percentage of fibrocytes in NANT from patients with chronic obstructive asthma is higher than that in asthmatic patients with normal lung function and healthy subjects, and is correlated with the annual decline rate of FEV_1 (Wang, Huang et al. 2008).

I proposed that increased circulating fibrocytes in the NANT cells from patients with severe asthma might result from rising bone marrow activity, heightened chemotaxis and exaggerated proliferation. It is possible that the bone marrows of

patients with severe asthma have an increased ability to produce or release circulating fibrocytes. It is worth noting that the age of patients with severe asthma recruited to this project is generally older compared to healthy subjects. More fibrocytes were observed in the peripheral blood of aged but otherwise healthy subjects (Mathai, Gulati et al. 2010), and ageing is associated with increased fibrocytes in bone marrow in an experimental mouse model (Sueblinvong, Neveu et al. 2014). Besides, increased fibrocytes in severe asthma may be related to escalated chemotaxis. Patients with severe asthma have more fibrocytes and higher level of CCL5/regulated on activation, normal T cell expressed and secreted (RANTES; ligand of CCR1, CCR3 and CCR5), CCL11/eotaxin-1 (ligand for CCR3, CCR5 and CXCR3) and CCL24/eotaxin-2 (ligand for CCR3) in their sputa, as well as increased expression of CCR3 and CCR5 in their fibrocytes (Isgro, Bianchetti et al. 2013). Additionally, fibrocytes from patients with severe asthma may have greater capacity to multiply in blood. Compared to asthmatic patients with normal lung function, patients with chronic obstructive asthma have more fibrocytes in their peripheral blood, with greater expression of epidermal growth factor receptors (EGFR) in these fibrocytes. EGFR inhibition prevents the proliferation and differentiation of fibrocytes (Wang, Huang et al. 2012). EGFR expression is increased in the airways of patients with severe asthma,

which is associated with more significant subepithelial fibrosis despite use of CS treatment (Puddicombe, Polosa et al. 2000, Hamilton, Torres-Lozano et al. 2003). It is possible that the expression of EGFR in fibrocytes from patients with severe asthma is also up-regulated. Further investigation regarding the expression of EGFR in fibrocytes from healthy subjects and patients with non-severe or severe asthma is indicated.

Although there was no difference amongst the three groups in terms of the number of Col I+/CD45+ fibrocytes in cultured NANT cells over time, there was an increase in the number of α-SMA+ differentiating fibrocytes from patients with severe asthma indicating an increased capacity of severe asthmatic fibrocytes to undergo myofibroblastic differentiation. The result is similar to an earlier report showing an increase in α-SMA+ cells in NANT from patients with chronic obstructive asthma, compared to healthy subjects and asthmatic patients with normal lung function (Wang, Huang et al. 2008). The increased number of differentiating fibrocytes in cultured NANT cells from patients with severe asthma may be a result of the higher proportion of fibrocytes in these patients' NANT cells. Nonetheless, it is also possible that this may occur due to an inherent difference in the signalling mechanisms of these cells in response to the mediators in the foetal bovine serum, or the effects of

autocrine or paracrine mediated by growth factors released from fibrocytes or other NANT cells (e.g. B cells, NK cells, etc.).

Apart from higher number of circulating fibrocytes in peripheral blood, an increased accumulation of fibrocytes in severe asthmatic airways may be related to exaggerated chemotaxis (Saunders, Siddiqui et al. 2009). Several chemokine receptors, including CCR7 (Sakai, Wada et al. 2006) and CXCR4 (Phillips, Burdick et al. 2004), mediate the homing of circulating fibrocytes to sites of disease. Blocking CCR7/CCL21-SLC axis reduces fibrocyte infiltration in mouse kidneys with unilateral ureter ligation and therefore attenuate renal fibrosis (Sakai, Wada et al. 2006). My data demonstrates that fibrocytes express CCR7, although CCR7 expression in fibrocytes from healthy subjects and asthmatic patients was not significantly different. The result is compatible with Kaur *et al.*'s report that airway fibroblasts, ASMC and myofibroblasts express CCR7 in human airways, but the proportion of CCR7+ cells was not different in healthy and asthmatic groups (Kaur, Saunders et al. 2006). Interestingly, CCR7 expression in human CD4+ T lymphocytes was even down-regulated in both CS-sensitive and CS-resistant asthmatic patients, compared to non-asthmatic non-atopic subjects (Syed, Blakemore et al. 1999). CCL19, the ligand of CCR7, is highly expressed by ASMC from asthmatic patients with

severe disease, and mast cells and vessels in asthmatic patients of all severities (Kaur, Saunders et al. 2006). It seems the increased recruitment of fibrocytes in asthmatic airways can be related to the higher level of CCL19/MIP-3-β expression in airways, rather than increased CCR7 expression in circulating fibrocytes.

In the subsequent chapters, I will investigate the effect of pro-inflammatory mediators and asthma medications on the number, myofibroblastic differentiation and CCR7 expression of fibrocytes derived from NANT cells, and also show their effects on fibrocytes derived from adherent PBMC (Bucala, Spiegel et al. 1994, Yang, Scott et al. 2002, Shao, Suresh et al. 2008). Many fibrocytes in adherent PBMC should be derived from CD14+ monocytes, since a large proportion of adherent PBMC are monocytes. Besides, fibrocytes in adherent PBMC may have been affected by TGF-β_1-releasing T cells, before removal of non-adherent fraction of PBMC (Yang, Scott et al. 2002). On the other hand, fibrocytes in NANT cells are less likely to be derived from monocytes and less affected by T cells, since these cells have been removed in the first place (Wang, Huang et al. 2008). It is possible that fibrocytes in adherent PBMC and NANT cells have different properties. To the best of my knowledge, no published study has compared the responses of fibrocytes in these two fractions of PBMC. I, therefore, optimised the isolation of fibrocytes from adherent

PBMC in order to compare their responses to NANT cell-derived fibrocytes.

There are a number of different protocols for isolating fibrocytes in adherent PBMC in the literature (Bucala, Spiegel et al. 1994, Pilling, Vakil et al. 2009, Curnow, Fairclough et al. 2010). However, technical details varied hugely amongst the different groups. Thus, I examined the effect of time in culture, as well as the use of FBS and fibronectin, on fibrocytes derived from adherent PBMC. The number of fibrocytes declined on day 10, while the number of differentiating fibrocytes peaked on day 6. Higher number of fibrocytes and differentiating fibrocytes were observed in PBMC in the presence of FBS, compared to the number of cells in the absence of FBS. Therefore, I picked day 6 as the time point to observe fibrocytes in adherent PBMC, and use FBS-containing media to support the growth of fibrocytes. I also confirmed that fibronectin increases the number of adherent PBMC, including fibrocytes and differentiating fibrocytes. Fibronectin, a glycoprotein existing in the ECM and plasma (Magnusson and Mosher 1998), has been used to support fibrocyte culture in some studies although the details were not mentioned (Hong, Belperio et al. 2007). Fibronectin is a product of most mesenchymal and epithelial cells. The functions of fibronectin include promoting cellular migration during wound healing and development, regulation of cell growth and differentiation and

thrombosis/haemostasis (Hocking 2002). Circulating endothelial progenitor cells within PBMC seeded on fibronectin appear earlier compared to those cells seeded on collagen (Colombo, Calcaterra et al. 2013). Since there is an increased deposition of ECM proteins, including fibronectin, in asthmatic airways, it may also be one of the possible mechanisms by which more fibrocytes are recruited in asthmatic airways than in healthy airways (Saunders, Siddiqui et al. 2009).

The number of differentiating fibrocytes in adherent PBMC from patients with severe asthma was not significantly higher than those from patients with non-severe asthma after 6 days in culture. The results seemed different from the observation in NANT cell model after 3 days in culture. Since the fibrocytes were derived from different fraction of PBMC, they might behave differently. Besides, the number of differentiating fibrocytes in NANT cells from patients with severe asthma was not significantly higher than those from patients with non-severe asthma on day 7 either. All surviving fibrocytes from patients with non-severe or severe asthma eventually acquire myofibroblastic phenotype in the long run. A larger population size will be needed to clarify this issue.

The airways of patients with severe asthma display more prominent airway remodelling (Macedo, Hew et al. 2009). Compared to healthy subjects and patients

with non-severe asthma, patients with severe asthma recruited to my project have more circulating fibrocytes which potentially migrate to their airway walls. Their fibrocytes also exert a greater capacity to differentiate into myofibroblasts, produce extracellular matrices and even modulate resident fibroblasts (Peng and Herzog 2012), contributing to extensive remodelling processes in severe asthma. The number of circulating fibrocytes is correlated with airflow limitation, similar to the observation in patients with chronic obstructive asthma (Wang, Huang et al. 2008). Increased number of α-SMA+ fibrocytes have been shown in bronchial lamina propria in severe asthma (Saunders, Siddiqui et al. 2009), whilst the number of recruited fibrocytes correlates with the thickness of reticular basement membrane (Nihlberg, Larsen et al. 2006). Therefore, fibrocytes may play an important role in the progress of airway remodelling in severe asthma.

Chapter 4: Effect of pro-fibrotic mediators on fibrocytes

4.1 Background

The inflammatory response in asthmatic patients is accompanied by an increased release of a range of inflammatory cytokines and growth factors that mediate the development of airway remodelling (Ohno, Nitta et al. 1996, Virchow, Julius et al. 1998, Chakir, Shannon et al. 2003). Specifically, the T_H2 cytokine interleukin (IL)-13 is increased (but IL-4 is reduced) in the airways of patients with severe asthma (Ohno, Nitta et al. 1996, Naseer, Minshall et al. 1997, Shannon, Ernst et al. 2008). Serum levels of neurotrophins, nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), are associated with severity of allergic asthma and childhood asthma, respectively (Bonini, Lambiase et al. 1996, Muller, Pitrez et al. 2010). Therefore, these mediators provide interesting therapeutic targets for attenuating airway remodelling in asthmatic patients.

IL-4 and IL-13 are released by T_H2 cells, group 2 innate lymphoid cells (ILC2s), eosinophils, basophils, mast cells and airway smooth muscle cells (ASMC) (Oliphant, Barlow et al. 2011). IL-4 and IL-13 induce subepithelial fibrosis and ASMC layer thickening in the remodelling airways (Takayama, Arima et al. 2006, Doherty and Broide 2007). In fibroblasts, IL-4 and IL-13 induce proliferation (Lewis, Sutherland et

al. 2003), myofibroblastic differentiation (Hashimoto, Gon et al. 2001) and production of CC chemokine ligand (CCL) 11/eotaxin (Richter, Puddicombe et al. 2001). IL-4 and IL-13 accelerate the differentiation of fibrocytes in healthy subjects (Shao, Suresh et al. 2008), and the production of collagen (type I, III, V) and cytokines (IL-6, IL-11, leukaemia inhibitory factor) in fibrocytes from patients with non-severe asthma (Bellini, Marini et al. 2012). The effect of T_H2 cytokines on fibrocytes from patients with severe asthma is currently unknown.

Neurotrophins are mainly produced by epithelial cells in inflamed lungs (Hahn, Islamian et al. 2006), and are also generated by nerves, endothelial cells, fibroblasts, ASMC, T cells, B cells, eosinophils, mast cells, monocytes and macrophages (Prakash, Thompson et al. 2010). Neurotrophins are increased in bronchoalveolar lavage fluid from patients with allergic asthma after allergen challenge (Virchow, Julius et al. 1998). Serum levels of neurotrophins, such as NGF and BDNF, are higher in patients with asthma (Bonini, Lambiase et al. 1996, Lommatzsch, Schloetcke et al. 2005). NGF and BDNF are implicated in airway remodelling (Renz and Kilic 2012, Prakash and Martin 2014). NGF is associated with collagen deposition and subepithelial fibrosis in animal models (Kilic, Sonar et al. 2011). NGF increases the migration, and myofibroblastic differentiation of pulmonary fibroblasts (Micera,

Vigneti et al. 2001), whilst BDNF enhances the proliferation of ASMC (Aravamudan, Thompson et al. 2012). Currently, there is no literature describing the effect of neurotrophins on fibrocyte behaviour.

T_H2 cytokines and neurotrophins could be important mediators of aberrant fibrocyte function in patients with severe asthma. In this chapter I investigated the impacts of IL-4, IL-13, NGF and BDNF on fibrocytes from healthy subjects and patients with asthma, which will clarify the significance of the pro-inflammatory mediators in fibrocyte-mediated airway remodelling in severe asthma, and may be useful to evaluate the effects of future anti-cytokine therapy.

4.2 Results

4.2.1 Effect of T_H2 cytokines on the number and differentiation of fibrocytes in non-adherent non-T cells from healthy subjects and patients with non-severe or severe asthma

To explore the effect of T_H2 cytokines on the number and myofibroblastic differentiation status of fibrocytes, non-adherent non-T (NANT) cells isolated from healthy subjects and patients with asthma were cultured in foetal bovine serum (FBS)-containing medium, as described in Chapter 3.2.2, and treated with IL-4 (0.1-10 ng/mL) or IL-13 (0.1-10 ng/mL). The concentrations of IL-4 and IL-13 used for treatment of fibrocytes were selected from previously published studies (Shao, Suresh et al. 2008, Bellini, Marini et al. 2012). NANT cells were recovered after 3 or 7 days in culture and the number of fibrocytes (Col I+/CD45+ cells), differentiating fibrocytes (α-SMA+ cells) and CCR7-expressing fibrocytes were determined by flow cytometry.

IL-4 increased the number of fibrocytes in cultured NANT cells from healthy subjects in a concentration-dependent manner, peaking after 3 days in culture (1.9-fold increase, $p < 0.01$, 1 ng/mL). IL-4 also increased the number of fibrocytes from patients with non-severe asthma but this effect reached a maximum of 1.5-fold

after 7 days ($p < 0.01$, 10 ng/mL). Conversely, the number of fibrocytes from patients with severe asthma did not change in response to IL-4 stimulation (Figure 4.1A-B). IL-4 also increased the number of differentiating fibrocytes from healthy subjects (1.6-fold, $p < 0.05$, 1 ng/mL) and patients with non-severe asthma (1.3-fold, $p < 0.05$, 1 ng/mL) after 7 days in culture. The number of differentiating fibrocytes in the NANT cells of patients with severe asthma were also unaffected by IL-4 (Figure 4.1C-D). Under FBS-free conditions, there was also a trend towards IL-4 increasing fibrocytes and differentiating fibrocytes within NANT cells from healthy subjects (Figure 4.2).

IL-4 did not affect the expression of CC chemokine receptor (CCR) 7 in fibrocytes (Figure 4.3).

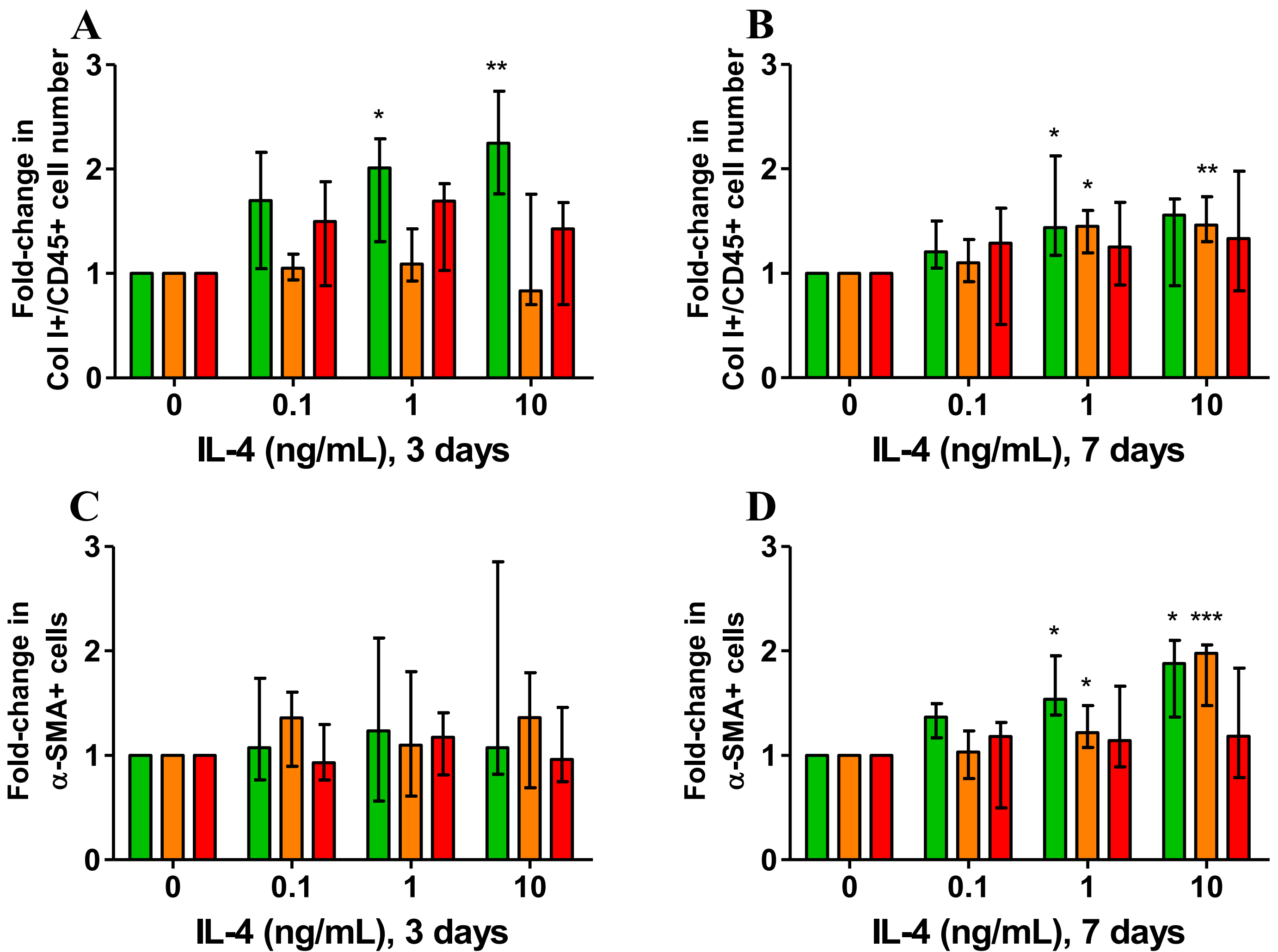


Figure 4.1: The effect of interleukin-4 on the number of fibrocytes and differentiating fibrocytes derived from non-adherent non-T cells. NANT cells from healthy subjects (■, n = 6) and patients with non-severe (■, n = 6) or severe asthma (■, n = 7) were treated with IL-4 (0.1-10 ng/mL) on day 0. Cells were harvested after 3 or 7 days in culture and the number of fibrocytes (**A-B**; Col I+/CD45+ cells) and differentiating fibrocytes (**B-D**; α-SMA+ cells) within the NANT cell population was determined by flow cytometry. Bars represent median with interquartile range. Data are expressed as fold-change with respect to untreated controls. The differences between the untreated control group and each treatment group were determined by Friedman test, followed by Dunn's post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to untreated controls.

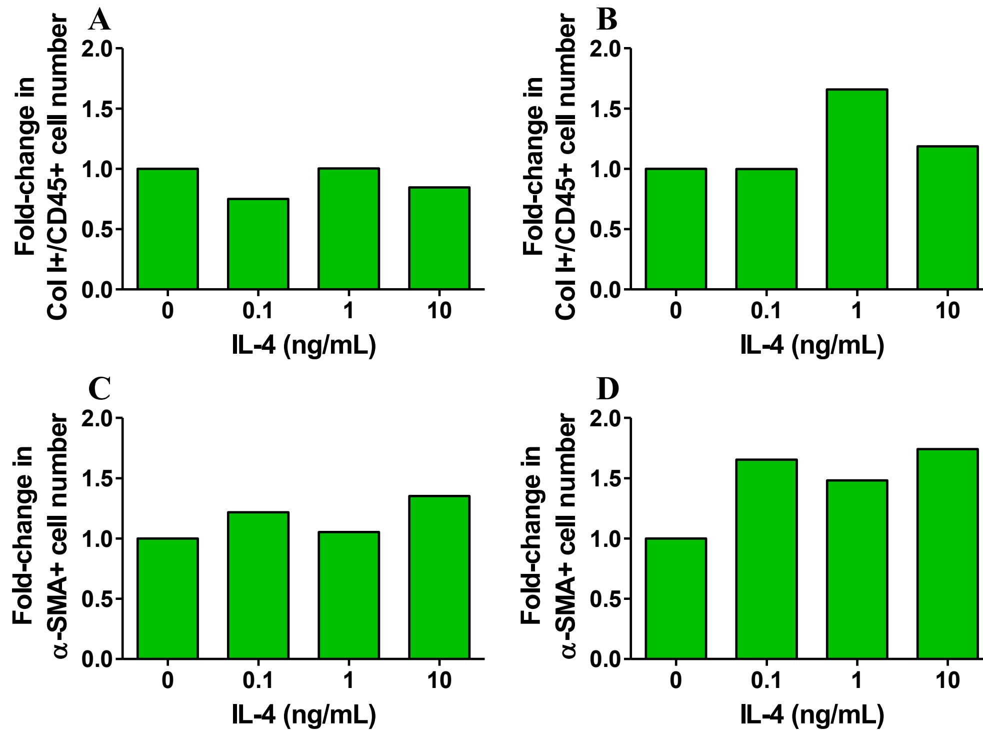


Figure 4.2: Effect of interleukin-4 on the number of fibrocytes and differentiating fibrocytes derived from non-adherent non-T cells under foetal bovine serum-free condition. NANT cells from healthy subjects (n = 2) were treated with IL-4 (0.1-10 ng/mL) on day 0. Cells were harvested after 3 or 7 days in culture and the number of fibrocytes (**A**-**B**; Col I+/CD45+ cells) and differentiating fibrocytes (**C-D**; α-SMA+ cells) within the NANT cell population was determined using flow cytometry. Data are expressed as fold-change with respect to untreated controls. Bars represent median values.

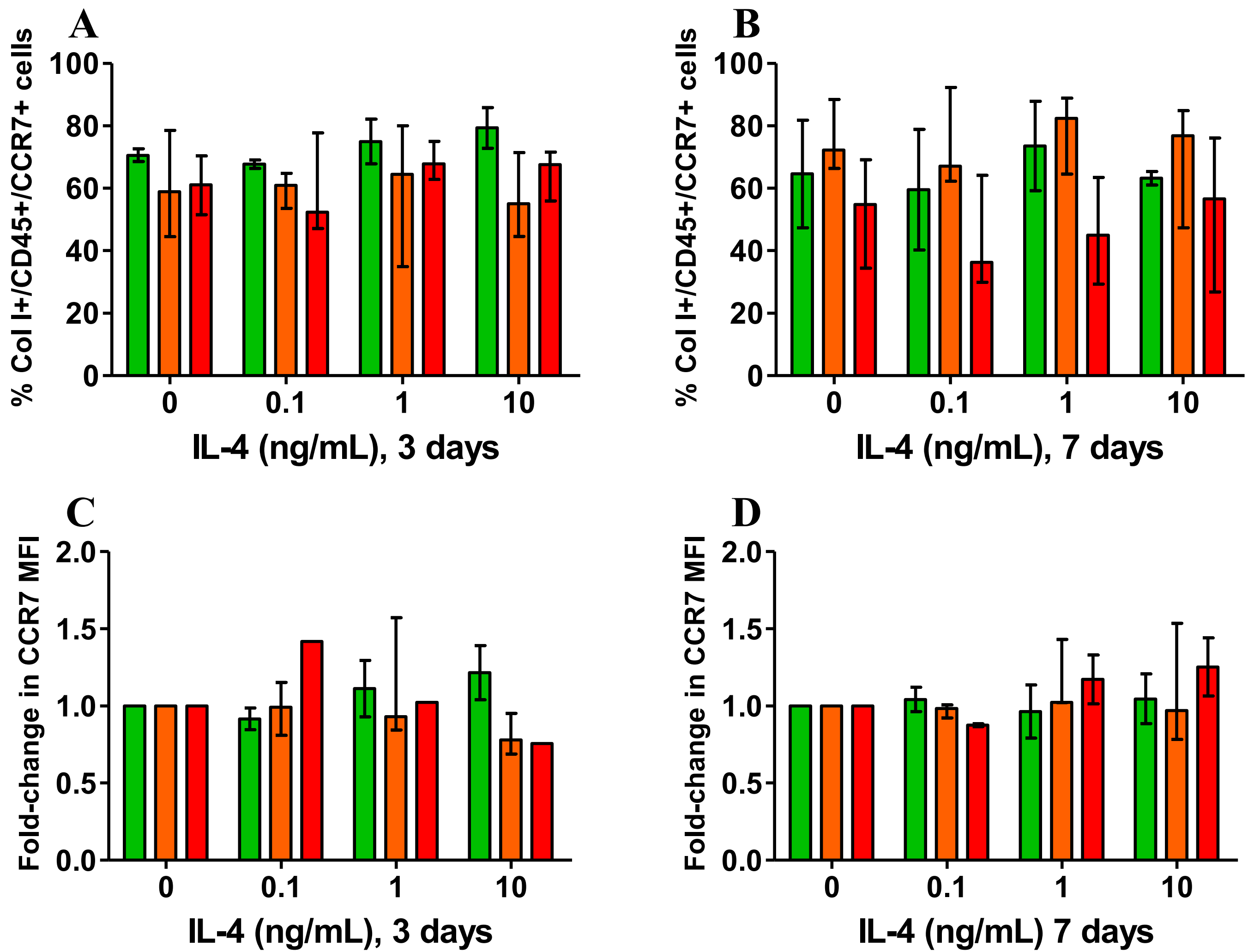


Figure 4.3: The effect of interleukin-4 on CC chemokine receptor 7 expression in fibrocytes derived from non-adherent non-T cells. NANT cells from healthy subjects (■, n = 6) and patients with non-severe (■, n = 6) or severe asthma (■, n = 7) were treated with IL-4 (0.1-10 ng/mL) on day 0. Cells were harvested after 3 or 7 days in culture and the percentage of CCR7+ fibrocytes within the NANT cell population (**A-B**; % Col I+/CD45+/CCR7+ cells) and CCR7 MFI (**C-D**) was determined by flow cytometry. Bars represent median with interquartile range. MFI data are expressed as fold-change with respect to untreated controls. The differences between the untreated control group and each treatment group were determined by Friedman test.

Treatment with IL-13 (10 ng/mL) increased the number of fibrocytes in the NANT cells from healthy subjects (1.8-fold, $p < 0.01$) and patients with non-severe asthma (1.6-fold, $p < 0.05$) in a concentration-dependent manner after 7 days in culture (Figure 4.4B). IL-13 also increased the number of differentiating fibrocytes from healthy subjects (1.3-fold, $p < 0.05$, 1 ng/mL) and patients with non-severe asthma (1.6-fold, $p < 0.05$, 10 ng/mL) after 7 days (Figure 4.4D). IL-13 (e.g. 10 ng/mL) did not affect the number of fibrocytes (day 3, $p = 0.81$; day 7, $p = 0.06$) and differentiating fibrocytes (day 3, $p = 0.63$; day 7, $p = 0.31$) in the NANT cells of patients with severe asthma.

IL-13 did not affect the expression of CCR7 in fibrocytes (Figure 4.5).

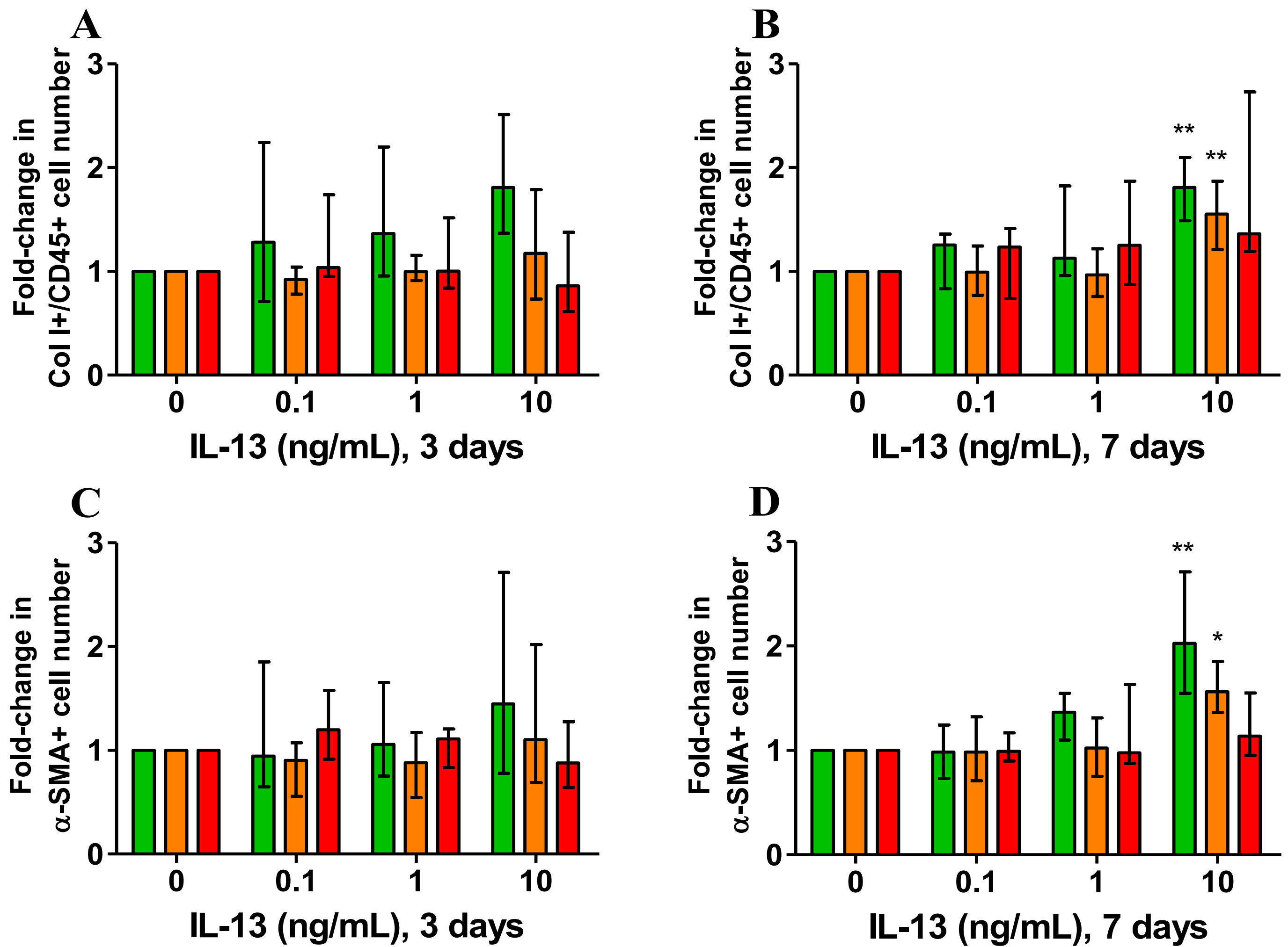


Figure 4.4: The effect of interleukin-13 on the number of fibrocyte and differentiating fibrocytes derived from non-adherent non-T cells. NANT cells from healthy subjects (■, n = 6) and patients with non-severe (■, n = 6) or severe asthma (■, n = 5) were treated with IL-13 (0.1-10 ng/mL) on day 0. Cells were harvested after 3 or 7 days in culture and the number of fibrocytes (**A**-**B**; Col I+/CD45+ cells) and differentiating fibrocytes (**C**-**D**; α-SMA+ cells) within the NANT cell population was determined by flow cytometry. Bars represent median with interquartile range. Data are expressed as fold-change with respect to untreated controls. The differences between the untreated control group and each treatment group were determined by Friedman test, followed by Dunn's post-hoc test. * $p < 0.05$, ** $p < 0.01$ compared to untreated controls.

