

Investigation of Corticosteroid Responsiveness in Airway Smooth Muscle Cells of Severe Asthma

By

Po-Jui Chang

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National Heart and Lung Institute, Imperial College London
Dovehouse Street, London, SW3 6LY

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Declaration of Originality

I declare that this thesis is entirely my own work.

Po-Jui Chang

To my wife, Chien-Hui, my son, Ching-Yen, and my parents

Abstract

Asthma is characterised by airway inflammation, hyper-responsiveness and airway remodelling. Features of airway remodelling include hypertrophy/hyperplasia of airway smooth muscle cells (ASMC), which possess an important synthetic capacity in addition to their contractile function. Patients with severe asthma respond poor to even high-dose corticosteroids and consume enormous healthcare resources. Corticosteroid insensitivity in peripheral blood mononuclear cells and alveolar macrophages of severe asthma has been reported. It is still unclear whether ASMC display a similar phenotype. I hypothesised that corticosteroid insensitivity exists in ASMC of severe asthma, and the underlying mechanisms were investigated. ASMC were obtained from endobronchial biopsy and were experimented with at passage 4-5.

TNF- α induced CCL11, CXCL8 and CXCL10 expression in ASMC. IFN- γ suppressed TNF α -induced CCL11 and CXCL8, through attenuation of NF- κ B (p65) recruitment to the gene promoters, but potentiated induced CXCL10. CX3CL1 was induced synergistically only by combined both cytokines. Baseline and TNF α -induced CCL11 expression in non-severe asthma were greater than the healthy and severe asthma, while IFN γ -induced CXCL10 was increased in severe asthma. Whereas TNF α -induced p65 expression was increased in severe asthma, there was no difference in nuclear translocation or recruitment to the gene promoters between groups. The effect of dexamethasone was reduced in terms of suppressing induced CCL11 and CXCL8 expression in severe asthma, while the potentiating effect on CX3CL1 was not different between groups. Of the mitogen-activated protein kinases (MAPK), p38

activation was heightened in severe asthma, and p38 inhibition restored the corticosteroid sensitivity. Expression of glucocorticoid receptor (GR) was decreased in asthma, while nuclear translocation of GR was impaired only in severe asthma.

In conclusion, there is differential expression of inflammatory chemokines in ASMC of asthma. Corticosteroid insensitivity exists in ASMC of severe asthma in terms of suppressing induced chemokines, partially attributed to heightened p38 MAPK activity and impaired nuclear translocation of GR.

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Table of Contents

Abstract	4
Acknowledgments	6
Table of Contents.....	7
List of Tables.....	14
List of Figures	15
Abbreviations.....	20
Chapter 1. Introduction.....	23
1.1 Asthma	23
1.1.1 Airway inflammation in asthma.....	24
1.1.1.1 T-lymphocytes.....	26
1.1.1.2 Mast cells.....	28
1.1.1.3 Eosinophils.....	28
1.1.1.4 Neutrophils	30
1.1.1.5 Structural cells	31
1.1.2 Airway obstruction.....	32
1.1.3 Airway hyper-responsiveness.....	33
1.1.4 Airway remodelling	33
1.1.5 Severe asthma.....	35
1.2 Airway smooth muscle cells (ASMC) and asthma.....	38
1.2.1 Pleiotropic role of ASMC	38
1.2.2 ASMC in asthma.....	40
1.3 Cytokines	41
1.3.1 Tumour necrosis factor-alpha (TNF-α)	41
1.3.2 Interferon-gamma (IFN-γ)	45
1.4 Chemokines	47
1.4.1 CCL11 (Eotaxin)	50
1.4.2 CXCL8 (IL-8)	51

1.4.3 CXCL10 (IP-10)	52
1.4.4 CX3CL1 (fractalkine)	53
1.5 Nuclear factor- κ B (NF- κ B)	55
1.5.1 NF- κ B Structure.....	56
1.5.2 NF- κ B activation	56
1.5.3 Post-translational modifications of NF- κ B	58
1.6 Mitogen-activated protein kinases (MAPK)	59
1.6.1 p38 MAPK	61
1.6.2 c-Jun N-terminal kinase (JNK).....	63
1.6.3 Extracellular signal-regulated kinase (ERK)	64
1.7 Corticosteroids and glucocorticoid receptor	65
1.7.1 Corticosteroids in treatment of asthma.....	65
1.7.2 Cellular effects of corticosteroids	66
1.7.3 Glucocorticoid receptor (GR)	68
1.7.3.1 Ligand binding and nuclear translocation	70
1.7.3.2 Transactivation.....	70
1.7.3.3 Transrepression	72
1.7.3.4 Destabilisation of inflammatory mRNA	73
1.7.3.5 Post-translational modification of GR: focus on phosphorylation	75
1.7.4 Corticosteroid insensitivity in severe asthma.....	78
1.8 Hypothesis and aims.....	79
Chapter 2. Materials and Methods	81
2.1 Materials.....	81
2.1.1 General reagents.....	81
2.1.2 Cell culture	81
2.1.3 Cell viability assay	81
2.1.4 Enzyme linked immunosorbent assays (ELISA)	82
2.1.5 RNA extraction, cDNA preparation and real-time quantitative PCR	82

2.1.6 Whole cell/cytoplasmic/nuclear protein extraction and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE)/Western blotting.....	82
2.2 Methods.....	83
2.2.1 Recruitment of healthy subjects and patients with non-severe and severe asthma.....	83
2.2.2 Human ASMC isolation and culture.....	85
2.2.3 Cell viability assay	86
2.2.4 ELISA	87
2.2.5 Determination of mRNA expression by RT-qPCR.....	89
2.2.5.1 RNA extraction	89
2.2.5.2 Reverse transcription	89
2.2.5.3 Real-time PCR	90
2.2.6 Chromatin immunoprecipitation (ChIP) assay	91
2.2.6.1 Optimisation of sonication	91
2.2.6.2 Chromatin immunoprecipitation	93
2.2.7 Determination of protein expression by Western blotting	94
2.2.7.1 Whole cell extraction	94
2.2.7.2 Cytoplasmic and nuclear extraction	94
2.2.7.3 Determination of protein concentration	95
2.2.7.4 Western blotting.....	96
2.2.8 Statistical analysis.....	97
Chapter 3. Regulation of chemokines in ASMC: effect of TNF- α and IFN- γ	100
3.1 Introduction	100
3.2 Results	102
3.2.1 Effect of TNF- α and IFN- γ on chemokine expression in ASMC	102
3.2.1.1 Effect of TNF- α and IFN- γ on CCL11 release.....	102
3.2.1.2 Effect of TNF- α and IFN- γ on CXCL8 release	104
3.2.1.3 Effect of TNF- α and IFN- γ on CXCL10 release	106
3.2.1.4 Effect of TNF- α and IFN- γ on CX3CL1 release	107

3.2.1.5 Effect of IFN- γ on TNF α -induced CCL11 in ASMC of healthy subjects and patients with non-severe and severe asthma	110
3.2.1.6 Effect of IFN- γ on TNF α -induced CXCL8 in ASMC of healthy subjects and patients with non-severe and severe asthma	110
3.2.1.7 Effect of IFN- γ on TNF α -induced CXCL10 in ASMC of the healthy subjects and patients with non-severe and severe asthma	115
3.2.1.8 Effect of TNF- α and IFN- γ on CX3CL1 in ASMC of the healthy subjects and patients with non-severe and severe asthma	115
3.2.1.9 Effect of TNF- α on recruitment of NF- κ B (p65) to the promoter of CCL11 in ASMC	119
3.2.1.10 Effect of TNF- α on recruitment of NF- κ B (p65) to the promoter of CXCL8 in ASMC	120
3.2.1.11 Effect of TNF- α and IFN- γ on recruitment of NF- κ B (p65) to the promoter of CX3CL1 in ASMC	121
3.2.1.12 Effect of IFN- γ on TNF α -induced p65 recruitment to the CCL11 promoter in ASMC of healthy subjects and patients and non-severe and severe asthma .	122
3.2.1.13 Effect of IFN- γ on TNF α -induced p65 recruitment to the CXCL8 promoter in ASMC of healthy subjects and patients and non-severe and severe asthma .	122
3.2.1.14 Effect of IFN- γ on TNF α -induced p65 recruitment to CX3CL1 promoter in ASMC of healthy subjects and patients and non-severe and severe asthma.....	125
3.2.2 Comparison of cytokine-induced chemokine release and mRNA expression in ASMC of healthy subjects and patients with non-severe and severe asthma.	127
3.2.2.1 Comparison of TNF α -induced CCL11 in ASMC of healthy subjects and patients with non-severe and severe asthma	127
3.2.2.2 Comparison of TNF α -induced CXCL8 in ASMC of healthy subjects and patients with non-severe and severe asthma	128
3.2.2.3 Comparison of cytokine-induced CXCL10 release in ASMC of healthy subjects and patients with non-severe and severe asthma.....	129
3.2.2.4 Comparison of TNF α and IFN γ -induced CX3CL1 in ASMC of healthy subjects and patients with non-severe and severe asthma.....	132
3.2.3 Investigation and comparison of NF- κ B (p65) regulation in ASMC	133
3.2.3.1 Comparison of TNF α -induced p65 protein and mRNA expression in ASMC of the healthy subjects and patients with non-severe and severe asthma.....	134
3.2.3.2 Effect of TNF- α on nuclear translocation of p65 in ASMC.....	135
3.2.3.3 Comparison of baseline nuclear p65 abundance in ASMC of healthy subjects and patients with non-severe and severe asthma.....	136

3.2.3.4 Comparison of nuclear translocation of p65 induced by TNF- α in ASMC of healthy subjects and patients with non-severe and severe asthma	137
3.2.3.5 Comparison of cytokine-induced p65 recruitment to chemokine promoters in ASMC of healthy subjects and patients with non-severe and severe asthma	138
3.3 Discussion	139
3.3.1 Modulating effect of IFN- γ on TNF α -induced inflammation	140
3.3.2 Differential expression of inflammatory chemokines in ASMC	144
3.3.3 Regulation and differential expression of NF- κ B (p65) in ASMC	146
Chapter 4. Corticosteroid responsiveness of ASMC of healthy subjects and patients with non-severe and severe asthma.....	150
4.1 Introduction	150
4.2 Results	152
4.2.1 Effect of dexamethasone on cell number and viability of ASMC.....	152
4.2.2 Comparison of the corticosteroid response in ASMC of healthy subjects and patients with non-severe and severe asthma	153
4.2.2.1 Comparison of the suppressive effect of dexamethasone on CCL11 release	153
4.2.2.2 Comparison of the suppressive effect of dexamethasone on CXCL8 release	155
4.2.2.3 Comparison of the suppressive effect of dexamethasone on CXCL10 release	157
4.2.2.4 Comparison of the potentiating effect of dexamethasone on CX3CL1 release	157
4.2.2.5 Comparison of the suppressive effect of dexamethasone on CCL11 mRNA expression.....	160
4.2.2.6 Comparison of the suppressive effect of dexamethasone on CXCL8 mRNA expression.....	162
4.2.2.7 Comparison of the potentiating effect of dexamethasone on CX3CL1 mRNA expression	164
4.2.3 Effect of IFN- γ on the suppressive effect of dexamethasone in ASMC of healthy subjects and patients with non-severe and severe asthma	166
4.2.3.1 Effect of IFN- γ on suppressive effect of TNF α -induced CXCL10 release in ASMC	166

4.2.3.2 Effect of IFN- γ on the suppressive effect of dexamethasone on TNF α -induced CCL11 release in ASMC	168
4.2.3.3 Effect of IFN- γ on suppressive effect of TNF α -induced CXCL8 release in ASMC	170
4.3 Discussion	172
4.3.1 Relative corticosteroid insensitivity in ASMC of patients with severe asthma	172
4.3.2 Impaired suppressive effect of dexamethasone by IFN- γ in ASMC.....	174
Chapter 5. Role of MAPK in corticosteroid insensitivity in ASMC of patients with severe asthma	179
5.1 Introduction	179
5.2 Results	181
5.2.1 Effect of TNF- α on MAPK activation in ASMC—Optimisation studies	181
5.2.2 Comparison of TNF α -induced p38 MAPK activation in ASMC of healthy subjects and patients with non-severe and severe asthma.....	182
5.2.3 Comparison of TNF α -induced JNK activation in ASMC of healthy subjects and patients with non-severe and severe asthma	183
5.2.4 Comparison of TNF α -induced ERK activation in ASMC of healthy subjects and patients with non-severe and severe asthma	184
5.2.5 Correlation between induced MAPK phosphorylation and suppressive effect of dexamethasone in ASMC	185
5.2.6 Effect of p38 inhibition on chemokine release and suppressive effect of dexamethasone in ASMC of patients with severe asthma.....	188
5.2.7 Effect of JNK inhibition on chemokine release and suppressive effect of dexamethasone in ASMC of patients with severe asthma.....	192
5.2.8 Effect of ERK inhibition on chemokine release and suppressive effect of dexamethasone in ASMC of patients with severe asthma.....	192
5.2.9 Effect of IFN- γ on MAPK activity induced by TNF- α in ASMC	196
5.3 Discussion	199
Chapter 6. Regulation of GR and NF- κ B (p65) by corticosteroids in ASMC	204
6.1 Introduction	204
6.2 Results	207
6.2.1 Investigation and comparison of GR regulation in ASMC	207

6.2.1.1 Comparison of GR abundance in ASMC of healthy subjects and patients with non-severe and severe asthma.....	207
6.2.1.2 Effect of dexamethasone on GR expression in ASMC	208
6.2.1.3 Effect of dexamethasone on nuclear translocation of GR in ASMC	209
6.2.1.4 Comparison of baseline nuclear abundance of GR in ASMC of healthy subjects and patients with non-severe and severe asthma.....	210
6.2.1.5 Comparison of nuclear translocation of GR induced by dexamethasone in ASMC of healthy subjects and patients with non-severe and severe asthma.....	211
6.2.1.6 Effect of dexamethasone on phosphorylation of GR at S211 in ASMC....	212
6.2.1.7 Comparison of phosphorylation of GR at S211 induced by dexamethasone in ASMC of healthy subjects and patients with non-severe and severe asthma.	213
6.2.2 Investigation of mechanisms of suppression of NF- κ B activity by corticosteroid	214
6.2.2.1 Comparison of the suppressive effect of dexamethasone on induced p65 protein and mRNA expression in ASMC of healthy subjects and patients with non-severe and severe asthma	214
6.2.2.2 Effect of dexamethasone on TNF α -induced nuclear translocation of p65 in ASMC of healthy subjects and patients with non-severe and severe asthma.....	215
6.2.2.3 Effect of dexamethasone on p65 recruitment to the CCL11 promoter in ASMC of healthy subjects and patients with asthma.....	217
6.2.3 Investigation of mechanisms by which TNF α -induced p38 activation modulates corticosteroid response in ASMC	218
6.2.3.1 Effect of TNF- α on dexamethasone-induced nuclear translocation of GR in ASMC of healthy subjects and patients with non-severe and severe asthma.....	218
6.2.3.2 Effect of p38 inhibition on dexamethasone-induced nuclear translocation of GR in ASMC	220
6.3 Discussion	221
6.3.1 Differential expression and regulation of GR in ASMC	221
6.3.2 Regulation of NF- κ B (p65) by dexamethasone in ASMC	223
6.3.3 Role of p38 MAPK activity for GR nuclear translocation in ASMC	224
Chapter 7. General discussion.....	227
Reference list	232