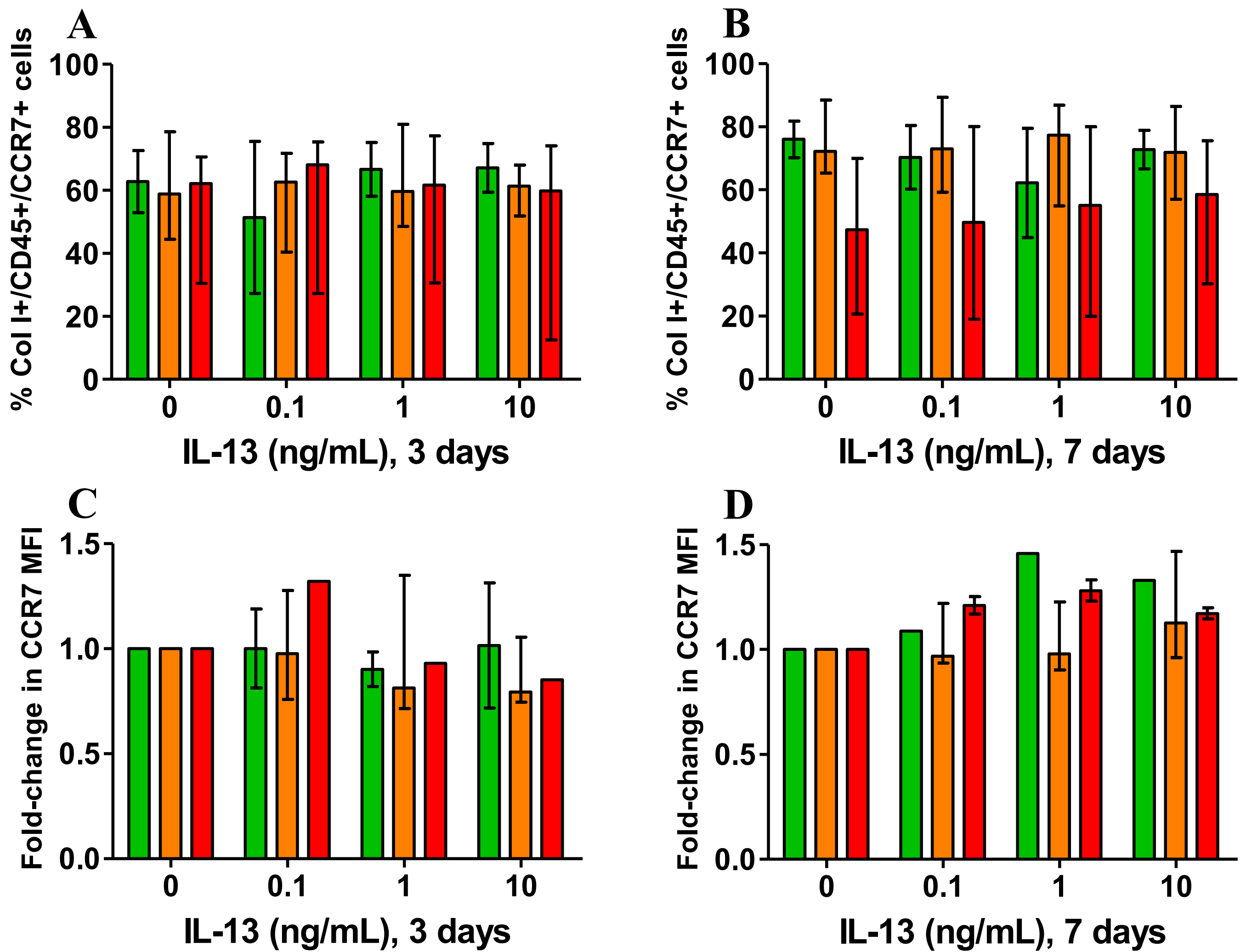


Figure 4.5: The effect of interleukin-13 on CC chemokine receptor 7 expression in fibrocytes derived from non-adherent non-T cells. NANT cells from healthy subjects (■, n = 6) and patients with non-severe (■, n = 6) or severe asthma (■, n = 5) were treated with IL-13 (0.1-10 ng/mL) on day 0. Cells were harvested after 3 or 7 days in culture and the percentage of CCR7+ fibrocytes within the NANT cell population (**A-B**; % Col I+/CD45+/CCR7+ cells) and CCR7 MFI (**C-D**) was determined by flow cytometry. Bars represent median with interquartile range. MFI data are expressed as fold-change with respect to untreated controls. The differences between the untreated control group and each treatment group were determined by Friedman test.

4.2.2 Effect of neurotrophins on the number and differentiation of fibrocytes in non-adherent non-T cells from healthy subjects and patients with non-severe or severe asthma

To explore the effect of neurotrophins on the number and myofibroblastic differentiation of fibrocytes, NANT cells isolated from healthy subjects and patients with asthma were cultured in FBS-containing medium, as described in Chapter 3.2.2, and treated with NGF (10-1000 ng/mL) or BDNF(10-100 ng/mL). The concentrations of NGF and BDNF used for treatment of fibrocytes were selected from previously published studies (Micera, Vigneti et al. 2001, Aravamudan, Thompson et al. 2012) .Cells were recovered after 3 or 7 days in culture and the number of fibrocytes and differentiating fibrocytes and CCR7-expressing fibrocytes were determined by flow cytometry.

Treatment with NGF modestly increased the number of fibrocytes from healthy subjects (300 ng/mL, 1.3-fold, $p < 0.05$ at 3 days; 30 ng/mL 1.2-fold, p < 0.05 at 7 days) and patients with non-severe (100 ng/mL, 1.5-fold, $p < 0.05$ at 3 days and 7 days) or severe asthma (300 ng/mL, 1.3-fold, $p < 0.05$ at 7 days; Figures 4.6A-B). NGF also increased the number of differentiating fibrocytes in the NANT cells from patients with non-severe asthma, reaching a maximum of approx. 1.7-fold after 3 days

in culture (100 ng/mL, $p < 0.05$), whilst it had less effect on fibrocytes from healthy subjects (1.3-fold, $p < 0.05$, 100 ng/mL at 3 days and 1.2-fold, $p < 0.05$, 300 ng/mL at 7 days) and patients with severe asthma (1.2-fold, $p < 0.05$, 1000 ng/mL at 7 days; Figure 4.6C-D).

Treatment with BDNF did not affect the number of fibrocytes and differentiating fibrocytes from healthy subjects or patients with asthma (Figure 4.8).

The expression of CCR7 in fibrocytes was not affected by NGF (Figure 4.7) and BDNF (Figure 4.9).

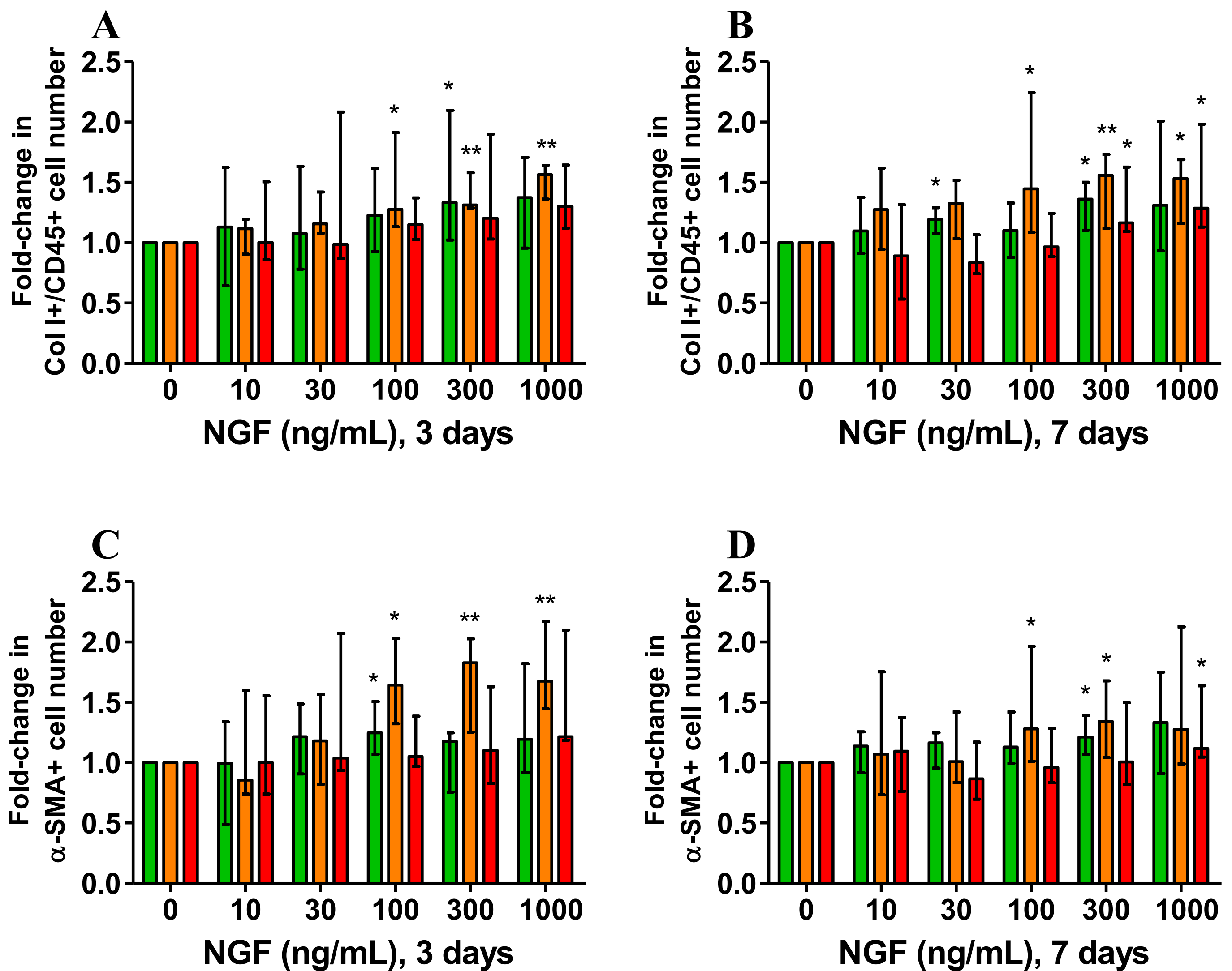


Figure 4.6: The effect of nerve growth factor on the number of fibrocyte and differentiating fibrocytes derived from non-adherent non-T cells. NANT cells from healthy subjects (■, n = 8) and patients with non-severe (■, n = 7) or severe asthma (■, n = 7) were treated with NGF (10-1000 ng/mL) on day 0. Cells were harvested after 3 or 7 days in culture and the number of fibrocytes (**A**-**B**; Col I+/CD45+ cells) and differentiating fibrocytes (**B-D**; α-SMA+ cells) within the NANT cell population was determined by flow cytometry. Bars represent median with interquartile range. Data are expressed as fold-change with respect to untreated controls. The differences between the untreated control group and each treatment group were determined by Friedman test, followed by Dunn's post-hoc test. * p < 0.05, ** p < 0.01 compared to untreated controls.

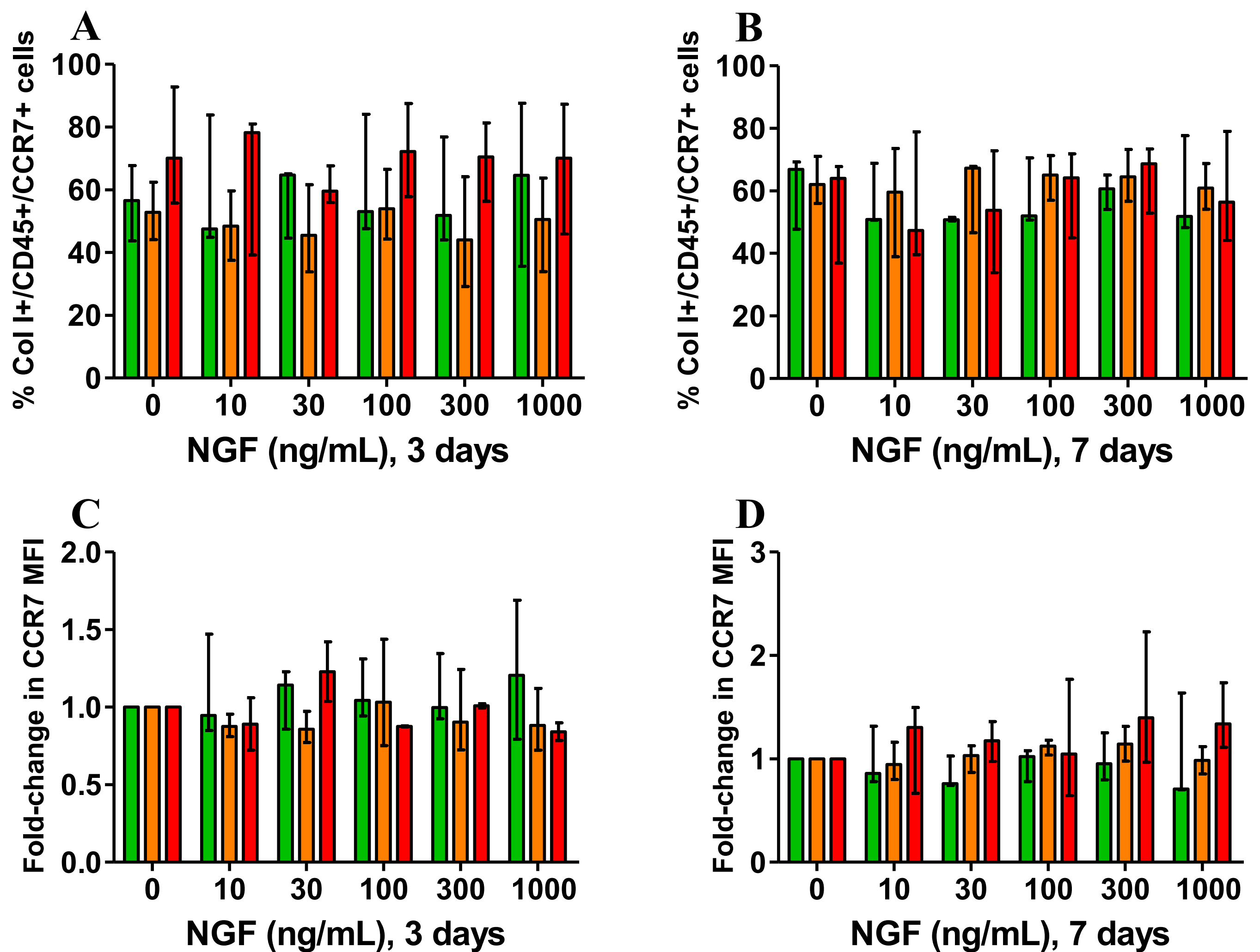


Figure 4.7: The effect of nerve growth factor on CC chemokine receptor 7 expression in fibrocytes derived from non-adherent non-T cells. NANT cells from healthy subjects (■, n = 8) and patients with non-severe (■, n = 7) or severe asthma (■, n = 7) were treated with NGF (10-1000 ng/mL) on day 0. Cells were harvested after 3 or 7 days in culture and the percentage of CCR7+ fibrocytes within the NANT cell population (**A-B**; % Col I+/CD45+/CCR7+ cells) and CCR7 MFI (**C-D**) was determined by flow cytometry. Bars represent median with interquartile range. MFI data are expressed as fold-change with respect to untreated controls. The differences between the untreated control group and each treatment group were determined by Friedman test.

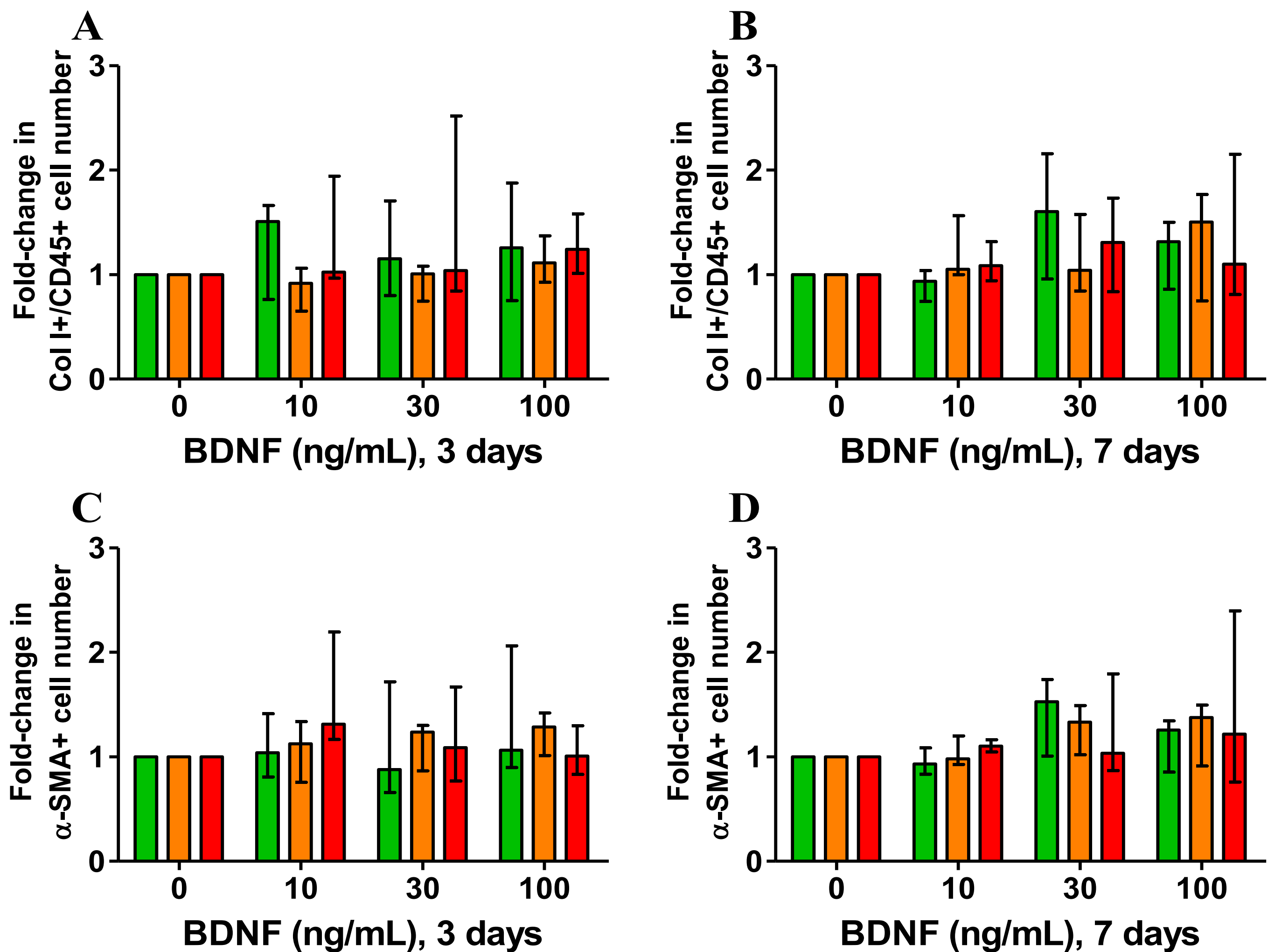


Figure 4.8: The effect of brain-derived neurotrophic factor on the number of fibrocyte and differentiating fibrocytes derived from non-adherent non-T cells. NANT cells from healthy subjects (■, n = 6) and patients with non-severe (■, n = 5) or severe asthma (■, n = 5) were treated with BDNF (10-100 ng/mL) on day 0. Cells were harvested after 3 or 7 days in culture and the number of fibrocytes (**A**-**B**; Col I+/CD45+ cells) and differentiating fibrocytes (**B-D**; α-SMA+ cells) within the NANT cell population was determined by flow cytometry. Bars represent median with interquartile range. Data are expressed as fold-change with respect to untreated controls. The differences between the untreated control group and each treatment group were determined by Friedman test.

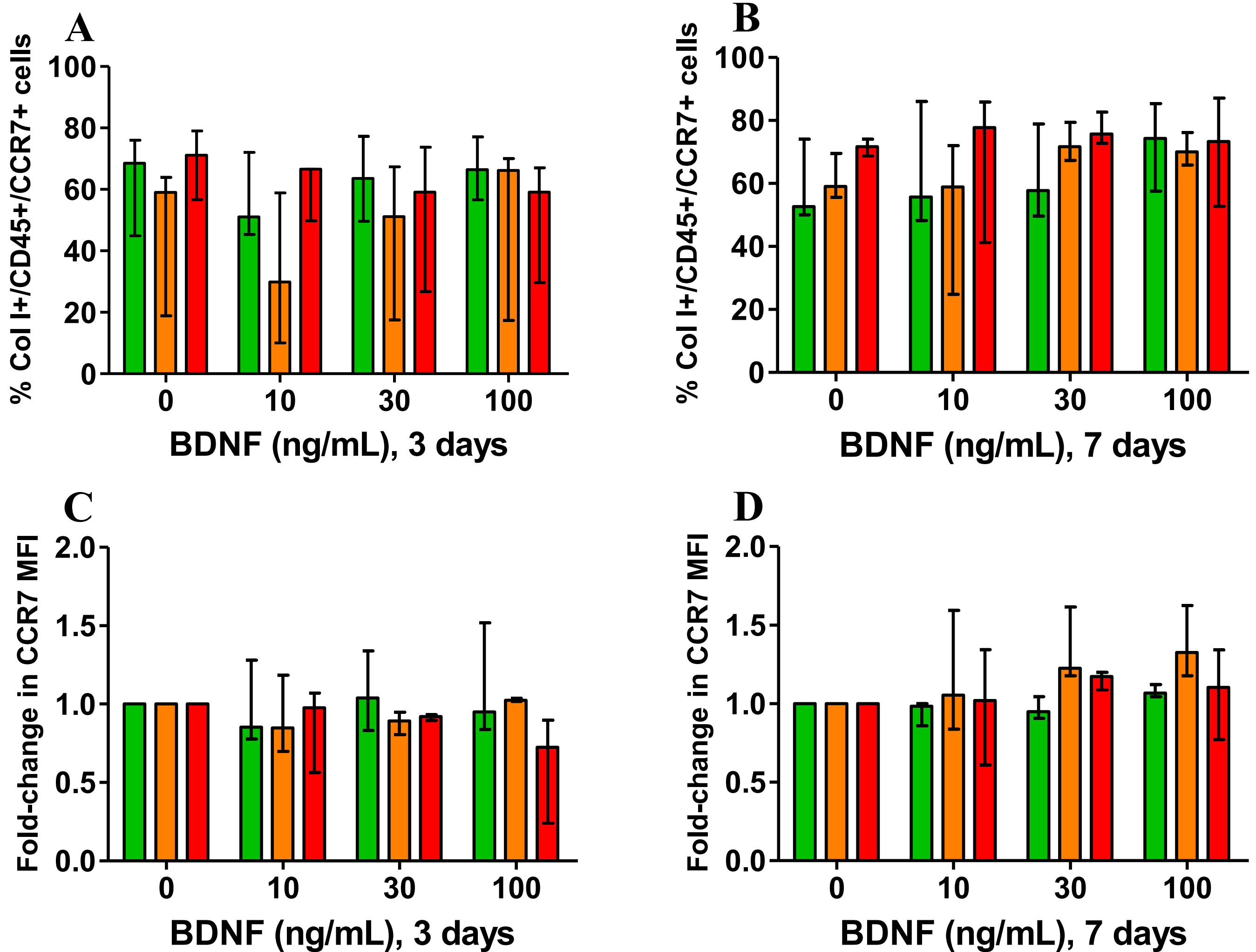


Figure 4.9: The effect of brain-derived neurotrophic factor on CC chemokine receptor 7 expression in fibrocytes derived from non-adherent non-T cells. NANT cells from healthy subjects (■, n = 6) and patients with non-severe (■, n = 5) or severe asthma (■, n = 5) were treated with BDNF (10-100 ng/mL) on day 0. Cells were harvested after 3 or 7 days in culture and the percentage of CCR7+ fibrocytes (**A-B**; % Col I+/CD45+/CCR7+ cells) within the NANT cell population and CCR7 MFI (**C-D**) was determined by flow cytometry. Bars represent median with interquartile range. MFI data are expressed as fold-change with respect to untreated controls. The differences between the untreated control group and each treatment group were determined by Friedman test.

4.2.3 The effect T_H2 cytokines and neurotrophins on non-adherent non-T cell death, differentiation and collagen I+/CD45+ fibrocyte proliferation

I investigated whether T_H2 cytokines and neurotrophins increased the number of fibrocytes through their anti-apoptotic, pro-myofibroblastic differentiation, or pro-proliferative effects on fibrocytes. NANT cells from healthy subjects were cultured in FBS-containing media. Annexin V/propidium iodide apoptosis assay (Chapter 2.2.4.4) was performed after 72 hr in culture. Annexin V^-/propidium iodide$^-$ were considered as live cells, Annexin V^+/propidium iodide$^-$ as apoptotic and annexin V^+/propidium iodide$^+$ as late apoptotic cells. IL-4 ($p < 0.05$) and IL-13 ($p < 0.05$) reduced the apoptosis in NANT cells, but there was a trend towards NGF increasing the apoptosis in NANT cells (Figure 4.10A-B). There was also a trend towards an increase in the expression of α-SMA mRNA by IL-4, IL-13 and NGF, suggesting myofibroblastic differentiation was induced by these mediators (Figure 4.10C). DNA synthesis, accessed by Click-iT® EdU cell proliferation assays, was increased by IL-13 and NGF after 24 hr in culture in one healthy subject (Figure 4.10D), suggesting these two mediators may promote cell proliferation.

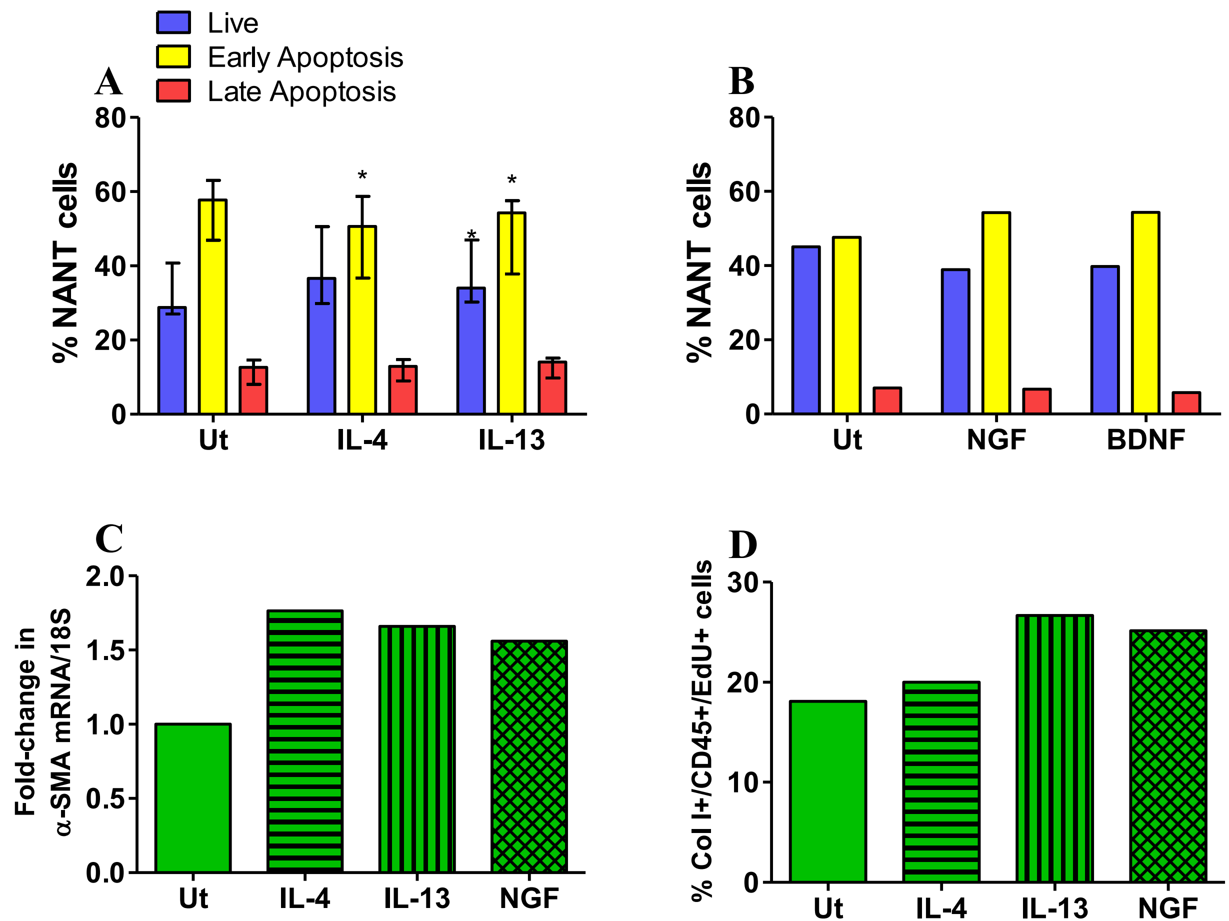


Figure 4.10: The effect of interleukin-4, interleukin-13 and nerve growth factor on apoptosis and α–smooth muscle actin expression in non-adherent non-T cells and proliferation of fibrocytes. NANT cells isolated from healthy subjects were incubated with IL-4 (10 ng/mL), IL-13 (10 ng/mL) or NGF (1000 ng/mL) in FBS-containing medium. The percentage of live (■, annexin V^-/propidium iodide$^-$), and early (■, annexin V^+/propidium iodide$^-$) and late apoptotic NANT cells (■, annexin V^+/propidium iodide$^+$) were accessed using annexin V/propidium iodide assay at 72 hr using flow cytometry (**A**, n = 4; **B**, n = 2). Bars represent median with interquartile range. Data are expressed as proportion in NANT cells. The differences between the untreated (Ut) control group and each treatment group were determined by Friedman test, followed by Dunn's post-hoc test. * $p < 0.05$ compared to Ut controls. Myofibroblastic differentiation of NANT cells was determined by the messenger RNA expression of α–SMA at 24 hr in culture (**C**, n = 2) Cell proliferation was determined by Click-iT® EdU cell proliferation assays at 24 hr in culture (**D**, n = 2). Bars represent median.

4.2.4 Effect of T_H2 cytokines and neurotrophins on fibrocytes derived from adherent peripheral blood mononuclear cells

I further investigated whether the effects of pro-inflammatory mediators on fibrocytes derived from adherent peripheral blood mononuclear cells (PBMC) were similar to their effects on fibrocytes derived from NANT cells. PBMC from healthy subjects were incubated in the presence of FBS in plates pre-coated with fibronectin. IL-4 (10 ng/mL), IL-13 (10 ng/mL), NGF (1000 ng/mL) or BDNF (100 ng/mL) was added on day 3 after changing culture media. Fibrocytes and differentiating fibrocytes in adherent cells were determined on day 6 days using flow cytometry.

IL-4 and IL-13 increased the number of fibrocytes (~1.5-fold, $p < 0.05$) and differentiating fibrocytes (~1.7-fold, $p < 0.05$) in cultured adherent PBMC (Figure 4.11A-B). NGF and BDNF did not affect the number of fibrocytes or differentiating fibrocytes in adherent cells. The results are compatible with the photos taken under light microscopy (Figure 4.11C-G).

The effect of these mediators in the absence of non-adherent PBMC (most T cells were removed; stimulated for 3 days in the presence of FBS; Figure 4.12A-B) and in the absence of FBS (stimulated for 6 days in the absence of FBS; Figure 4.12C-D) were similar but less significant.

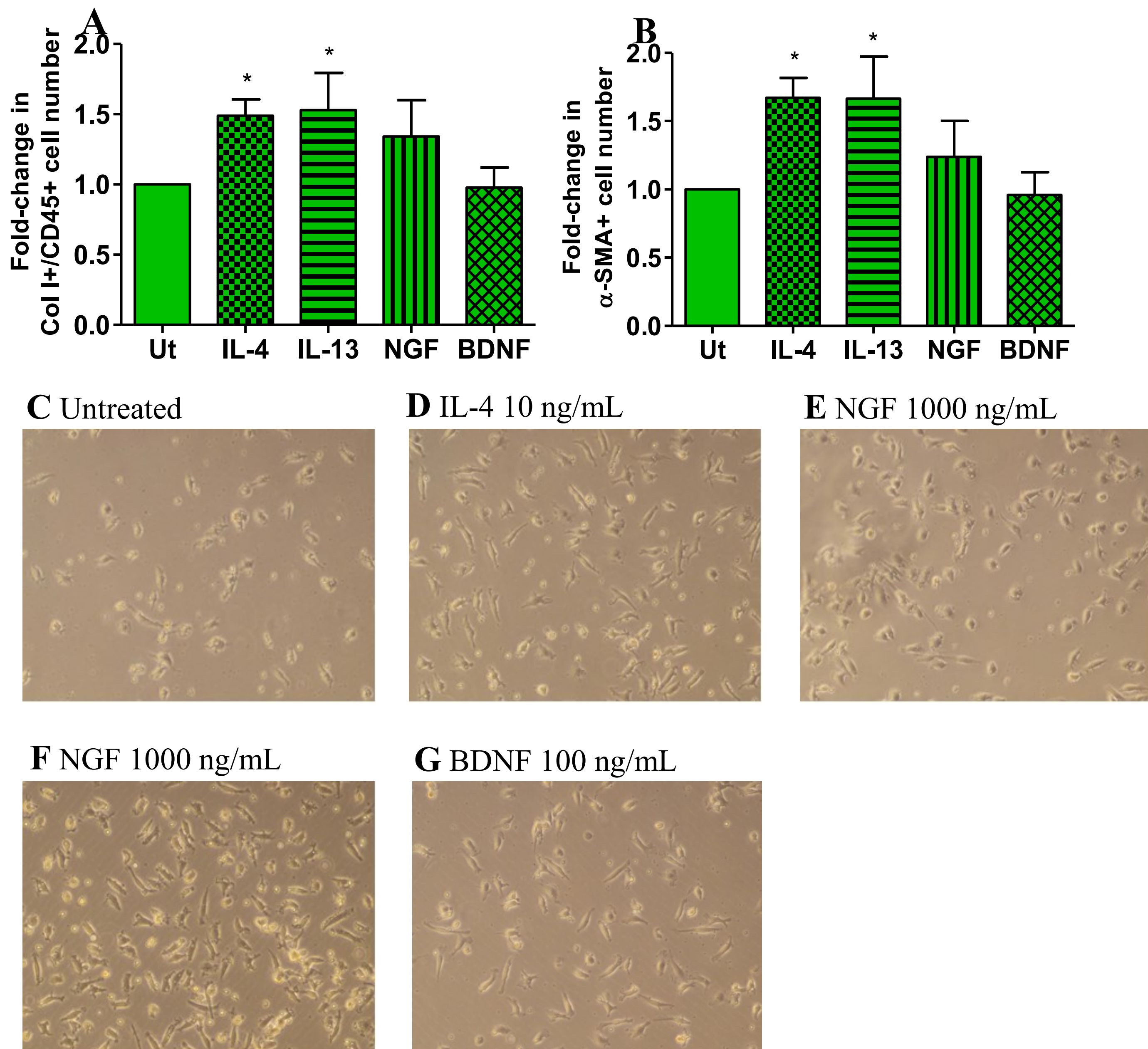


Figure 4.11: The effect of T_H2 cytokines and neurotrophins on fibrocytes derived from adherent peripheral blood mononuclear cells (stimulated on day 3 after removal of adherent cells). PBMC from 6 healthy subjects were seeded in plates pre-coated with fibronectin in the presence of FBS. IL-4 (10 ng/mL), IL-13 (10 ng/mL), NGF (1000 ng/mL) or BDNF (100 ng/mL) was added after changing medium on day 3. Adherent cells were harvested and counted after 6 days in culture. Fibrocytes (Col I+/CD45+ cells; **A**) and differentiating fibrocytes (α-SMA+ cells; **B**) were determined by flow cytometry. Bars represent median with interquartile range. Data of mediator effects were presented as fold-change in cell number with respect to untreated (Ut) controls. The differences between the untreated control group and each treatment group were determined by Friedman test, followed by Dunn's post-hoc test. * $p < 0.05$ compared to untreated controls. Photos of cells from a healthy subject treated with IL-4 (**D**), IL-13 (**E**), NGF (**F**) or BDNF (**G**) were taken at day 6.

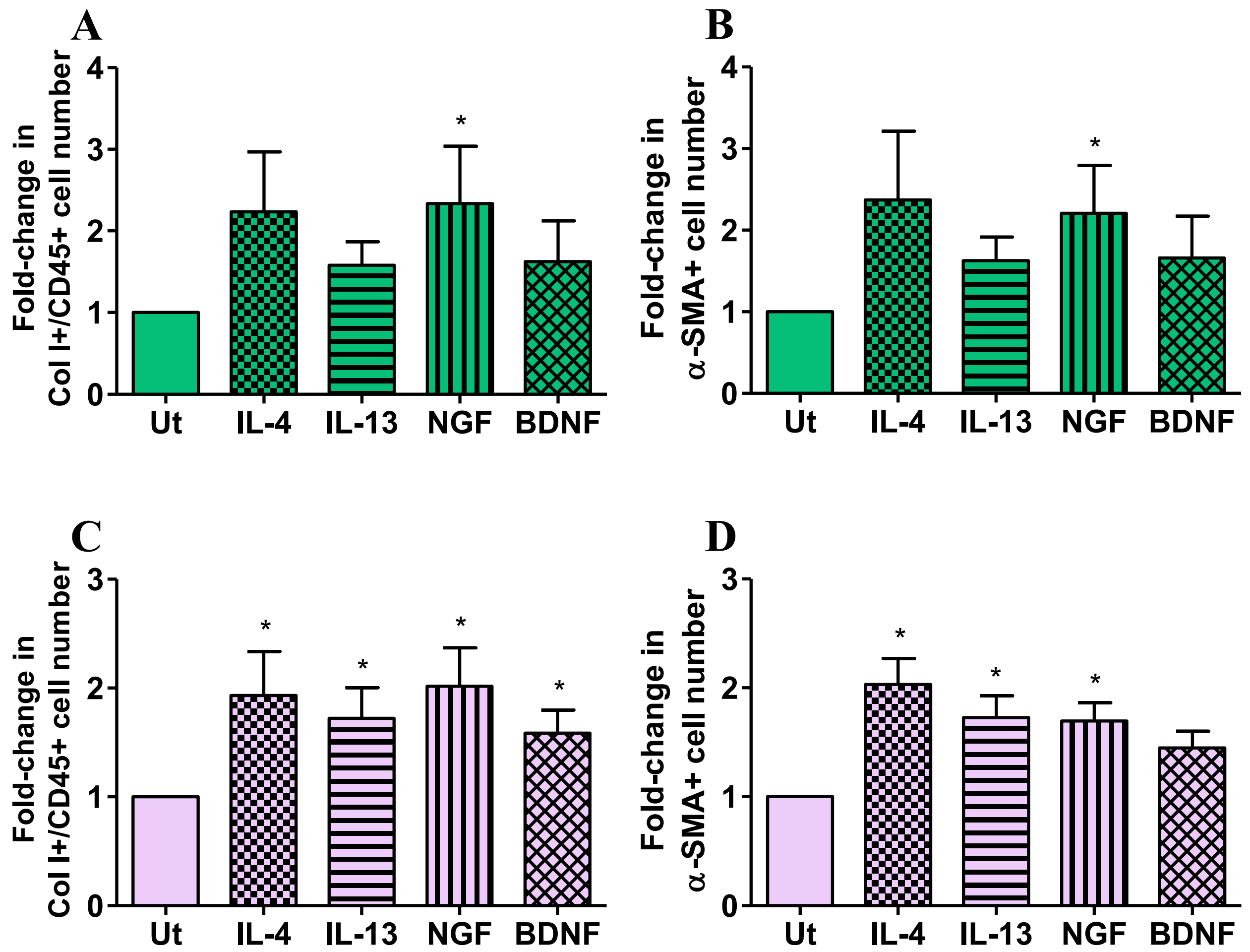


Figure 4.12: Effect of T_H2 cytokines and neurotrophins on fibrocytes derived from adherent peripheral blood mononuclear cells (stimulated on day 0 in the presence of adherent cells). PBMC from 6 healthy subjects were incubated with IL-4 (10 ng/mL), IL-13 (10 ng/mL), NGF (1000 ng/mL) or BDNF (100 ng/mL) in plates pre-coated with fibronectin under FBS-free (■, **A**-**B**) or FBS-containing (■, **C**-**D**) condition. Medium was not changed before adherent cells were harvested on day 6. Fibrocytes (**A**, **C** Col I+/CD45+ cells) and differentiating fibrocytes (**B**, **D** α-SMA+ cells) were determined by flow cytometry. Data are presented as fold-change in cell number with respect to untreated controls. Bars represent median with interquartile range. The differences between the untreated control group and each treatment group were determined by Friedman test.* $p < 0.05$. Ut: untreated; IL-4: interleukin-4; IL-13: interleukin-13; NGF: nerve growth factor; BDNF: brain-derived neurotrophic factor.

4.3 Discussion

In this chapter, responsiveness of fibrocytes to T_H2 cytokines and neurotrophins in patients with severe asthma was compared to that in patients with non-severe asthma and healthy subjects. I showed that the number of fibrocytes and differentiating fibrocytes in NANT cells from healthy subjects and patients with non-severe asthma was increased by IL-4, IL-13 and NGF. These mediators had a similar effect on fibrocytes in adherent PBMC, and did not increase the expression of CCR7. BDNF did not affect fibrocyte function in all three groups. Unexpectedly, the number of fibrocytes from patients with severe asthma was neither affected by IL-4 and IL-13, nor more significantly increased by NGF.

IL-4 and IL-13 are implicated in increased extracellular matrix deposition and increased ASMC layer thickness in the remodelling of asthmatic airways (Takayama, Arima et al. 2006, Doherty and Broide 2007). These two T_H2 cytokines increase the number of spindle-shaped fibrocytes in cultured PBMC from healthy subjects (Shao, Suresh et al. 2008), and the collagen production in fibrocytes from patients with non-severe asthma (Bellini, Marini et al. 2012). Therefore, I investigated whether fibrocytes from patients with severe asthma are more responsive to IL-4 and IL-13. My data showed that IL-4 and IL-13 increased the number of fibrocytes and

differentiating fibrocytes from healthy subjects and patients with non-severe asthma. IL-4 and IL-13 reduced the apoptosis in NANT cells from healthy subjects. It has been reported that IL-4 and IL-13 have anti-apoptotic effects on human conjunctival fibroblasts through the phosphoinositide 3-kinase (PI3K) pathway (Fujitsu, Fukuda et al. 2005). IL-4 and IL-13 increased α–SMA expression in NANT cells. The production of α-SMA is increased by IL-4 and IL-13 in fibroblasts from lung (via c-Jun N-terminal kinase (Hashimoto, Gon et al. 2001)) and by IL-4 in fibroblasts from liver (Pissaia, Aoudjehane et al. 2010). My data also showed that IL-13 increased the proliferation of fibrocytes, although the size of patient population was small.

Intriguingly, my data showed that IL-4 and IL-13 did not increase the number of fibrocytes and differentiating fibrocytes in severe asthma. I propose two possible reasons. Firstly, the unstimulated NANT cells from patients with severe asthma are more resistant to apoptosis (Chapter 5.2.3) and more ready to differentiate into myofibroblasts than NANT cells from patients with non-severe asthma and healthy subjects (Chapter 3.2). IL-4 and IL-13 could hardly further prolong their survival or promote their differentiation. Besides, the expression of IL-4 receptor-α chain (IL-4Rα), shared by the receptors of IL-4 and/or IL-13 (IL-4Rα/IL-13Rα_1, IL-4Rα/IL-4Rγ and IL-4Rα/IL-13Rα_2), may have been altered in fibrocytes from

patients with severe asthma (Chiaramonte, Mentink-Kane et al. 2003, Gibejova, Mrazek et al. 2003). Patients with severe asthma are on high dose inhaled CS, sometimes systemic CS as well. Up-regulation of IL-4Rα induced by polyinosinic:polycytidylic acid in epithelial cells and by phorbol myristate acetate in PBMC is inhibited by dexamethasone (Mozo, Gayo et al. 1998, Tsuji, Yamamoto et al. 2005). Although asthma is commonly known as a T_H2 cytokine disease, interferon (IFN)-γ is increased and IL-4 is decreased in severe asthmatic airways (Shannon, Ernst et al. 2008). IL-4Rα is up-regulated by IL-4 and down-regulated by IFN-γ (So, Park et al. 2000, Bonder, Davies et al. 2002). Circulating fibrocytes might have been exposed to higher levels of CS and IFN-γ and lower level of IL-4 before being isolated from patients with severe asthma, leading to a lower expression of IL-4Rα. Further comparison of the expression of IL-4 and IL-13 receptors in fibrocytes from different asthmatic severity groups is warranted.

NGF and BDNF have been implicated in airway remodelling (Renz and Kilic 2012, Prakash and Martin 2014). The effect of neurotrophins on fibrocytes has not been reported. I investigated whether fibrocytes from patients with severe asthma are more responsive to NGF and BDNF. My data showed that NGF increases the number of fibrocytes and differentiating fibrocytes from both healthy subjects and patients

with non-severe or severe asthma. Furthermore, NGF increases proliferation in fibrocytes and facilitates α-SMA expression in NANT cells from healthy subjects. It has been reported that NGF increases the production of α-SMA in human dermal and pulmonary fibroblasts (Micera, Vigneti et al. 2001) and the proliferation of human dermal microvascular endothelial cells (Raychaudhuri, Raychaudhuri et al. 2001). In contrast to IL-4 and IL-13, there was a trend towards NGF inducing apoptosis in NANT cells. NGF differentially affects cell death in different cells: withdrawal of NGF induces apoptosis in rat-1 fibroblast via p38 pathway (Kummer, Rao et al. 1997), whilst NGF is pro-apoptotic for myofibroblasts from rodent fibrotic liver (Kendall, Hennedige et al. 2009) and human conjunctiva (Micera, Puxeddu et al. 2012). The lower affinity p75 neurotrophin receptor (p75NTR) promotes survival through interactions with the high affinity tropomyosin-receptor-kinase (Trk) receptors, and promotes apoptosis through association with sortilin death signalling complex (Kaplan and Miller 2004, Kraemer, Yoon et al. 2014). NGF had a greater pro-mitotic effect compared to its pro-apoptotic effect in NANT cells. On the other hand, BDNF did not affect the number of fibrocytes and differentiating fibrocytes. It is not known whether fibrocytes express TrkA and TrkB receptors, which preferentially bind to NGF and BDNF, respectively.

My data did not show that NGF increases fibrocytes in severe asthma more significantly than in non-severe asthma. It is possible that at baseline fibrocytes from patients with severe asthma were already more differentiated, so NGF could not further increase their proliferation and differentiation. Furthermore, severe asthmatic fibrocytes may have been exposed to higher dose of CS *in vivo*. It has been reported that CS reduce the expression of TrkA and p75NTR in medullar thymic epithelial cells (Perez-Pinera, Garcia-Suarez et al. 2006). It is also possible that previous exposure to CS down-regulates the expression of TrkA in fibrocytes from patients with severe asthma, leading to less response to NGF. Further measurement of the expression of p75NTR or TrkA in fibrocytes from asthmatic patients with different severity is of interest.

Although it has been reported that IL-4 increases CCR7 expression in human CD8+ lymphocytes (Seneviratne, Black et al. 2007), IL-4, IL-13, NGF and BDNF did not up-regulate the expression of CCR7 in fibrocytes. IL-4 and IL-13 prime the migration of haematopoietic progenitor cells to CXCL12/stromal cell-derived factor 1 (SDF-1) by enhancing CXCR4 incorporation into lipid rafts (Punia, Smith et al. 2012). Investigating whether these mediators promote fibrocyte migration via other chemokine receptors/chemokine axes, such as CXCR4/CXCL12-SDF-1 is warranted.

T_H2 cytokines and neurotrophins exert similar effects on fibrocytes in both the adherent fraction and the NANT fraction of PBMC from healthy subjects recruited to my study. It has been reported that IL-4 and IL-13 increase spindle-shaped fibrocytes derived from PBMC in the presence of non-adherent fraction (e.g. T cells) under FBS-free medium conditions (Shao, Suresh et al. 2008). I showed a similar trend towards IL-4, IL-13 and NGF increasing the number of fibrocytes in adherent cells using the same model, and the effect of these mediators was even more potentiated in the presence of FBS. However, the addition of T_H2 cytokines and neurotrophins to the whole PBMC population might induce the release of mediators from coexisting non-adherent PBMC (mainly T cells), and indirectly affect the fibrocytes in adherent PBMC in a paracrine manner. Although it has been reported the non-monocyte fraction of PBMC, mainly T cells, increases the differentiation of co-cultured adherent fibrocytes via the release of TGF-β, I demonstrated that IL-4, IL-13 and NGF increased the number of fibrocytes and differentiating fibrocytes derived from both adherent fraction and NANT fraction of PBMC even in the absence of T cells.

In summary, IL-4, IL-13 and NGF increase the number of fibrocytes more significantly in non-severe asthmatic patients than in severe asthmatic patients *in vitro*. Although the existence of pro-fibrotic mediators in bronchial biopsies or

broncho-alveolar lavage samples was not determined in my project, increased airway remodelling in severe asthma may not be the result of a heightened responsiveness to pro-fibrotic IL-4, IL-13, NGF or BDNF in severe asthmatic fibrocytes. Increasing the patient population is required to further clarify whether these mediators increase fibrocytes by affecting apoptosis, differentiation and proliferation. This will aid understanding of how these mediators manipulate signalling pathways in fibrocytes.

Chapter 5: Do fibrocytes from patients with severe asthma display corticosteroid insensitivity?

5.1 Background

Patients with severe asthma respond poorly to asthma therapy which mainly consists of corticosteroids (CS) and long acting-β_2 agonists (LABA). Although inflammation may be partly resolved by medication, airway remodelling has been shown not to be reversed by treatment. The molecular and cellular mechanisms behind CS insensitivity are unclear.

Immune and structural cells isolated from patients with severe asthma have been reported to show relative CS insensitivity. CS are less effective in suppressing the release of pro-inflammatory cytokines and/or chemokines from peripheral blood mononuclear cells (PBMC) (Hew, Bhavsar et al. 2006), alveolar macrophages (Bhavsar, Hew et al. 2008) and airway smooth muscle cells (ASMC) (Chang, Bhavsar et al. 2012) from patients with severe asthma than cells from patients with non-severe asthma and healthy subjects, which might be secondary to heightened p38 mitogen-activated protein kinase (MAPK) activation. Furthermore, ASMC from asthmatic patients are resistant to the anti-proliferative effect of CS (Roth, Johnson et al. 2004). Several molecular mechanisms have been reported to mediate CS

insensitivity in cells from patients with severe asthma. For example, activation of MAPK pathways and reduced nuclear translocation in PBMC (Takayama, Arima et al. 2006), up-regulation of glucocorticoid receptor (GR)-β subunit in PBMC, airway T cells and skin cells (Hamid, Wenzel et al. 1999, Sousa, Lane et al. 2000) and decreased histone deacetylase activity in PBMC (Hew, Bhavsar et al. 2006) are postulated to be involved in mechanisms leading to CS insensitivity in severe asthma. Furthermore, reduced total GR (majorly GR-α) expression has been found in PBMC from CS-insensitive nephritis and systemic lupus erythematosus patients (Du, Li et al. 2009, Hammad, Yahia et al. 2013).

In the previous chapter, I demonstrated that patients with severe asthma have a higher number of circulating fibrocytes which have a greater capacity to differentiate *in vitro* (Chapter 3.2.3). However, the effect of CS on the function of fibrocytes from asthmatic patients and whether fibrocytes isolated from patients with severe asthma display CS insensitivity is currently unknown.

In this chapter I will investigate the effect of CS on fibrocyte survival, gene expression, and the expression of the CC chemokine receptor (CCR) 7 which is known to be involved in the recruitment of fibrocytes (Sakai, Wada et al. 2006). I will also determine GR expression both at baseline and in response to CS treatment,

inflammatory cytokines and antioxidant. Finally, I investigated the effect of MAPK inhibition on CS function.

5.2 Results

5.2.1 Effect of systemic corticosteroids on circulating fibrocytes from patients with severe asthma

I investigated whether systemic CS treatment affects the numbers of circulating fibrocytes. To this end, I divided patients with severe asthma into two subgroups: severe asthmatic patients on inhaled CS alone and severe asthmatic patients on inhaled CS and oral CS. Collagen I (Col I)+/CD45+ fibrocytes in freshly isolated non-adherent non-T (NANT) cells from patients with severe asthma were determined by flow cytometry. Patients with severe asthma on oral CS treatment did not have more, or fewer circulating fibrocytes compared to patients with severe asthma who were on inhaled CS alone ($p = 0.44$, Figure 5.1), suggesting that systemic CS therapy may not affect the number of circulating fibrocytes.

p = 0.4417

Col I+/CD45+ cells/mL ($\times 10^4$)

15

10

5

0

SA (Inhaled CS alone)

SA (Oral CS)

Figure 5.1: Effect of systemic corticosteroids on circulating fibrocytes from patients with severe asthma. The number of fibrocytes (Col I+/CD45+ cells) in freshly isolated NANT cells from patients with severe asthma (SA) on oral CS (▼, n = 26) or not (▲, n = 12) was determined by flow cytometry. Horizontal lines represent the median values for each group. Results were analysed using Mann-Whitney test.

5.2.2 Effect of dexamethasone on the number of fibrocytes and differentiating fibrocytes within non-adherent non-T cells from healthy subjects and patients with non-severe or severe asthma

To explore the effect of CS on the number and myofibroblastic differentiation of fibrocytes, NANT cells isolated from healthy subjects and asthmatic patients were cultured in foetal bovine serum (FBS)-containing medium, as previously described (Chapter 2.2.2 and Chapter 3.2.2), and treated with dexamethasone (10^{-8} - 10^{-5} M). Cells were recovered after 3 days and the number of fibrocytes and α-smooth muscle actin (α-SMA)+ differentiating fibrocytes were determined by flow cytometry.

Dexamethasone had an inhibitory effect on the number of fibrocytes ($p < 0.01$, 10^{-7} M) and differentiating fibrocytes ($p < 0.01$, 10^{-7} M) from healthy subjects, as well as the number of fibrocytes ($p < 0.01$, 10^{-7} M) and differentiating fibrocytes ($p < 0.001$, 10^{-8} M) from patients with non-severe asthma (Figure 5.2A-B). Dexamethasone also reduced the total number of NANT cells in healthy subjects ($p < 0.01$, 10^{-7} M) and patients with non-severe asthma ($p < 0.05$, 10^{-8} M; Figure 5.2C), suggesting the effect of dexamethasone was not specific to fibrocytes but also to other non-fibrocytes NANT cells from healthy subjects and patients with non-severe asthma.

In severe asthma, dexamethasone only reduced fibrocyte number at a very high concentration of 10^{-5}M ($p < 0.05$; Figure 5.2A) and did not affect the number of differentiating fibrocytes ($p = 0.25$) and NANT cells ($p = 0.08$; Figure 5.2B-C).

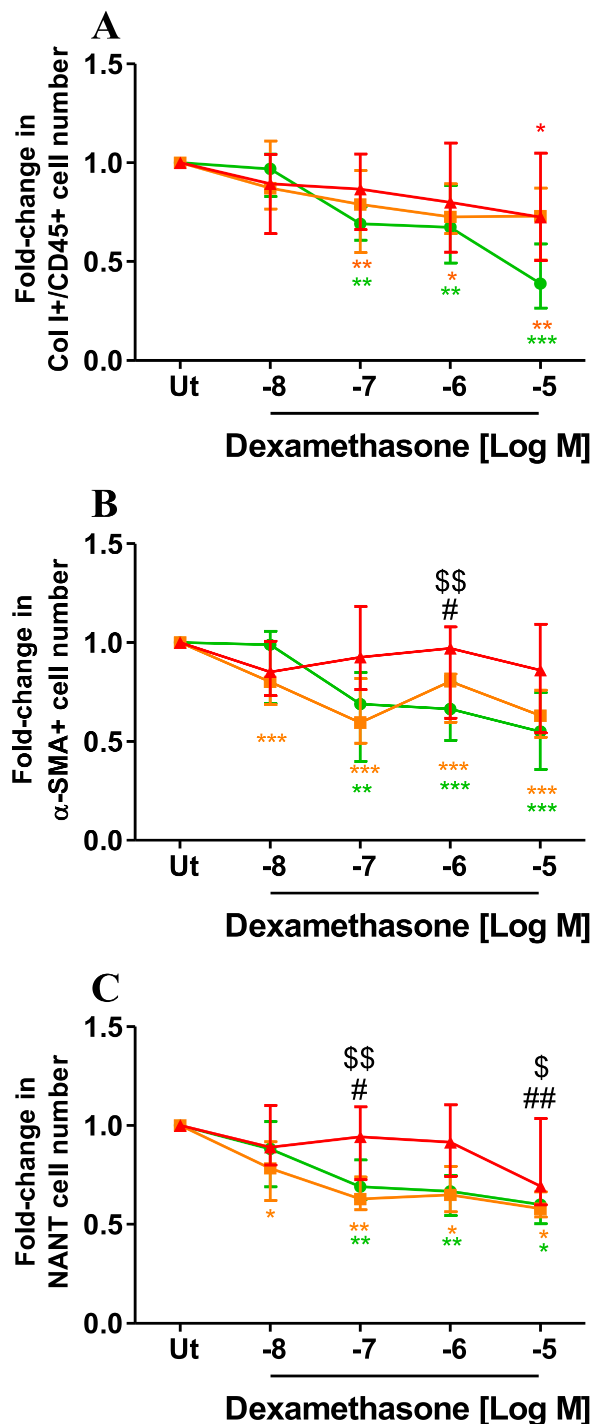


Figure 5.2: Effect of dexamethasone on the number of fibrocytes derived from non-adherent non-T cells. NANT cells from healthy subjects (●, n = 9) and patients with non-severe (■, n = 10) or severe asthma (▲, n = 12) were cultured for 3 days in the presence of dexamethasone (10^{-8} - 10^{-5} M). The number of NANT cells were counted **(C)** and fibrocytes (Col I+/CD45+ cells) **(A)** and differentiating fibrocytes (α-SMA+ cells) **(B)** were determined by flow cytometry. Data are expressed as fold-change compared to untreated controls (Ut). Data points represent median with interquartile range. The differences between control group and each treatment group were determined by Friedman test, followed by Dunn's post-hoc test. The differences between disease groups were determined by Kruskal-Wallis test, followed by Dunn's post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs Ut for each group. # $p < 0.05$, ## $p < 0.01$ vs healthy subjects, \$ $p < 0.05$, \$\$ $p < 0.01$ vs patients with non-severe asthma.

5.2.3 Effect of dexamethasone on the number of fibrocytes and differentiating fibrocytes within adherent fraction of peripheral blood mononuclear cells from healthy subjects and patients with non-severe or severe asthma

I also looked at the effect of dexamethasone on fibrocytes derived from adherent PBMC. PBMC were placed in plates pre-coated with fibronectin in the presence of FBS. Media were changed and dexamethasone (10^{-7} M) was added on day 3. The number of adherent cells and percentage of fibrocytes and differentiating fibrocytes were determined by flow cytometry on day 6.

As shown in the NANT cell model, the number of fibrocytes and differentiating fibrocytes were reduced by dexamethasone in healthy subjects and patients with non-severe asthma ($p < 0.05$), but not in patients with severe asthma (Figure 5.3A-B). Morphologically, dexamethasone significantly reduced the number of spindle-shaped cells in adherent PBMC from healthy subjects and patients with non-severe asthma, but not spindle-shaped cells in adherent PBMC from patients with severe asthma (Figure 5.3C-H).

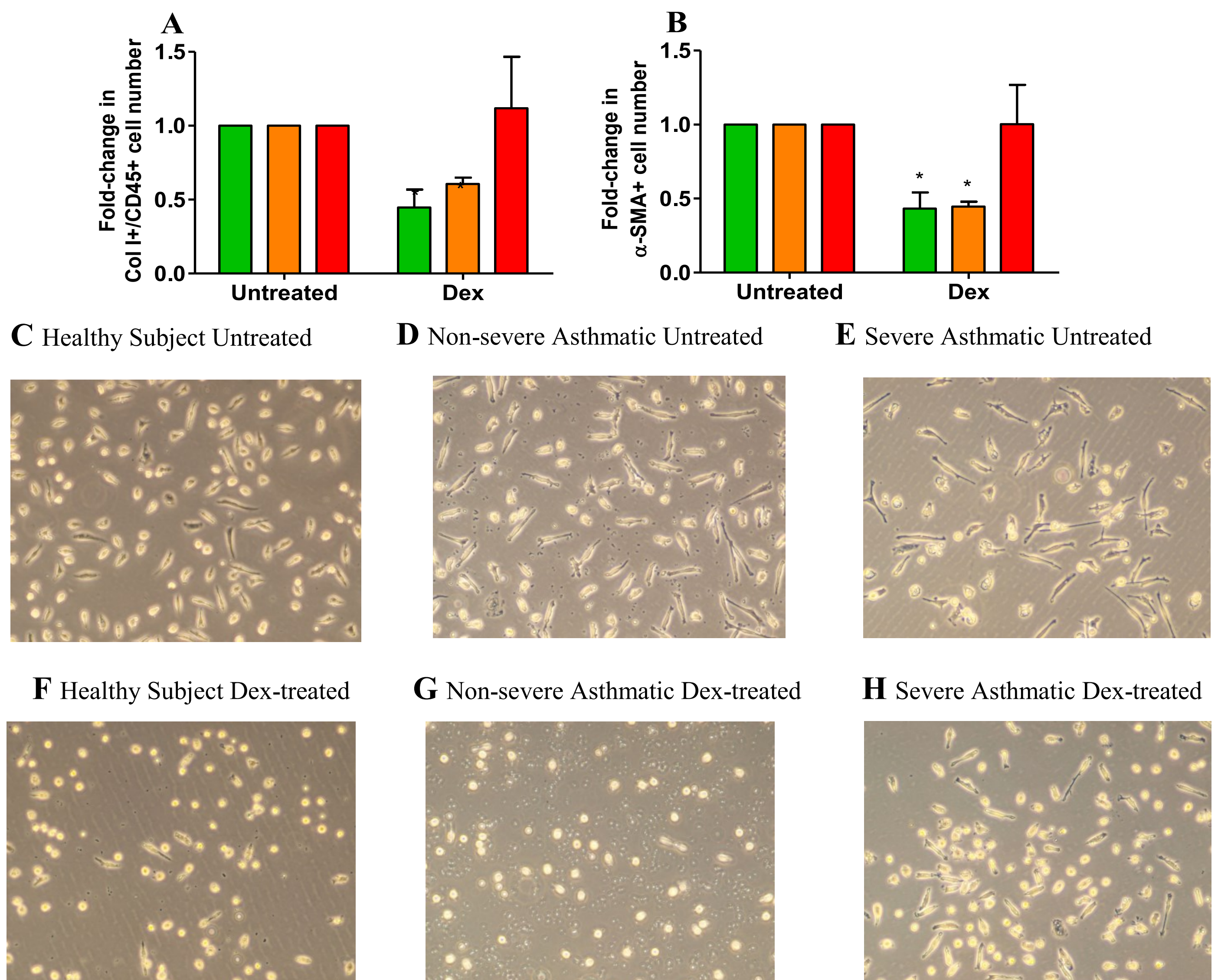


Figure 5.3: Effect of dexamethasone on the number of fibrocytes derived from adherent peripheral blood mononuclear cells. Adherent PBMC from healthy subjects (green, n = 6) and patients with non-severe (orange, n = 6) or severe asthma (red, n = 6) were cultured in plates pre-coated with fibronectin for 3 days followed by incubation with dexamethasone (Dex, 10^{-7}M) for a further 3 days. The number of fibrocytes (Col I+/CD45+ cells; **A**) and differentiating fibrocytes (α-SMA+ cells; **B**) were determined by flow cytometry. Data are presented as fold-change in fibrocyte and differentiating fibrocyte numbers with respect to untreated controls. Bars represent median with interquartile range. Photos of cells from healthy subjects (**C**, **F**) and patients with non-severe (**D**, **G**) or severe asthma (**E**, **H**) were taken after 6 days in culture in the absence (**C**-**E**) and presence (**F**-**H**) of dexamethasone treatment. The differences between the control group and each treatment group were determined by Friedman test, followed by Dunn's post-hoc test. * $p < 0.05$ vs untreated for each group.

5.2.4. Effect of dexamethasone on non-adherent non-T cell apoptosis

I determined the proportion of apoptotic cells in NANT cells in freshly isolated, and dexamethasone-treated, NANT cells by the annexin V/propidium iodide assay (chapter 2.2.4.4). Annexin V^-/propidium iodide$^-$ were considered live cells, Annexin V^+/propidium iodide$^-$ as apoptotic and annexin V^+/propidium iodide$^+$ as late apoptotic cells. Immediately after isolation, the percentage of apoptotic cells was similar in all groups (28.5-30.17%, including 0.2-0.95% in late apoptotic stage; Figure 5.4A). After 72 hr in culture, untreated NANT cells from patients with severe asthma had a higher percentage of live cells ($p < 0.01$) and a lower percentage of early apoptotic cells ($p < 0.05$) compared to NANT cells from healthy subjects (Figure 5.4B), suggesting increased survival of severe asthmatic NANT cells. Dexamethasone (10^{-7} M) treatment further increased late apoptotic NANT cells in the healthy subjects group ($p < 0.05$) and early apoptotic NANT cells in the non-severe asthmatic group ($p < 0.05$), and reduced the live cells in both group ($p < 0.05$; Figure 5.4C-D). The percentage of live cells ($p = 0.31$), early ($p = 0.69$) and late apoptotic cells ($p = 0.07$) were not significantly affected by dexamethasone in NANT cells from patients with severe asthma (Figure 5.4E). Thus, NANT cells, including fibrocytes, from patients with

severe asthma showed increased survival and were resistant to the pro-apoptotic effect of dexamethasone.