List of Tables

Table 1.1. ATS Workshop Consensus for severe/refractory asthma	36
Table 1.2. Summary of effects of GR phosphorylation at S201, S211 and S226.....	77
Table 2.1. Cytokines and inhibitors used in the project.	81
Table 2.2. Characteristics of studied subjects	84
Table 2.3. Conditions used for ELISA.	87
Table 2.4. Primers used for RT-qPCR.	91
Table 2.5. Primer sequences for ChIP assays.	93
Table 2.6. Antibodies used for Western blot.	98
 Table 6.1. Mechanisms of glucocorticoid action by GR and sites of regulation in corticosteroid insensitivity	 205

List of Figures

Chapter 1

Figure 1.1. Inflammatory cells involved in asthma	25
Figure 1.2. Interaction between Th1/Th2 lymphocytes in immunopathogenesis of asthma.	27
Figure 1.3. Structures of airways from subjects with and without asthma	34
Figure 1.4. Pleiotropic role of ASMC	39
Figure 1. 5. TNF- α biology and signalling	42
Figure 1.6. The normal airway and the effects of TNF- α on the asthmatic airway.....	44
Figure 1.7. Classification and structure of chemokines.....	48
Figure 1.8. Role of membrane bound and soluble CX3CL1 and the receptor CX3CR1	54
Figure 1.9. NF- κ B signalling pathways	57
Figure 1.10. MAPK cascades.....	60
Figure 1.11. Schematic overview of MAPK pathways regulating ASMC functions.....	62
Figure 1.12. Cellular effects of corticosteroids in the airway	67
Figure 1.13. Overview of mechanisms of glucocorticoid-GR regulation of gene expression	69
Figure 1.14. Corticosteroid activation of anti-inflammatory gene expression (transactivation)	71
Figure 1.15. Corticosteroid suppression of activated inflammatory genes expression (transrepression).....	72
Figure 1.16. Destabilisation of inflammatory mRNA by corticosteroids through suppression of p38 MAPK by induction of MKP-1	74
Figure 1.17. Structure and post-translational modifications of GR	75

Chapter 2

Figure 2.1. Optimisation of sonication for ChIP assay	92
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Chapter 3

Figure 3.1. Effect of TNF- α and IFN- γ on CCL11 release in ASMC	103
Figure 3.2. Effect of TNF- α and IFN- γ on CXCL8 release in ASMC.....	105
Figure 3.3. Effect of TNF- α and IFN- γ on CXCL10 release in ASMC.....	107

Figure 3.4. Effect of TNF- α and IFN- γ on CX3CL1 release in ASMC	109
Figure 3.5. Effect of IFN- γ on TNF α -induced CCL11 release in ASMC of healthy subjects and patients with non-severe and severe asthma	111
Figure 3.6. Effect of IFN- γ on TNF α -induced CCL11 mRNA expression in ASMC of healthy subjects and patients with non-severe and severe asthma	112
Figure 3.7. Effect of IFN- γ on TNF α -induced CXCL8 release in ASMC of healthy subjects and patients with non-severe and severe asthma	113
Figure 3.8. Effect of IFN- γ on TNF α -induced CXCL8 mRNA expression in ASMC of healthy subjects and patients with non-severe and severe asthma	114
Figure 3.9. Effect of IFN- γ on TNF α -induced CXCL10 release in ASMC of healthy subjects and patients with non-severe and severe asthma	116
Figure 3.10. Effect of TNF- α and IFN- γ on CX3CL1 release in ASMC of healthy subjects and patients with non-severe and severe asthma	117
Figure 3.11. Effect of TNF- α and IFN- γ on CX3CL1 mRNA expression in ASMC of healthy subjects and patients with non-severe and severe asthma	118
Figure 3.12. Effect TNF- α on p65 NF- κ B recruitment to the CCL11 promoter in ASMC....	119
Figure 3.13. Effect TNF- α on p65 NF- κ B recruitment to the CXCL8 promoter in ASMC ...	120
Figure 3.14. Effect TNF- α and IFN- γ on p65 NF- κ B recruitment to the CX3CL1 promoter in ASMC	121
Figure 3.15. Effect of IFN- γ on TNF α -induced p65 recruitment to the CCL11 promoter in ASMC	123
Figure 3.16. Effect of IFN- γ on TNF α -induced p65 recruitment to the CXCL8 promoter in ASMC	124
Figure 3.17. Effect of IFN- γ on TNF α -induced p65 recruitment to the CX3CL1 promoter in ASMC	126
Figure 3.18. Comparison of TNF α -induced CCL11 in ASMC of healthy subjects and patients with non-severe and severe asthma.....	128
Figure 3.19. Comparison of TNF α -induced CXCL8 in ASMC of healthy subjects and patients with non-severe and severe asthma.....	130
Figure 3.20. Comparison of cytokine-induced CXCL10 in ASMC of healthy subjects and patients with non-severe and severe asthma	131
Figure 3.21. Comparison of TNF α and IFN γ -induced CX3CL1 in ASMC of healthy subjects and patients with non-severe and severe asthma	133
Figure 3.22. Comparison of induced p65 protein and mRNA expression in ASMC of healthy subjects and patients with non-severe and severe asthma	134

Figure 3.23. Effect of TNF- α on p65 NF- κ B nuclear translocation in ASMC	135
Figure 3.24. Comparison of baseline abundance of nuclear p65 in ASMC of healthy subjects and patients with non-severe and severe asthma	136
Figure 3.25. Comparison of TNF α -induced p65 nuclear translocation in ASMC of healthy subjects and patients with non-severe and severe asthma	137
Figure 3.26. Comparison of cytokine-induced p65 NF- κ B recruitment to chemokine promoters in ASMC of healthy subjects and patients with non-severe and severe asthma	139

Chapter 4

Figure 4.1. Effect of dexamethasone, TNF- α , and IFN- γ on cell number and viability of ASMC	152
Figure 4.2. Comparison of the suppressive effect of dexamethasone on CCL11 release in ASMC of healthy subjects and patients with non-severe and severe asthma	154
Figure 4.3. Comparison of the suppressive effect of dexamethasone on CXCL8 release in ASMC of healthy subjects and patients with non-severe and severe asthma	156
Figure 4.4. Comparison of the suppressive effect of dexamethasone on CXCL10 release in ASMC of healthy subjects and patients with non-severe and severe asthma	158
Figure 4.5. Comparison of the potentiating effect of dexamethasone on CX3CL1 release in ASMC of healthy subjects and patients with non-severe and severe asthma	159
Figure 4.6. Effect of dexamethasone on induced CCL11 mRNA expression in ASMC	160
Figure 4.7. Comparison of the suppressive effect of dexamethasone on CCL11 mRNA expression	161
Figure 4.8. Effect of dexamethasone on induced CXCL8 mRNA expression in ASMC	162
Figure 4.9. Comparison of the suppressive effect of dexamethasone on CXCL8 mRNA expression	163
Figure 4.10. Effect of dexamethasone on induced CX3CL1 mRNA expression in ASMC	164
Figure 4.11. Comparison of the potentiating effect of dexamethasone on CX3CL1 mRNA expression	165
Figure 4.12. Effect of IFN- γ on suppression of TNF α -induced CXCL10 release by dexamethasone in ASMC	167
Figure 4.13. Effect of IFN- γ on the suppressive effect of TNF α -induced CCL11 release by dexamethasone in ASMC	169
Figure 4.14. Effect of IFN- γ on the suppression of TNF α -induced CXCL8 release by dexamethasone in ASMC	171

Chapter 5

Figure 5.1. Regulation of MAPK in ASMC	181
Figure 5.2. Comparison of induced p38 MAPK phosphorylation in ASMC of healthy subjects and patients with non-severe and severe asthma	182
Figure 5.3. Comparison of induced JNK phosphorylation in ASMC of healthy non-severe and severe asthmatics	183
Figure 5.4. Comparison of induced ERK phosphorylation in ASMC of healthy subjects and patients with non-severe and severe asthma	184
Figure 5.5. Correlation between suppression of induced CCL11 and CXCL8 release by dexamethasone and phosphorylation of p38 MAPK induced by TNF- α in ASMC	186
Figure 5.6. Correlation between suppression of induced CCL11 and CXCL8 release by dexamethasone and phosphorylation of JNK induced by TNF- α in ASMC	187
Figure 5.7. Correlation between suppression of induced CCL11 and CXCL8 release by dexamethasone and phosphorylation of ERK induced by TNF- α in ASMC.....	188
Figure 5.8. Effect of GW-856553 on MAPK activities in ASMC	190
Figure 5.9. Effect of p38 MAPK inhibitor on chemokine release in ASMC of patients with severe asthma.....	191
Figure 5.10. Effect of p38 MAPK inhibition on the response of dexamethasone in ASMC of patients with severe asthma.....	191
Figure 5.11. Effect of JNK inhibitor on chemokine release in ASMC of patients with severe asthma.....	194
Figure 5.12. Effect of JNK inhibitor on the response of dexamethasone in ASMC of patients with severe asthma	194
Figure 5.13. Effect of ERK inhibitor on chemokine release in ASMC of patients with severe asthma.....	195
Figure 5.14. Effect of ERK inhibitor on chemokine release in ASMC of patient with severe asthma.....	195
Figure 5.15. Effect of IFN- γ on TNF α -induced p38 MAPK phosphorylation	197
Figure 5.16. Effect of IFN- γ on TNF α -induced JNK phosphorylation.....	198
Figure 5.17. Effect of IFN- γ on TNF α -induced ERK phosphorylation	199

Chapter 6

Figure 6.1. Mechanisms of glucocorticoid action by GR and sites of regulation in corticosteroid insensitivity	205
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Figure 6.2. Comparison of baseline GR expression in ASMC of healthy subjects and patients with non-severe (NSA) and severe asthma (SA).....	207
Figure 6.3. Effect of dexamethasone on GR expression in ASMC.....	208
Figure 6.4. Effect of dexamethasone on nuclear translocation of GR in ASMC	209
Figure 6.5. Comparison of baseline abundance of nuclear GR in ASMC of healthy subjects and patient with non-severe (NSA) and severe asthma (SA).....	210
Figure 6.6. Comparison of dexamethasone-induced GR nuclear translocation in ASMC of healthy subjects and patients with non-severe and severe asthma	211
Figure 6.7. Effect of dexamethasone on GR phosphorylation at S211 in ASMC.....	212
Figure 6.8. Comparison of dexamethasone-induced GR phosphorylation at S211 in ASMC of healthy subjects and patients with non-severe and severe asthma	213
Figure 6.9. Comparison of the suppressive effect of dexamethasone on induced p65 expression in ASMC of healthy subjects and patients with non-severe and severe asthma	215
Figure 6.10. Effect of dexamethasone on TNF α -induced nuclear translocation of p65 NF- κ B in ASMC.....	216
Figure 6.11. Effect of IFN- γ on TNF α -induced p65 recruitment to the CCL11 promoter in ASMC of healthy subjects and patients with asthma	217
Figure 6.12. Effect of TNF- α on induced nuclear translocation of GR in ASMC	219
Figure 6.13. The effect of p38 α inhibitor on nuclear translocation of GR in ASMC.....	220

Abbreviations

3'-UTR	three prime untranslated region
ADAM	A Disintegrin And Metalloproteinase
AMV	avian myeloblastosis virus
AP-1	activated protein-1
ARE	adenylate-uridylylate-rich elements
ASMC	airway smooth muscle cells
ATP	adenosine triphosphate
ATF	activating transcription factor
ATS	American Thoracic Society
BAL	bronchoalveolar lavage
BCA	bicinchoninic acid
BSA	bovine serum albumin
CBP	CREB-binding protein
CD	cluster of differentiation
C/EBP	CAAT/enhancer binding protein
ChIP	chromatin immunoprecipitation
COPD	chronic obstructive pulmonary disease
CREB	cAMP response element-binding
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
ELR	glutamate-leucine-arginine
ERK	extracellular signal-regulated kinase
ERS	European Respiratory Society
FCS	foetal calf serum
FEV ₁	forced expiratory volume in 1 second
hsp	heat shock protein
GINA	Global Initiative for Asthma Guideline
GR	glucocorticoid receptor
GRE	glucocorticoid-responsive element
GRO	growth regulated oncogene
HAT	histone acetyltransferase
HBSS	Hank's Balanced Salt Solution
IFN	interferon
Ig	immunoglobulin
IκB	inhibitor of NF-κB
IKK	IκB kinase

IL	interleukin
IP	interferon-induced protein
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
kDa	kilodalton
LPS	lipopolysaccharide
PBMC	peripheral blood mononuclear cells
MAPK	mitogen-activated protein kinases
MAPKK	mitogen-activated protein kinase kinases
MAPKKK	mitogen-activated protein kinase kinase kinases
MKP	mitogen-activated protein kinase phosphatase
MSK	mitogen and stress-activated protein kinase
MTT	methylthiazolyldiphenyl-tetrazolium bromide
NAEPP	National Asthma Education and Prevention Programme
NF- κ B	nuclear factor-kappa B
NK	natural killer
pCAF	p300/CBP activating factor
PDGF	platelet-derived growth factor
PG	prostaglandin
PMSF	phenylmethanesulphonyl fluoride
RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
RT-qPCR	reverse transcription-quantitative polymerase chain reaction
SAP	serum response factor accessory protein
SRC	steroid receptor coactivator
STAT	signal transducer and activator of transcription
TACE	TNF α -converting enzyme
TBP	TATA box-binding protein
TCR	T-cell receptor
Th	T helper
TGF	transforming growth factor
TLR	Toll-like receptor
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
Treg	T-regulatory cells
WHO	World Health Organisation

Chapter 1.

Introduction

Chapter 1. Introduction

1.1 Asthma

Asthma is a disorder defined by its clinical, physical, and pathological characteristics. The predominant feature of the clinical history is episodic shortness of breath, particularly at night, often accompanied by cough. Wheezing appreciated on auscultation of the chest is the most common physical finding. The main physiological feature of asthma is episodic airway obstruction characterised by expiratory airflow limitation. The dominant pathological feature is airway inflammation, sometimes associated with airway structural changes.

A common referenced formal definition is that published in the Global Initiative for Asthma Guideline (GINA 2011), which proposes an operational description of asthma as:

A chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation is associated with airway hyper-responsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread, but variable, airflow obstruction within the lung that is often reversible either spontaneously or with treatment.

Asthma is one of the commonest diseases and affects patients of all ages and both genders. Generally beginning early in life, asthma can have its onset at any age.

Global burden of disease estimates suggest that at least 300 million people worldwide have asthma, and the frequency of this disease has increased greatly since the 1980s (Bousquet, Khaltaev et al. 2007), with the potential for an additional 100 million more cases by the year 2025 (Masoli, Fabian et al. 2004). The World Health Organization (WHO) estimates that 15 million disability-adjusted life years are lost annually due to asthma, representing 1% of the total global disease burden, and annual worldwide deaths from asthma are estimated at 250,000/year (Cote, Cartier et al. 1997).

The pathophysiology of asthma includes persistent airway inflammation, reversible airway obstruction, airway hyper-responsiveness, and airway remodelling. These characteristics are described below.

1.1.1 Airway inflammation in asthma

The clinical spectrum of asthma is highly variable, and different cellular patterns have been observed, but the presence of airway inflammation in asthma is persistent even though symptoms are episodic (Cohn, Elias et al. 2004). The inflammation affects all airways including the upper respiratory tract and the nose but its physiological effects are most pronounced in medium-sized bronchi (Jeffery 1999).