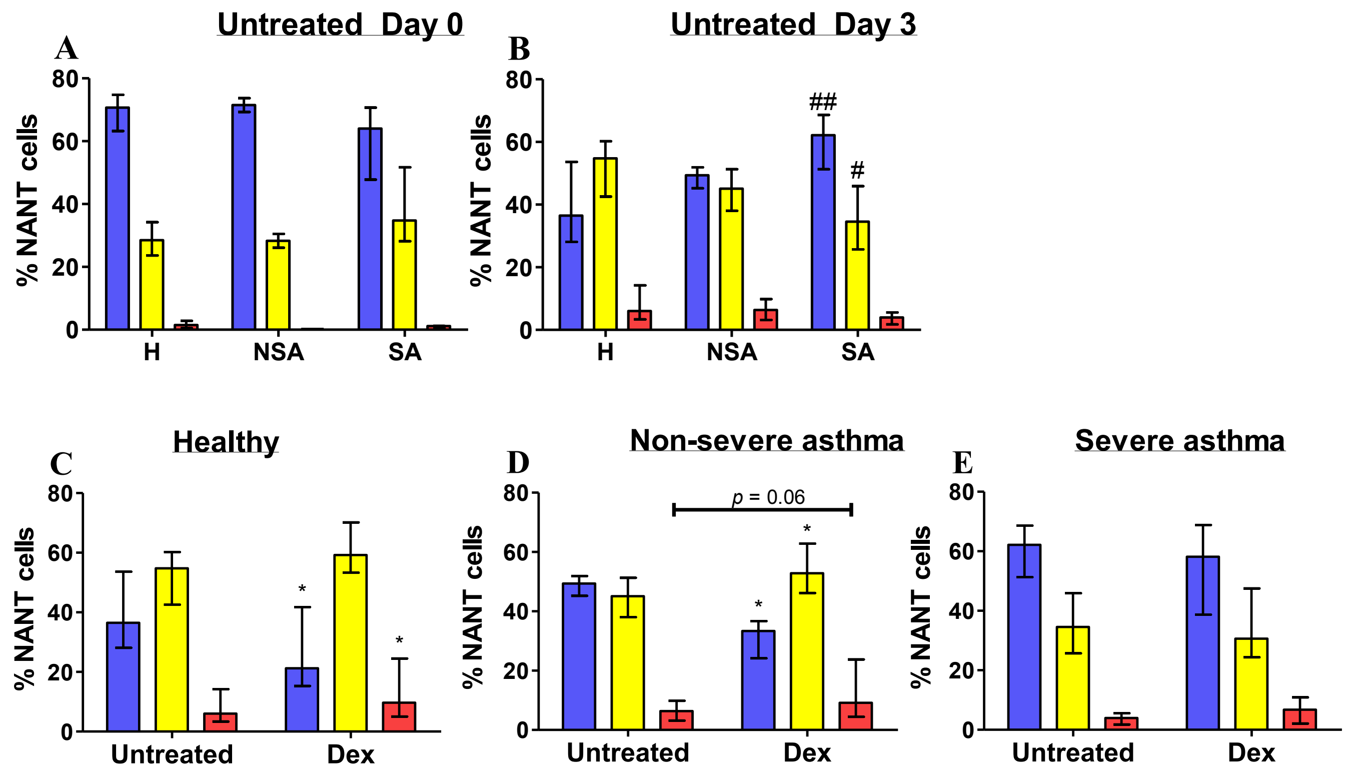


Figure 5.4: Effect of dexamethasone on non-adherent non-T cell apoptosis in healthy subjects and patients with non-severe or severe asthma. The percentage of live (■, annexin V⁻/propidium iodide⁻), and early (■, annexin V⁺/propidium iodide⁻) and late apoptotic NANT cells (■, annexin V⁺/propidium iodide⁺) from healthy subjects (H, n = 4 on day 0, n = 6 on day 3) and patients with non-severe (NSA, n = 2 on day 0, n = 6 on day 3) or severe asthma (SA, n = 4 on day 0, n = 6 on day 3) was determined by flow cytometry immediately after isolation (**A**) or after 3 days in culture in the absence (**B**) or presence of dexamethasone (Dex, 10^{-7} M, **C**-**E**). Bars represent median with interquartile range. The differences amongst disease groups were determined by Kruskal-Wallis test, followed by Dunn's post-hoc test. The differences between the untreated control groups and treatment groups were determined by Wilcoxon matched pairs test. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs healthy subjects. * $p < 0.05$ compared to untreated controls for each group.

5.2.5 Effect of dexamethasone on the gene expression of fibrocyte markers

NANT cells from healthy subjects and patients with severe asthma were cultured in the presence of FBS. Dexamethasone (10^{-7} M) was added at either the beginning (treated for 24 hr) or at the 20 hr time point (treated for 4 hr). The expression of Col I, CD45 and α–SMA mRNAs in NANT cells was determined after 24 hr.

Treatment with dexamethasone for 4 hr ($p < 0.05$) and 24 hr ($p < 0.01$) reduced CD45 mRNA expression in NANT cells from healthy subjects (Figure 5.5B). There was also a trend towards dexamethasone reducing Col I and α-SMA mRNA expression in healthy subjects (Figure 5.5 A and C, respectively). However, the expression of these genes was not affected by dexamethasone in NANT cells from patients with severe asthma.

5.2.6 Effect of dexamethasone on the expression of pro-apoptotic and anti-inflammatory genes

NANT cells from healthy subjects and patients with severe asthma were cultured in the presence of FBS. Dexamethasone (10^{-7} M) was added at either the beginning (treated for 24 hr) or at the 20 hr time point (treated for 4 hr). The expression of

mRNAs of two CS-inducible genes, B-cell lymphoma 2 interacting mediator of cell death-extra long (Bim_{EL}) and glucocorticoid-induced leucine zipper (GILZ), in NANT cells was determined after 24 hr.

Treatment with dexamethasone for 4 hr increased the expression of Bim_{EL} mRNA in NANT cells from patients with severe asthma ($p < 0.05$; Figure 5.6A), and the expression of GILZ mRNA in NANT cells from healthy subjects ($p < 0.05$) and patients with severe asthma ($p < 0.05$; Figure 5.6B). Treatment with dexamethasone for 4 hr induced more GILZ mRNA expression in healthy subjects than in patients with severe asthma ($p = 0.08$). However, dexamethasone effect on Bim_{EL} mRNA expression was not different between healthy subjects and patients with severe asthma ($p = 0.65$).

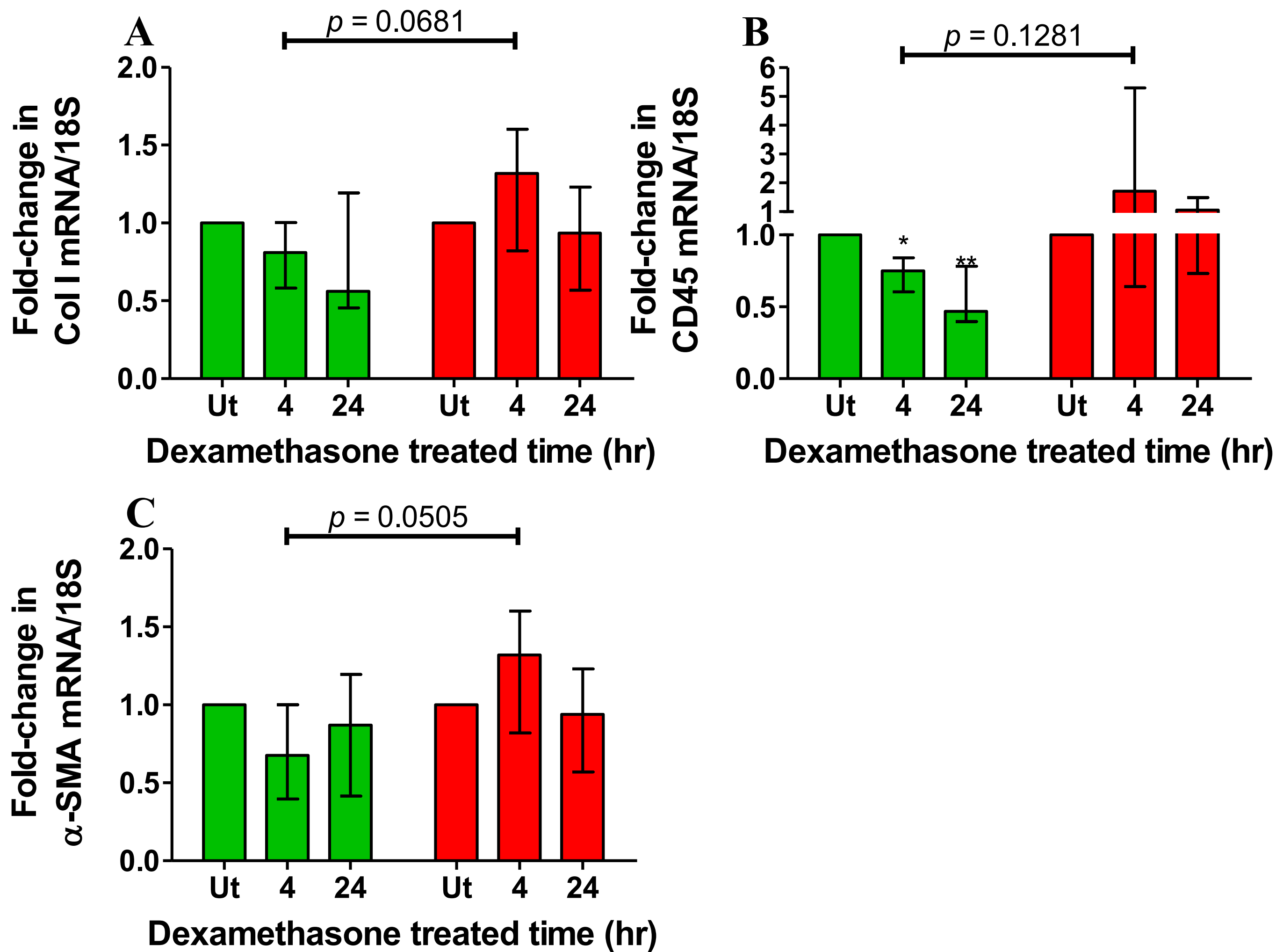


Figure 5.5: Expression of fibrocyte markers in dexamethasone-treated non-adherent non-T cells from healthy subjects and patients with severe asthma. NANT cells isolated from healthy subjects (green, n = 6) and patients with severe asthma (red, n = 3) were incubated for 24 hr. Dexamethasone (10^{-7} M) was added at either the beginning (treated for 24 hr) or at the 20 hr time point (treated for 4 hr). Data are expressed as fold-change in mRNA of Col I (**A**), CD45 (**B**) and α-SMA (**C**) normalized to 18S mRNA, with respect to untreated controls (Ut). Bars represent median with interquartile range. The differences between the untreated control group and each treatment group were determined by Friedman test, followed by Dunn's post-hoc test. The differences between healthy subject group and severe asthmatic group were determined by Mann-Whitney test.

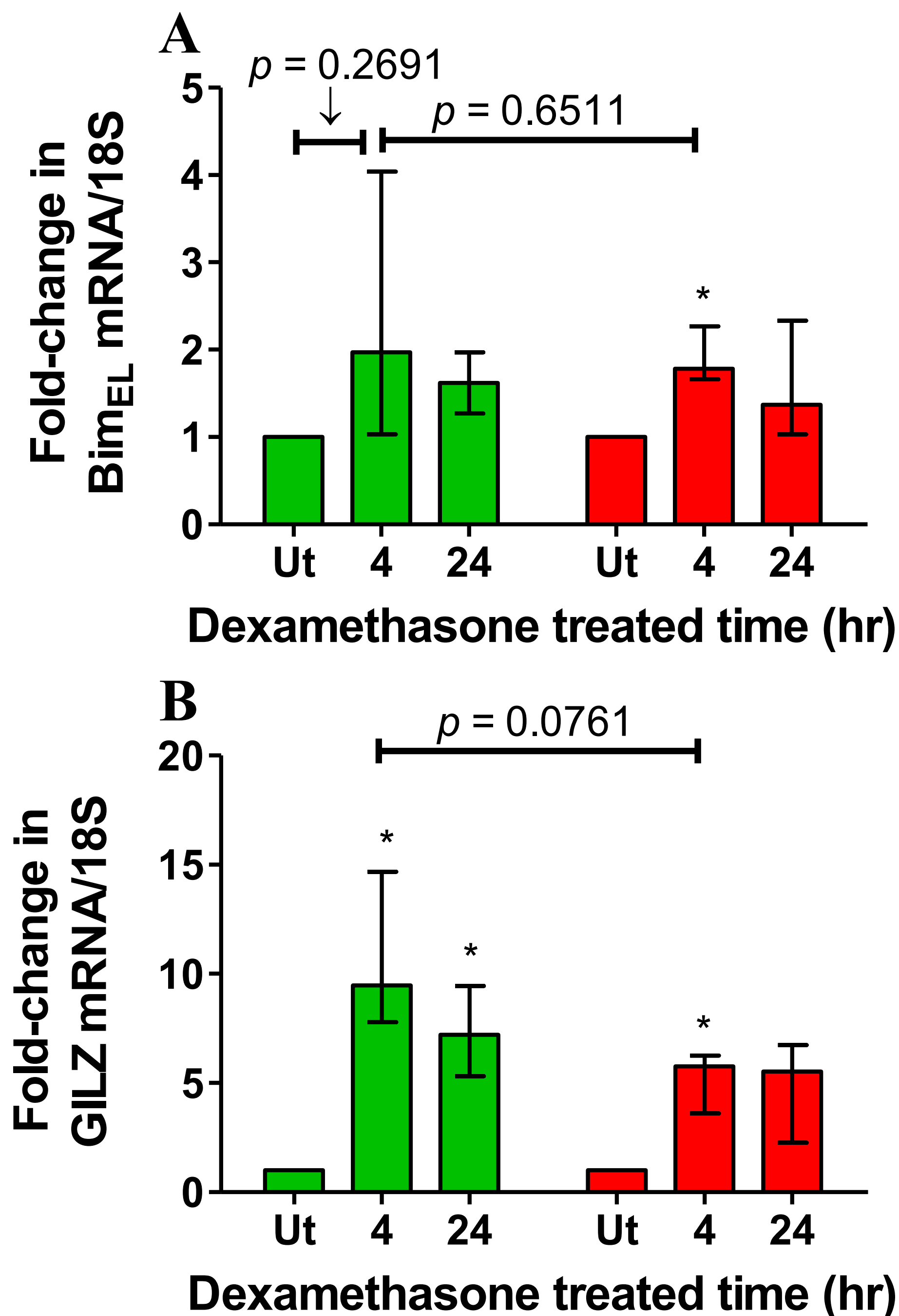


Figure 5.6: Expression of corticosteroid-inducible genes in dexamethasone-treated non-adherent non-T cells from healthy subjects and patients with severe asthma. NANT cells isolated from healthy subjects (■, n = 4) and patients with severe asthma (■, n = 3) were incubated for 24 hr. Dexamethasone (10^{-7} M) was added at either the beginning (treated for 24 hr) or at the 20 hr time point (treated for 4 hr). Data are expressed as fold-change in mRNA of Bim_{EL} (**A**) and GILZ (**B**) normalized to 18S mRNA, with respected to untreated controls (Ut). Bars represent median with interquartile range. The differences between the untreated control group and each treatment group were determined by Friedman test, followed by Dunn's post-hoc test. The differences between healthy subject group and severe asthmatic group were determined by Mann-Whitney test. * $p < 0.05$ compared to Ut control.

5.2.7 Role of the glucocorticoid receptor in corticosteroid action

To confirm that CS act on fibrocytes and NANT cells through the glucocorticoid receptor (GR), NANT cells isolated from healthy subjects were cultured in FBS-containing medium, as described before. NANT cells were pre-treated with the GR antagonist RU486 (mifepristone, 10^{-6} - 10^{-5} M) 1 hr prior to addition of dexamethasone (10^{-7} M). Cells were recovered and counted after 3 days and fibrocytes and differentiating fibrocytes were determined by flow cytometry.

In terms of the number of NANT cells, fibrocytes and differentiating fibrocytes, the effect of dexamethasone was completely blocked by RU486 (Figure 5.7). RU486 itself did not affect the number of NANT cells, including fibrocytes and differentiating fibrocytes, from healthy subjects at the concentration of 10^{-6} - 10^{-5}M.

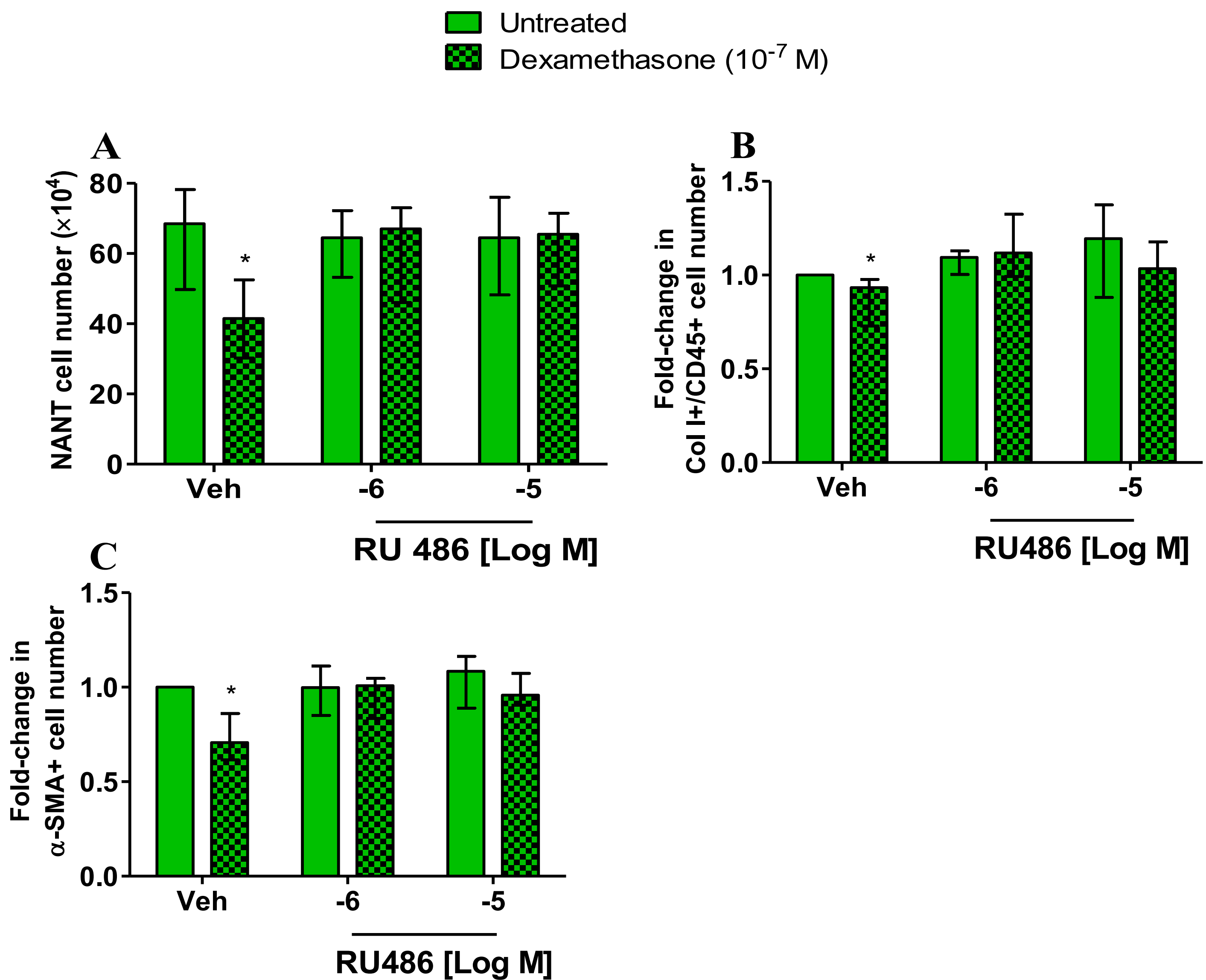


Figure 5.7: Effect of glucocorticoid receptor antagonism on dexamethasone-induced reduction in the number of fibrocytes derived from non-adherent non-T cells. NANT cells from healthy subjects (n = 6) were incubated with either vehicle (Veh; DMSO) or the GR antagonist RU486 (10^{-6} - 10^{-5} M) 1 hr prior to dexamethasone (10^{-7} M). The number of NANT cells was counted and fibrocytes (Col I+/CD45+ cells) and differentiating fibrocytes (α-SMA+ cells) were determined by flow cytometry after 3 days. Data were presented as either total cell number for NANT cells (**A**) or fold-change in the number of fibrocytes (**B**) and differentiating fibrocytes (**C**) compared to vehicle-treated controls. Bars represent median ± interquartile range. The differences between the vehicle-treated control group and each treatment group were determined by Friedman test, followed by Dunn's post-hoc test. * $p < 0.05$ compared to Veh-treated controls.

I subsequently looked at the effect of RU486 on dexamethasone-treated fibrocytes in adherent PBMC model. The effect of dexamethasone (10^{-7} M) on the number of adherent PBMC, including fibrocytes and differentiating fibrocytes, was also inhibited by RU486 (10^{-6} M) in healthy subjects (Figure 5.8A-C). Morphologically, the reduction of spindle-shaped cells caused by dexamethasone was restored by RU486 (Figure 5.8D-H).

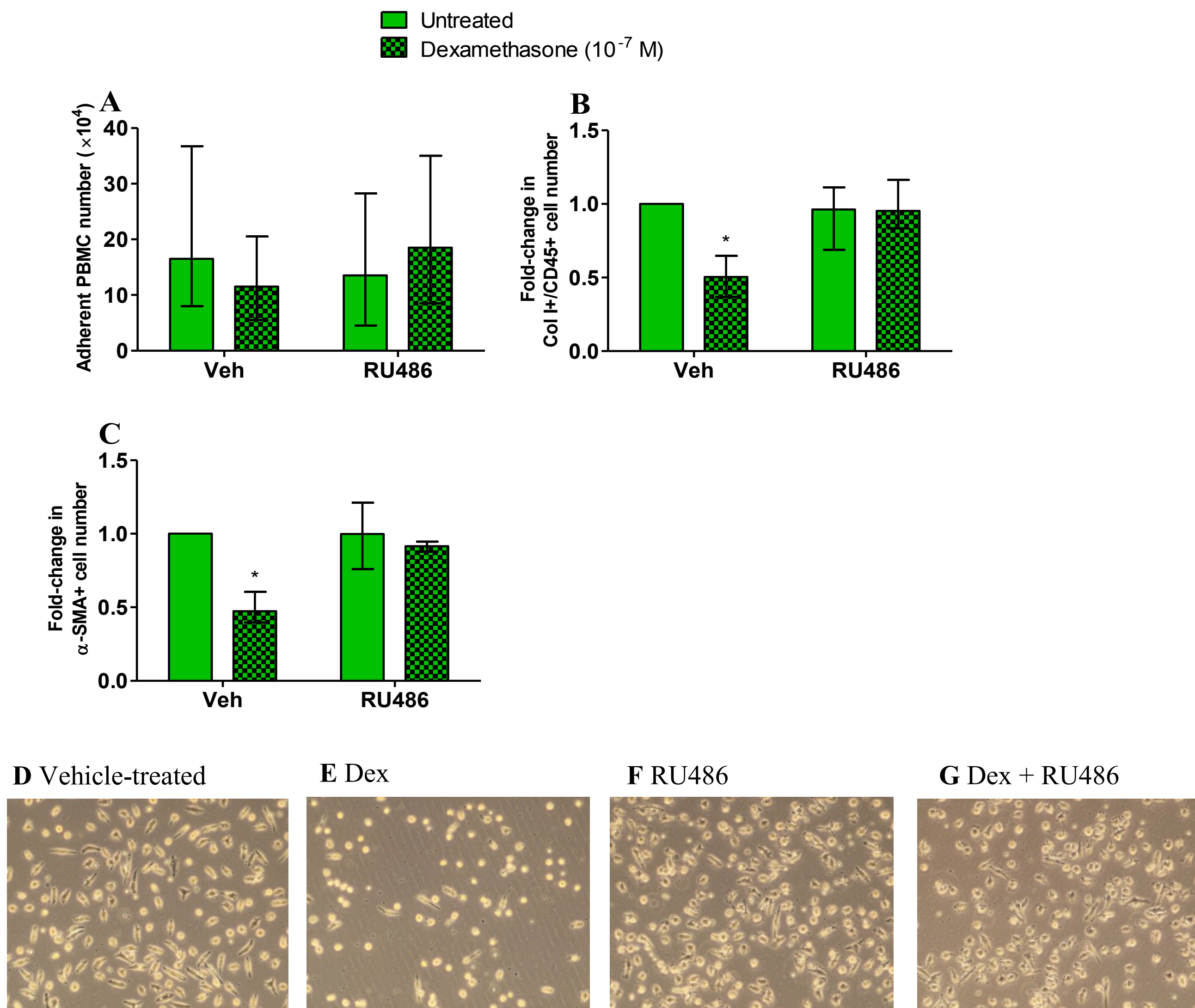


Figure 5.8: Effect of glucocorticoid receptor antagonism on dexamethasone-induced reduction in the number of fibrocytes derived from adherent peripheral blood mononuclear cells. PBMC from healthy subjects (n = 6) were cultured for 3 days, followed by incubation with the GR antagonist RU486 (10^{-6} M) or vehicle (Veh; DMSO) 1 hour prior to dexamethasone (Dex; 10^{-7} M) for a further 3-day period. The number of adherent PBMC were counted (**A**) and fibrocytes (Col I+/CD45+ cells; **B**) and differentiating fibrocytes (α-SMA+ cells; **C**) were determined by flow cytometry. Data are presented as either total cell number for adherent PBMC or fold-change in the number of fibrocytes and differentiating fibrocytes compared to vehicle-treated controls. Bars represent median with interquartile range. The differences between the vehicle-treated control group and each treatment group were determined by Friedman test, followed by Dunn's post-hoc test. * $p < 0.05$ compared to vehicle-treated cells. Photos were taken after 6 days in culture with vehicle (**D**), dexamethasone (**E**), RU486 (**F**), dexamethasone plus RU486 (**G**).

5.2.8 The expression of glucocorticoid receptor in fibrocytes isolated from healthy subjects and patients with non-severe or severe asthma

To investigate a mechanism for reduced CS responsiveness in fibrocytes from patients with severe asthma, the GR expression in fibrocytes in freshly isolated NANT cells from healthy subjects and patients with non-severe or severe asthma were determined by flow cytometry. Compared to healthy subjects and non-severe asthmatics, both the proportion of GR+ fibrocytes (% Col I+/CD45+/GR+ cell; Figure 5.9B) and median fluorescence intensity (MFI) ratio (background fluorescence of isotype control was subtracted from the MFI of the sample, representing the intensity of GR expression; Figure 5.9C) were significantly lower in patients with severe asthma (all $p < 0.01$). The proportion of GR+ NANT cells (Figure 5.9 D) and the GR MFI ratio (Figure 5.9 E) were also reduced in the whole NANT cell isolated from patients with severe asthma compared to healthy subjects ($p < 0.001$ and $p < 0.05$) and patients with non-severe asthma ($p < 0.05$ and $p < 0.05$).

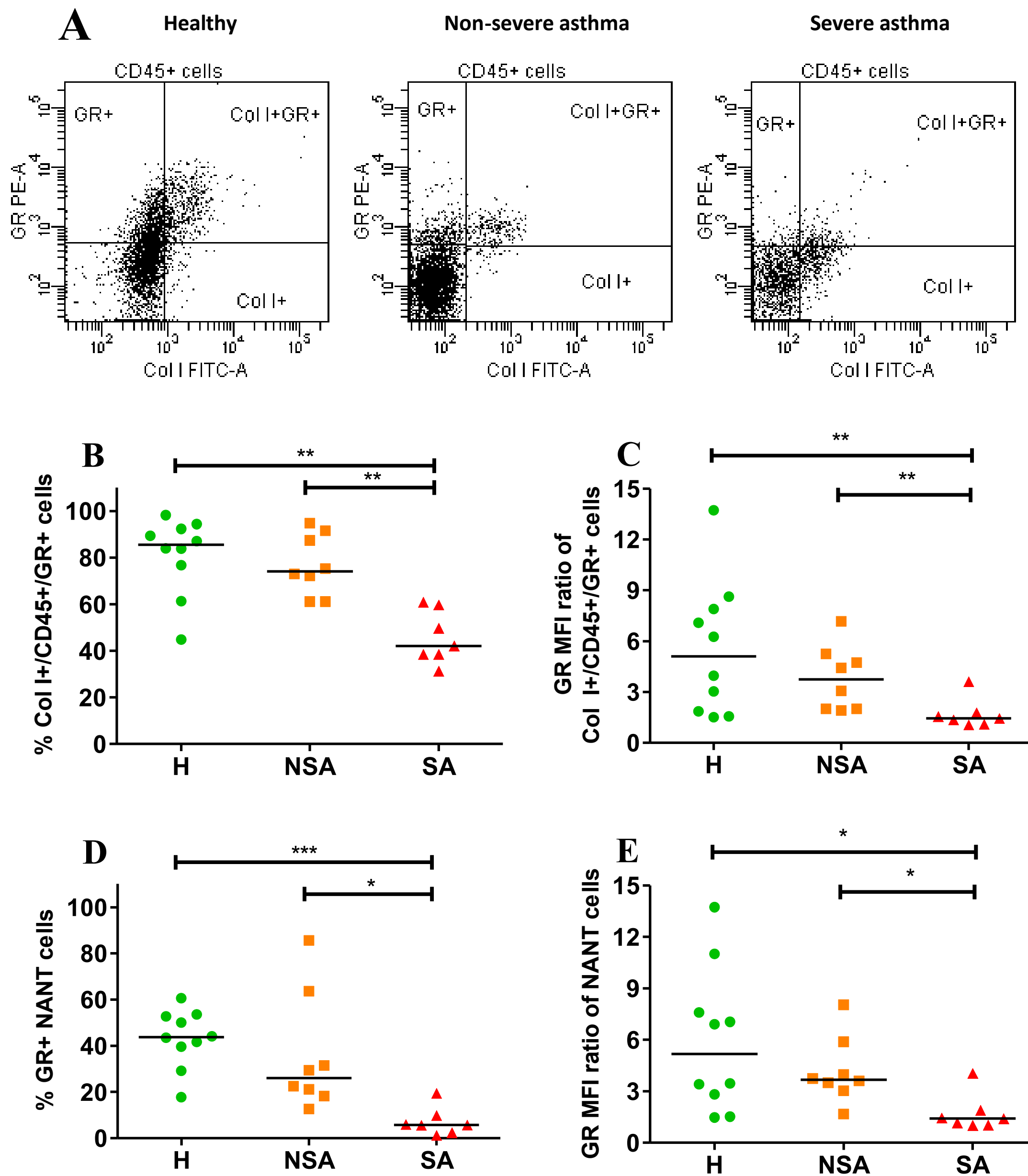


Figure 5.9: Glucocorticoid receptor expression in fibrocytes from healthy subjects and patients with non-severe or severe asthma. Freshly isolated NANT cells from healthy subjects (H, ●, n = 10) and patients with non-severe (NSA, ■, n = 8) or severe asthma (SA, ▲, n = 7) were analysed for the expression of GR. Representative flow cytometric scatter plots from one experiments per group are shown (**A**). The percentage (**B**) and MFI ratio of GR+ fibrocytes (Col I+/CD45+/GR+ cells; **C**), and the percentage (**D**) and MFI ratio of GR+ NANT cells (**E**) were determined. The differences between disease groups were determined by Kruskal-Wallis test, followed by Dunn's post-hoc test. The horizontal lines indicate median values. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.2.9 Effect of T_H2 cytokines, corticosteroids, β_2-adrenoceptor agonist and antioxidant on glucocorticoid receptor expression

To determine whether reduced GR expression in severe asthma is a result of an exposure to pro-inflammatory mediators or asthma medications, NANT cells were incubated with either interleukin(IL)-4 (10 ng/mL), IL-13 (10 ng/mL), dexamethasone (10^{-7} M) and/or salmeterol (10^{-8} M), tiotropium (10^{-6} M) or N-acetylcysteine (10^{-2} M) in FBS-containing medium for a 3-day period. The concentrations of these stimuli used for treatment of fibrocytes were selected from previously published studies (Linden 1992, Micera, Vigneti et al. 2001, Baouz, Giron-Michel et al. 2005, Pieper, Chaudhary et al. 2007, Shao, Suresh et al. 2008, Aravamudan, Thompson et al. 2012, Bellini, Marini et al. 2012). The expression of GR in fibrocytes was quantified by flow cytometry.

The proportion of GR+ fibrocytes and GR MFI ratio were not affected by IL-4 and IL-13 in healthy subjects (Figure 5.10A-B) and by dexamethasone and/or salmeterol and tiotropium in healthy subjects and patients with non-severe or severe asthma (Figure 5.10C-F). Interestingly, N-acetylcysteine increased the proportion of GR+ fibrocytes (% Col I+/CD45+/GR+ cell) and the GR MFI ratio in patients with

severe asthma ($p < 0.05$), but not in healthy subjects and patients with non-severe asthma (Figure 5.10G-H).