It is evident that no single inflammatory cell is able to account for the complex pathophysiology of asthma, but some cells predominate in asthmatic inflammation. The characteristic pattern of inflammation found in allergic diseases is exemplified by

asthma, with activated mast cells, increased numbers of activated eosinophils, increased number of T cell receptor invariant natural killer (NK) T cells and T helper 2 lymphocytes, which release mediators that contribute to symptoms. Structural cells of the airways also produce inflammatory mediators and contribute to the persistence of inflammation in various ways (Fig 1.1).

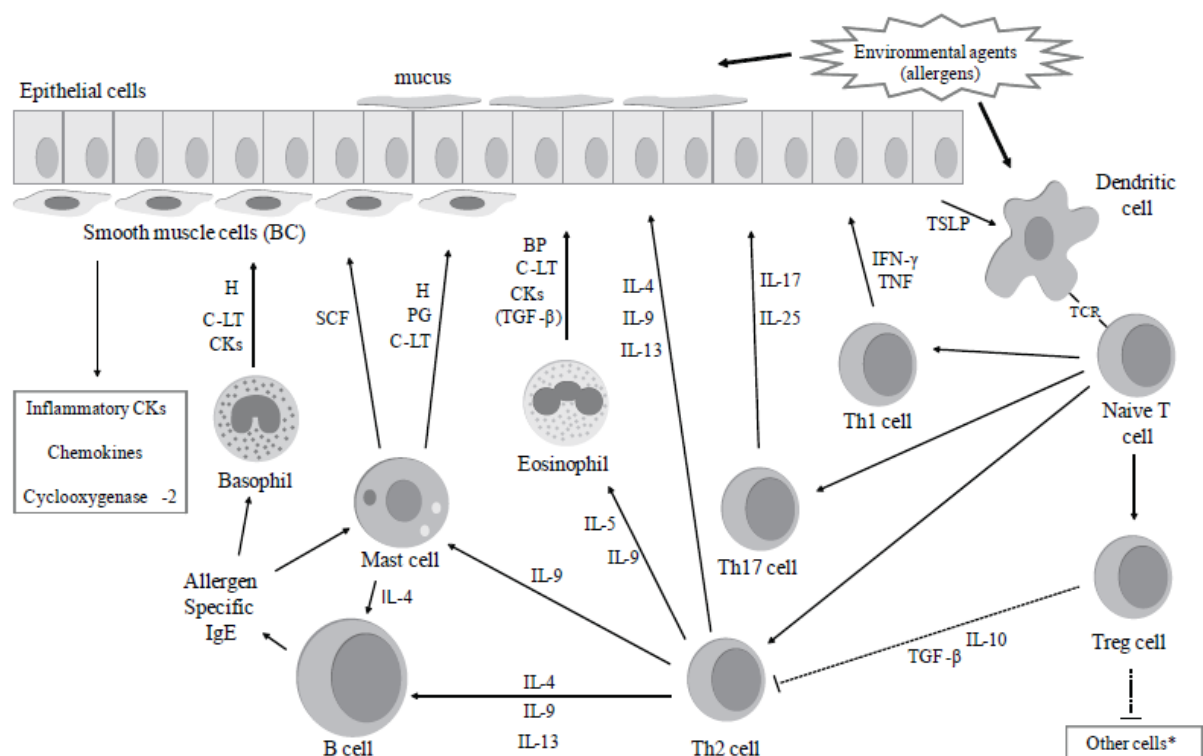


Figure 1.1. Inflammatory cells involved in asthma. Epithelial cells play an important role in orchestrating the inflammation of asthma through release of multiple cytokines (CKs), including stem cell factor (SCF; which maintains mast cells in the airways) and thymic stromal lymphopoietin (TSLP; which acts on dendritic cells to chemoattract Th2 cells). Th2 cells release CKs to stimulate B cells to synthesise immunoglobulin E (IgE), attract eosinophils (which cause tissue damage through release of major basic protein (BP)) and stimulate mast cell proliferation. Mast cells play an important role in asthma through release of histamine (H), cysteinyl-leukotrienes (C-LT), and prostaglandin (PG), which causes bronchoconstriction (BC) (Finiasz, Otero et al. 2011).

1.1.1.1 T-lymphocytes

An increased understanding of the development and regulation of airway inflammation in asthma follows the discovery and description of subpopulations of lymphocytes, most importantly T helper 1 (Th1) and T helper 2 (Th2) cells, with distinct inflammatory mediator profiles and effects on airway function (Fig. 1.2). After the discovery of these distinct lymphocyte subpopulations in animal models of allergic inflammation, evidence emerges that, in human asthma, a predilection towards the Th2-cytokine profile results in the eosinophilic inflammation characteristic of asthma (Cohn, Elias et al. 2004). In addition, generation of Th2 cytokines, such as interleukin-4 (IL-4), IL-5 and IL-13, also accounts for overproduction of IgE, presence of eosinophils and development of airway hyper-responsiveness. Although it is an oversimplification of a complex process as a Th2 disease, recognizing the importance of the families of cytokines and chemokines has advanced our understanding of the development of airway inflammation in asthma.

A subset of cluster of differentiation (CD) 4⁺ T-cells expressing CD25, termed T-regulatory cells (Treg), are capable of suppressing both Th1 and Th2-mediated adaptive immune responses through release of transforming growth factor-beta (TGF- β) or IL-10 (Taylor, Verhagen et al. 2006). An imbalance between allergen-specific Treg and effector Th2 cells is shown in allergic diseases (Ling, Smith et al. 2004).

Another recently described CD4⁺ T-cell subpopulation, Th17, secrete IL-17 and several other cytokines. Th17 cells are characterised by the production of IL-17A and IL-17E (also named IL-25) (Finiasz, Otero et al. 2011). These cytokines have been

linked to neutrophilic inflammation by inducing the release of CXCL1 and CXCL8 from airway epithelial cells (Laan, Lotvall et al. 2001), which may be associated with pathogenesis of severe asthma (Al-Ramli, Prefontaine et al. 2009). Th17 cells also produce IL-21, which is important for the differentiation of these cells and thus acts as a positive auto-regulatory mechanism (Barnes 2008) (Al-Ramli, Prefontaine et al. 2009).

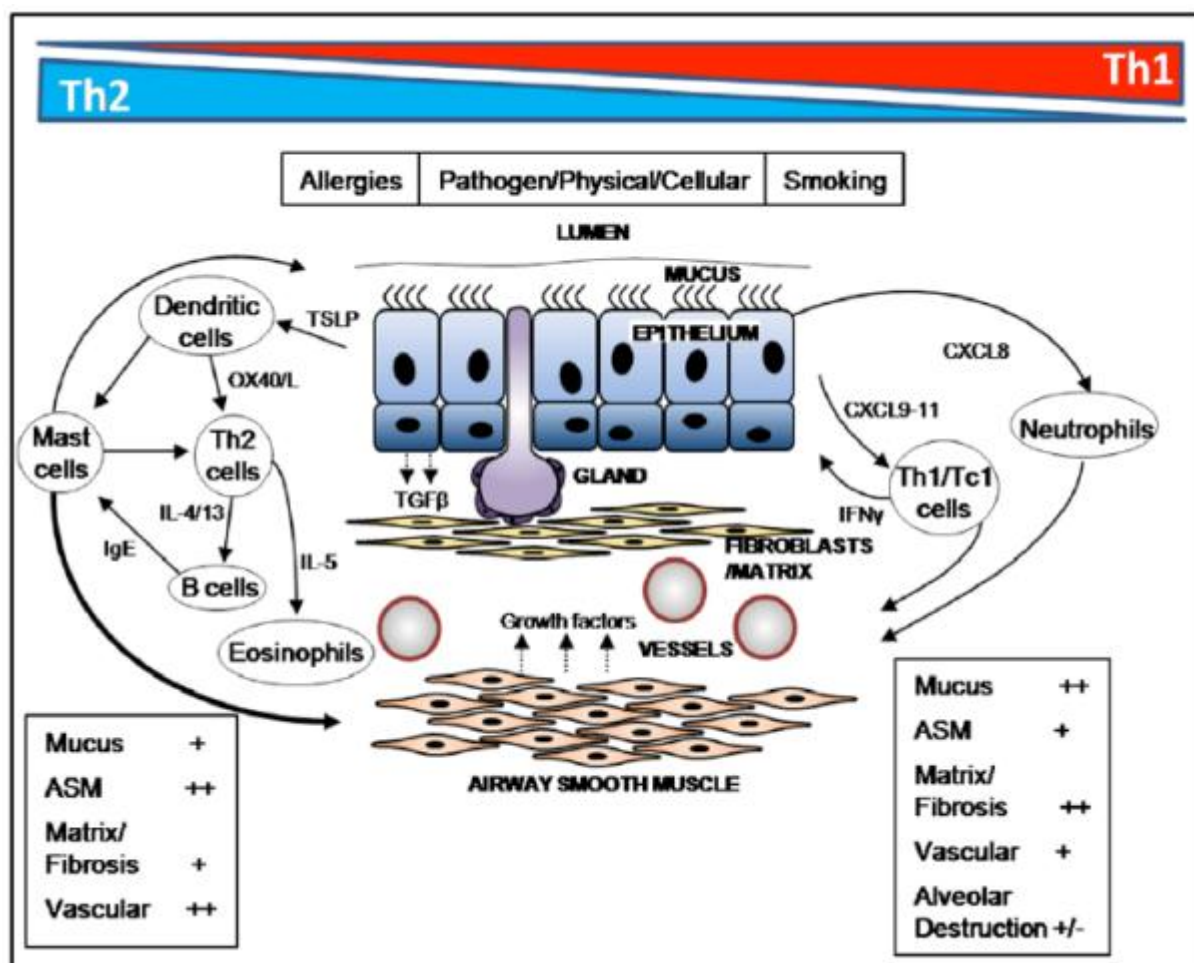


Figure 1.2. Interaction between Th1/Th2 lymphocytes in immunopathogenesis of asthma. Th2 cells predominate in most patients with asthma and differentiate from uncommitted precursor T cells on exposure to allergens, orchestrating allergic inflammation through release of IL-4, IL-5 and IL-13. Smoking or viral infection activates Th1 as well as cytotoxic T cells through CXCR3 chemokines CXCL9-11. Both inflammatory profiles may either counter-regulate or co-exist to varying degrees in asthma (Brightling, Gupta et al. 2011).

1.1.1.2 Mast cells

Mast cells are important in initiating the acute bronchoconstrictor response to allergen and other indirect stimuli, such as exercise and hyperventilation. Classically, mast cells are activated by allergens through an IgE-dependent mechanism via interactions with high-affinity IgE receptors (FcεRI and FcεRII) on the cell surface (Mauad, Poon et al. 2011), as well as by osmotic stimuli, which accounts for exercise-induced bronchoconstriction. Upon allergic inflammation, mast cells release pre- and newly formed mediators, such as histamine, cysteinyl-leukotrienes and prostaglandins, that play essential roles in the induction of acute allergic responses and the development of chronic allergic inflammation. Activation of mast cells plays a key role in the symptoms of asthma in the early phase and during acute exacerbation. In patients with asthma, there is infiltration of airway smooth muscle by mast cells (Brightling, Bradding et al. 2002), which is linked to hyper-responsiveness in this disease (Robinson 2004).

1.1.1.3 Eosinophils

Eosinophil infiltration is a characteristic feature of asthmatic airways and differentiates asthma from other non-infectious inflammatory conditions of the airway. Indeed, asthma was described as “chronic eosinophilic bronchitis” as early as 1916. Increased numbers of eosinophils exists in the airways of most, but not all, patients with asthma. In addition, numerous studies show that treating asthma with corticosteroids reduces circulating and airway eosinophils in parallel with clinical improvement.

Allergen inhalation results in a marked increase in eosinophils in bronchoalveolar lavage (BAL) fluid at the time of the late phase reaction, and there is a correlation between eosinophil counts in peripheral blood or BAL and airway hyper-responsiveness. Eosinophils are linked to the development of airway hyper-responsiveness through the release of basic protein and oxygen-derived free radicals (Simon and Simon 2009). In addition, the number of eosinophils in the diverse lung compartments is associated with disease severity and airway obstruction (Mauad, Poon et al. 2011).

Eosinophils are derived from bone marrow precursors; the Th2 cytokine IL-5 is a unique mediator of eosinophil differentiation and survival in response to allergen provocation. After allergen challenge, eosinophils appear in BAL fluid during the late response, and this appearance is associated with a decrease in peripheral eosinophil counts and with the appearance of eosinophil progenitors in the circulation. The signal for increased eosinophil production is likely to be IL-5 derived from the inflamed airway. Eosinophil recruitment initially involves adhesion of eosinophils to vascular endothelial cells in the airway circulation, their migration into the submucosa, and their subsequent activation. There are several mediators involved in the migration of eosinophils from the circulation to the surface of the airway. Cysteinyl leukotrienes, through stimulation at the sys-LT_1 -receptor, are in part responsible for eosinophilopoiesis in human airways. Other mediators, such as chemokine CCL11 (eotaxin), also play a role by promoting recruitment to tissues in response to allergic provocation, via CCR3 receptors.

1.1.1.4 Neutrophils

Although neutrophils are not a predominant cell type observed in the airways of most patients with mild-to-moderate asthma, they appear to be a more prominent cell type in airways and induced sputum of patients with severe asthma (Wenzel 2005). Cluster analyses of patients with asthma from the Severe Asthma Research Program study have shown that neutrophilia is associated with an absence of atopy, female sex and obesity in severe asthma (Moore, Meyers et al. 2010). These patients present with the lowest lung function, worse asthma control, and need more healthcare.

Evidence suggests that airway neutrophilia may be associated with a more severe form of asthma. For example, in patients who die suddenly of asthma, large numbers of neutrophils are found in their airways (Sur, Crotty et al. 1993). In addition, there is an increase in neutrophil number in the sputum of patients with severe asthma compared to those with mild asthma and healthy subjects (Jatakanon, Uasuf et al. 1999). Furthermore, rapid withdrawal of corticosteroids results in the appearance of neutrophils in the airways of patients with asthma (Maneechotesuwan, Essilfie-Quaye et al. 2007). The mechanisms of neutrophilic inflammation are not yet well understood, but several mediators, including CXCL8 (IL-8), are likely to be involved. A recent study suggests the neutrophilic inflammatory phenotype may be associated with systemic inflammation in asthma, exemplified by elevated plasma C-reactive protein (CRP) and IL-6 in asthmatic patients with increased neutrophil count and CXCL8 levels in the sputum (Wood, Baines et al. 2012).

1.1.1.5 Structural cells

Airway epithelial cells sense their mechanical environment, express multiple inflammatory proteins in asthma, and release cytokines, chemokines, and lipid mediators. Viruses and air pollutants also interact with epithelial cell to produce more inflammatory mediators or to injure the epithelium itself (Proud and Leigh 2011). The repair process following injury to the epithelium may be abnormal, thus furthering the obstructive lesions that occur in asthma (Holgate 2011).

Airway smooth muscle cells (ASMC) are not only a target of the asthma response by undergoing contraction to result in airflow obstruction and airway hyper-responsiveness, but also contribute to the disease via production of pro-inflammatory mediators (John, Clarke et al. 2008). As a consequence of airway inflammation and the generation of growth factors and cytokines, ASMC undergo proliferation, contraction and hypertrophy (Panettieri 2008); these events can influence airway dysfunction in asthma. The detailed role of ASMC in asthma will be described later in this chapter.

Other cells involved in airway inflammation in asthma include: endothelial cells of the bronchial circulation recruits inflammatory cells from the circulation into the airway (Lee, Ma et al. 2011); fibroblasts and myofibroblasts produce connective tissue components, such as collagens and proteoglycans, which are involved in airway remodelling (Halwani, Al-Muhsen et al. 2010); cholinergic nerves may be activated by reflex triggers in the airways and cause bronchoconstriction and mucus secretion (Canning 2006), whilst sensory nerves, sensitized by inflammatory stimuli including substance P and neurotrophins, cause symptoms such as cough and chest tightness (Groneberg, Quarcoo et al. 2004).

1.1.2 Airway obstruction

In asthma, the dominant physiological event leading to clinical symptoms is airway narrowing and a subsequent interference with airflow. Several factors contribute to the development of airway obstruction in asthma.

In acute exacerbations of asthma, bronchial smooth muscle contraction occurs quickly to narrow the airways in exposure to allergens or irritants. Allergen-induced acute bronchoconstriction results from an IgE-dependent release of mediators from mast cells, including histamine, tryptase, leukotrienes and prostaglandins, which directly contract airway smooth muscle. Aspirin and other non-steroidal anti-inflammatory drugs can also cause acute airway obstruction in some patients through a non-IgE-dependent reaction. In addition, other stimuli, such as exercise, cold air, and irritants, can cause acute airflow obstruction. The mechanisms regulating the airway response to these agents are less well defined, but the intensity of the response appears related to underlying airway inflammation. Furthermore, psychosocial stress may also play a role in precipitating asthma exacerbations (Ritz, Simon et al. 2011) and is negatively correlated with physiological markers of airway function (Vig, Forsythe et al. 2006).

As the disease becomes more persistent and inflammation more progressive, other factors further limit airflow. Airway oedema due to increased microvascular leakage in response to inflammatory mediators may be particularly important during acute exacerbations. Mucus hypersecretion and formation of inspissated mucus plugs may lead to luminal occlusion. Airway remodelling due to structural changes is important in more severe disease (Halwani, Al-Muhsen et al. 2010), and these changes may not be reversed by the usual treatments.

1.1.3 Airway hyper-responsiveness

Hyper-responsiveness is a characteristic functional abnormality of asthma and results in airway narrowing in exposure to stimuli that would be innocuous in normal people. In turn, this airway narrowing leads to variable airflow limitation and intermittent symptoms. The degree of airway hyper-responsiveness observed in response to methacholine correlates with the clinical severity of asthma (Cockcroft 2010). The underlying mechanisms are multi-factorial and include excessive contraction of airway smooth muscle due to increased volume and contractility of the cells. Moreover, uncoupling of airway contraction as a result of inflammatory changes in the airway wall may lead to excessive airway narrowing and a loss of the maximal plateau of contraction found in normal airways when bronchoconstrictor substances are inhaled (DiCosmo, Geba et al. 1994).

1.1.4 Airway remodelling

In some patients who have asthma, airflow limitation may be only partially reversible. Permanent structural changes can occur in the airway, and these are associated with a progressive loss of lung function that may not be fully prevented or reversed by current therapy. Airway remodelling involves activation of many of the structural cells, leading to permanent changes in the airways and deterioration of airway obstruction and airway hyper-responsiveness, and thus renders the patients less responsive to therapy.

Firstly, subepithelial fibrosis results from the deposition of collagen fibres and proteoglycans under the basement membrane and is seen in all patients with asthma, even before the onset of symptoms. Additionally, hypertrophy and hyperplasia of airway smooth muscle contribute to the increased thickness of the airway wall. This process relates to disease severity (Pepe, Foley et al. 2005). Other factors such as blood vessel proliferation and dilatation, hyperplasia of goblet cells in the epithelium, as well as submucosal glands, also contribute to increased airway wall thickness (Halwani, Al-Muhsen et al. 2010) (Fig. 1.3).

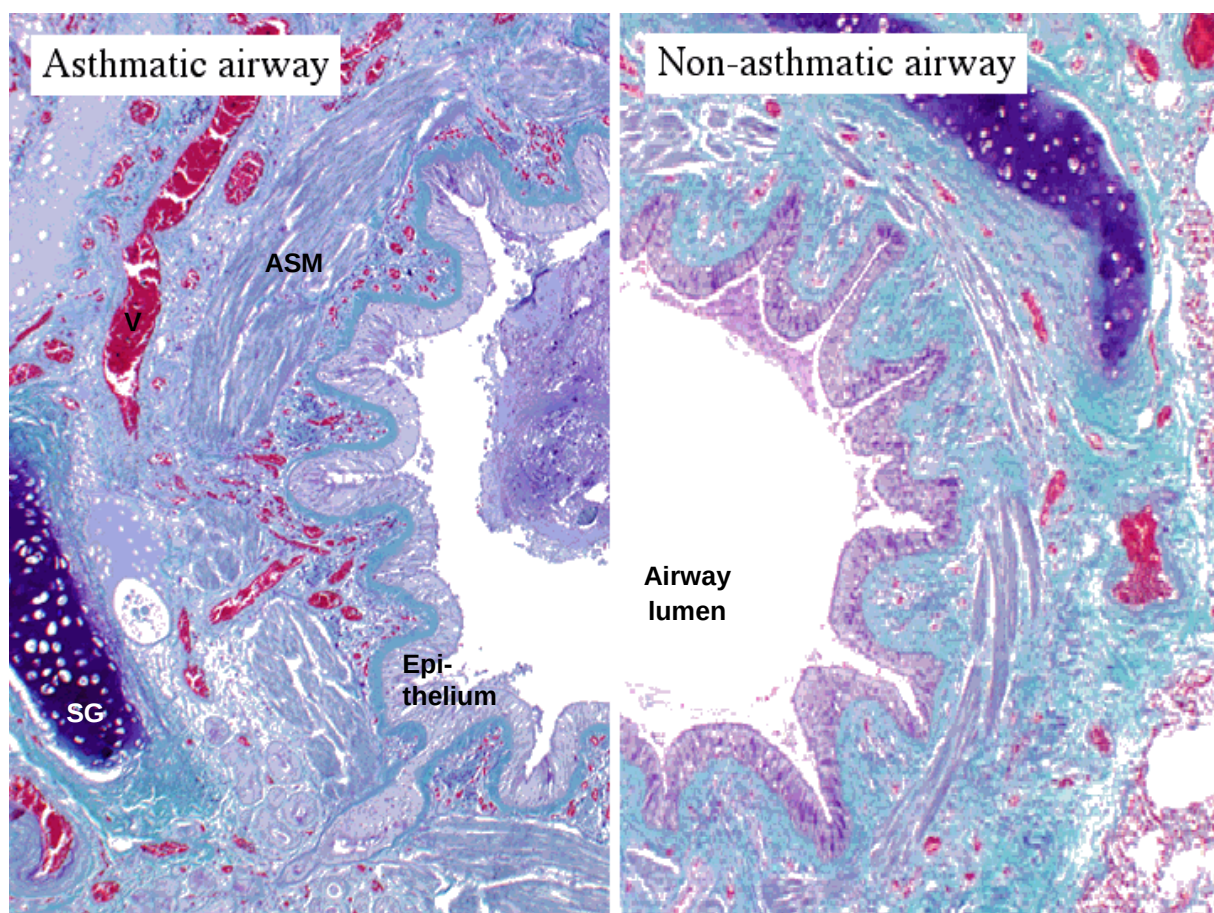


Figure 1.3. Structures of airways from subjects (left) with and (right) without asthma. Features of airway remodelling in asthma include subepithelial fibrosis, hypertrophy and hyperplasia of airway smooth muscle (ASM), blood vessel (V) proliferation and dilatation, and hyperplasia of goblet cells and submusocal gland (SG). Cited from <http://sydney.edu.au/medicine/pharmacology/rrg/images/content/karens.gif>.

1.1.5 Severe asthma

While most patients with asthma demonstrate a good therapeutic response to inhaled corticosteroid therapy, a proportion of patients do not acquire adequate control even under high-dose oral steroids. These patients, labelled as severe asthmatics, comprise of 5-10% of all asthmatic population. They account for more than half of the healthcare spending on asthma because patients with severe asthma consume more expensive drugs and are more likely to be hospitalized or require additional medical attention (Chung, Gibeon et al. 2011). The characteristics of these patients include the presence of frequent or severe exacerbations, low baseline lung function, reliance on high doses of inhaled or even oral corticosteroids and near daily symptoms. Quality of life is impacted and numerous comorbidities are often present either as a result of treatment or of the disease itself (Bousquet, Mantzouranis et al. 2010).

In the GINA 1995 and 2002 updates and the National Asthma Education and Prevention Programme (NAEPP) 1997 guidelines, overall asthma severity was primarily based on the patient's clinical characteristics prior to commencing treatment. Off-treatment severity was classified into mild intermittent, mild persistent, moderate persistent and severe persistent, based on symptoms, short-acting β_2 -agonist use, night time awakening and peak expiratory flow or the percentage predicted forced expiratory volume in 1 second (FEV_1). This initial classification was used to determine the patient's initial treatment but did not take into account disease responsiveness to treatment (Bel, Sousa et al. 2011).

In 1999, a European Respiratory Society (ERS) Task Force defined “difficult/therapy-resistant asthma” as poorly controlled asthma and a continued requirement for short-acting β 2-agonists despite delivery of a reasonable dose of inhaled corticosteroids and follow-up by a respiratory specialist for over six months (Chung, Godard et al. 1999). In 2000, an American Thoracic Society (ATS) Workshop adopted the term “refractory asthma” and developed a definition by consensus. The definition included one of the two major criteria (continuous high-dose inhaled corticosteroids or oral corticosteroids for over 50% of the time during the previous year), with two out of seven additional minor criteria (ATS. 2000) (Table 1.1). This definition was adopted by the National Institute of Health/National Heart, Lung and Blood Institute-sponsored Severe Asthma Research Program network (Moore, Bleecker et al. 2007).

The definition of refractory asthma requires one or both major criteria and two minor criteria	
Major criteria	
To achieve control to level of mild-moderate persistent asthma:	
1. Treatment with continuous or near continuous (≥ 50 percent of year) oral glucocorticoids	
2. Treatment with high-dose inhaled glucocorticoids:	
Beclomethasone >1260 mcg/day	
Budesonide >1200 mcg/day	
Flunisolide >2000 mcg/day	
Fluticasone >880 mcg/day	
Triamcinolone >2000 mcg/day	
Minor criteria	
1. Requirement for additional daily treatment with a controller medication, eg, 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 airflow limitation (FEV1 <80 percent predicted; diurnal peak expiratory flow variability >20 percent)	
4. One or more urgent care visits for asthma per year	
5. Three or more oral glucocorticoid bursts per year	
6. Prompt deterioration with ≤ 25 percent reduction in oral or inhaled glucocorticoid dose	
7. Near-fatal asthma event in the past	

Table 1.1. ATS Workshop Consensus for severe/refractory asthma (ATS. 2000).

The European Network for Understanding Mechanisms of Severe Asthma (ENFUMOSA) defined “severe asthma” in 2003 as confirmed asthma (typical asthma symptoms, reversibility FEV₁ or airway hyper-responsiveness) plus occurrence of one or more exacerbation in the previous year despite oral or high-dose inhaled corticosteroids (ENFUMOSA. 2003). In 2007, an international workshop was organised in Paris to discuss the important questions in severe asthma. This workshop agreed that a diagnosis of “severe asthma” should be reserved for those patients who have refractory asthma after an extensive re-evaluation of the correct diagnosis, aggravating comorbidities and environmental factors and an appropriate observation period of at least six months (Chanez, Wenzel et al. 2007).

In 2010, the WHO proposed a uniform definition of asthma severity, control and exacerbations. Severe asthma was defined as “Uncontrolled asthma which can result in risk of frequent severe exacerbations (or death) and/or adverse reactions to medications and/or chronic morbidity” (Bousquet, Mantzouranis et al. 2010). Severe asthma was further classified into three groups: i) untreated severe asthma; ii) difficult-to-treat severe asthma and iii) treatment-resistant severe asthma. The last group comprises the current concept of refractory and corticosteroid-resistant asthma and asthma that can be controlled only at the highest doses of treatment.

1.2 Airway smooth muscle cells (ASMC) and asthma

1.2.1 Pleiotropic role of ASMC

Airway remodelling is one of the key features of asthma, being characterised by thickening of the reticular basement membrane, an increase in smooth muscle mass through hypertrophy and hyperplasia of airway smooth muscle cells (ASMC), mucous gland hyperplasia, extracellular matrix deposition, and an increased mass of vessels. Airway remodelling is correlated with airflow limitation, airway hyper-responsiveness and air trapping in asthma, suggestive of a close link between structural and functional changes (Horvath, Sorkness et al. 2011). Of these, increased mass of ASMC has been implicated in pathophysiology of asthma and has been correlated with disease severity (Macedo, Hew et al. 2009).

ASMC were originally thought to be a passive partner in airway inflammation, contracting in response to pro-inflammatory mediators and neurotransmitters, and relaxing in response to endogenous and exogenous bronchodilators. It is now clear that ASMC have several other important properties of relevance to airway diseases including asthma (Fig.1.4). These functions include the ability to proliferate, undergo hypertrophy and migrate, and thereby contribute to the dysfunctional repair mechanisms that cause airway remodelling. In addition, ASMC can synthesise cytokine and growth factor and express cell-surface molecules, allowing their communication with the extracellular matrix and inflammatory cells (Howarth, Knox et al. 2004). Whereas the contraction of ASMC used to be regarded as an event secondary to the presence of inflammatory cells and mediators, it is accepted that

the cell itself can produce all these factors. ASMC are now considered to play a central role in orchestrating the inflammatory response within the bronchial wall.

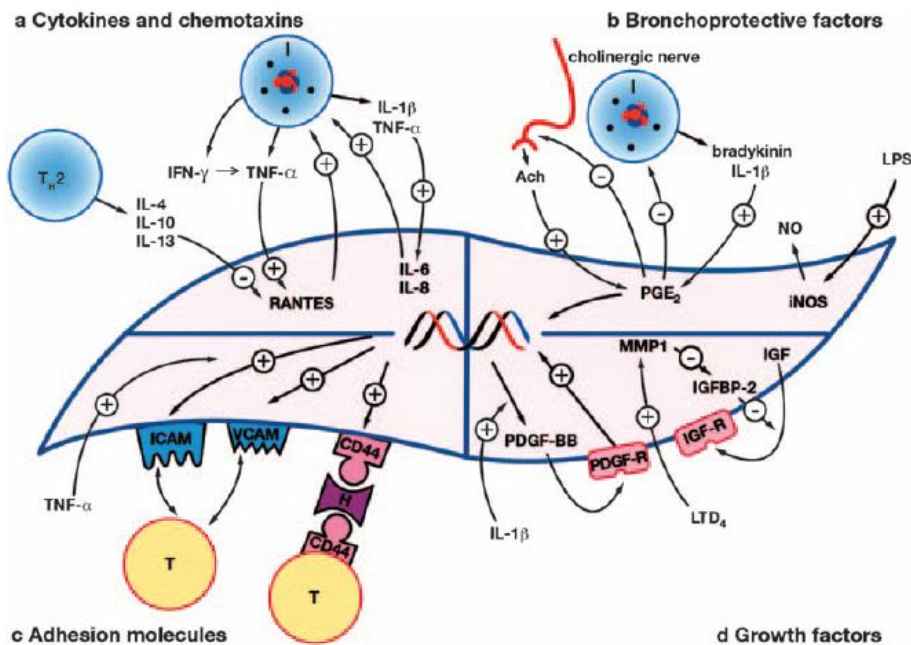


Figure 1.4. Pleiotropic role of ASMC. (A) Cytokines and chemotaxins: Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES) in response to TNF- α , which is inhibited by Th2 cytokines; IL-8 and IL-6 in response to IL-1 β and TNF- α , which causes further chemotaxis and cell activation. (B) Bronchoprotective factors: PGE₂ in response to bradykinin, IL-1 β acetylcholine (ACh); nitrogen oxide (NO) in response to lipopolysaccharide (LPS). (C) Adhesion molecules: intercellular adhesion molecule (ICAM) and vascular cell-adhesion molecule (VCAM) up-regulated by TNF- α . T-lymphocyte adhesion is promoted via these integrins and the non-integrin mechanism involving CD44 and hyaluronate. (D) Growth factors: platelet-derived growth factor (PDGF) in response to IL-1 β stimulates growth by an autocrine mechanism; insulin-like growth factor (IGF) is up-regulated by leukotriene D4 (LTD₄) (Howarth, Knox et al. 2004).