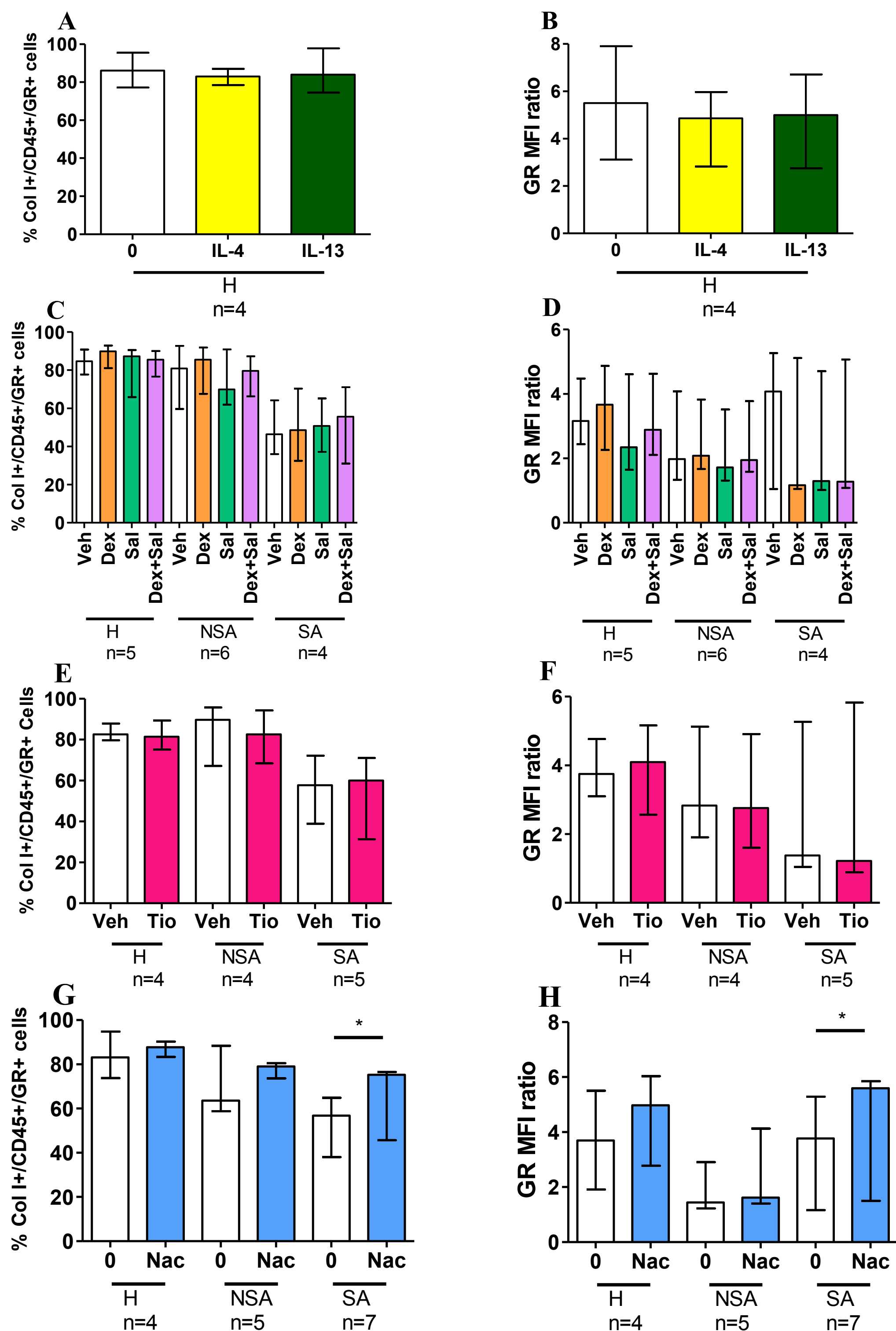


Figure 5.10: Effect of T_H2 cytokines, asthma medications and anti-oxidant on the expression of glucocorticoid receptor in fibrocytes. NANT cells were incubated with IL-4 (10 ng/mL), IL-13 (10 ng/mL) (**A**-**B**), dexamethasone (Dex; 10^{-7} M) and/or salmeterol (Sal; 10^{-8} M) (**C**-**D**), tiotropium (Tio; 10^{-6} M; **E**-**F**) and N-acetylcysteine (Nac; 10^{-4} M; **G**-**H**). The proportion of GR+ fibrocytes within the NANT cell population (% Col I+/CD45+/GR+ cells) and MFI ratio was determined by flow cytometry after 3 days. Bars represent median with interquartile range. For intra-group comparison, results were analysed using the Friedman test, followed by Dunn's post-hoc test (**A**-**D**), or Wilcoxon matched pairs test (**E**-**H**). H: healthy subjects, NSA: non-severe asthmatics, SA: severe asthmatics, Veh: vehicle (DMSO), * $p < 0.05$.

5.2.10 Effect of dexamethasone on CC chemokine receptor 7 expression in fibrocytes isolated from healthy subjects and patients with non-severe or severe asthma

As CCR7 mediates the chemotaxis of fibrocytes to diseased sites (Sakai, Wada et al. 2006), I therefore investigated whether CS treatment affects CCR7 expression in fibrocytes of asthma. NANT cells from patients with non-severe or severe asthma were treated with dexamethasone (10^{-8} - 10^{-5} M) in FBS-containing medium. The expression of CCR7 in fibrocytes was determined by flow cytometry after 3 days in culture (Figure 5.11A-C). Dexamethasone reduced the proportion of CCR7+ fibrocytes ($p < 0.05$, 10^{-7} M) and CCR7 MFI ratio ($p < 0.05$, 10^{-7} M) in non-severe asthma in a concentration-dependent manner, but not in severe asthma, suggesting the expression of CCR7 in fibrocytes is less sensitive to CS inhibition in severe asthma.

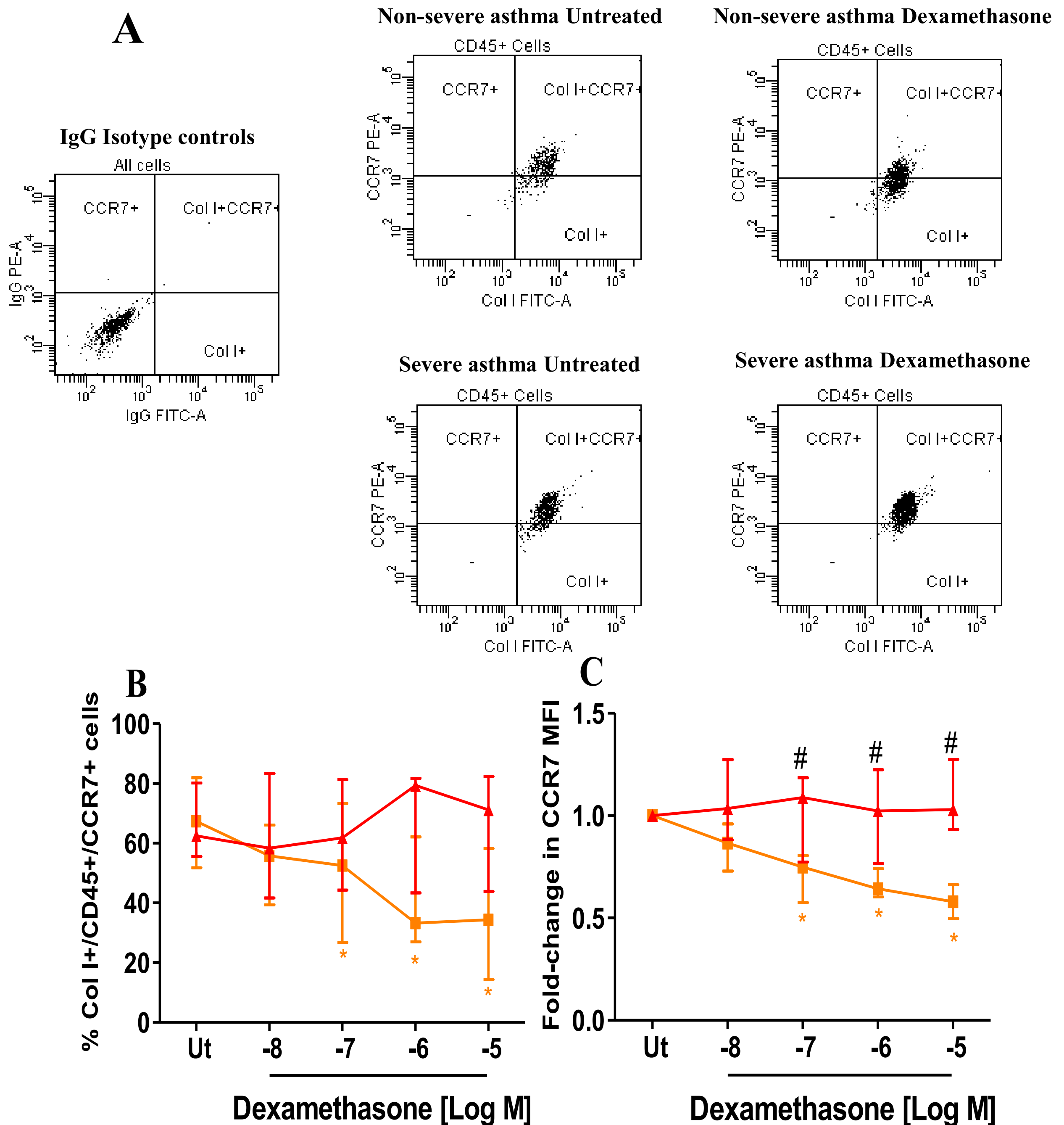


Figure 5.11: Effect of dexamethasone on CC chemokine receptor 7 expression in fibrocytes from patients with non-severe or severe asthma. Cultured NANT cells isolated from patients with non-severe (■, n = 8) or severe asthma (▲, n = 9) were treated with dexamethasone (10^{-8} -10^{-5} M) for a 3-day period. **(A)** Representative flow cytometric scatter plots from one experiment per group are shown. Percentage of CCR7+ fibrocytes within the NANT cell population (% Col I+/CD45+/CCR7+ cells; **B)** and fold-change in MFI (**C**) after dexamethasone treatment was determined on NANT cells from the same patients. Data points represent median with interquartile range. The differences between the untreated control group and each treatment group were determined by Friedman test, followed by Dunn's post-hoc test. The differences between non-severe asthmatic and severe asthmatic patients were determined by Mann-Whitney test. * $p < 0.05$, ** $p < 0.01$ vs untreated (Ut) control. # $p < 0.05$ vs patients with non-severe asthma.

5.2.11 Role of kinases on fibrocytes and their corticosteroid responsiveness

The phosphorylation status of GR is associated with less CS inhibition of induced pro-inflammatory mediators in alveolar macrophages, PBMC, ASMC in severe asthma (Hew, Bhavsar et al. 2006, Bhavsar, Hew et al. 2008, Chang, Bhavsar et al. 2012). Here I investigated the role of three MAPKs: p38 MAPK, c-jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) as well as phosphatidylinositol 3'-kinase (PI3K) in CS-induced fibrocyte reduction in in severe asthma.

NANT cells from patients with non-severe or severe asthma were incubated with dexamethasone (10^{-7} M) and/or kinase inhibitors (p38 MAPK inhibitor SB202190 at 10^{-6} M; JNK inhibitor SP600125 at 10^{-6} M; ERK inhibitor U0126 at 10^{-6} M; PI3K inhibitor LY294002 at 10^{-5} M; or vehicle DMSO) in the presence of FBS for a 3-day period. Fibrocytes and differentiating fibrocytes were identified by flow cytometry.

5.2.11.1 p38 mitogen-activated protein kinase

Dexamethasone reduced the number of fibrocytes and differentiating fibrocytes from patients with non-severe asthma (Figure 5.12A, C) but not the number of these cells from patients with severe asthma (Figure 5.12B, D). SB202190 alone did not affect

the number of these cells from patients with either non-severe or severe asthma (Figure 5.12A-D). In the presence of SB202190, dexamethasone did not reduce the number of fibrocytes ($p = 0.31$; Figure 5.12B) and differentiating fibrocytes ($p = 0.38$; Figure 5.12D) from patients with severe asthma.

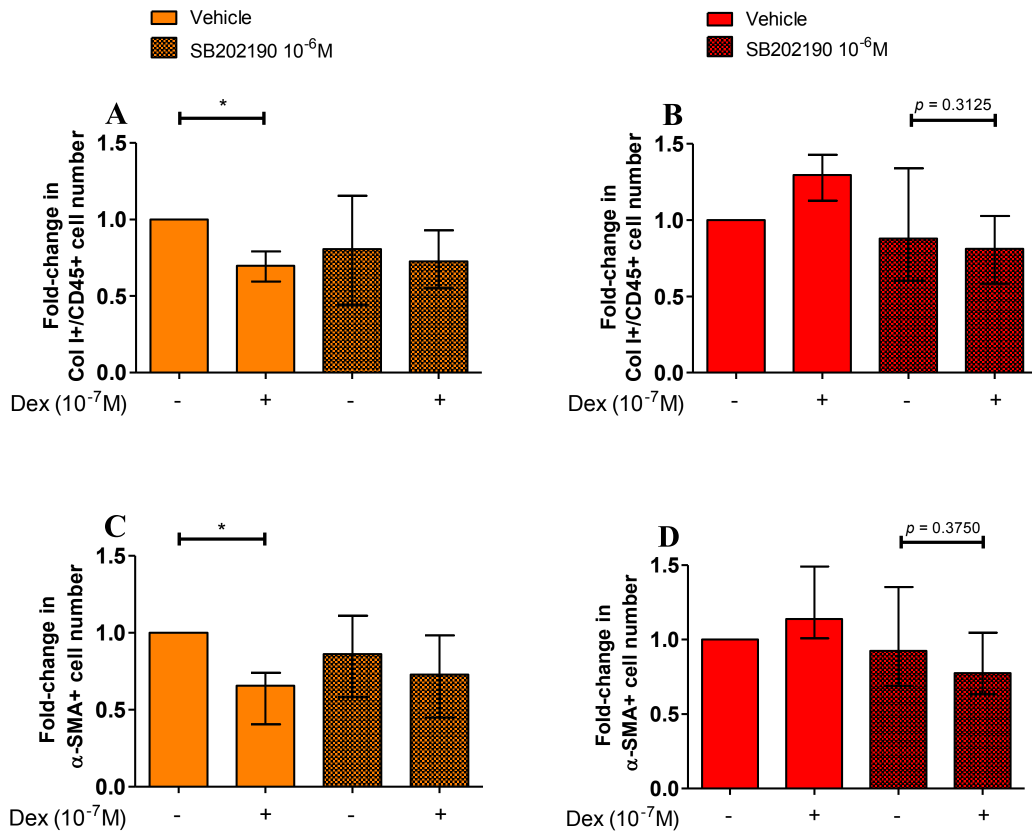


Figure 5.12: Effect of p38 mitogen-activated protein kinase inhibitor on corticosteroid sensitivity of fibrocytes in non-adherent non-T cells from patients with asthma. Cultured NANT cells, isolated from patients with non-severe (**A**, **C**, ■, n = 6) or severe asthma (**B**, **D**, ■, n = 7), were treated with p38 MAPK inhibitor SB202190 (10^{-6} M) ± dexamethasone (10^{-7} M) for a 3-day period. Fibrocytes (Col I+/CD45+ cells; **A**-**B**) and differentiating fibrocytes (α-SMA+ cells; **C**-**D**) were determined by flow cytometry. Data are expressed as fold-change in cell number with respect to vehicle-treated controls. Bars represent median with interquartile range. The differences between the vehicle-treated control group and each treatment group were determined by Friedman test, followed by Dunn's post-hoc test. * $p < 0.05$.

5.2.11.2 c-Jun N-terminal kinase

Dexamethasone reduced the number of fibrocytes and differentiating fibrocytes from patients with non-severe asthma (Figure 5.13A, C) but not the number of these cells from patients with severe asthma (Figure 5.13B, D). SP600125 alone reduced the number of differentiating fibrocytes ($p < 0.05$; Figure 5.13C), but not fibrocytes ($p = 0.09$; Figure 5.13A) from patients with non-severe asthma. SP600125 did not affect the number of cells from patients with severe asthma (Figure 5.13D). In the presence of SP600125, dexamethasone significantly reduced the numbers of fibrocytes ($p < 0.05$; Figure 5.13B), but not differentiating fibrocytes ($p = 0.58$, Figure 5.13D), from patients with severe asthma.

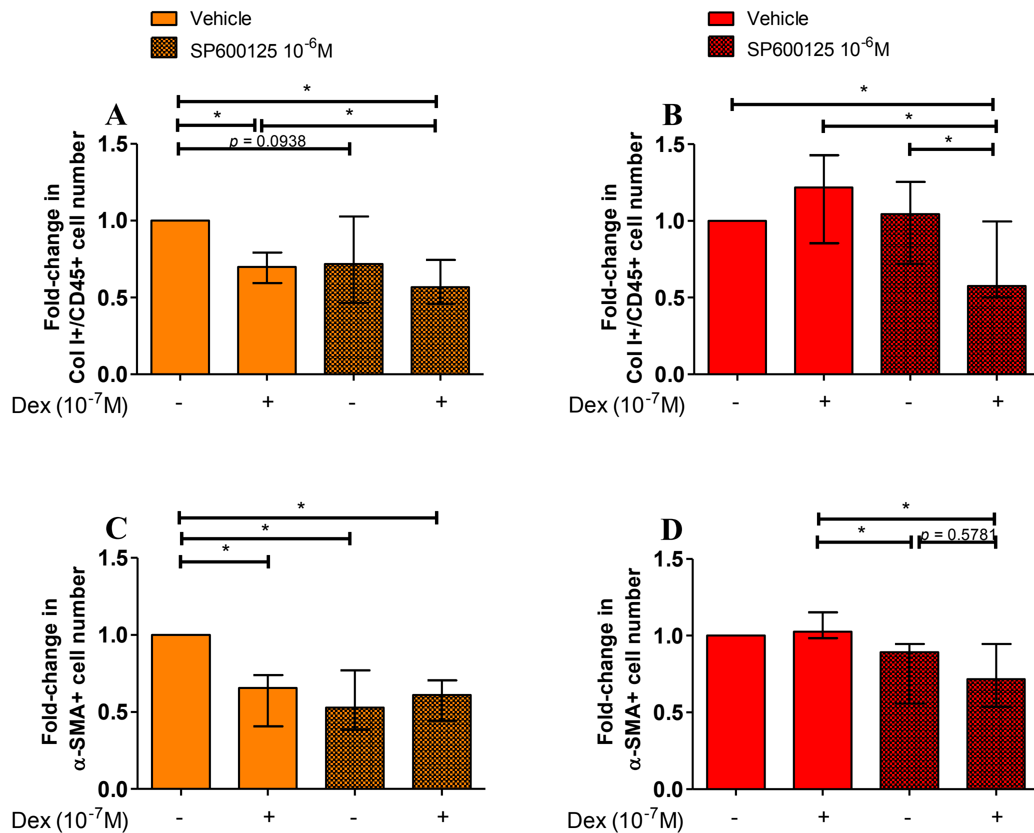


Figure 5.13: Effect of c-jun N-terminal kinase inhibitor on corticosteroid sensitivity of fibrocytes in non-adherent non-T cells from patients with asthma. Cultured NANT cells, isolated from patients with non-severe (**A**, **C**, ■, n = 6) or severe asthma (**B**, **D**, ■, n = 7), were treated with JNK inhibitor SP600125 (10^{-6} M) ± dexamethasone (10^{-7} M) for a 3-day period. Fibrocytes (Col I+/CD45+ cells; **A**-**B**) and differentiating fibrocytes (α-SMA+ cells; **C**-**D**) were determined by flow cytometry. Data are expressed as fold-change in cell number with respect to vehicle-treated controls. Bars represent median with interquartile range. The differences between the vehicle-treated control group and each treatment group were determined by Friedman test, followed by Dunn's post-hoc test. * $p < 0.05$.

5.2.11.3 Extracellular signal-regulated kinase

Dexamethasone reduced the number of fibrocytes and differentiating fibrocytes from patients with non-severe asthma (Figure 5.14A, C) but not the number of these cells from patients with severe asthma (Figure 5.14B, D). U0126 alone reduced the number of fibrocytes from patients with non-severe ($p < 0.01$; Figure 5.14A) and severe asthma ($p < 0.05$; Figure 5.14B), and differentiating fibrocytes from non-severe ($p < 0.01$; Figure 5.14C); however its effect on the number of differentiating fibrocytes from patients with severe asthma was less significant ($p = 0.13$; Figure 5.14D). In the presence of U0126, dexamethasone did not affect the numbers of fibrocytes ($p = 0.94$; Figure 5.14B) and differentiating fibrocytes ($p = 0.47$; Figure 5.14D) from patients with severe asthma.

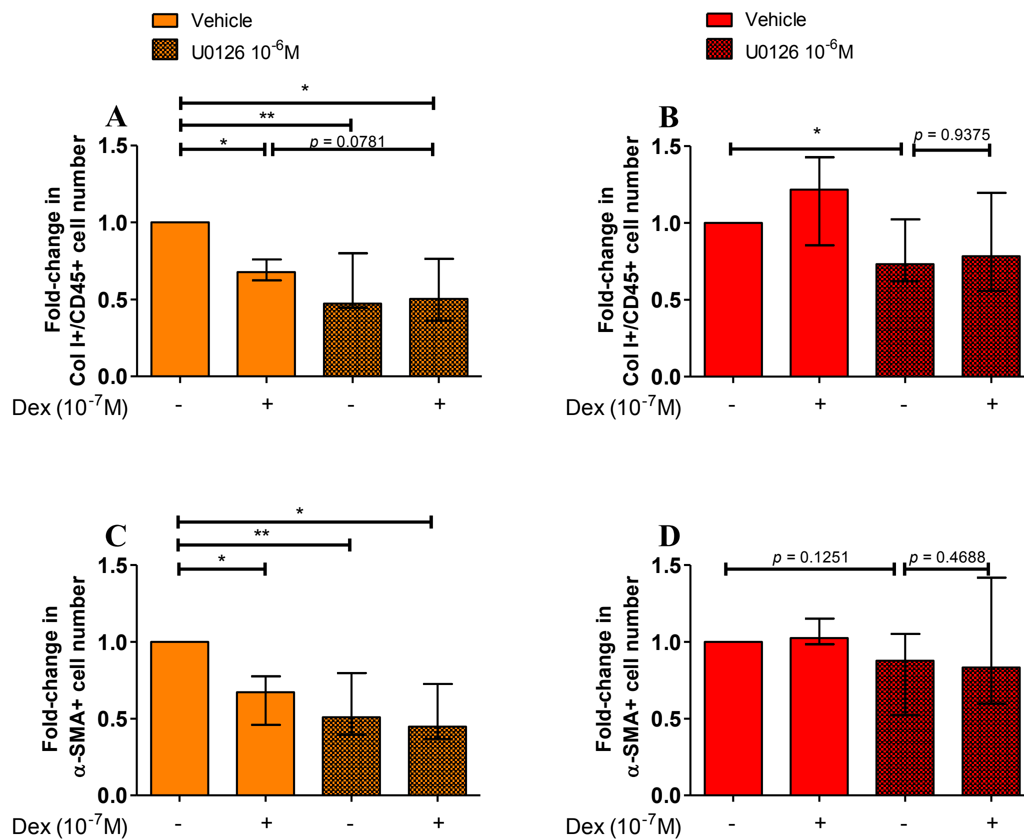


Figure 5.14: Effect of extracellular signal-regulated kinase inhibitor on corticosteroid sensitivity of fibrocytes in non-adherent non-T cells from patients with asthma. Cultured NANT cells, isolated from patients with non-severe (**A**, **C**, ■, n = 7) or severe asthma (**B**, **D**, ■, n = 7), were treated with ERK inhibitor U0126 (10^{-6} M) ± dexamethasone (10^{-7} M) for a 3-day period. Fibrocytes (Col I+/CD45+ cells; **A**-**B**) and differentiating fibrocytes (α-SMA+ cells; **C**-**D**) were determined by flow cytometry. Data are expressed as fold-change in cell number with respect to vehicle-stimulated controls. Bars represent median with interquartile range. The differences between the vehicle-treated control group and each treatment group were determined by Friedman test, followed by Dunn's post-hoc test. * $p < 0.05$, ** $p < 0.01$.

5.2.11.4 Phosphatidylinositol 3'-kinase

Dexamethasone reduced the number of fibrocytes and differentiating fibrocytes from patients with non-severe asthma (Figure 5.15A, C) but not the number of these cells from patients with severe asthma (Figure 5.15B, D). LY294002 alone reduced the number of fibrocytes from patients with non-severe ($p < 0.05$; Figure 5.15A) or severe asthma ($p < 0.01$; Figure 5.15B), and differentiating fibrocytes from patients with non-severe ($p < 0.05$; Figure 5.15C) or severe asthma ($p < 0.01$; Figure 5.15D). In the presence of LY294002, dexamethasone did not affect the numbers of fibrocytes ($p = 1.00$; Figure 5.15B) and differentiating fibrocytes ($p = 0.69$; Figure 5.15D) from patients with severe asthma.

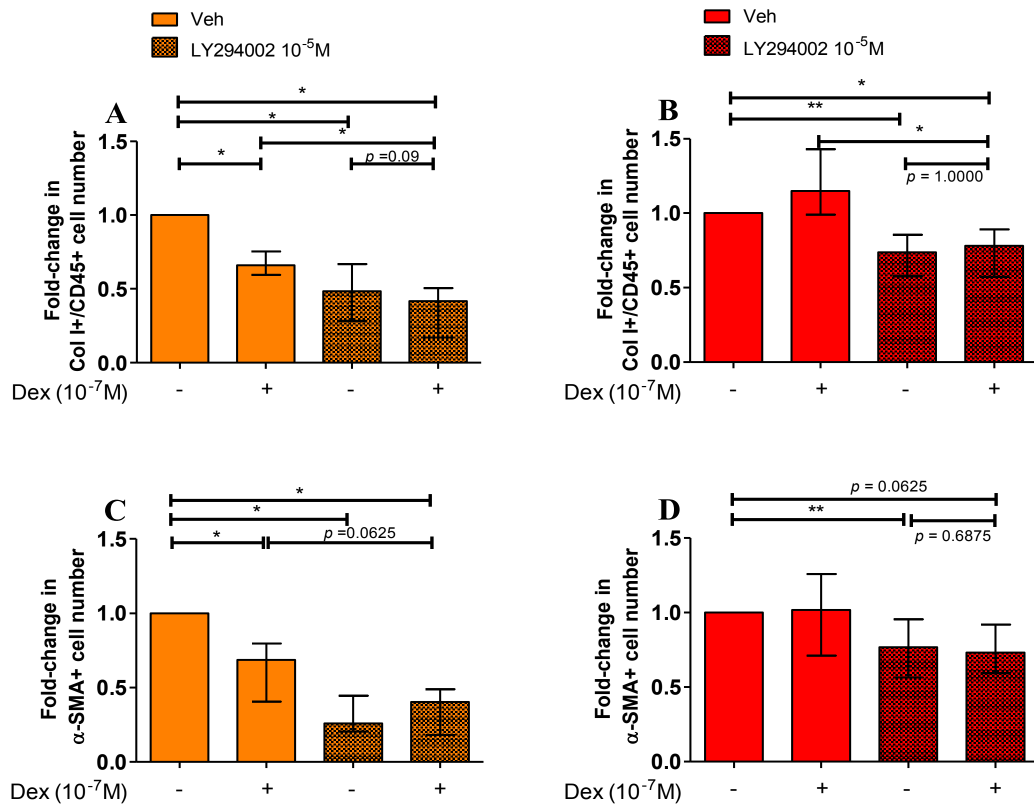


Figure 5.15: Effect of phosphoinositide 3-kinases inhibitor on corticosteroid sensitivity of fibrocytes in non-adherent non-T cells from patients with asthma. Cultured NANT cells, isolated from patients with non-severe (**A**, **C**, ■, n = 6) or severe asthma (**B**, **D**, ■, n = 6), were treated with PI3K inhibitor LY294002 (10^{-5} M) ± dexamethasone (10^{-7} M) for a 3-day period. Fibrocytes (Col I+/CD45+ cells; **A-B**) and differentiating fibrocytes (α-SMA+ cells; **C-D**) were determined by flow cytometry. Data are expressed as fold-change in cell number with respect to vehicle-treated controls. Bars represent median with interquartile range. The differences between the vehicle-treated control group and each treatment group were determined by Friedman test, followed by Dunn's post-hoc test. * $p < 0.05$, ** $p < 0.01$.

5.3 Discussion

The results of this chapter showed that dexamethasone induced the apoptosis in NANT cells, including fibrocytes and differentiating fibrocytes, from healthy subjects and patients with non-severe asthma, as well as the expression of CCR7 in fibrocytes from patients with non-severe asthma. However, dexamethasone did not either induce cell apoptosis or reduce CCR7 expression in fibrocytes from patients with severe asthma. GR mediates the effect of dexamethasone on fibrocytes; GR expression was lower in fibrocytes from patients with severe asthma but was increased in the presence of antioxidant N-acetylcysteine. JNK inhibition restored the suppressive effect of dexamethasone in terms of reducing fibrocyte number, in severe asthma.

In asthma, CS are prescribed as controller medications which reduce asthmatic symptoms, exacerbations and mortality, and thus improve lung function and quality of life (Bateman, Hurd et al. 2008). CS attenuate airway inflammation by reducing pro-inflammatory cells and mediators, and increasing anti-inflammatory mediators (Chung and Barnes 1999); these effects are mediated by the nuclear translocation of cytoplasmic liganded GR (Barnes 2010). The role of CS in asthmatic airway remodelling is unclear (van Essen-Zandvliet, Hughes et al. 1994, Hoshino 2004, Ward and Walters 2005). Circulating fibrocytes invade the airways, transform into

myofibroblasts, and therefore play a role in airway remodelling (Schmidt, Sun et al. 2003, Saunders, Siddiqui et al. 2009). It has been reported that dexamethasone reduces the proliferation, α-SMA expression, and release of mediators (IL-13, IL-6, IL-10) in fibrocytes from healthy subjects (Hayashi, Kawakita et al. 2013, Hayashi, Kawakita et al. 2014). Nonetheless, the effect of CS on fibrocytes from patients with non-severe or severe asthma has not been previously reported.

I showed that dexamethasone can induce apoptosis in NANT cells, including fibrocytes and differentiating fibrocytes, which is consistent with the literature showing CS-induced apoptosis in T lymphocytes (Brunetti, Martelli et al. 1995), monocytes (Schmidt, Lugering et al. 2001) and basophils (Yoshimura, Miyamasu et al. 2001). Apoptosis, also referred to as programmed cell death, is a result of the activation of the caspase cascade through signalling of either the extrinsic pathway or the intrinsic pathway. The extrinsic pathway involves membrane-bound "death signal" receptors. The intrinsic pathway, leading to increased permeability of mitochondria, induces the release of cytochrome c and other proteins which neutralise the endogenous inhibitors of apoptosis (Figure 5.16). CS initiate apoptosis via the intrinsic pathway in most cell types (Schlossmacher, Stevens et al. 2011). In some cases, activated GR may also induce apoptosis through rapid activation of protein

kinases such as MAPK, PI3K, protein kinase B (AkT), which, in turn, activate endothelial nitric oxide synthase (Limbourg and Liao 2003).

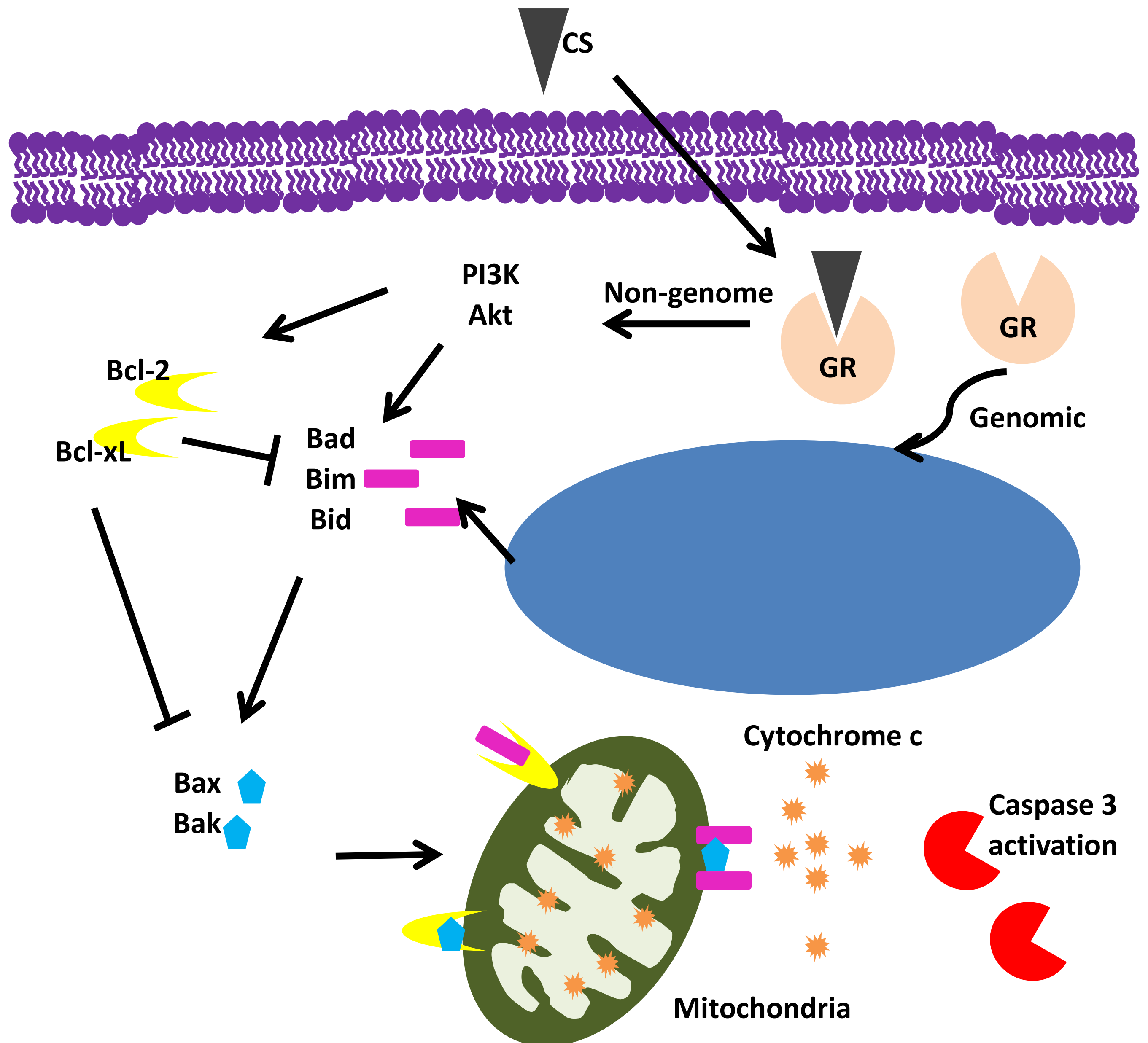


Figure 5.16: Corticosteroid-induced apoptosis. CS exert their effects either genomically or non-genomically. It is proposed that CS act via the intrinsic pathway, which tips the balance from anti-apoptotic to pro-apoptotic Bcl-2 family, to cause the release of cytochrome c from mitochondria and the subsequent caspase activation. CS: corticosteroid; GR: glucocorticoid receptor; PI3K: phosphoinositide 3-kinase; Akt: protein kinase B; Bcl-2: B cell lymphoma 2; Bcl-$_{XL}$: B cell lymphoma extra large; Bad: Bcl-2-associated death promoter; Bim: B-cell lymphoma 2 interacting mediator of cell death; Bid: BH3-interacting-domain death agonist; Bax: Bcl-2-associated X protein; Bak: Bcl-2 antagonist/killer-1. Modified from Schlossmacher *et al.*, Journal of Endocrinology 2011; 211:17.