1.2.2 ASMC in asthma

With the help of the availability of techniques to culture ASMC (Panettieri, Murray et al. 1989), as well as access to newer technologies such as laser capture microdissection and gene microarrays, the understanding of the biology and pharmacology of ASMC, in both the healthy and the diseased, has increased exponentially over the last two decades. Additionally, with the ability to culture ASMC via biopsies derived from patients with asthma, studies reveal important differences in the *in vitro* behaviour of ASMC of asthmatic and non-asthmatic volunteers. Firstly, ASMC of patients with asthma proliferate more rapidly than the non-asthmatic counterparts (Johnson, Roth et al. 2001). Interestingly, although glucocorticoids can inhibit proliferation of ASMC from healthy subjects, they fail to do so in cells of patients of asthma (Roth, Johnson et al. 2004). Secondly, ASMC of patients with asthma release increased connective tissue growth factor (CTGF) (Burgess, Johnson et al. 2003), decreased prostaglandin E₂ (PGE₂) (Chambers, Black et al. 2003), and greater levels of chemokines such as CCL11 (Chan, Burgess et al. 2006), CXCL8 (John, Zhu et al. 2009) and CXCL10 (Brightling, Ammit et al. 2005). Furthermore, there is greater pro-inflammatory response to rhinovirus infection in ASMC of patients with asthma (Oliver, Johnston et al. 2006). Nevertheless, there are still few studies investigating the differential phenotype in ASMC of patients with asthma of different severities.

1.3 Cytokines

Cytokines are signalling proteins, usually less than 80 kilodalton (kDa) in size, which regulate a wide range of biological functions including innate and acquired immunity, hematopoiesis, inflammation and repair, and proliferation through mostly extracellular signalling. They are secreted by many inflammatory cells (macrophages, mast cells, eosinophils and lymphocytes) as well as structural cells (epithelial, endothelial and smooth muscle cells) and act on closely adjacent cells in a paracrine fashion. They may also act at a distance by secretion of soluble products into the circulation (endocrine or systemic effect) and may have influence on the cell of origin itself (autocrine effect).

1.3.1 Tumour necrosis factor-alpha (TNF- α)

TNF- α is the most widely studied pleiotropic cytokine of the TNF superfamily. TNF- α is an important cytokine in the innate immune response, which plays a key role in the immediate host defence against invading microorganisms before activation of the adaptive immune system. It is principally produced by macrophages in response to activation of membrane-bound pattern-recognition molecules, such as Toll-like receptors (TLR), which detect common bacterial cell-surface products, such as lipopolysaccharides (LPS). TNF- α is also produced by several other cells, such as fibroblasts, epithelial cells, and ASMC (Brightling, Berry et al. 2008).

TNF- α is initially produced as a biologically active, 26-kd membrane-anchored precursor protein (mTNF- α), which is subsequently cleaved by TNF α -converting enzyme (TACE) to release the 17-kd free protein (Zheng, Saftig et al. 2004). These proteins form biologically active homotrimers that act on the ubiquitously expressed TNF receptor (TNFR) 1 and 2 (Brockhaus, Schoenfeld et al. 1990). This receptor-ligand interaction causes intracellular signalling without internalization of the complex, leading to phosphorylation of I κ B α and thus activation of nuclear factor-kappa B (NF- κ B), which then interacts with the DNA chromatin structure to increase transcription of pro-inflammatory genes, such as IL-1 β , IL-6, CXCL8, and TNF- α itself (Fig. 1.5). The response to TNF- α activation is balanced by shedding of the extracellular domain of the TNF- α receptors (Brightling, Berry et al. 2008).

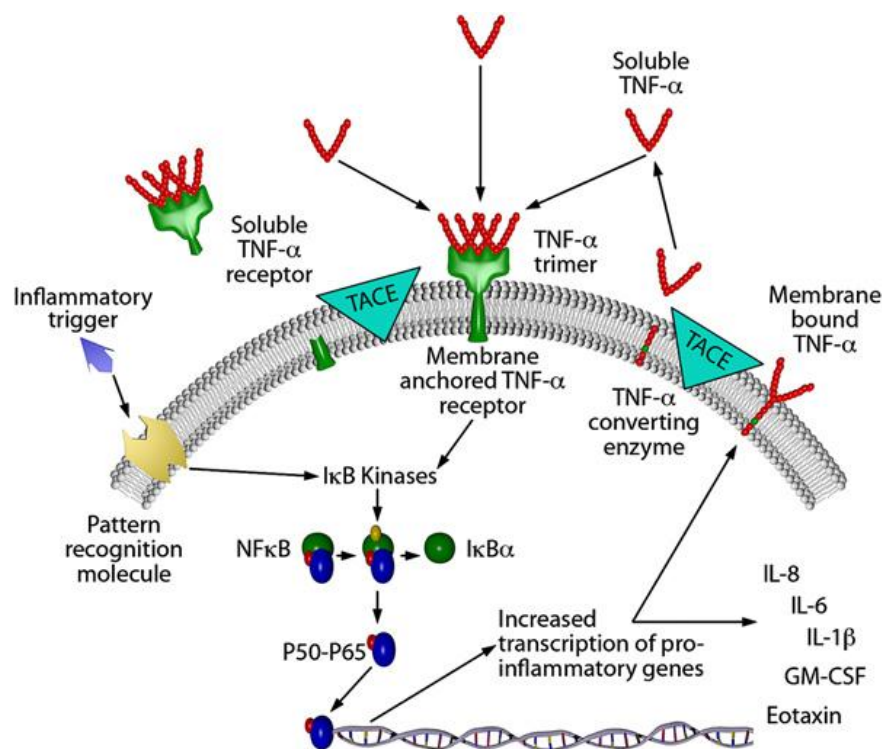


Figure 1.5. TNF- α biology and signalling. mTNF- α is cleaved by TACE to release soluble TNF- α , which forms biologically active homotrimers and binds TNFR 1 and 2, causing intracellular signalling, including phosphorylation of I κ B α and activation of NF- κ B heterodimer, which interacts with the DNA to increase transcription of pro-inflammatory genes (Brightling, Berry et al. 2008).

It is observed that TNF- α mRNA (Ying, Robinson et al. 1991) and protein (Bradding, Roberts et al. 1994) are increased in the airways of patients with asthma, implicating a possibility of this cytokine to be involved in the inflammation of asthma. Extensive genetic, biologic, and physiological evidence indicates that TNF- α plays a critical role in the initiation and amplification of airway inflammation in asthma (Fig. 1.6). Preformed TNF- α is stored by mast cells and rapidly released during IgE-mediated reactions that typify the asthmatic response to allergens. TNF- α perpetuates and amplifies inflammation by up-regulating adhesion molecules, which leads to increased migration of eosinophils and neutrophils into the airway. These key effector cells are activated by TNF- α to release cytotoxic mediators and reactive nitrogen and oxygen species that result in airway injury (Erzurum 2006). In addition to its effect on inflammation, TNF- α has direct effects on airway hyper-reactivity to methacholine and allergen, as shown in isolated tracheal-ring models (Pennings, Kramer et al. 1998). Importantly, administration of inhaled recombinant TNF- α leads to development of airway hyper-responsiveness and influx of neutrophils in normal subjects (Thomas, Yates et al. 1995) and patients with mild asthma (Thomas and Heywood 2002).

Further studies reveal the correlation between the expression of TNF- α to the severity of asthma. The level of TNF- α mRNA and protein is greater in the airways of patients with refractory asthma than those of normal subjects and patients with mild asthma. (Howarth, Babu et al. 2005). In addition, as compared with patients with mild-to-moderate asthma and controls, patients with refractory asthma have increased expression of membrane-bound TNF- α , TNF- α receptor 1, and TACE by peripheral-blood monocytes (Berry, Hargadon et al. 2006). Moreover, administration

of etanercept, a soluble TNF- α receptor-IgG1Fc fusion protein, in patients with refractory asthma improves airway measures, symptom scores and airway hyper-responsiveness (Howarth, Babu et al. 2005; Berry, Hargadon et al. 2006).

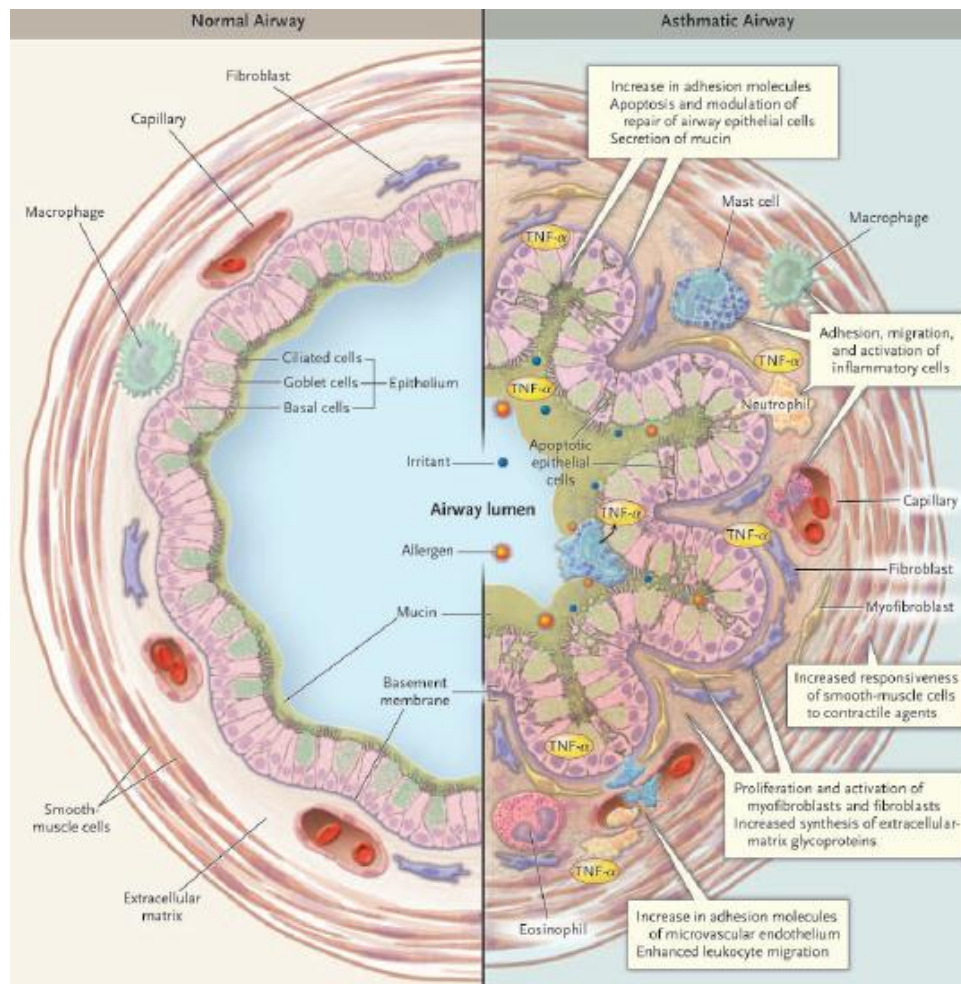


Figure 1.6. The normal airway and the effects of TNF- α on the asthmatic airway. Multiple actions of TNF- α contribute to asthma pathogenesis. Preformed TNF- α alone with histamine is immediately released from mast cells during allergic reponse. Through paracrine and autocrine effects, TNF- α perpetuates and amplifies inflammation by up-regulating adhesion molecules, which increase migration of activated leukocytes. TNF- α also induces epithelial cells to secrete mucin and fibroblasts, increasing production of glycoproteins. Independent of its pro-inflammatory effects, TNF- α increases response of smooth muscles to contractile agents (Erzurum 2006).

1.3.2 Interferon-gamma (IFN- γ)

The IFNs were originally discovered in 1957 as agents that interfere with viral replication (Isaacs and Lindenmann 1957). Initially, they were classified by the secreting cell type but now classified into type I and type II according to receptor specificity and sequence homology. The type I IFNs, in human, are comprised of multiple IFN- α subtypes (depending on species), IFN- β , IFN- ϵ , IFN- κ and IFN- ω (Platanias 2005). By contrast, IFN- γ is the sole type II IFN. While all IFNs have anti-proliferative, anti-viral, and immunomodulatory effects, IFN- α and IFN- β predominantly contain the first two effects, whereas IFN- γ is predominantly an immunomodulatory mediator (Peters 1996).

The observation that viral infection, the common inducer of IFNs, is a well-known trigger for asthma supports the concept that IFNs could play a negative role in the disease (Gern 2000). Typically, viral syndromes are characterised by intense inflammatory responses in the airways, with marked leukocyte trafficking and production of Th1-type cytokines such as IFNs. The subsequent interaction of IFNs with airway resident cells could initiate asthma exacerbations via multiple mechanisms, including production of cytokines/chemokines, or recruitment of different inflammatory cells (Proud and Chow 2006).

IFN- γ is structurally unrelated to type I IFNs, binds to a different receptor, and is encoded by a separate chromosomal locus. Increased levels of IFN- γ have been detected in asthmatic airways (Cembrzynska-Nowak, Szklarz et al. 1993), and IFN- γ expression, assessed by immunostaining, is greater in subepithelium of patients with

severe asthma compared to those with moderate asthma (Shannon, Ernst et al. 2008). Nevertheless, the immunopathogenic role of the IFN-associated pathways in asthma is still controversial. Previous *in vivo* evidence shows that IFN- γ could have suppressive activities against key features of allergic responses including IgE production, airway hyper-responsiveness and eosinophilic influx (Chung and Barnes 1999). In contrast, recent evidence shows that IFNs could be detrimental to the pathogenesis of asthma. A chronic model of allergic asthma, by using blocking antibodies, reveals that IFN- γ is a major player in mediating ovalbumin-induced airway hyper-responsiveness to methacholine (Kumar, Herbert et al. 2004). Another study targets expression of IFN- γ in the airways of IFN-knockout mice significantly increases allergen-induced responses including IL-5 and IL-13 expression and BAL eosinophilia (Koch, Witzentrath et al. 2006). Furthermore, an elegant study shows a critical role of IFN- γ and pulmonary dendritic cells in enhancing Th-2 dependent allergic responses after viral infection (Dahl, Dabbagh et al. 2004).

1.4 Chemokines

Leukocyte recruitment represents a refinement of a fundamental property of a motile cell to detect a chemical gradient, orient itself, and move along the gradient. This process, known as chemotaxis, has been elegantly described and analysed in primitive single-celled organisms and is an essential property of cells at different stages of their life history in multicellular organisms. In higher animals, this property has been adapted to trap specific leukocyte types on the inner surface of small blood vessels and induce migration through the vessel wall into the tissues. Pivotal to the process of chemoattraction of leukocytes are the chemokines.

Chemokines are small chemoattractant cytokines (8-14 kDa) with diverse effects on cellular recruitment, activation and differentiation. Chemokines are classified into four subfamilies, C, CC, CXC and CX3C on the basis of their sequence homology and the position of conserved cysteine residues within the protein (Zlotnik and Yoshie 2000) (Fig. 1.7). Most of the known chemokines belong to CC chemokine (28 in number) and CXC chemokine (16 members) families. In both families, the first cysteine forms a disulphide bond with the third, and the second with the fourth cysteine. CC chemokines act upon the majority of leukocytes, but generally have little activity on neutrophils. In contrast, CXC chemokines are characteristically chemotactic for neutrophils and lymphocytes. CXC chemokines are further subdivided into two groups according to the presence or absence of a glutamate-leucine-arginine (ELR) motif immediately before the first cysteine. Members of the former, such as CXCL1 (growth regulated oncogene- α , GRO- α), CXCL5 (epithelial neutrophil activating protein, ENA-78), CXCL6 (granulocyte chemotactic

Chemokines are thought to bind to presenting molecules on the luminal surface of the vascular endothelium, such as glycosaminoglycans, where the chemokines engage their receptors on the leukocyte surface. Leukocytes typically roll on the endothelium through low affinity intermolecular interactions mediated by selectins. Stimulation of chemokine receptors induces the upregulation of adhesion molecules on the leukocyte surface and cytoskeletal changes necessary for firm attachment, followed by emigration. Chemokines can recruit and activate a range of leukocyte subtypes, which include eosinophils, Th2 lymphocytes, neutrophils and mast cells to the bronchi (Lukacs, Hogaboam et al. 2005). Chemokines can also activate or modulate the phenotype of structural cells within the airways.

Following stimulation with inflammatory mediators, ASMC release a wide variety of chemokines. These include the CC chemokines CCL2 (monocyte chemotactic protein-1, MCP-1), CCL8 (MCP-2), CCL7 (MCP-3) (Pype, Dupont et al. 1999), CCL5 (Regulated upon Activation, Normal T-cell Expressed, and Secreted, RANTES) (John, Hirst et al. 1997), CCL11 (eotaxin) (Chung, Patel et al. 1999), CCL17 (thymus and activation regulated chemokine, TARC) (Faffe, Whitehead et al. 2003) and CCL19 (macrophage inflammatory protein-3 beta, MIP-3 β) (Kaur, Saunders et al. 2006); the CXC chemokines CXCL1 (GRO- α), CXCL2 (GRO- β), CXCL3 (GRO- γ) (Jarai, Sukkar et al. 2004), CXCL8 (IL-8) (Pang and Knox 1998), CXCL10 (IFN γ -induced protein 10, IP-10) (Hardaker, Bacon et al. 2004); and the CX3C chemokine CX3CL1 (fractalkine) (Sukkar, Issa et al. 2004).

1.4.1 CCL11 (Eotaxin)

CCL11 is a highly potent chemoattractant for eosinophils (Jose, Griffiths-Johnson et al. 1994) acting via a single chemokine receptor, CCR3, which is highly expressed on eosinophils (Ponath, Qin et al. 1996).

Animal studies have highlighted the production of both CCL11 mRNA and protein in the lung during early response to allergen exposure (Jose, Adcock et al. 1994; Ganzalo, Jia et al. 1996). In both animal and human studies of allergic airway disease, airway epithelium, microvascular endothelium, tissue macrophages, and sputum cells are shown to express increased CCL11, which is associated with airway hyper-responsiveness (Ying, Robinson et al. 1997; Ying, Meng et al. 1999; Zeibecoglou, Macfarlane et al. 1999). Furthermore, mice deficient in CCL11 show a reduction in ovalbumin-induced lung eosinophilia (Rothenberg, MacLean et al. 1997). These studies suggest a ubiquitous and essential role of CCL11 in the pathogenesis of asthma.

CCL11 is released from ASMC constitutively and increased by TNF- α , IL-1 β (Chung, Patel et al. 1999; Ghaffar, Hamid et al. 1999), IL-4 and IL-13 (Hirst, Hallsworth et al. 2002; Moore, Church et al. 2002). The human CCL11 promoter contains CAAT/enhancer binding protein (C/EBP), activated protein-1 (AP-1), signal transducer and activator of transcription 6 (STAT6) and NF- κ B binding sites. Induction of CCL11 by the Th2 cytokines IL-4 and IL-13 is mediated via STAT6 activation, whereas TNF α -induced transcription is dependent on the NF- κ B pathway (Clifford, Coward et al. 2011). In addition, histone H4 acetylation following TNF- α stimulation is a key event in regulating binding of NF- κ B to the CCL11 promoter and subsequent transcription of CCL11 (Clarke, Sutcliffe et al. 2008).

1.4.2 CXCL8 (IL-8)

CXCL8, a member of the ELR+ CXC chemokines, binds to G protein-coupled serpentine receptors CXCR1 and CXCR2 (Li, Zhang et al. 2002) on surface membrane of the target cells. CXCL8 is a potent chemoattractant for neutrophils.

The level of CXCL8 in the sputum is increased in patients with asthma, with the highest levels in severe asthma (Jatakanon, Uasuf et al. 1999). In addition, CXCL8 expression, detected by immunostain, is increased in both airway epithelium and subepithelium (Shannon, Ernst et al. 2008), as well as in the smooth muscle (Pepe, Foley et al. 2005), in patients with severe asthma compared to those with moderate asthma. Similarly, there are more neutrophils in the epithelium and subepithelium, along with more CXCL8 mRNA-positive cells in the subepithelium of patients with severe exacerbation of asthma (Qiu, Zhu et al. 2007).

ASMC release CXCL8 in large quantities after stimulation by TNF- α , IL-1 β (Watson, Grix et al. 1998), TGF- β (Fong, Pang et al. 2000) and bradykinin (Zhu, Bradbury et al. 2003). The CXCL8 promoter contains binding motifs for the transcription factors NF- κ B, AP-1, and C/EBP (Mukaida, Okamoto et al. 1994). In ASMC, NF- κ B is the major transcription factor involved in TNF α or bradykinin-induced CXCL8 gene transcription although AP-1 and C/EBP also contribute to maximal activation of the promoter (Clifford, Coward et al. 2011). In addition, baseline and TNF α -induced CXCL8 release and mRNA expression is increased in ASMC of patients with asthma. Although there is no global increase of NF- κ B activity, there is increased recruitment of p65 and C/EBP β as well as RNA polymerase II to the promoter of CXCL8 in ASMC of patients with asthma (John, Zhu et al. 2009).

1.4.3 CXCL10 (IP-10)

CXCL10, a member of the ELR- CXC chemokines, elicits its effects by binding to CXCR3 (Loetscher, Pellegrino et al. 2001) on the surface of the target cell. As a chemoattractant, CXCL10 attracts several cell types including monocytes/macrophages, T cells, natural killer (NK) cells, mast cells and dendritic cells (Groom and Luster 2011).

The observation of increased airway hyper-responsiveness and Th2 inflammatory response in the airways of CXCL10 transgenic mice and an opposite effect after deletion of CXCL10 in a mouse model of asthma suggests the role for this cytokine in the pathogenesis of asthma (Medoff, Sauty et al. 2002). In human studies, CXCL10 is preferentially expressed in ASMC of patients with asthma and is elevated in the BAL and airway mucosa of patients with asthma compared to healthy subjects (Brightling, Ammit et al. 2005). In addition, this chemokine in BAL is increased in patients with asthma following segmental allergen challenge (Bochner, Hudson et al. 2003). Furthermore, migration of human lung mast cell migration to asthmatic ASMC can be mediated by the CXCL10/CXCR3 axis (Brightling, Ammit et al. 2005). CXCL10 may also play a role in virus-induced asthma exacerbation. In patients with acute clinical asthma, serum CXCL10 is elevated in those with virus-induced than non-virus attack. In addition, increased serum CXCL10 levels are predictive of virus-induced asthma and strongly associated with more severe airway obstruction (Wark, Bucchieri et al. 2007).

In ASMC, CXCL10 is induced by TNF- α and IL-1 β , mediated by NF- κ B activation (Alrashdan, Alkhouri et al. 2012), and IFN- γ , through activation of the JAK2/STAT1 pathway (Clarke, Clifford et al. 2010) .

1.4.4 CX3CL1 (fractalkine)

CX3CL1 is a unique member of the CX3C chemokine and exists in two forms, a membrane bound and a soluble form, each mediating distinct biological actions. It is synthesised as a membrane-spanning adhesion molecule with the chemokine domain presented on a mucin-like stalk and serve as an adhesion protein promoting the retention of monocytes and T-cells. Soluble CX3CL1 can be released by proteolysis at a membrane-proximal region by TACE (A Disintegrin And Metalloproteinase 17; ADAM17) and ADAM10, and this soluble form resembles more a conventional chemokine which exhibits efficient chemotactic activity for monocyte, NK cells and T lymphocytes (Bazan, Bacon et al. 1997). Constitutive shedding occurs primarily via ADAM10, while increased shedding under inflammatory and excitotoxic conditions is mediated primarily by ADAM17 (Ludwig, Hundhausen et al. 2005). Both chemotaxis and adhesion are mediated by a highly selective G-protein-coupled receptor CX3CR1 (Imai, Hieshima et al. 1997) (Fig. 1.8).

In the airways, CX3CL1 is expressed in epithelial cells (Lucas, Chadwick et al. 2001), endothelial cells (Rimaniol, Till et al. 2003) and ASMC (Sukkar, Issa et al. 2004). CX3CL1 is involved in the pathogenesis of chronic inflammatory airway diseases such as asthma (Bisset and Schmid-Grendelmeier 2005) and chronic obstructive pulmonary disease (COPD) (Zhang and Patel 2010). CX3CL1 expression is increased in the epithelium, submucosa, and airway smooth muscle in patients with asthma, which contributes to recruitment of mast cells to the smooth muscle layer (El-Shazly, Berger et al. 2006). Compared with healthy subjects, patients with asthma have increased circulating CX3CL1 levels, and in the BAL fluid of these

patients, soluble CX3CL1 concentration is increased after segmental allergen challenge (Rimaniol, Till et al. 2003).

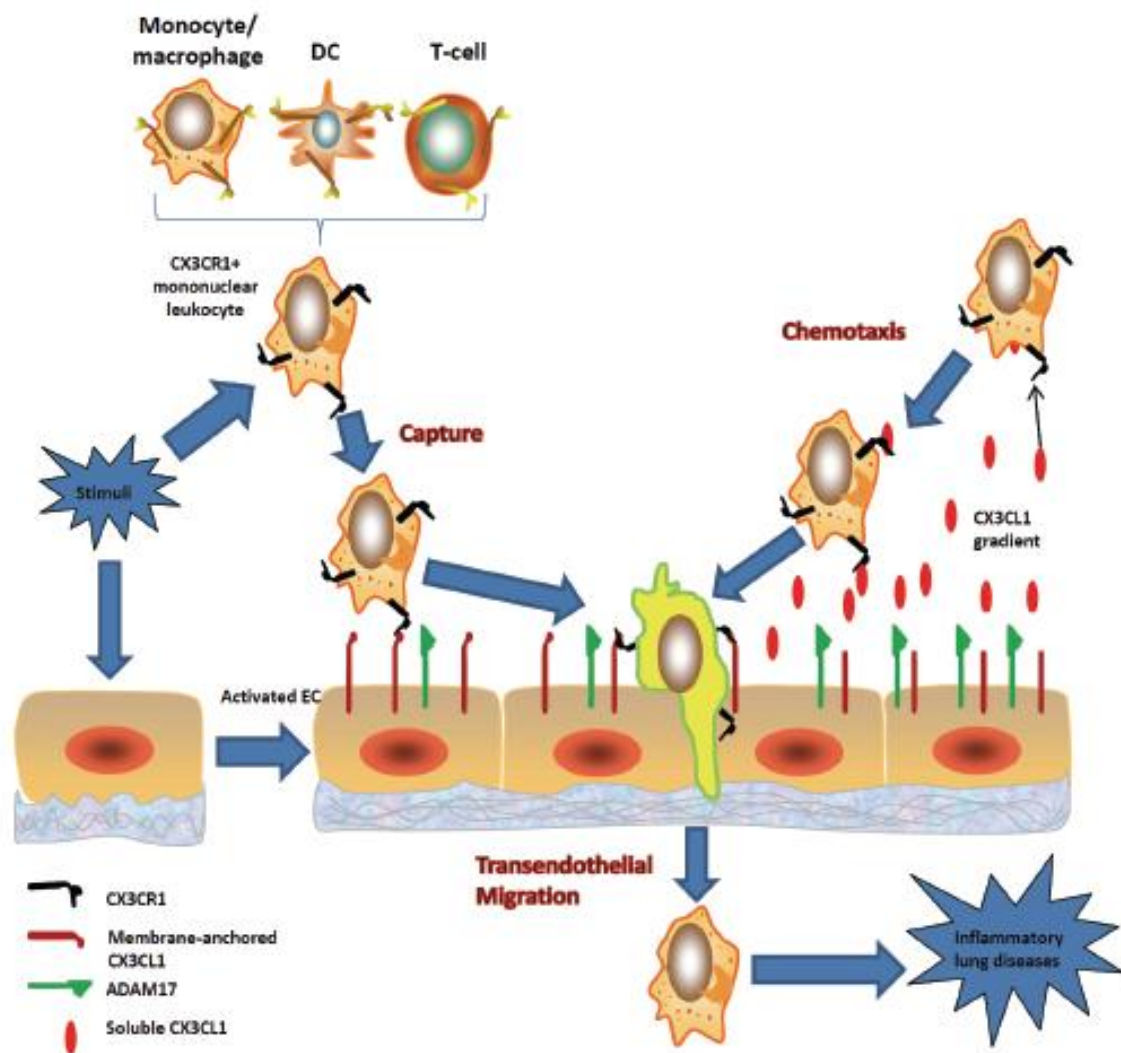


Figure 1.8. Role of membrane bound and soluble CX3CL1 and the receptor CX3CR1. The membrane-anchored CX3CL1 functions as an adhesion molecule. The soluble CX3CL1, released by proteolysis at a membrane-proximal region by ADMA17, is chemotactic for monocytes, dendritic cells (DC) and T cells, which expresses CX3CR1 on their cell surface (Zhang and Patel 2010).

1.5 Nuclear factor- κ B (NF- κ B)

Chronic inflammation in asthma is due to increased expression of multiple inflammatory proteins (cytokines, enzymes, receptors, adhesion molecules). In many cases these inflammatory proteins are induced by transcription factors, DNA-binding factors that mediate the transcription of selected target genes. Transcription factors bind to DNA-regulatory sequences (enhancers and silencers), usually localised in the 5'-upstream region of target genes, to modulate the rate of gene transcription, protein synthesis, and subsequent altered cellular function. Transcription factor activation is complex and may involve multiple intracellular signal transduction pathways, including protein kinase A (PKA), PKC, mitogen-activated protein kinases (MAPK) and Janus kinase (JAK), stimulated by cell-surface receptors (Clifford, Coward et al. 2011). They may also be directly activated by ligands such as glucocorticoids and vitamin A and D. Although numerous transcription factors are activated during the inflammatory response, NF- κ B is regarded of paramount importance in asthmatic inflammation because it is activated by all the stimuli considered important in the inflammatory response to allergen exposure (Adcock, Ito et al. 2004).

NF- κ B is activated in response to a number of stimuli, such as physical and chemical stress, LPS, double-stranded RNA, T and B cell mitogens and pro-inflammatory cytokines. NF κ B-induced gene expression is controlled by a complex series of enzymatic signalling events at multiple levels. A multitude of studies in ASMC implicate a role of NF- κ B in regulation of inflammatory cytokines, chemokines and adhesion molecules (Clarke, Damera et al. 2009).