Activated GR induces the expression of a variety of pro-apoptotic genes (Distelhorst 2002, Schlossmacher, Stevens et al. 2011), such as Bim_{EL} in the dexamethasone-treated NANT cells shown in this chapter. The apoptotic mitochondrial events are regulated by the balance of pro-apoptotic and anti-apoptotic members of the B cell lymphoma 2 (Bcl-2) family (Bcl-2 rheostat) (Ploner, Rainer et al. 2008). The anti-apoptotic members (e.g. Bcl-2 and Bcl-extra large (Bcl-x_L)) conserve three or four characteristic regions of Bcl-2 homology (BH) 1-4 domains. The pro-apoptotic Bcl-2 family members are further divided into two subgroups: those with two or three BH domains (e.g. Bcl-2-associated X protein (Bax), Bcl-2 antagonist/killer-1 (Bak)), and those with only the BH3 domain (BH3-only proteins, e.g. Bim/Bcl-2-related ovarian death (Bod), Bcl-2-associated death promoter (Bad), BH3-interacting-domain death agonist (Bid)) (Huang and Strasser 2000). BH3-only proteins are activated by stimuli such as drugs (e.g. CS), radiation, toxins, hypoxia, hyperthermia, or deprivation of growth factors, cytokines and hormones which maintain the survival of the cells. BH3-only proteins in turn activate Bax and Bak, which dimerise, insert into the mitochondrial membrane, and form channels resulting leakage of cytochrome c and other mitochondrial proteins into the cytoplasm. When cells are exposed to growth factors and survival signals, anti-apoptotic Bcl-2 and

Bcl-xL are synthesized to antagonise Bax and Bak and limit the leakage from mitochondria. Bim_{EL}, one of three isoforms of Bim, contains additional regulatory regions that interact with the dynein light chain LC8, which modulates their pro-apoptotic activity (Puthalakath, Huang et al. 1999). In this study I found Bim_{EL} was up-regulated by CS in NANT cells, although its expression was not significantly different in healthy subjects and patients with severe asthma after treatment at the 4-hr or 24-hr time points. Dexamethasone treatment induces Bim expression in biopsies from children with CS-sensitive acute lymphoblastic leukemia (ALL) but not in biopsies from children with CS-resistant ALL (Bachmann, Gorman et al. 2005). Lack of Bim induction in these CS-resistant biopsies is accompanied by maintenance of mitochondrial transmembrane potential and failure to activate effector caspases. However, GR expression and nuclear translocation in response to dexamethasone was not correlated with CS sensitivity in these biopsies; in contrast to the present study, expression of GR was generally lower in NANT cells, including fibrocytes, from the relative CS-insensitive severe asthmatic subjects, suggesting different mechanisms form the basis for CS insensitivity in different cell types. Furthermore, as Bim is not the only Bcl-2 gene that is differentially regulated between CS-sensitive and

CS-insensitive cells, a comparison of the expression of other Bcl-2 family proteins in NANT cells from CS-sensitive and CS-resistant patients may be of interest.

Dexamethasone also up-regulates the expression of the GILZ gene in the NANT cells. GILZ induces CD4+/CD8+ thymocyte apoptosis (Delfino, Agostini et al. 2004); down-regulation of GR-α inhibits dexamethasone-induced GILZ transactivation (Vazquez-Tello, Halwani et al. 2013). Here I also demonstrated a trend showing a smaller extent of dexamethasone-induced GILZ expression in NANT cells from patients with severe asthma, which may be associated with lesser CS-induced apoptosis in NANT cells from severe asthmatic patients compared to that in NANT cells from healthy subjects.

I showed dexamethasone reduced the mRNA expression of CD45 (and Col I and α-SMA mRNA to a lesser extent) in healthy subjects. Some studies demonstrated inhaled CS reduce collagen deposition and myofibroblastic differentiation of fibroblasts in the airways (Olivieri, Chetta et al. 1997, Hoshino, Nakamura et al. 1998), whilst others failed to show these effects (Jeffery, Godfrey et al. 1992). To suppress gene expression, liganded GR interacts with coactivators and recruits co-repressors (e.g. histone deacetylase 2) to the activated transcription complex, leading to reduction of histone acetylation, chromatin remodelling and polymerase 2

action (trans-repression). Less frequently, activated GR binds to the negative glucocorticoid response element in the promoters to repress gene expression (cis-repression) (Barnes 2010). Decreased mRNA of fibrocyte markers in the NANT cells may either reflect the transrepression or cis-repression induced by dexamethasone, or simply be due to the reduction in the number of total cells. Nevertheless, the mRNA expression of these genes was not decreased by dexamethasone in the NANT cells from patients with severe asthma.

It has previously been shown that PBMC (Hew, Bhavsar et al. 2006), alveolar macrophages (Bhavsar, Hew et al. 2008) and ASMC (Chang, Bhavsar et al. 2012) from patients with severe asthma display relative CS insensitivity in terms of reduced suppression of induced cytokine release, and here I report fibrocytes from patients with severe asthma also responded differentially to dexamethasone. Several mechanisms are implicated in the reduction of CS responsiveness in severe asthma: altered nuclear translocation of GR (Takayama, Arima et al. 2006), increased GR-β expression (Hamid, Wenzel et al. 1999, Sousa, Lane et al. 2000) and defective histone acetylation (Hew, Bhavsar et al. 2006). I propose lower expression of (total) GR may play a role in CS insensitivity in circulating fibrocytes from patients with severe asthma. Lower expression of total GR in CD3+ and CD14+ cells has been described

in CS-resistant nephrotic syndrome children (Hammad, Yahia et al. 2013) and systemic lupus erythematosus adults (Du, Li et al. 2009). CS-induced apoptosis of thymocytes is increased in transgenic mice with over-expression of GR (Reichardt, Umland et al. 2000) and decreased in transgenic mice with lower GR expression (Pazirandeh, Xue et al. 2002). It has been reported that the expression of GR-β is significantly increased in PBMC from CS-resistant asthmatic patients (Goleva, Jackson et al. 2012). In my study I could only detect total GR because commercial antibodies specific for GR-α for flow cytometry are currently unavailable. Further investigation of the ratio of GR-β/GR-α in circulating fibrocyte may be indicated.

I showed dexamethasone reduced CCR7 expression in fibrocytes from patients with non-severe asthma. Dexamethasone reduces the expression of CCR7 and the homing to drainage lymph nodes in mouse dendritic cells; the effect can be abolished by the GR antagonist RU486 (Xing, ML et al. 2002, Vizzardelli, Pavelka et al. 2006, Larange, Antonios et al. 2012). CC chemokine ligand 21-secondary lymphoid-tissue chemokine (SLC)/CCR7 signalling mediates the infiltration of fibrocytes in skin and fibrotic kidneys in mouse models (Abe, Donnelly et al. 2001, Sakai, Wada et al. 2006). Since dexamethasone reduced the expression of CCR7 in fibrocytes from patients with non-severe asthma *in vitro*, CS may also help to decrease the airway

remodelling induced by circulating fibrocytes. However, severe asthmatic fibrocytes also showed resistance to dexamethasone-induced inhibition of CCR7 expression. Failure of CS to down-regulate CCR7 expression in fibrocytes may allow for their accumulation and migration into the airways of patients with severe asthma despite being under treatment with CS, thus leading to greater degree of airway wall remodelling.

Since the expression of GR in fibrocytes from patients with severe asthma was lower, I screened for mediators and medications which might affect the expression of GR. It is known that patients with severe asthma have a higher level of IL-13 in their airways compared to patients with non-severe asthma (Naseer, Minshall et al. 1997, Saha, Berry et al. 2008, Macedo, Hew et al. 2009). IL-2 and IL-4 exposure reduces GR-α expression in PBMC (Vazquez-Tello, Halwani et al. 2013). CS, β_2-agonists and muscarinic antagonists are frequently prescribed in asthma; the expression of GR was down-regulated by CS in leukaemia cells (Kfir, Sionov et al. 2007). However, my data showed neither T_H2 cytokines (e.g. IL-4, IL-13) nor asthma medications (e.g. dexamethasone, salmeterol, tiotropium) altered the expression of GR in circulating fibrocytes. However, anti-oxidative N-acetylcysteine increased the expression of GR in fibrocytes from patients with severe asthma. Asthmatic patients have increased

reactive nitrogen species and reactive oxygen species released by inflammatory cells in their airways (Chung and Marwick 2010). An oxidant-antioxidant imbalance may be involved in many fibrotic diseases, including idiopathic pulmonary fibrosis, alcoholic hepatitis and asthmatic airway remodelling. It has been shown that hydrogen peroxide increases the number and myofibroblastic differentiation of fibrocytes via up-regulation of epidermal growth factor receptor, and these effects can be abolished by N-acetylcysteine (Wang, Huang et al. 2012). The addition of N-acetylcysteine to prednisolone provides better outcomes for patients with idiopathic pulmonary fibrosis or acute alcoholic hepatitis, compared to standard regimens without N-acetylcysteine (Demedts, Behr et al. 2005, Nguyen-Khac, Thevenot et al. 2011). Oxidative stress reduces CS sensitivity in patients with chronic obstructive pulmonary disease (COPD) or severe asthma and asthmatic smokers; the known mechanisms include histone deacetylase 2 inhibition or GR-α phosphorylation (Barnes and Adcock 2009). Since N-acetylcysteine increases the expression of GR in severe asthmatic fibrocytes, it is worth exploring whether down-regulation of GR is also one of the mechanisms involving oxidative stress-induced CS insensitivity.

Many external inflammatory signals in asthma (e.g. such as pathogens, allergens, cytokines, growth factors, oxidative stress, etc.) activate intracellular kinase cascades

to modulate the proliferation, differentiation, gene expression, migration and survival of cells (Adcock, Chung et al. 2006). MAPK family proteins (p38MAPK, JNK, ERK) and PI3K regulate airway inflammation through activation of pro-inflammatory transcriptional factors or modulation of mRNA half-life (Haluzikova, Dostalova et al. 2009). These kinases display heightened activity in asthma; therefore targeting MAPKs and PI3K might potentially allow for selective treatment of inflammation and remodelling in asthmatic airways (Liu, Liang et al. 2008, Takeda, Ito et al. 2010). It has been reported that the inhibition of p38 MAPK (Kokubo, Sakai et al. 2012), JNK (Hong, Belperio et al. 2007), ERK (Nikam, Wecker et al. 2011) and PI3K (Mehrad, Burdick et al. 2009) down-regulates the expression of pro-collagen I, α-SMA, integrins and CXCR4 in fibrocytes respectively. In patients with non-severe asthma recruited to my project, the number of fibrocytes was reduced by inhibition of ERK and PI3K and differentiating fibrocytes were reduced by inhibition of JNK, ERK and PI3K. It has been demonstrated that TGF-β_1 induces α-SMA expression in fibrocytes through the stress-activated protein kinases/JNK pathway (Hong, Belperio et al. 2007). Whilst there is little in the literature regarding whether inhibition of ERK and PI3K affects the proliferation and myofibroblastic differentiation of fibrocytes, some studies have shown that these kinase inhibitors reduce the number and α–SMA

expression of other cell types. For example, ERK inhibition induces cell apoptosis in PBMC (Blank, Burger et al. 2002) and reduces endothelin-1/TGF-β_1-induced collagen production in pulmonary arterial smooth muscle cells (Lambers, Roth et al. 2013) and skin fibroblasts from patients with scleroderma (Chen, Leask et al. 2008). Inhibition of PI3K (p100γ and p100α isoforms) attenuates TGF-β-induced proliferation and α-SMA expression in pulmonary fibroblasts (Conte, Fruciano et al. 2011). Intriguingly, only PI3K inhibition, but not JNK or ERK inhibition, reduced the number of fibrocytes and differentiating fibrocytes from patients with severe asthma. Further comparison about the phosphorylation status of these kinases in fibrocytes from asthmatic patients with different severity may help to find out the mechanism leading to more cultured cells in patients with severe asthma at day 3.

Post-translational modification of GR, such as phosphorylation, nitrosylation and ubiquitination, reduce nuclear translocation and transactivational activity of GR (Barnes 2010). The alteration in phosphorylation status of GR is related to CS insensitivity in severe asthma (Adcock and Lane 2003). However, whether these kinases affect CS insensitivity in fibrocytes has not been investigated. I showed JNK inhibition improved the suppressive effect of dexamethasone on fibrocytes in severe asthma. It has also been shown that toxic shock toxin-1 reduces the inhibitory effect

of prednisolone on the proliferation of PBMC, which can be restored by JNK inhibition (Fukushima, Hirano et al. 2007). Although the inhibition of p38MAPK (Bhavsar, Hew et al. 2008, Chang, Bhavsar et al. 2012, Mercado, Hakim et al. 2012) and PI3K (Rossios, To et al. 2012) has been reported to reverse CS insensitivity in severe asthma, inhibition of these two kinases or ERK did not improve CS sensitivity in fibrocytes from patients with severe asthma recruited to my project. The discrepancy might come from different parameters used to evaluate the inhibitory effect of CS: I observed "the reduction in the number of fibrocytes and differentiating fibrocytes induced by dexamethasone", whilst others evaluated "suppression of cytokine/chemokine release". I propose that "impaired CS suppression of release function" is more related to p38 MAPK-phosphorylated GR in PBMC, alveolar macrophages and ASMC, whilst "failure to induce cell apoptosis by CS" may be more related to lower GR expression in fibrocytes.

Fibrocytes from patients with severe asthma are less sensitive to CS-induced apoptosis and CS-inhibition of CCR7 expression, which may contribute to increased recruitment of fibrocytes and more remarkable airway remodelling in severe asthmatic airways despite high dose CS treatment (Saunders, Siddiqui et al. 2009). CS insensitivity in fibrocytes from patients with severe asthma may be related to lower

GR expression, which could be reversed by antioxidants, or heightened JNK activity. Since CS could not provide satisfactory inhibition on fibrocytes in severe asthma, it is worth investigating the effect of other classes of anti-asthmatic medications, such bronchodilators, on fibrocyte function.

Chapter 6: The effect of bronchodilators on fibrocyte function

6.1 Background

Bronchodilators are widely used in asthma. Apart from the relief of bronchospasm, bronchodilators also provide anti-inflammatory effects (Remington and Digiovine 2005, Meurs, Dekkers et al. 2013). Two classes of inhaled bronchodilators are frequently prescribed: β_2-adrenoreceptor (β_2-AR) agonists (or β_2-agonists) and muscarinic antagonists.

β_2-AR agonists act on β_2-AR, which are widely expressed in the airways. Activation of β_2-AR results in increased intracellular 3',5'-cyclic adenosine monophosphate (cAMP), leading to relaxation of airway smooth muscles (ASM). Salmeterol xinafoate, a long-acting β_2-AR agonists (LABA), inhibits the proliferation of ASM cells (ASMC) and peripheral blood mononuclear cells (PBMC) (Young, Skinner et al. 1995, Oddera, Silvestri et al. 1997) and the expression of α–smooth muscle actin (α–SMA) in lung fibroblasts (Baouz, Giron-Michel et al. 2005), suggesting LABAs may have the ability to reduce airway remodelling. The addition of LABA to inhaled corticosteroids (CS) improves the symptom control and lung

function and reduces the need of high dose inhaled CS (Greening, Ind et al. 1994). LABAs have been shown to induce glucocorticoid receptor (GR) nuclear translocation in ASMC and fibroblasts (Eickelberg, Roth et al. 1999) and enhance CS actions *in vitro* and *in vivo* (Pang and Knox 2000, Roth, Johnson et al. 2002, Usmani, Ito et al. 2005). CS insensitivity seen in the peripheral blood mononuclear cells (PBMC) from patients with severe asthma is associated with reduced GR nuclear translocation and with hyperphosphorylation of GR, which are reversed by LABA through p38 mitogen-activated protein kinase (MAPK)-γ inhibition (Mercado, To et al. 2011).

Muscarinic antagonists prevent cholinergic nerve-induced bronchospasms and mucus hypersecretion and are sometimes prescribed with short-acting β_2-adrenoceptor agonist (SABA) as an add-on bronchodilator in acute exacerbation of asthma (Gross 2006) or as an alternative reliever for SABA-intolerant asthmatic patients (Bateman, Hurd et al. 2008). Acetylcholine signals the muscarinic receptors expressed in respiratory organs through both parasympathetic nerves (Gosens, Zaagsma et al. 2006) and non-neuronal cells (Wessler and Kirkpatrick 2001). Non-neuronal cells such as epithelial cells, fibroblasts and macrophages also express muscarinic receptors (Meurs, Dekkers et al. 2013). M_2 receptor, the predominant subtype of muscarinic

receptors in lung tissues such as fibroblasts, couples preferentially to inhibition of adenylyl cyclase (Racke, Haag et al. 2008). In terms of airway remodelling, tiotropium bromide, a long-acting muscarinic antagonist (LAMA), attenuates hypertrophy of ASMC and mucus glands in ovalbumin-challenged experimental guinea pigs (Gosens, Bos et al. 2005, Bos, Gosens et al. 2007). Muscarinic antagonists block muscarinic receptor-induced proliferation (Matthiesen, Bahulayan et al. 2007), collagen I (Col I) production (Haag, Matthiesen et al. 2008) and α-SMA expression (Milara, Serrano et al. 2012) in lung fibroblasts .

In this chapter I will explore the impact of salmeterol and tiotropium on *in vitro* cultured fibrocytes, which have not been previously reported in the literature. As I have previously showed that CS cannot reduce the number of fibrocytes (Col I+/CD45+ cells) and differentiating fibrocytes (α-SMA+ cells) from patients with severe asthma (Chapter 5), I will further investigate whether these bronchodilators affect fibrocyte survival.

6.2 Results

6.2.1 Effect of β₂-adrenoceptor agonist on fibrocytes

6.2.1.1 Effect of salmeterol on fibrocytes derived from non-adherent non-T cells

To explore the effect of LABA on the number and myofibroblastic differentiation status of fibrocytes, non-adherent non-T cells (NANT cells; 10^6 cells/well) isolated from healthy subjects and patients with asthma were cultured in foetal bovine serum (FBS)-containing medium, as previously described (chapter 2.2.2), and treated with either vehicle (dimethyl sulfoxide; DMSO) or salmeterol (10^{-9}-10^{-7} M). The concentrations of salmeterol used for treatment of fibrocytes were selected from previously published studies (Linden 1992, Baouz, Giron-Michel et al. 2005). The number of fibrocytes and differentiating fibrocytes, and CC chemokine receptor (CCR) 7 expression, were determined by flow cytometry on day 3.

Salmeterol decreased the number of fibrocytes from healthy subjects ($p < 0.01$, 10^{-9} M) and patients with non-severe asthma ($p < 0.05$, 10^{-9} M), but not the number of fibrocytes from patients with severe asthma (Figure 6.1A). Salmeterol also reduced the number of differentiating fibrocytes from healthy subjects ($p < 0.01$, 10^{-9} M) and patients with non-severe asthma ($p < 0.05$, 10^{-9} M; Figure 6.1B).

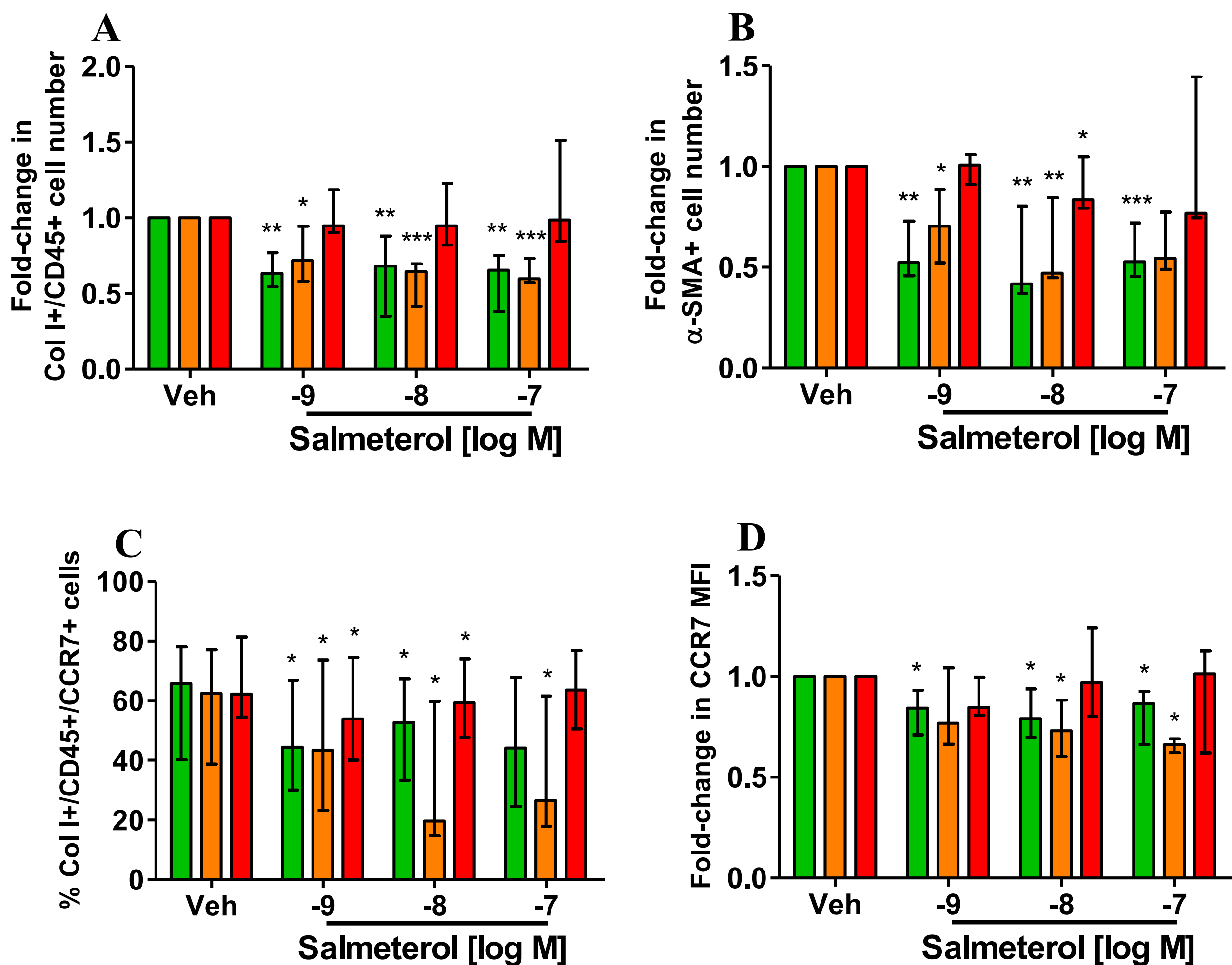


Figure 6.1: Effect of salmeterol on fibrocytes derived from non-adherent non-T cells. NANT cells from healthy subjects (green, n = 9) and patients with non-severe (orange, n = 7) or severe asthma (red, n = 8) were treated with salmeterol (10^{-9}- 10^{-7} M) on day 0. Cells were harvested after 3 days in culture and the number of fibrocytes (**A**; Col I+/CD45+ cells) and differentiating fibrocytes (**B**; α-SMA+ cells) within the NANT cell population, as well as the percentage of CCR7+ fibrocytes within fibrocytes (**C**) and CCR7 MFI (**D**) was determined by flow cytometry. Bars represent median with interquartile range. (**A**, **B**, **D**) Data are expressed as fold-change with respect to vehicle (Veh, DMSO)-treated controls. The differences between the Veh-treated control group and each treatment group were determined by Friedman test, followed by Dunn's post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to Veh-treated cells for each group.

Salmeterol reduced the proportion of Col I+/CD45+/CCR7+ cells in Col I+/CD45+ cells (% Col I+/CD45+/CCR7+ cells) from healthy subjects and patients with non-severe or severe asthma (all $p < 0.05$, 10^{-9} M; Figure 6.1C). Salmeterol also reduced CCR7 expression in CCR7+ fibrocytes (CCR7 median fluorescence intensity (MFI), Chapter 2.2.3) from healthy subjects ($p < 0.05$, 10^{-9} M) and patients with non-severe asthma ($p < 0.05$, 10^{-8} M) but not from patients with severe asthma (Figure 6.1D).

6.2.1.2 Effect of salmeterol and/or dexamethasone on fibrocytes derived from non-adherent non-T cells

To investigate the effect of the combination of CS and LABA on fibrocytes, I treated NANT cells from healthy subjects and patients with asthma with a submaximal dose of salmeterol (10^{-8} M) and/or dexamethasone (10^{-7} M) for a 3-day period. As previously mentioned, treatment with either salmeterol or dexamethasone only had an inhibitory effect on the number of fibrocytes and differentiating fibrocytes from healthy subjects and patients with non-severe asthma (Figure 6.2A-B). The combination of salmeterol and dexamethasone did not show a further inhibitory effect on the number of cells from healthy subjects and patients with non-severe asthma. The combination had no effect on the number of fibrocytes (p = 0.17) and differentiating fibrocytes (p = 0.14) from severe asthmatic patients compared to vehicle treatment, but reduced more fibrocytes compared to dexamethasone alone (p = 0.0568), and more differentiating fibrocytes compared to salmeterol alone ($p < 0.01$).

Although salmeterol did reduce the proportion of CCR7+ fibrocytes in both healthy subjects and asthmatic patients ($p < 0.05$), the combination of salmeterol and dexamethasone did not significantly affect the proportion of CCR7+ fibrocytes (p =

0.91) or CCR7 MFI ($p = 0.47$) in fibrocytes from patients with severe asthma (Figure 6.2C-D).

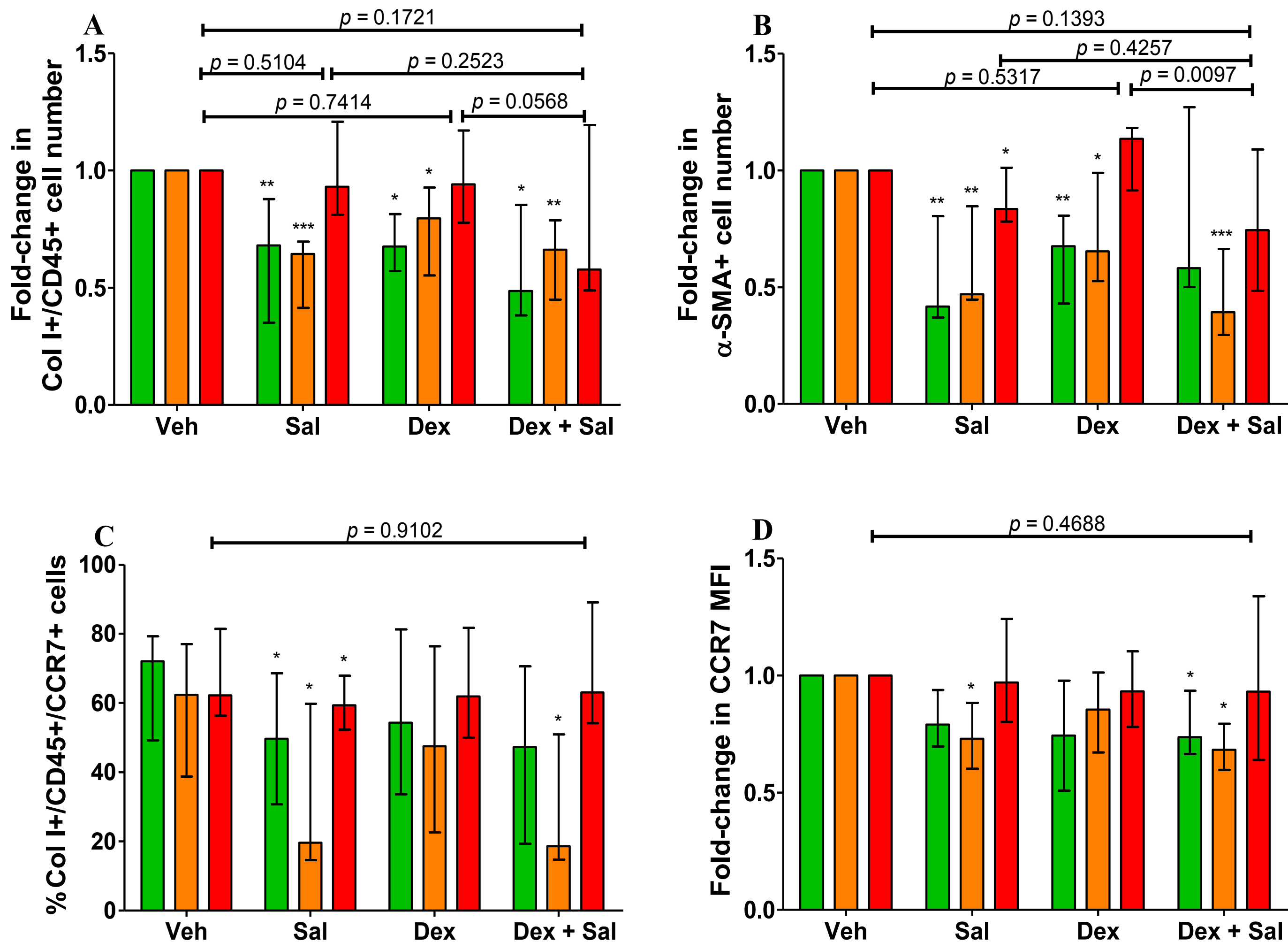


Figure 6.2: Effect of salmeterol ± dexamethasone on fibrocytes derived from non-adherent non-T cells. NANT cells from healthy subjects (■, n = 9) and patients with non-severe (■, n = 7) or severe asthma (■, n = 7) were treated with salmeterol (Sal; 10^{-9}- 10^{-7} M) or/and dexamethasone (Dex; 10^{-7}M) on day 0. Cells were harvested after 3 days in culture and the number of fibrocytes (**A**; Col I+/CD45+ cells) and differentiating fibrocytes (**B**; α-SMA+ cells) within the NANT cell population, as well as the percentage of CCR7+ fibrocytes within fibrocytes (**C**) and CCR7 MFI (**D**) was determined by flow cytometry. Bars represent median with interquartile range. (**A**, **B**, **D**) Data are expressed as fold-change with respect to vehicle (Veh, DMSO)-treated controls. The differences between the Veh-treated control group and each treatment group were determined by Friedman test, followed by Dunn's post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to Veh-treated cells for each group.

6.2.1.3 Effect of salmeterol and/or dexamethasone on non-adherent non-T cell apoptosis

Annexin V/propidium iodide staining (Chapter 2.2.4.4) was performed to determine whether salmeterol induced apoptosis in NANT cells in the presence or absence of dexamethasone (Figure 6.3). Annexin V^-/propidium iodide$^-$ were considered as live cells, Annexin V^+/propidium iodide$^-$ as apoptotic and annexin V^+/propidium iodide$^+$ as late apoptotic cells. After 3 days of incubation in the presence of dexamethasone, the proportion of apoptotic cells increased in NANT cells from healthy subjects and patients with non-severe asthma (Chapter 5.2.3). Salmeterol neither increased apoptosis in NANT cells, nor augmented the dexamethasone-induced apoptosis in NANT cells from healthy subjects and patients with non-severe or severe asthma, suggesting that salmeterol reduced fibrocyte numbers through either the inhibition of the proliferation of fibrocytes or differentiation of fibrocytes from NANT cells, rather than induction of apoptosis.

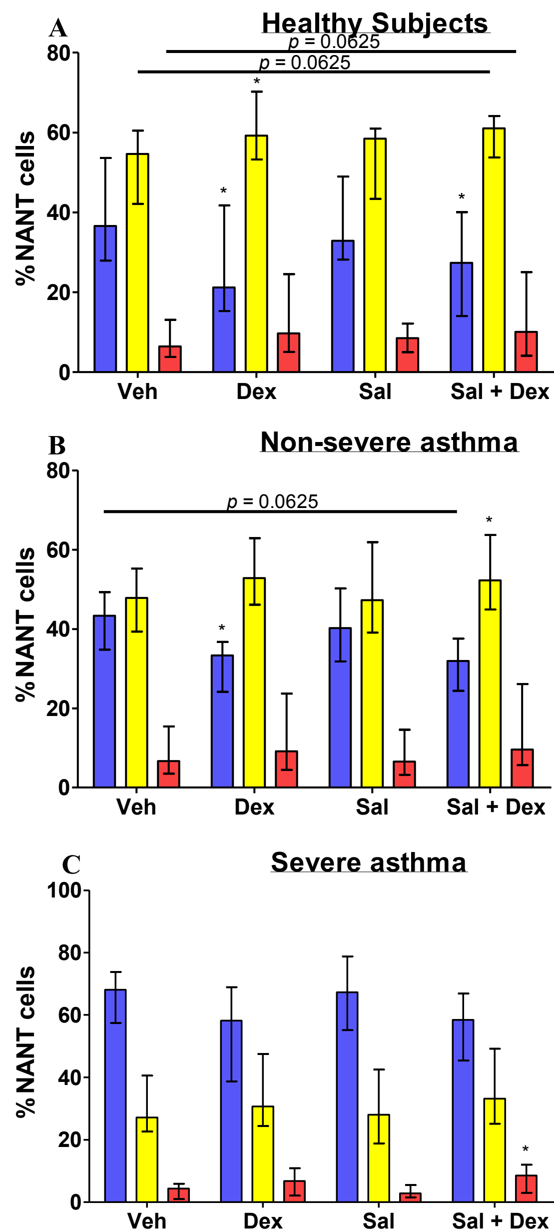


Figure 6.3: Effect of salmeterol ± dexamethasone on non-adherent non-T cell apoptosis in healthy subjects and patients with non-severe or severe asthma. The percentage of live (■, annexin V$^-$/propidium iodide$^-$), and early (■, annexin V$^+$/propidium iodide$^-$) and late apoptotic NANT cells (■, annexin V$^+$/propidium iodide$^+$) from healthy subjects (**A**; n = 6) and patients with non-severe (**B**; n = 6) or severe asthma (**C**; n = 6) was determined by flow cytometry after 3 days in culture in the presence of dexamethasone (Dex; 10^{-7}M) and/or salmeterol (Sal; 10^{-8} M). Bars represent median with interquartile range. The differences between the untreated control group and each treatment group were determined by Wilcoxon matched pairs test. * $p < 0.05$ compared to vehicle (Veh: vehicle; DMSO***)***-treated cells for each group.