1.5.1 NF- κ B Structure

NF- κ B is made up of a hetero- or homodimer of members of the DNA-binding Rel family of proteins which contains five known mammalian members: p50 (NF- κ B1, precursor of which is p105), p65 (Rel A, NF- κ B3), p52 (NF- κ B2, precursor of which is p100), c-Rel and Rel B. While p52, c-Rel and Rel B are restricted to specific differentiated cell types (Siebenlist, Franzoso et al. 1994), the p65 and p50 subunits are ubiquitously expressed and have been extensively investigated, of which p50 increases DNA binding and p65 confers transcriptional regulation (Hayden and Ghosh 2008).

1.5.2 NF- κ B activation

In resting cells, the majority of NF- κ B is bound to the inhibitor of NF- κ B (I κ B). I κ B associates via its ankyrin repeats domain with NF- κ B, which thus masks the nuclear localisation signals (NLS) of both NF- κ B and I κ B and holding NF- κ B in the cytoplasm. Upon cellular stimulation, I κ B is phosphorylated, ubiquitinated, and degraded by the proteosomal pathway. With I κ B removed, NF- κ B translocates to the nucleus and mediates gene transcription. An overview of the NF- κ B cascades is depicted in Fig.1.9.

I κ B phosphorylation and activation of Rel proteins can occur via the classical (canonical) or non-classical (non-canonical) pathway. In the classical pathway, a critical phosphorylation of I κ B is performed by the I κ B kinase (IKK) complex, which consists of at least three subunits, including two catalytic subunits IKK- α and β , also known as IKK-1 and 2, and one regulatory subunit IKK- γ , known as NF- κ B essential

modulator (NEMO). Of the two catalytic subunits, IKK- β is 20-fold more active than IKK- α in the phosphorylation of I κ B and is critical for NF- κ B activation. Stimuli of the classical pathway include the TLR/IL-1R family members, ligation of the T-cell receptor (TCR), and TNFR signalling.

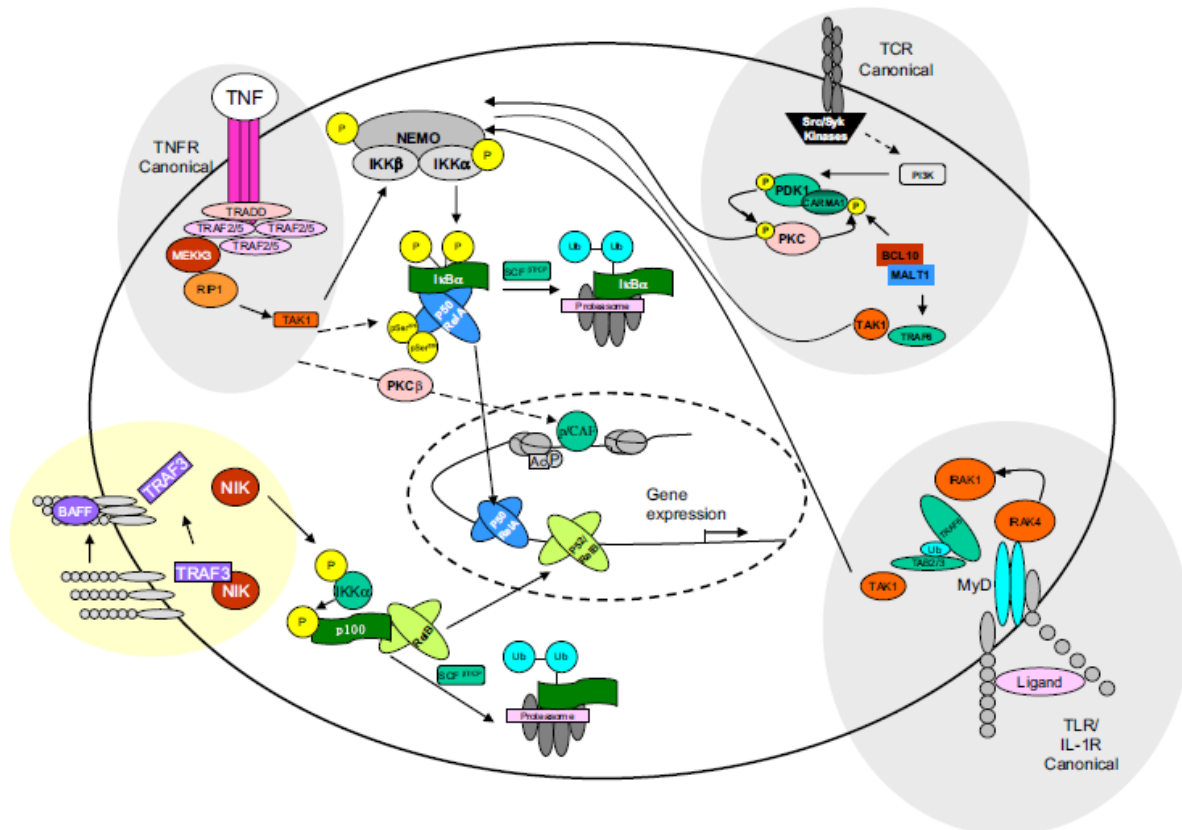


Figure 1.9. NF- κ B signalling pathways. The canonical pathways signal through i) TLR/IL-1 receptors, leading to IRAK activation and IKK- β phosphorylation; ii) intracellular viral receptors including RNA helicases and PKR, which activate IKK- β and iii) the TCR pathway, leading to IKK- α / β activation; TNFR pathway, which signals via TRADD to activate IKK- β . The alternative pathway is induced by CD40-CD40L activation, lymphotoxin- β or RANKL, which leads to activation of NIK and IKK- α . IRAK: IL-1 receptor-associated kinase, PKR: dsRNA-dependent protein kinase R, TNFR: TNF receptor, TRADD: TNFR-1 associated death domain, RANKL: receptor activator of NF- κ B ligand, NIK: NF κ B-inducible kinase (Edwards, Bartlett et al. 2009).

In addition to the classical pathway, an alternative non-canonical pathway is described mainly in B cells. This latter pathway can be activated by different stimuli, including lymphotoxin β , CD40 ligand, and receptor activator of NF- κ B ligand. The alternative pathway is characterised by the inducible phosphorylation and processing of p100-p52, and subsequent nuclear translocation of the heterodimer p52:rel B, which is independent of IKK- γ or IKK- β but only requires the IKK- α subunit. This pathway is believed to play key roles in adaptive immunity (Clarke, Damera et al. 2009).

1.5.3 Post-translational modifications of NF- κ B

Subsequent to the activation process, NF- κ B activity is substantially modulated by various post-translational modifications, including acetylation, SUMOylation, and phosphorylation. The intracellular control of NF- κ B transactivation, subcellular localisation, DNA binding affinity and the interaction with cofactors and I κ B via post-translational modifications forms the intricate web for NF- κ B regulation (Beck, Vanden Berghe et al. 2009). For example, phosphorylation of p65 enhances transcription, yet phosphorylation of p105 reduces its processing into p50 and hence reduces activation (Naumann and Scheidereit 1994). Acetylation of the Rel proteins also modulates the function of NF- κ B (Spange, Wagner et al. 2009; Ghizzoni, Haisma et al. 2011). Additionally, covalent modification of the chromatin environment, which regulates the access of transcription factors to gene promoters, also alters NF κ B-dependent transcription. This control is achieved by recruitment of protein complexes that alter chromatin structure via enzymatic modifications of histone tails and/or nucleosome remodelling. NF- κ B activation requires several cofactor histone acetyltransferases (HAT), including CREB-binding protein (CBP), p300, p300/CBP activating factor (pCAF) and steroid receptor coactivator (SRC)-1.

1.6 Mitogen-activated protein kinases (MAPK)

Many external inflammatory signals important in the inflammatory response seen in asthma, such as viral and bacterial infection, allergen, cytokines and growth factors, can activate intracellular kinases following binding to transmembrane receptors on responsive cells. The rapid amplification of the initiating signal is due to the number of enzymes involved in each kinase cascade. Protein kinases are classified as either tyrosine or serine/threonine kinases although dual kinases also exist. All kinases transfer the γ -phosphate of ATP to hydroxyl acceptor groups of the residues of target protein (Adcock, Chung et al. 2006).

Mitogen-activated protein kinases (MAPK) are a family of serine/threonine kinases that transduce extracellular signals to the nucleus. The pathways are organised as parallel kinase cascades, consisting of MAPK, MAPK kinases (MAPKK, or MKK), and MAPK kinase kinases (MAPKKK, or MKKK) (Fig. 1.10). Activation of MAPK requires dual phosphorylation of threonine and tyrosine by upstream kinases and occurs in response to different stimuli. Once activated, MAPK phosphorylate selected intracellular proteins including transcription factors. The resulting changes in gene expression affect fundamental cellular processes such as proliferation, differentiation, survival and inflammation. In mammalian cells, there are five distinct subfamilies including p38 MAPK, c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), EKK 3/4 and ERK 5. Among the five distinctive MAPK pathways, p38 MAPK, JNK and ERK have been best characterised in ASMC (Clarke, Damera et al. 2009).

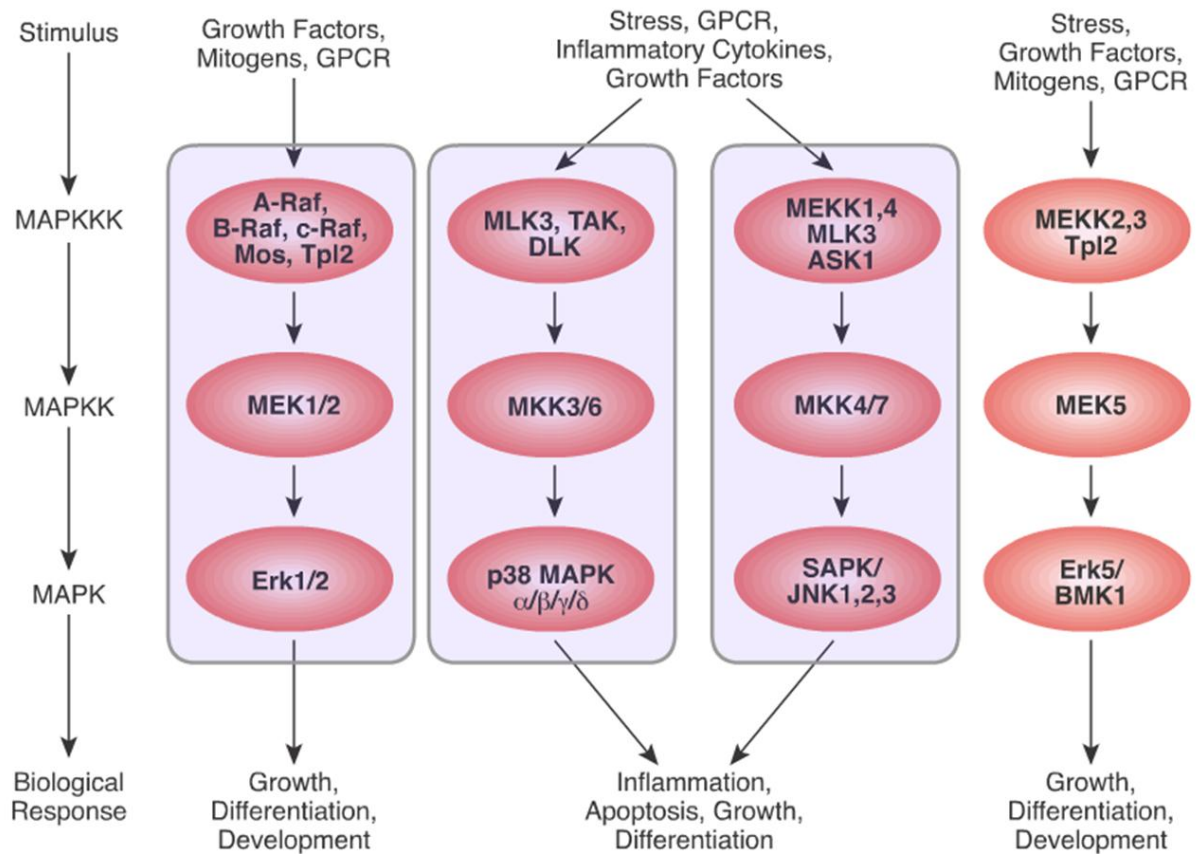


Figure 1.10. MAPK cascades (Cited from <http://www.cellsignal.com/pathways/map-kinase.jsp>). Each group of cascades is composed of a set of three evolutionarily conserved, sequentially acting kinases: a MAPK, a MAPKK and a MAPKKK. MAPKKK activation leads to the phosphorylation and activation of a MAPKK, which then stimulates MAPK activity through dual phosphorylation on threonine and tyrosine residues.

1.6.1 p38 MAPK

p38 MAPK signalling is activated in response to physical and chemical challenges, such as oxidative stress, ultraviolet irradiation, hypoxia, ischemia, as well as various cytokines. The downstream effectors of this cascade are transcription factors such as E26-like kinase 1 (Elk-1), serum response factor accessory protein (SAP)-1, activating transcription factor (ATF)-2, cAMP response element-binding (CREB), C/EBP homologous protein (CHOP), and myc-associated factor X (Max) (Fig. 1.11).

There are four members of the p38 kinase family: α , β , γ and δ . All isoenzymes are activated by the same upstream MAPK kinases MKK3 or MKK6 but differ in their tissue distribution, regulation of kinase activation and subsequent phosphorylation of downstream substrates (Adcock, Chung et al. 2006). The α and β isoforms are effective in activating kinases downstream of p38 MAPK, such as MAPK activated protein kinase (MAPKAPK2/3) and mitogen and stress-activated protein kinase (MSK) 1 and 2. The substrates of p38 α and β include transcription factors, other protein kinases that in turn phosphorylate transcription factors, cytoskeletal proteins and translational components. In contrast, the γ and δ isozymes preferentially target transcription factors such as ATF-2, Elk-1, and SAP-1 (Chung 2011).

In ASMC, p38 MAPK is involved in regulation of contractile response, proliferation, as well as inflammatory responses. For instance, p38 contributes to maximal acetylcholine-induced isometric contractile responses and in the increased contractile response of murine intrapulmonary airways after ozone exposure (Li, Zhang et al. 2011). In addition, p38 mediates proliferation of ASMC induced by basic fibroblast growth factor (bFGF) (Fernandes, Ravenhall et al. 2004) or IL-1 β (Zhai, Eynott et al. 2004). Importantly, p38 mediates expression of many inflammatory

genes in response to multiple stimuli. For example, p38 mediates IL17A-induced IL-6, CXCL8 and CCL11 as well as bradykinin-induced IL-6 secretion and expression. p38 also regulates expression of IL-5R in response to IL1- β , TNF- α and IFN- γ . p38 appears to have both positive and negative regulatory effects on cytokine-induced inflammatory response: while it augments TNF α -induced IL-6 and CCL5 and IL1 β -induced CCL11 release, this kinase inhibits TNF α -induced ICAM-1 expression and IL1 β -induced granulocyte macrophage-colony stimulating factor (GM-CSF) (Clarke, Damera et al. 2009). This suggests a gene specific role for p38 MAPK in regulating specific transcriptional outcomes.