6.2.1.4 Effect of salmeterol and/or dexamethasone on fibrocytes derived from the adherent fraction of peripheral blood mononuclear cells

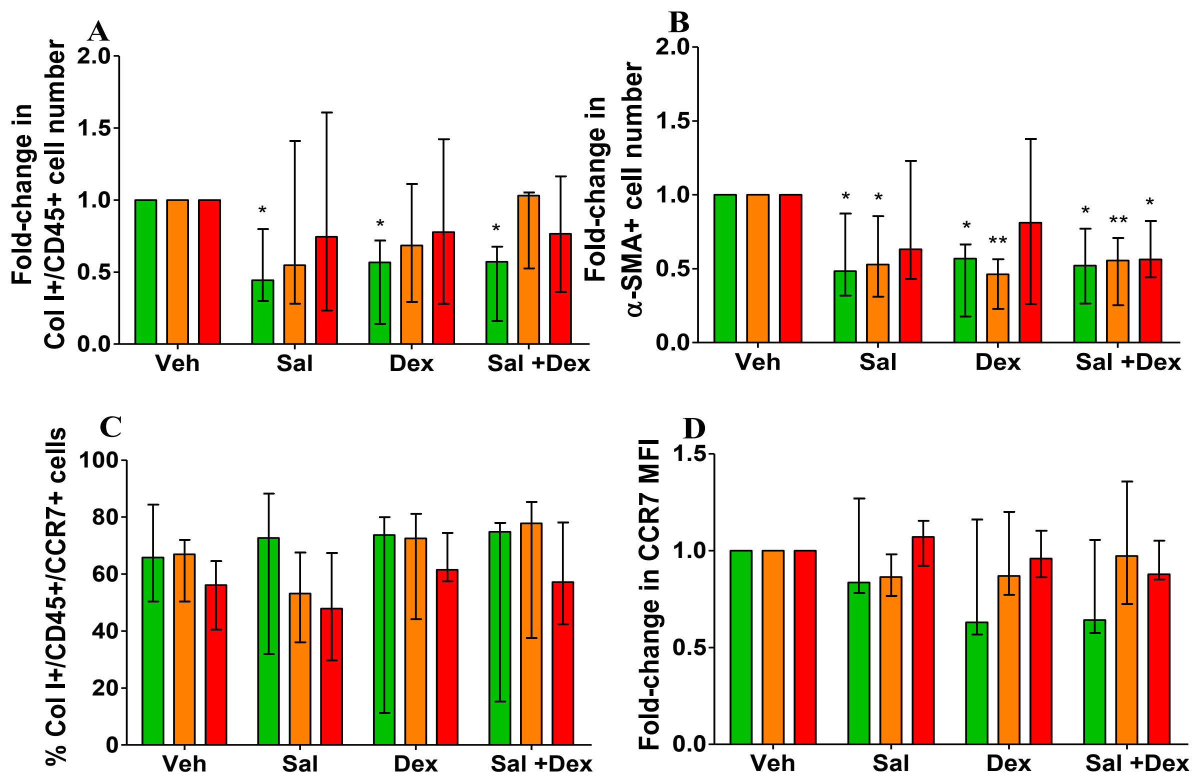
PBMC from healthy subjects and patients with asthma were incubated in the presence of FBS in plates pre-coated with fibronectin. Salmeterol (10^{-8} M) ± dexamethasone (10^{-7} M) was added on day 3 after changing culture medium. The number of fibrocytes and differentiating fibrocytes in adherent cells and CCR7 expression were determined on day 6 days by flow cytometry (Figure 6.4).

As described in chapter 5.2.2, treatment with dexamethasone alone reduced the number of fibrocytes and differentiating fibrocytes in adherent PBMC from healthy subjects, and differentiating fibrocytes in adherent PBMC from patients with non-severe asthma (Figure 6.4A-B). Treatment with salmeterol also reduced the number of fibrocytes from healthy subjects ($p < 0.05$), and differentiating fibrocytes from healthy subjects ($p < 0.05$) and patients with non-severe asthma ($p < 0.05$), but did not affect the number of fibrocytes from patients with non-severe ($p = 0.57$) or severe asthma ($p = 0.69$) and differentiating fibrocytes ($p = 0.44$) from patient with severe asthma. Salmeterol in combination with dexamethasone did not provide an additional effect on the number of cells from healthy subjects and patients non-severe

asthma. In severe asthma, the combination of drugs did not reduce the number of fibrocytes ($p = 0.22$) but reduced the number of differentiating fibrocytes ($p < 0.05$). The results are similar to that observed with the NANT cell model.

The effect of either salmeterol or dexamethasone on CCR7 expression in fibrocytes from healthy subjects and patients with non-severe asthma in adherent PBMC model was not as significant as that seen in fibrocytes in NANT cell model (Figure 6.4 C-D).

Morphologically (Figure 6.4E-H), both dexamethasone and salmeterol significantly reduced the number of spindle-shaped cells derived in adherent PBMC from healthy subjects after 6 days in culture. The combination of drugs did not demonstrate an additional effect.



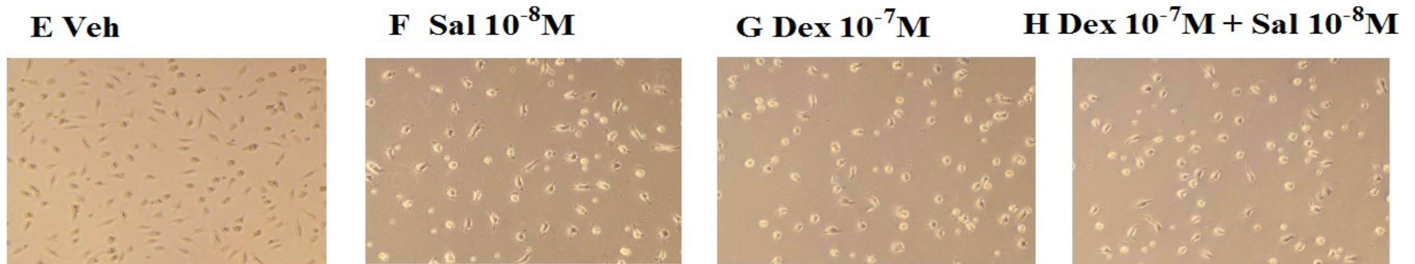


Figure 6.4: Effect of salmeterol ± dexamethasone on fibrocytes derived from adherent peripheral blood mononuclear cells. PBMC from healthy subjects (green, n = 5) and patients with non-severe (orange, n = 5) or severe asthma (red, n = 6) were stimulated with vehicle (Veh; DMSO), salmeterol (Sal; 10^{-8} M) and/or dexamethasone (Dex; 10^{-7} M) after changing medium on day 3. Adherent cells were harvested for flow cytometry after a further 3 days in culture. The number of fibrocytes (**A**; Col I+/CD45+ cells) and differentiating fibrocytes (**B**; α-SMA+ cells), as well as the percentage of CCR7+ fibrocytes within fibrocytes (**C**) and fold-change in CCR7 MFI (**D**) was determined by flow cytometry. Bars represent median with interquartile range. (**A**, **B**, **D**) Data are expressed as fold-change with respect to vehicle (Veh, DMSO)-treated controls. The differences between the Veh-treated control group and each treatment group were determined by Friedman test, followed by Dunn's post-hoc test.* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to Veh-treated cells for each group. Photos of cells from a healthy subject with Veh (**E**), Sal (**F**), Dex (**G**), Sal + Dex (**H**) treatment were taken on day 6.

6.2.2 Effect of muscarinic antagonist on fibrocytes

6.2.2.1 Effect of acetylcholine on fibrocytes derived from non-adherent non-T cells

To explore the effect of acetylcholine on fibrocytes *in vitro*, NANT cells isolated from healthy subjects and patients with asthma were cultured in FBS-containing medium, as previously described, and treated with acetylcholine (10^{-9} - 10^{-7} M). The concentrations of tiotropium used for treatment of fibrocytes were selected from previously published studies (Pieper, Chaudhary et al. 2007). Cells were recovered after 3 days in culture. The numbers of fibrocytes and differentiating fibrocytes was determined by flow cytometry (Figure 6.5).

Acetylcholine (10^{-9} M) increased the number of fibrocytes ($p < 0.05$, Figure 6.5A) and also slightly increased the number of differentiating fibrocytes ($p = 0.09$; Figure 6.5B; although the change did not reach significance) from patients with non-severe asthma. The effect of acetylcholine on the number of fibrocytes and differentiating fibrocytes from healthy subjects and patients with severe asthma was not significant.

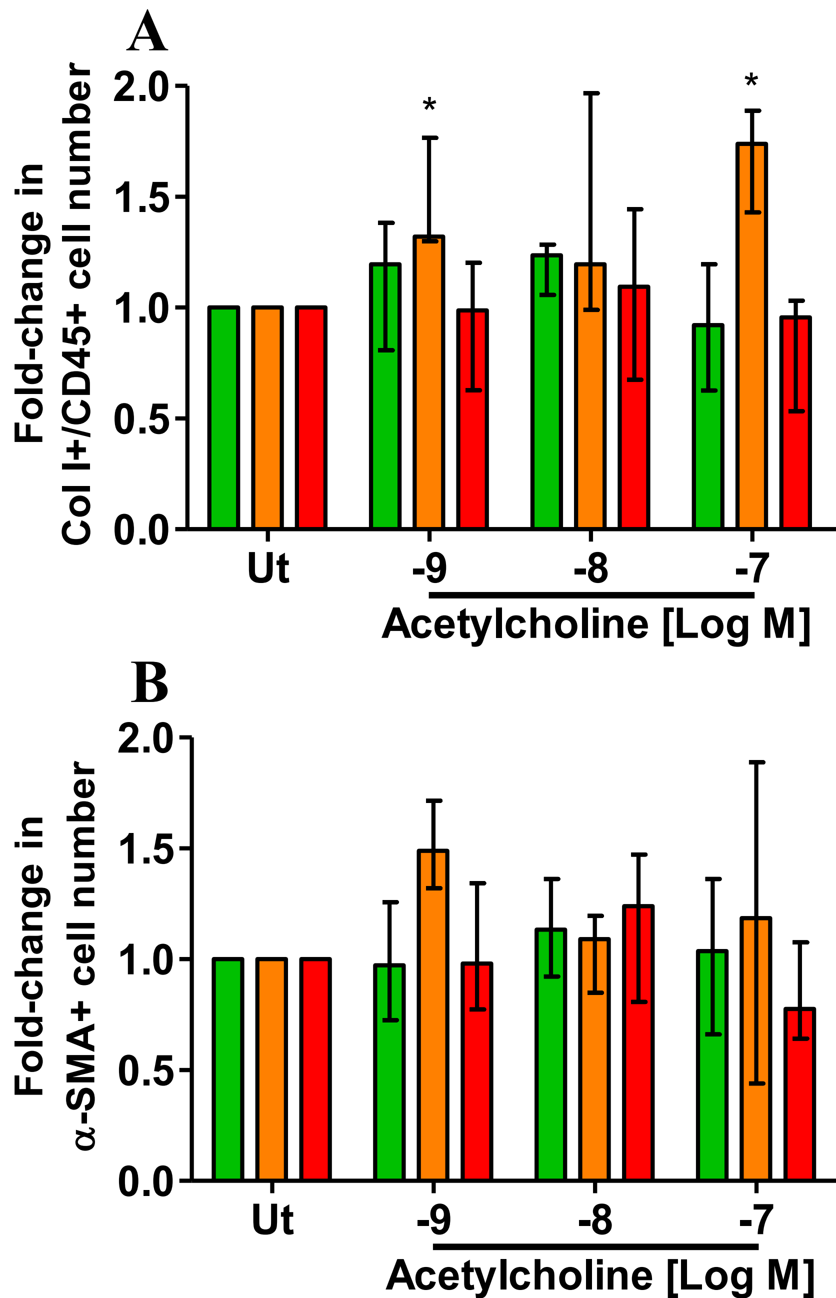


Figure 6.5: Effect of acetylcholine on fibrocytes derived from non-adherent non-T cells. NANT cells from healthy subjects (■, n = 6) and patients with non-severe (■, n = 4) or severe asthma (■, n = 6) were treated with acetylcholine (10^{-9}- 10^{-7} M) on day 0. Cells were harvested after 3 days in culture and the number of fibrocytes (**A**; Col I+/CD45+ cells) and differentiating fibrocytes (**B**; α-SMA+ cells) within the NANT cell population was determined using flow cytometry. Bars represent median with interquartile range. Data expressed as fold-change with respect to untreated (Ut) controls. The differences between the Ut control group and each treatment group were determined by Friedman test, followed by Dunn's post-hoc test. * $p < 0.05$ compared to Ut cells for each group.

6.2.2.2 Effect of tiotropium on fibrocytes derived from non-adherent non-T cells

To explore the effect of muscarinic antagonists on fibrocytes *in vitro*, NANT cells isolated from healthy subjects and patients with asthma were cultured in FBS-containing medium, as previously described, and treated with tiotropium (10^{-8} - 10^{-6} M). The concentrations of tiotropium used for treatment of fibrocytes were selected from a previously published study (Pieper, Chaudhary et al. 2007). Cells were recovered after 3 days in culture and the numbers of fibrocytes and differentiating fibrocytes and CCR7 expression were determined by flow cytometry (Figure 6.6).

Tiotropium reduced the number of fibrocytes and differentiating fibrocytes from healthy subjects ($p < 0.05$, 10^{-8} M) and patients with non-severe ($p < 0.05$, 10^{-6} M) or severe asthma ($p < 0.05$, 10^{-8} M; Figure 6.6A-B). Tiotropium did not change either the proportion of CCR7+ fibrocytes within fibrocytes or the CCR7 MFI of CCR7+ fibrocytes (Figure 6.6C-D).

6.2.2.3 Effect of tiotropium on non-adherent non-T cell apoptosis

Cell death was analysed by annexin V/propidium iodide apoptosis assay (Figure 6.7). Tiotropium did not affect the apoptosis in NANT cells from healthy subjects and patients with non-severe or severe asthma.

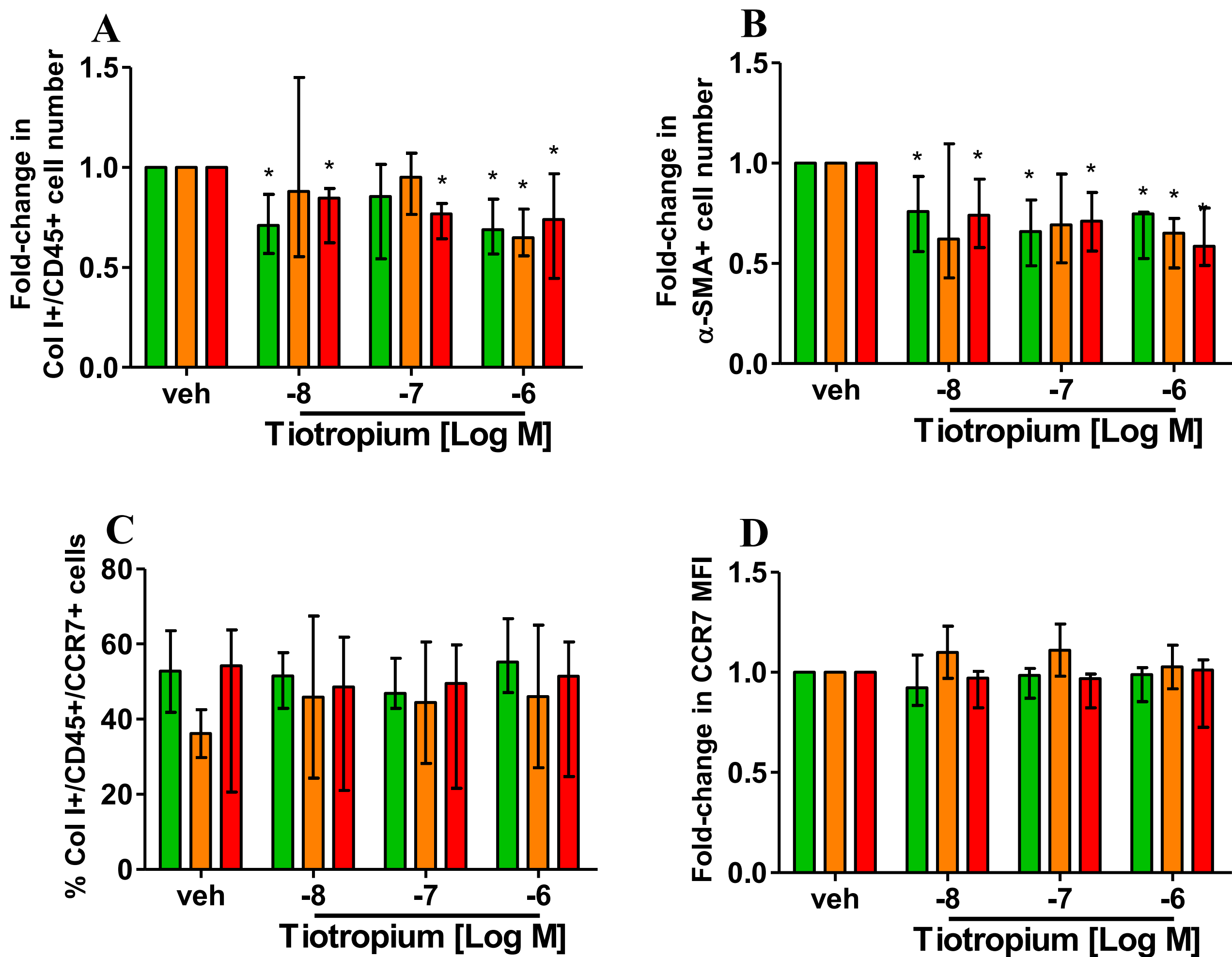


Figure 6.6: Effect of tiotropium on fibrocytes derived from non-adherent non-T cells. NANT cells from healthy subjects (■, n = 6) and patients with non-severe (■, n = 6) or severe asthma (■, n = 7) were treated with tiotropium (10^{-8}- 10^{-6} M) on day 0. Cells were harvested after 3 days in culture and the number of fibrocytes (**A**; Col I+/CD45+ cells) and differentiating fibrocytes (**B**; α-SMA+ cells) within the NANT cell population, as well as the percentage of CCR7+ fibrocytes within fibrocytes (**C**) and CCR7 median fluorescence intensity (MFI; **D**) was determined using flow cytometry. Bars represent median with interquartile range. (**A**, **B**, **D**) Data are expressed as fold-change with respect to vehicle (Veh, DMSO)-treated controls. The differences between the Veh-treated control group and each treatment group were determined by Friedman test, followed by Dunn's post-hoc test * $p < 0.05$ compared to Veh-treated cells for each group.

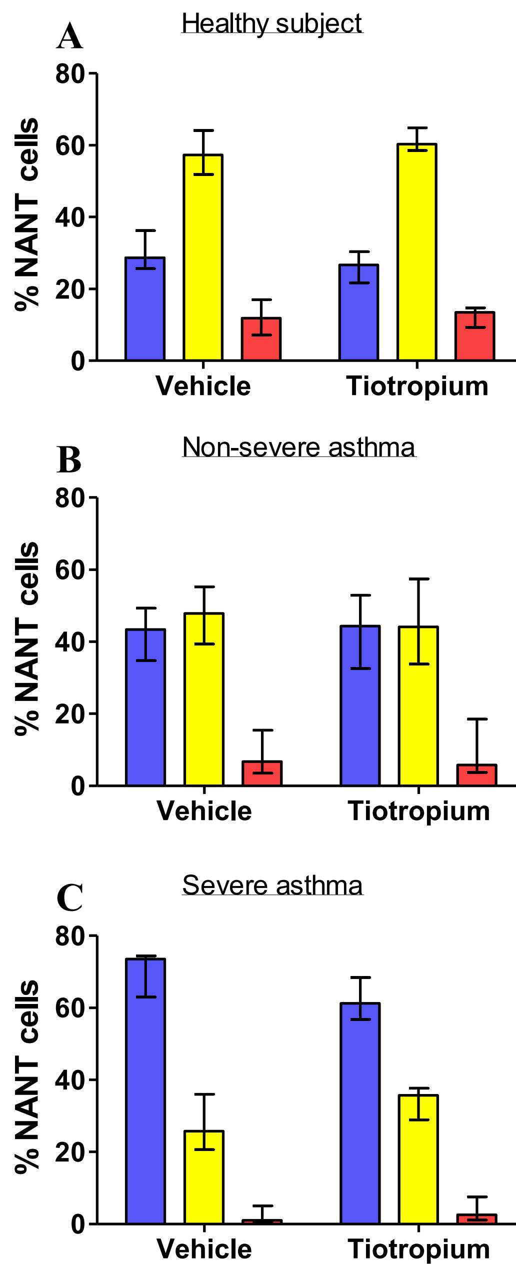


Figure 6.7: Effect of tiotropium on non-adherent non-T cell apoptosis in healthy subjects and patients with non-severe or severe asthma. The percentage of live (■, annexin V^-/propidium iodide$^-$), and early (■, annexin V^+/propidium iodide$^-$) and late apoptotic NANT cells (■, annexin V^+/propidium iodide$^+$) from healthy subjects (**A**; n = 3) and patients with non-severe (**B**; n = 6) or severe asthma (**C**; n = 3) was determined using flow cytometry after 3 days in culture in the presence of tiotropium (10^{-6} M). Bars represent median with interquartile range. The differences between the untreated control group and each treatment group were determined by Wilcoxon matched pairs test.

6.2.2.4 Effect of tiotropium on fibrocytes derived from the adherent fraction of peripheral blood mononuclear cells

PBMC from healthy subjects and patients with asthma were incubated in the presence of FBS in plates pre-coated with fibronectin. Tiotropium (10^{-6} M) was added on day 3 after changing culture medium. The number of fibrocytes and differentiating fibrocytes in adherent cells and CCR7 expression were determined on day 6 days by flow cytometry (Figure 6.8).

Tiotropium reduced the number of fibrocytes (Figure 6.8A) derived from adherent PBMC from healthy subjects ($p < 0.05$). There was also a trend towards tiotropium reducing differentiating fibrocytes from healthy subjects ($p = 0.07$), although the reduction did not reach significance (Figure 6.8B). Tiotropium did not affect the number of fibrocytes and differentiating fibrocytes derived from patients with non-severe or severe asthma; this may be a result of the study being under powered if at all.

Although CCR7 on fibrocytes in NANT cells was not affected by tiotropium, tiotropium reduced the proportion of CCR7+ fibrocytes ($p < 0.05$) and CCR7 expression in CCR7+ fibrocytes ($p < 0.05$) derived from adherent PBMC from healthy subjects (Figure 6.8C-D).

Morphologically, tiotropium significantly reduced the number of spindle-shaped cells derived from healthy subjects' adherent PBMC (Figure 6.8E-F).

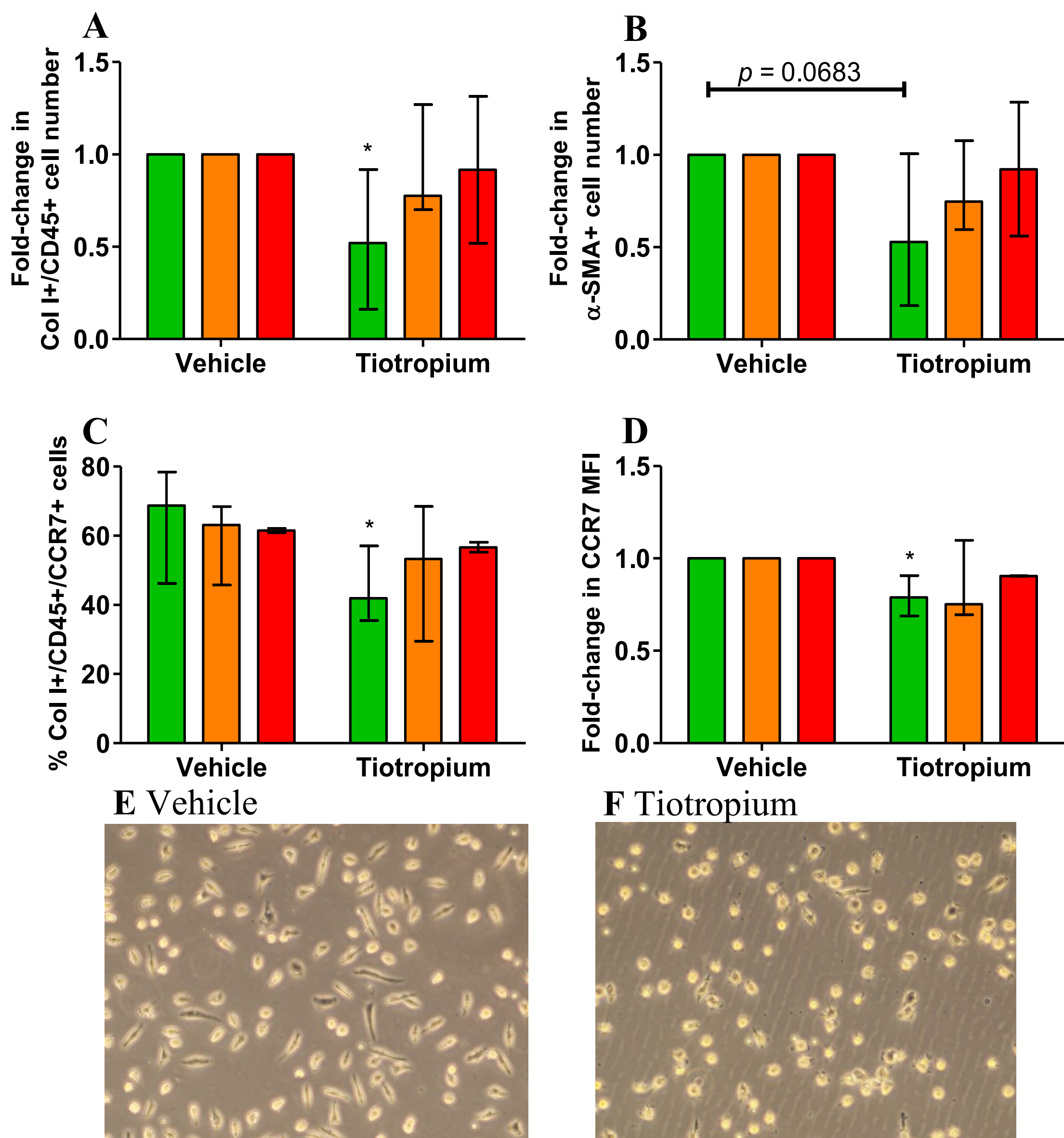


Figure 6.8: Effect of tiotropium on fibrocytes derived from adherent peripheral blood mononuclear cells. PBMC from healthy subjects (■, n = 6) and patients with non-severe (■, n = 4) or severe asthma (■, n = 2) were stimulated with vehicle (DMSO) or tiotropium (10^{-6} M) after changing medium on day 3. Adherent cells were harvested after a further 3 days in culture. The number of fibrocytes (**A**; Col I+/CD45+ cells) and differentiating fibrocytes (**B**; α-SMA+ cells), as well as the percentage of CCR7+ fibrocytes within fibrocytes (**C**) and CCR7 MFI (**D**) was determined using flow cytometry. Bars represent median with interquartile range. (**A**, **B**, **D**) Data are expressed as fold-change with respect to vehicle (Veh, DMSO)-treated controls. The differences between the vehicle-treated control group and each treatment group were determined by Friedman test, followed by Dunn's post-hoc test. * $p < 0.05$ compared to vehicle-treated cells for each group. Photos of cells from a healthy subject with vehicle (**E**) or tiotropium (**F**) treatment were taken on day 6.

6.2.3 The role of 3',5'-cyclic adenosine monophosphate on fibrocytes derived from non-adherent non-T cells

Adenylyl cyclase, which catalyses the conversion of adenosine triphosphate to cAMP, is activated by β_2-AR and inhibited by M_2 receptor (Racke, Haag et al. 2008). To investigate whether cAMP acts on fibrocytes, NANT cells isolated from healthy subjects and patients with severe asthma were cultured in the presence of rolipram (10^{-6} -10^{-5} M), which blocks the degradative action of phosphodiesterase type IV (PDE4) on cAMP, or the cAMP analogue 8-bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP; 10^{-4} - 10^{-3}M). The concentrations of rolipram and 8-Br-cAMP used for treatment of fibrocytes were selected from previously published studies (Wang and Adjaye 2011, Michalski, Kanaji et al. 2012). The number of fibrocytes and differentiating fibrocytes were determined by flow cytometry on day 3.

Rolipram (10^{-6} M; Figure 6.9A-B) reduced the number of fibrocytes from healthy subjects ($p < 0.05$) and severe asthmatic patients ($p < 0.05$), and differentiating fibrocytes from healthy subjects ($p < 0.01$) and severe asthmatic patients ($p < 0.05$). 8-Br-cAMP (10^{-4} M; Figure 6-9C-D) reduced the number of fibrocytes from healthy subjects ($p < 0.01$) and severe asthmatic patients ($p < 0.05$),

and the number of differentiating fibrocytes from healthy subjects ($p < 0.01$) and patients with severe asthma ($p < 0.05$).

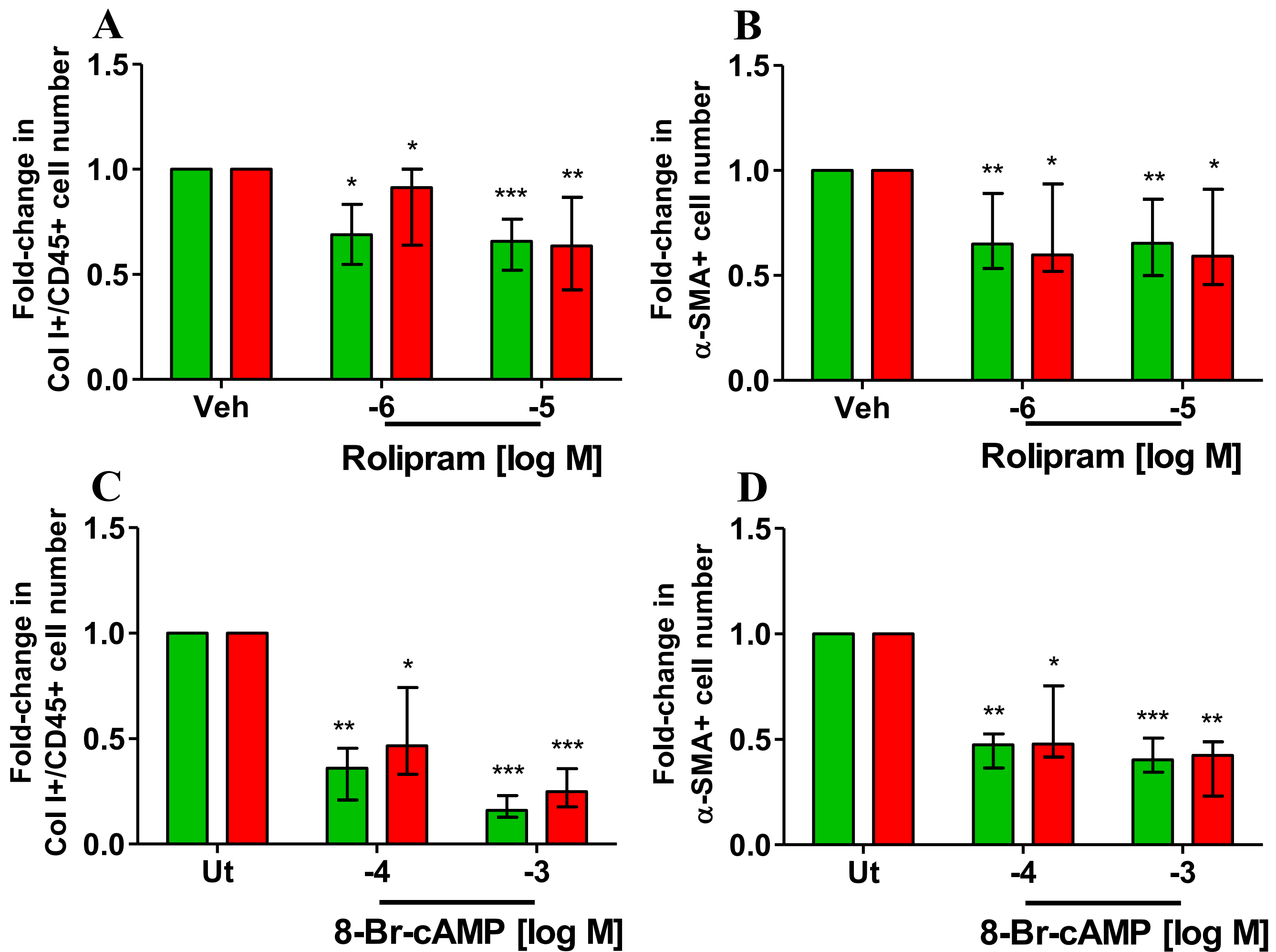


Figure 6.9: The effect of rolipram and 8-bromoadenosine 3′,5′-cyclic monophosphate on fibrocytes derived from non-adherent non-T cells. NANT cells from healthy subjects (green, n = 7 for rolipram, n = 4 for 8-Br-cAMP) and patients with severe asthma (red, n = 6 for rolipram, n = 4 for 8-Br-cAMP) were treated with phosphodiesterase type IV inhibitor rolipram (10^{-6} - 10^{-5} M) or cyclic adenosine monophosphate analogue 8-BR-cAMP (10^{-4}- 10^{-3} M) on day 0. Cells were harvested for flow cytometry after 3 days in culture. The number of fibrocytes (**A**, **C**; Col I+/CD45+ cells) and differentiating fibrocytes (**B**, **D**; α-SMA+ cells) after rolipram or 8-Br-cAMP treatment was determined on NANT cells from the same patients. Bars represent median with interquartile range. Data are expressed as fold-change with respect to vehicle (Veh)-treated (**A-B**) or untreated (Ut) controls (**C-D**). The differences between the control group and each treatment group were determined by Friedman test, followed by Dunn's post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to Veh-treated (**A-B**) or Ut (**C-D**) cells for each group.

6.3 Discussion

In this chapter the effect of two long-acting bronchodilators, salmeterol and tiotropium, was examined on *in vitro* cultured fibrocytes from healthy subjects and patients with asthma. Both salmeterol and tiotropium reduced the number of fibrocytes and differentiating fibrocytes from healthy subjects and patients with non-severe asthma. The numbers of fibrocytes from patients with severe asthma were only reduced by tiotropium, but not salmeterol. The expression of CCR7 in fibrocytes derived from non-adherent non-T cells was reduced by salmeterol, but not by tiotropium.

Salmeterol and tiotropium might reduce the number of cultured fibrocytes and differentiating fibrocytes through several possible mechanisms: i) inhibiting the proliferation of fibrocytes; ii) inducing apoptosis or reducing the survival of fibrocytes; and iii) decreasing NANT cell-to-fibrocyte differentiation (reducing the production of Col I in NANT cells). Salmeterol does not reduce collagen deposition in airways (Roberts, Bradding et al. 1999, Vanacker, Palmans et al. 2002), but reduces α-SMA production in myofibroblasts (Baouz, Giron-Michel et al. 2005), inhibits the proliferation of PBMC (Oddera, Silvestri et al. 1997), myoblasts and myotubes (Duranti, La Rosa et al. 2011), and ASMC (Young, Skinner et al. 1995) and induce

apoptosis in myoblasts and myotubes (Duranti, La Rosa et al. 2011). Tiotropium attenuates ASM remodelling in animal model of allergic asthma (Gosens, Bos et al. 2005). Muscarinic antagonists block the proliferation (Matthiesen, Bahulayan et al. 2007, Pieper, Chaudhary et al. 2007) and the production of collagen and α-SMA in pulmonary fibroblasts (Milara, Serrano et al. 2012), and induce CD8+ T cell apoptosis (Profita, Riccobono et al. 2012). Currently, little is known about the effect of bronchodilators on fibrocytes. Unlike the dexamethasone induced apoptosis of NANT cells, including fibrocytes in healthy subjects and non-severe asthmatics, my data showed neither salmeterol nor tiotropium can induce NANT cell apoptosis. Future studies could focus on the effect of bronchodilators on i) the expression of mRNA expression of Col I in NANT cells, which reflects NANT cell-to-fibrocyte differentiation, and ii) fibrocyte proliferation using 5-ethynyl-2'-deoxyuridine cell proliferation assay (Chapter 2.2.4.5).

I also described that the number of fibrocytes from patients with severe asthma was less affected by treatment with salmeterol. Cells from patients with severe asthma were more resistant to apoptosis, as described in chapter 5.2.4. However, salmeterol did not induce NANT cell apoptosis. A PDE4 inhibitor (rolipram) and a cAMP analogue (8-Br-cAMP) could reduce the number of fibrocytes and differentiating

fibrocytes from patients with severe asthma. It seems that the downstream signalling of adenylyl cyclase in these patients was not altered. The clinical responses to β_2-AR agonists can be affected by the β_2-AR polymorphisms (ADRB2). In retrospective and prospective studies in patients with asthma not taking inhaled CS, regular use of SABA, such as salbutamol (albuterol), was associated with lower lung function in individuals homozygous for arginine at the 16th amino acid position (B16 Arg/Arg) than in individuals homozygous for glycine at that position (B16 Gly/Gly) (Israel, Drazen et al. 2000, Israel, Chinchilli et al. 2004). B16 Arg/Arg polymorphism was not determined in my cohort of patients with severe asthma. Future studies could determine whether these patients with severe asthma have β_2-AR B16 Arg/Arg polymorphisms, lower β_2-AR expression or altered β_2-AR function.

My data did not support the hypothesis that salmeterol provides an additional effect when used with dexamethasone to restore CS sensitivity in fibrocytes from patients with severe asthma. The combination of LABA and low dose inhaled CS achieves better asthma control than single drug treatment or higher-dose inhaled CS alone (Reynolds, Lyseng-Williamson et al. 2005). The combination of CS and LABA leads to a reduction of constitutive and transforming growth factor-β-induced expression of α-SMA and an inhibition of fibroblast-to-myofibroblast differentiation

(Baouz, Giron-Michel et al. 2005). Mercado *et al*. reported that in PBMC from patients with severe asthma, LABAs (formoterol and salmeterol) can restore the inhibitory effect of CS on tumour necrosis factor-α-induced CXCL8/interleukin (IL)-8 production, reverse reduced GR nuclear translocation and p38 MAPK-γ-associated hyperphosphorylation of GR (Mercado, To et al. 2011). The experimental cell model used by Mercado and colleagues differs from the fibrocytes in the NANT cell fraction in PBMC used in my study. In addition, there are other differences between my experiments and those from Mercado and colleagues. Firstly, I focused on the suppressive effect of CS on the number of fibrocytes and differentiating fibrocytes from NANT fraction of PBMC, not cytokine released from the total PBMC population. Secondly, I detected lower GR expression in the fibrocytes from patients with severe asthma (Chapter 5.2.8), but the expression of GR-α and GR-β was not significantly different amongst three groups in Mercado's study. Thirdly, p38 MAPK inhibition did not improve CS sensitivity in fibrocytes from patients with severe asthma (Chapter 5.2.11).

β_2-AR and muscarinic M_2 receptors are linked by guanosine nucleotide-binding proteins (G proteins), to adenylyl cyclases, which catalyse the conversion of adenosine triphosphate to cAMP, and pyrophosphate, in a stimulatory and inhibitory

manner, respectively (Racke, Haag et al. 2008) (Figure 6.10). Increase in cAMP signalling exerts an anti-fibrotic effect by inhibiting of the formation and action of pro-fibrotic mediators, such as connective tissue growth factor, reducing proliferation, myofibroblastic differentiation and motility of fibroblasts, promoting cell death of fibroblasts, and decreasing the synthesis, release and function of extracellular matrix components (Insel, Murray et al. 2012). Intracellular cAMP can be raised by cAMP analogues, agonists/antagonists of G-protein coupled receptors and activation of adenylyl cyclases. The downstream effects of cAMP include activation of protein kinase A and exchange protein activated by cAMP (Epac). In human fibroblasts, selective cAMP analogue studies demonstrated proliferation can be blocked by the Epac inhibitor 8-pCPT-2′-O-Me-cAMP (Haag, Warnken et al. 2008) and collagen synthesis can be suppressed by protein kinase A inhibitor 6-Bnz-cAMP (Huang, Wettlaufer et al. 2007). PDE4 is responsible for the hydrolysis of intracellular cAMP (Burnouf and Pruniaux 2002). PDE4 inhibitors, such as roflumilast and rolipram, are also capable of preventing fibrotic and vascular remodelling in the lung, as shown in clinical trials and animal studies (Rabe, Bateman et al. 2005, Shepherd 2006, Cortijo, Iranzo et al. 2009). More specifically, knockdown of PDE4 isoform PDE4A and PDE4B attenuates fibroblast proliferation induced by the combination of basic

fibroblast growth factor and IL-1β, whilst knockdown of PDE4B and PDE4D inhibits fibroblast-to-myofibroblast differentiation induced by TGF-β (Selige, Hatzelmann et al. 2011). It has been reported that rolipram overcomes β_2-AR desensitisation in ASMC (Hu, Nino et al. 2008). Moreover, rolipram increases GR-α expression and inhibits GR-β expression in human pulmonary artery endothelial cells and attenuates CS insensitivity induced by cigarette smoke extract (Ortiz, Milara et al. 2013). Further investigation regarding the effect of PDE4 inhibitors combined with LABA or CS on LABA-insensitive and CS-insensitive fibrocytes is indicated.

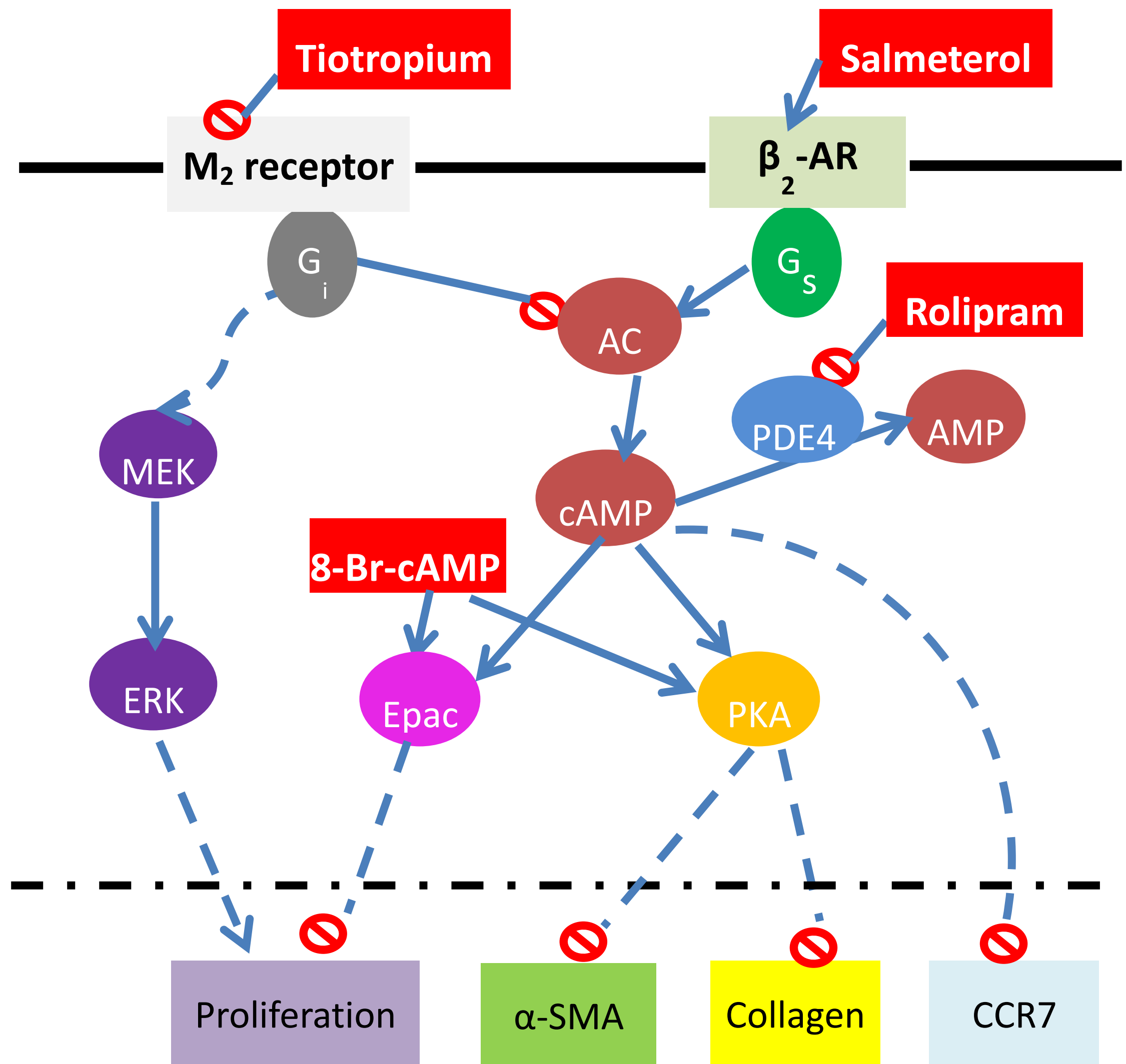


Figure 6.10: Possible effect of β₂-adrenoceptor agonist, muscarinic antagonist, phosphodiesterase inhibitor and cyclic adenosine monophosphate analogue on fibrocyte function. Salmeterol stimulates adenylyl cyclase (AC) whilst tiotropium attenuates the inhibition of AC. Rolipram inhibits the degradative action of phosphodiesterase type IV (PDE4) on 3',5'-cyclic adenosine monophosphate (cAMP). 8-bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP) is a non-selective cAMP analogue. These drugs increase intracellular cAMP accumulation, leading to activation of downstream exchange protein activated by cAMP (Epac), which reduces cell proliferation, and protein kinase A (PKA), which suppresses the production of α–smooth muscle actin (α-SMA) and collagen. cAMP also reduces the expression of CC chemokine receptor 7 (CCR7). Tiotropium may also inhibit cell proliferation via mitogen-activated protein kinase activating enzyme (MEK)/ extracellular signal-regulated kinase (ERK) pathway.

I found acetylcholine only slightly increased the number of fibrocytes and differentiating fibrocytes in healthy subjects and patients with severe asthma. In contrast, tiotropium reduced the number of fibrocytes and differentiating fibrocytes in all three groups. It has been reported that acetylcholine increases fibroblast and myofibroblast number in FBS-free medium and the effect is abolished by tiotropium (Pieper, Chaudhary et al. 2007). Since my culture medium contained 30% FBS, it is possible that either certain muscarinic receptor agonists were present in FBS which overwhelmed the effect of acetylcholine treatment, or cholinesterases in FBS degraded acetylcholine. Future studies could replace acetylcholine by carbachol, a cholinomimetic drug targeting both muscarinic and nicotinic receptors. As carbachol cannot be hydrolysed by acetylcholinesterase, this leads to a longer duration of action compared to acetylcholine (Nathanson, Klein et al. 1978). A quantification of the level of cholinesterase and acetylcholine could help to explain why fibrocyte number was not significantly affected by acetylcholine whilst it was reduced by tiotropium.

In this study, the number of fibrocytes from patients with severe asthma was reduced by tiotropium, but not by salmeterol. There is a growing interest in the use of anti-muscarinic agents in selected patients with poorly controlled asthma already receiving combination high-dose inhaled CS and LABA therapy. Patients with

polymorphisms of β_2-AR (ADRB2 16Arg/Arg and 16 Arg/Gly) and with a predominance of sputum neutrophils particularly benefit from tiotropium treatment (Bollmeier and Lee 2013). As mentioned earlier, activation of muscarinic receptors inhibits to adenylyl cyclases. Inactivation of Gi/o proteins on muscarinic receptors reduces the degradation of the intracellular cAMP level and prevents the proliferation of human pulmonary fibroblasts (Matthiesen, Bahulayan et al. 2006). Apart from this, muscarinic receptors also mediate Col I synthesis and proliferation of fibroblasts through ERK/MAPK pathway, which can be blocked by PD98059, an inhibitor of MAPK-activating enzyme (Matthiesen, Bahulayan et al. 2007). Although tiotropium is known to have a relatively short M_2 dissociation time, it still inhibits collagen synthesis and proliferation of lung fibroblasts, which are known to be M_2-predominant (Matthiesen, Bahulayan et al. 2007, Haag, Matthiesen et al. 2008). My future studies could investigate the expression of M_1, M_2 and M_3 receptors on fibrocytes.

I found CCR7 expression on fibrocytes is down-regulated by salmeterol, but not by tiotropium. To the best of my knowledge, studies investigate the effect of bronchodilators on CCR7 expression are limited. Activation of β_2-AR in bacterial toll-like receptor agonist-treated dendritic cells leads to down-regulation of IL-12 and

up-regulation of IL-10, with subsequent inhibition of CCR7 expression and dendritic cell migration (Maestroni 2006). Although nicotine up-regulates CCR7 expression in dendritic cells (Vassallo, Tamada et al. 2005), there is no evidence that muscarinic receptors are involved in CCR7 expression. It seems that cAMP activation inhibits CCR7 expression. Intriguingly, Giordano *et al.* demonstrated that cAMP analogues 8-Br-cAMP hampered LPS-induced expression of CCR7 on dendritic cells and the migration of dendritic cells toward CC chemokine ligand 19/macrophage inflammatory protein-3-β (MIP-3-β) (Giordano, Magaletti et al. 2003). Further study into signalling pathways involved in CCR7 production will help to clarify the mechanism.

To summarise, bronchodilators are important components in anti-asthmatic regimens, and I show that bronchodilators reduce the number of fibrocytes. However, the possibility of salmeterol and tiotropium-induced NANT cell apoptosis has been excluded by the annexin V/propidium iodide apoptosis assay. It is still not clear whether the cell reduction effect of these two bronchodilators is through inhibition of proliferation and/or NANT cell-to-fibrocyte differentiation. EdU proliferative assay and Col I and α–SMA mRNA detection may provide direction for further investigation about the mechanisms of action of these drugs on fibrocytes. β_2-AR

detection on fibrocytes and β_2-AR polymorphism studies for patients with severe asthma may help to explain why fibrocytes from patients with severe asthma only responded to tiotropium. PDE4 inhibitors and cAMP analogues may bypass the problems caused by β_2-AR polymorphisms and be the solution of fibrocytes-related airway remodelling in severe asthma.

Chapter 7: Conclusion

7.1 Findings of study and contribution to the field

Asthmatic airways are characterised by subepithelial fibrosis and airway smooth muscle thickening, characteristics of airway remodelling, which is even more pronounced in patients with severe asthma (Macedo, Hew et al. 2009) and may be associated with airflow limitation and airway hyperresponsiveness (Tsurikisawa, Oshikata et al. 2010). The role of fibroblasts, myofibroblasts and airway smooth muscle cells (ASMC) in airway remodelling has been extensively studied (Al-Muhsen, Johnson et al. 2011). Circulating fibrocytes are progenitor cells of fibroblasts and myofibroblasts (Grieb and Bucala 2012). Since the identification of fibrocytes as a sub-population of leukocytes in 1994, there is a growing research interest in their participation in the repair and the fibrotic process, including airway remodelling (Bucala, Spiegel et al. 1994). Circulating fibrocytes are recruited to the airways upon allergen exposure (Schmidt, Sun et al. 2003). Elevated concentrations of chemokines in the airways, higher expression of the respective chemokine receptors in fibrocytes (Isgro, Bianchetti et al. 2013) and increased number of fibrocytes in peripheral blood and bronchial biopsies (Saunders, Siddiqui et al. 2009) have been observed in severe asthma.

In this project, I have demonstrated that patients with severe asthma have a greater pool of circulating fibrocytes which can readily undergo differentiation (Chapter 3.2.3) and exert an inherent resistance to apoptosis (Chapter 5.2.4), thus contributing to the increased number of myofibroblasts and consequently the augmented subepithelial fibrosis and ASM thickness observed in the airways of these patients. Furthermore, I found that severe asthmatic fibrocytes were relatively insensitive to the inhibitory effect of corticosteroids (CS) on the expression of CC chemokine receptor (CCR) 7 (Chapter 5.2.10), a key mediator of their migration, as well as the induction of apoptosis (Chapter 5.2.2-4). This suggests that despite the use of high doses of inhaled and sometimes oral CS, patients with severe asthma have a high numbers of circulating fibrocytes that can migrate to the airways. Intriguingly, these cells were also resistant to the suppressive effect of long-acting β2-adrenoceptor (β2-AR) agonists (LABAs, Chapter 6.2.1). Therefore, it is not surprising that there is a higher number of infiltrating fibrocytes and differentiating fibrocytes in the airways of severe asthma patients despite the use of high dose CS and LABAs (Saunders, Siddiqui et al. 2009). However, severe asthmatic fibrocytes were sensitive to long-acting muscarinic antagonists (LAMA, Chapter 6.2.2), which are widely used amongst patients with chronic obstructive pulmonary disease but are currently not

used as a mainstay treatment for asthma. My study not only highlights fibrocytes as an important therapeutic target to control airway remodelling, but also provides a model to investigate other CS-insensitive fibrotic diseases associated with the activity of fibrocytes.

One way of targeting fibrocytes would be to reduce their numbers in the circulation. Angiotensin II receptor antagonists, such as valsartan, have been shown to reduce the number of fibrocytes in the bone marrow of a mice model of renal fibrosis and the production of collagen and transforming growth factor (TGF)-β in human fibrocytes (Sakai, Wada et al. 2008). Moreover, targeting chemokine receptors such as CCR7, CCR3, CCR5 and CXCR4 or their ligands using monoclonal antibodies would be a way of reducing fibrocyte numbers in the airway tissue by blocking their migration. Interestingly, sirolimus (rapamycin), which inhibits mammalian target of rapamycin (mTOR) has been shown to down-regulate CXCR4 expression on fibrocytes in animal models of pulmonary fibrosis (Mehrad, Burdick et al. 2009).

Having shown that fibrocytes from patients with severe asthma show an inherent resistance to apoptosis, specifically inducing fibrocyte apoptosis could be another way of reducing their numbers. Comparison of the differential expression of the pro-apoptotic and anti-apoptotic members of B cell lymphoma 2 (bcl-2) family genes

in fibrocytes from patients with non-severe or severe asthma by microarrays might provide candidates for further gene therapy; e.g. overexpressing the pro-apoptotic genes or interfering the anti-apoptotic genes by inhaled liposomal vectors. Phosphatidylinositol 3'-kinase (PI3K) inhibition reduced the number of fibrocytes and differentiating fibrocytes even in severe asthma (Chapter 5.2.11). Further determination of isoforms (PI3K α, β, δ or γ) involving the proliferation, differentiation and survival of fibrocytes will help to select specific PI3K inhibitor to prevent fibrocyte-mediated airway remodelling in severe asthma. Although T_H2 cytokines and neurotrophins did not show significant effect on severe asthmatic fibrocytes in this project (Chapter 4.2.1-2), oxidative stress, transforming-growth factor-β (TGF-β), endothelin-1 and epidermal growth factor (EGF) are associated with increased proliferation and myofibroblastic differentiation of fibrocytes in chronic obstructive asthma (Wang, Huang et al. 2008, Wang, Huang et al. 2012, Weng, Chen et al. 2013). Moreover, N-acetylcysteine decreases the expression of epidermal growth factor receptor and the number and myofibroblastic differentiation of fibrocytes from asthmatic patients with chronic obstruction (Wang, Huang et al. 2012), suggesting that targeting reactive oxygen species using antioxidants could reduce fibrocyte proliferation. Leukotriene modifiers, such as montelukast, inhibited

the expression of TGF-β and collagen deposition in the airways of an asthmatic mouse model (Ochkur, Protheroe et al. 2013). Leukotriene modifiers also enhance the activity of fibrocytes' pro-matrix metalloproteinase-9 (Hayashi, Kawakita et al. 2014), which represses subepithelial fibrosis through degradation of collagen in remodelled airways (Mattos, Lim et al. 2002). Therefore, the effect of N-acetylcysteine, leukotriene modifiers, endothelin receptor antagonists (such as bosentan, used in idiopathic pulmonary fibrosis) on fibrocyte-mediated airway remodelling would be worth investigating.

Overcoming the relative CS insensitivity observed in fibrocytes from severe asthma patients could also lead to reduction in fibrocytes and thus improvement of airway remodelling. N-acetylcysteine, which up-regulates the expression of glucocorticoid receptor (GR) in severe asthmatic fibrocytes (Chapter 5.2.9), may reverse CS insensitivity, although signs of anaphylaxis, such as bronchospasm, should be cautioned (Rogers 2007). Besides, c-jun N-terminal kinase (JNK) inhibition also restores the suppressive effect of CS on the number of fibrocytes in severe asthma (Chapter 5.2.11). Several JNK inhibitors (CC-401, for rheumatoid arthritis) or p38 mitogen-activated protein kinase/JNK inhibitors (CNI-149, for Crohn's disease) have

been investigated in clinical trials (Adcock, Chung et al. 2006). These agents may also overcome CS insensitivity in fibrocytes in severe asthma.

In contrast to dexamethasone and salmeterol, the muscarinic antagonist tiotropium, led to a reduction in the number of fibrocytes in vitro. Thus, LAMAs in combination with inhaled CS and LABAs could be beneficial to patients with severe asthma with evidence of fibrocyte-mediated airway remodelling. The effect of tiotropium on fibrocytes could be, at least partly, through an increase in 3',5'-cyclic adenosine monophosphate (cAMP) levels. Indeed, I have shown that both cAMP analogues and phosphodiesterase (PDE) type IV inhibitor exert suppressive effects on severe asthmatic fibrocytes (Chapter 6.2.3). Hence, compounds that elevate cAMP could reduce fibrocyte numbers. Apart from theophylline (non-selective PDE inhibitor) and roflumilast (PDE4 inhibitor), a couple of agents, such as RPL554 (PDE3/4 inhibitor) and CHF6001 (PDE4 inhbitor), are under clinical trial for asthma treatment. Downstream effectors of cAMP, such as exchange protein directly activated by cAMP and protein kinase A (Epac), could also be targets of new drug development for the prevention of fibrocyte-mediated airway remodelling in severe asthma.

The drawback of all the above treatments is that they lack specificity for fibrocytes as they can act on other cell types. Fibrocyte-targeted gene therapy, by delivering DNA constructs directly to fibrocytes or using a fibrocyte-specific promoter, could be an appealing way of specifically modulating fibrocyte function. However, more work into identifying fibrocyte-specific target genes and promoters is required before these treatments can be developed.

7.2 Advantages and Limitations of study and future work

A limitation of my study is that the recruitment of fibrocytes in bronchial biopsies from patients with severe asthma was not determined by immunohistochemical staining; however, this has been demonstrated previously by other groups (Saunders, Siddiqui et al. 2009). Given that only 0.5% of leukocytes in peripheral blood are fibrocytes (although cultured CD14+ cells acquire fibrocyte phenotype characterised by collagen expression) (Grieb and Bucala 2012), the clinical significance of fibrocytes in the development airway remodelling in severe asthmatic patients is open to debate. However, allergen exposure induces the recruitment of fibrocytes into the bronchial tissue in allergic asthmatic patients and also in a mouse model of allergic asthma (Schmidt, Sun et al. 2003). The migration of fibrocytes towards CC chemokine ligand (CCL) 19/macrophage inflammatory protein-3-β (MIP-3-β) and

CXC chemokine ligand (CXCL) 12/stromal cell-derived factor 1 (SDF-1) *in vitro* has recently been reported (Wang et al. J Allergy Clin Immunol 2014, in press). Blocking the chemotaxis of fibrocytes prevents fibrosis in the lungs (CXCL12/ CXCR4), peritoneum (CCL2-monocyte chemotactic protein-1 (MCP-1)/ CCR2) and kidneys (CCL21-secondary lymphoid-tissue chemokine (SLC)/CCR7) in animal models (Sakai, Wada et al. 2006, Mehrad, Burdick et al. 2009, Kokubo, Sakai et al. 2012). A severe asthma model has been established using animals sensitised to house dust mite mixed with complete Freund's adjuvant which involves recruitment of steroid-insensitive neutrophils and eosinophils to the lung (Nakada, Shan et al. 2014). Further observation of the impact of anti-chemokine antibodies or anti-chemokine receptor antibodies on the accumulation of fibrocytes, and the subsequent collagen (Col) I deposition, and ASM thickness in the airways of an animal model of severe asthma will be of interest.

My experimental model is based on the flow cytometric analysis of Col I+/CD45+ fibrocytes and α-smooth muscle actin (α-SMA)+ differentiating fibrocytes within non-adherent non-T (NANT) fraction of peripheral blood mononuclear cells (PBMC). Since the fibrocytes observed in adherent PBMC or CD14+ cell models are usually recovered and purified after a prolonged period of culture (5-14 days), the

NANT cell model has several advantages. Firstly, the effect of treatment on fibrocytes can be observed at an earlier stage of differentiation (day 3) when the functional properties of fibrocytes are probably more similar to those in peripheral blood. Secondly, flow cytometry requires a relatively smaller number of cells compared to other methods (e.g. Western blotting), and quantifies fibrocytes with a higher specificity compared to counting under microscope according to morphology.

The major limitation is that neither NANT cells nor adherent PBMC represent a homogenous fibrocyte population. The subpopulations in NANT cells and adherent PBMC have not been identified. The major components of PBMC include monocytes (10%), lymphocytes (70-85%), B cells (10-15%) NK cells (10%), natural killer T cells (10%) (Abbas and Lichtman 2003). Isolation of human monocytes by the adherence method usually provides a purity of the monocyte population of > 90%, with 60% - 90% recovery (Hashimoto, Gon et al. 2001). Since the monocytes and T cells have been largely removed by attachment and CD3+ MicroBeads T cell depletion respectively, the NANT cell fraction of PBMC should consist of B cells, NK cells, very few non-adherent monocytes and some unclassified progenitor cells apart from fibrocytes (Wang, Hsieh et al. 1999). Therefore, studying changes in the function of fibrocytes cannot be specifically evaluated. It is still feasible to block

certain pathways by chemically inhibiting or transfecting the whole NANT cell population, followed by identifying fibrocytes within NANT cells by flow cytometry. However, we could still not rule out the possibility that non-fibrocyte NANT cells interfere with fibrocyte function in a paracrine manner. A similar problem cannot be avoided with the adherent PBMC model if cells are stimulated before purification of fibrocytes on day 5-14 in culture. The function of fibrocytes isolated from the NANT fraction and the adherent fraction of PBMC has not been previously compared. In this study, I have, for the first time, shown that fibrocytes derived from both fractions of PBMC had similar responses to pro-inflammatory mediators and asthma medications, which means that data generated from NANT cell model should be reproducible in the adherent cell model.

The ideal method to conduct fibrocyte studies would be to obtain purified Col I+/CD45+ fibrocytes immediately after isolation of PBMC. I propose to develop two novel methods for isolating fibrocytes from peripheral blood: the first requires a customised magnetic beads-conjugated anti-Col I antibody, which is not currently available, combined with an anti-CD45 (or anti-CD34) MicroBeads (Miltenyl Biotec). Fibrocytes labelled by these two antibodies could be purified from freshly isolated PBMC using a magnetic separator and magnetic columns. The alternative method

requires sorting Col I+/CD45+ cells by a fluorescence-activated cell sorting machine. It is worth noting that Col I is usually stained with antibody after permeabilisation, a process which may be detrimental to the survival of fibrocytes (Wang, Huang et al. 2008, Moeller, Gilpin et al. 2009, Bellini, Marini et al. 2012).

To the best of my knowledge, this project is the first one to investigate i) the effect of neurotrophins, β2-AR agonist, muscarinic antagonist and PDE4 inhibitor on fibrocytes from healthy subjects and asthmatic patients, ii) the effect of CS on fibrocytes from asthmatic patients, and iii) the effect of T_H2 cytokines on fibrocytes from severe asthmatic patients. To further investigate the mechanisms involved during the change in fibrocyte number upon treatment, future work should include:

i) Determine why fibrocytes from patients with severe asthma have more circulating fibrocytes with a greater capacity to differentiate. The number of fibrocytes in peripheral blood could be related to their abundance in the bone marrow, chemotaxis, proliferation and cell survival. The fibrocytes in the bone marrow could be determined in a severe asthma animal model. The level of mediators that are known to mediate fibrocyte proliferation and differentiation such as EGF, TGF-β, endothelin-1, connective tissue growth factor (CTGF) and chemokines (CCL19/MIP-3-β, CCL21/SLC,

CXCL12/SDF-1, CCL5/regulated on activation, normal T cell expressed and secreted (RANTES), CCL11/eotaxin, CCL24/eotaxin-2) could be measured in sera and in the airways, whilst the expression of their respective receptors would be determined on fibrocytes from healthy subjects and patients with non-severe or severe asthma. The proliferation of fibrocytes, using 5-ethynyl-2'-deoxyuridine incorporation assay (Chapter 2.2.4.5), could be compared amongst the three patient groups.

ii) Determine the molecular mechanisms behind CS insensitivity. Severe asthmatic fibrocytes show reduced GR expression. It is worth investigating whether overexpressing GR restores CS sensitivity. Also, if a pure fibrocyte population is available, JNK activity and the phosphorylation status and nuclear translocation of GR, could also be compared amongst healthy subjects and patients with non-severe or severe asthma. Other mechanisms related to CS responsiveness, such as histone deacetylase activity and histone acetylation, could also be explored.

iii) Elucidate the mechanisms behind the resistance to salmeterol. Compare the expression of β_2-adrenoceptor (β_2-AR) and its activity by determining

cAMP production, in fibrocytes from healthy subjects and patients with non-severe or severe asthma.

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