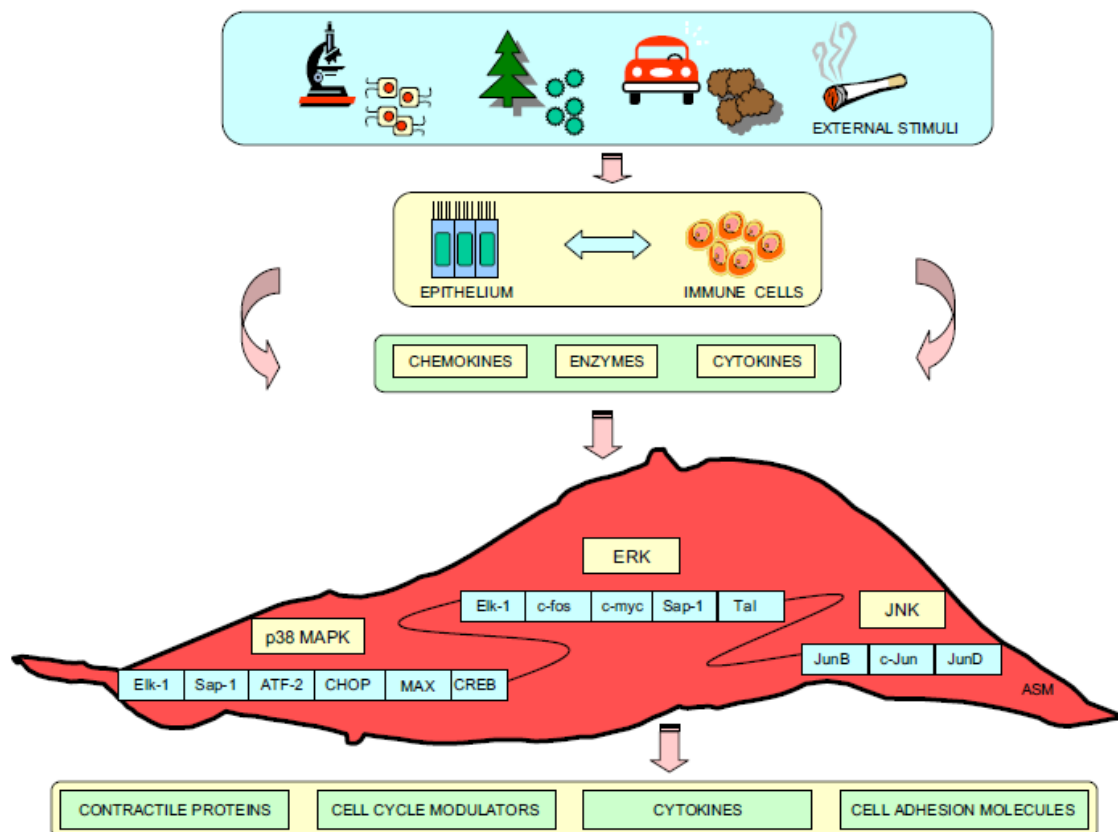


Figure 1.11. Schematic overview of MAPK pathways regulating ASMC functions. Various external stimuli activate immune cells or airway epithelial cells to release a variety of biological mediators. These mediators transduce their effects through p38, JNK or ERK signalling cascades, leading to expression of genes that modulate the contractile, proliferative and secretory responses of airway smooth muscles (Clarke, Damera et al. 2009).

1.6.2 c-Jun N-terminal kinase (JNK)

JNK signalling is activated by environmental stress, pro-inflammatory cytokines and genotoxic agents. JNK consists of three isoforms, encoded by three different genes, of which JNK1 and 2 isoforms are widely distributed, while JNK3 is expressed only in neurons, cardiac myocytes and testes (Bennett 2006). JNK are phosphorylated and activated by the dual specificity MAPK kinases MKK4 and MKK7. Following activation, JNK enhances the transcriptional activity of AP-1 by phosphorylation of the AP-1 component c-Jun and thereby increasing AP-1 association with the basal transcriptional complex. These transcriptional factors modulate gene expression responsible for many biological responses, including migration, proliferation, differentiation and cell death. (Adcock, Chung et al. 2006).

In murine studies, administration of the JNK inhibitor SP600125 after allergen challenge prevents T cell and eosinophil-mediated airway inflammation and ASMC proliferation, indicating a role for JNK signalling in allergic airway inflammation and remodelling (Eynott, Nath et al. 2003; Nath, Eynott et al. 2005).

JNK activation is involved in expression of inflammatory mediators in human ASMC, as shown by the increased phosphorylation of JNK and c-Jun following TNF, IL-1 β or IL-4/IL-13 stimulation, and the inhibition of GM-CSF, CCL5 (Oltmanns, Issa et al. 2003), CXCL1 (Issa, Xie et al. 2006), CXCL10 (Alrashdan, Alkhouri et al. 2012) and CX3CL1 (Sukkar, Issa et al. 2004) expression after treatment with the JNK inhibitor SP600125.

1.6.3 Extracellular signal-regulated kinase (ERK)

ERK signalling induces downstream activation of different intracellular transcription factors such as Elk-1, c-fos, c-myc, Sap-1 and Tal (Fig. 1.11), and consequently modulates regulation of meiosis, mitosis and post-mitotic functions in differentiated cells, in response to a variety of stimuli including growth factors, cytokines, virus infection, ligands for heterotrimeric G protein-coupled receptors, transforming agents and carcinogens (Shaul and Seger 2007). Activation of Raf leads to the activation of the MAPK kinases MEK1 and MEK2, which in turn activate ERK1/2.

In ASMC, activation of ERK signalling is elicited by various stimuli, such as PDGF, epidermal growth factor (EGF), thrombin, oncostatin M, leukemia inhibitory factor (LIF), insulin-like growth factor-1 (IGF-1), and 5-hydroxy-tryptamine (Clarke, Damera et al. 2009). Cytokines are also important activators of ERK signalling. IL1 β -activated phosphorylation of ERK1/2 leads to production of eosinophil-activating cytokines including PGE₂, CCL5, CCL11 and GM-CSF (Hallsworth, Moir et al. 2001). ERK is also involved in mediating release of CCL11 and CXCL8 in response to Th2 cytokines IL-4, IL9 and IL13, and the Th17 cytokine IL-17 (Clarke, Damera et al. 2009).

1.7 Corticosteroids and glucocorticoid receptor

1.7.1 Corticosteroids in treatment of asthma

Corticosteroids are primary stress hormones that function to maintain homeostasis. They are synthesised and released by the adrenal cortex following stress-induced activation of the hypothalamic-pituitary-adrenal axis and affect nearly every organ and tissue in the body. Corticosteroids regulate a plethora of biological processes, including immune function, skeletal growth, reproduction, cognition, behaviour, cell proliferation and survival. Because of their powerful anti-inflammatory and immunosuppressive actions, synthetic corticosteroids are widely prescribed for the treatment of acute and chronic inflammatory diseases, autoimmune diseases, organ transplant rejection, and malignancies of the lymphoid system (Oakley and Cidlowski 2011).

Corticosteroids are by far the most effective controllers used in the treatment of asthma and the only drugs that can effectively suppress the characteristic inflammation in asthmatic airways. They improve asthma control with a reduction in the need for reliever medications, such as a short-acting β_2 -adrenergic agonist, and lung function measurements, such as peak expiratory flow rate or FEV₁. Corticosteroids also consistently reduce airway hyper-responsiveness in asthma to both direct and indirect challenges including histamine, cholinergic agonists, allergens, exercise and cold air and reduce numbers of exacerbations, prevent irreversible airway changes and decrease mortality (Chung, Gibeon et al. 2011). Inhaled corticosteroids are now recommended as first-line therapy for all patients with persistent symptoms and the need to use a β_2 -agonist inhaler for symptom control more than three times weekly (GINA 2011).

1.7.2 Cellular effects of corticosteroids

At the cellular level, corticosteroids reduce the numbers of inflammatory cells in the airways, including eosinophils, T-lymphocytes, mast cells, and dendritic cells. These effects are produced through inhibition of inflammatory cell recruitment into the airway by suppressing the production of chemotactic mediators and adhesion molecules and by reducing the survival of the inflammatory cells in the airways. Structurally, corticosteroids reverse the shedding of epithelial cells, goblet cell hyperplasia and basement membrane thickening in the asthmatic airway. They also reduce the increased airway wall vascularity and mucosal blood flow, as well as the increased thickness of the sub-basement membrane representing deposition of the extracellular matrix, which are present in patients with asthma (Chung, Gibeon et al. 2011) (Fig. 1.12). The suppression of mucosal inflammation is relatively rapid with a significant reduction in eosinophils detectable within six hours (Gibson, Saltos et al. 2001). Reversal of airway hyper-responsiveness may take several months to reach a plateau, probably reflecting recovery of structural changes in the airway (Juniper, Kline et al. 1990).

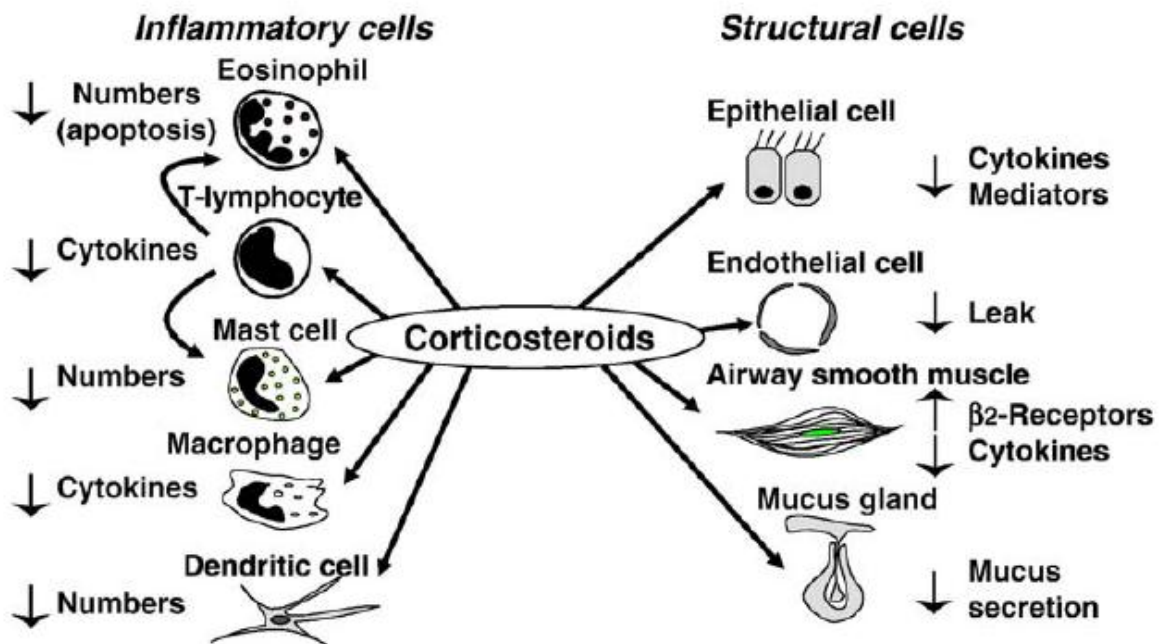


Figure 1.12. Cellular effects of corticosteroids in the airway. Corticosteroids inhibit the recruitment of inflammatory cells into the airway by suppressing production of chemotactic mediators and adhesion molecules and by inhibiting survival of inflammatory cells in the airway. Corticosteroids also inhibit cytokine release from airway structural cells such as epithelial cells and ASMC, reduce permeability of endothelial cells, and decrease mucus secretion from the mucus gland (Barnes 2006).

1.7.3 Glucocorticoid receptor (GR)

Most anti-inflammatory effects of glucocorticoids are mediated via an intracellular protein, the glucocorticoid receptor (GR). GR belongs to the steroid/thyroid/retinoic acid nuclear receptor superfamily of transcription factor proteins and functions as a ligand-dependent transcription factor (Nicolaidis, Galata et al. 2010) which suppresses expression of inflammatory genes through several mechanisms.

Transactivation occurs via direct binding of activated GR to a DNA sequence termed the glucocorticoid-responsive element (GRE), which is present on the promoter of anti-inflammatory genes. In contrast, transrepression defines an interaction of activated GR with different transcription factors, such as NF- κ B and AP-1, thus repressing expression of pro-inflammatory genes. Furthermore, *cis*-repression occurs when GR binds negative GRE, leading to gene suppression. There are few well documented examples of the last category, but some are relevant to side effects of corticosteroids, such as genes that regulate the hypothalamic-pituitary axis (proopiomelanocortin and corticotrophin releasing factor), bone metabolism (osteocalcin) and skin structure (keratin) (Barnes 2006) (Fig. 1.13).

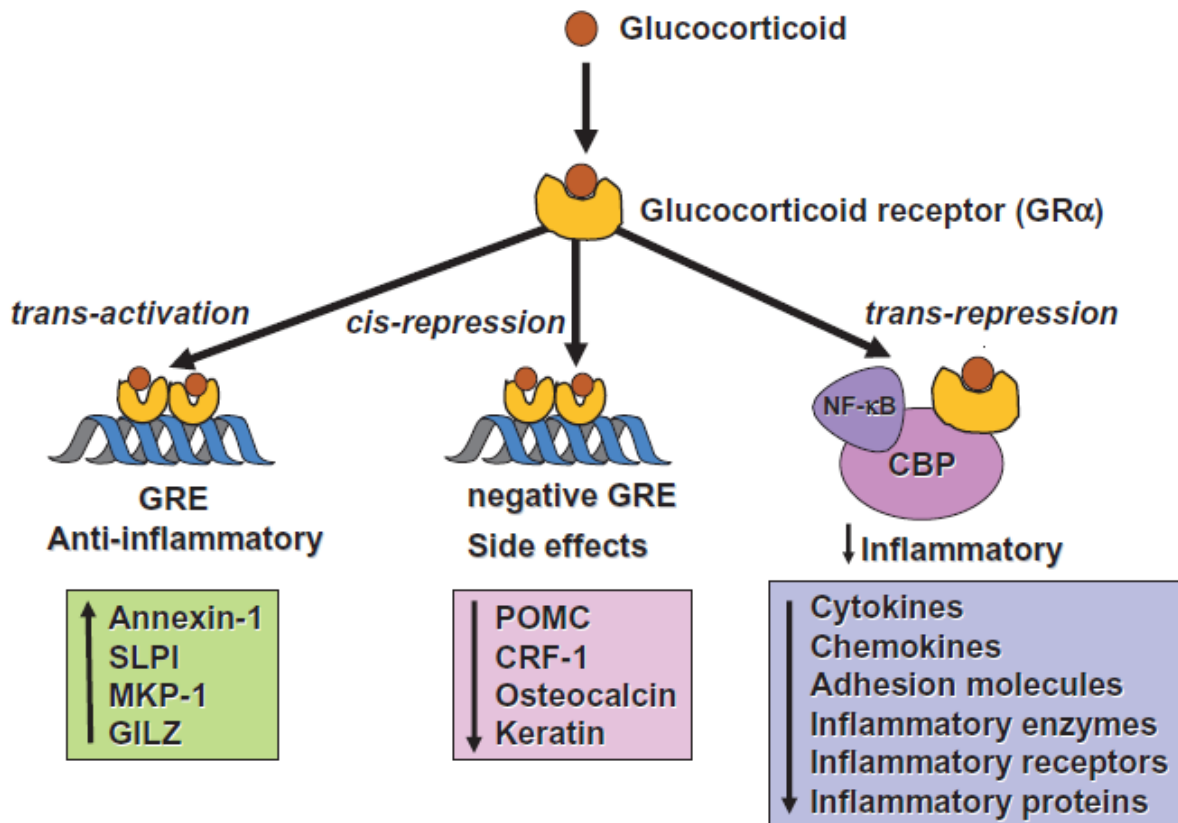


Figure 1.13. Overview of mechanisms of glucocorticoid-GR regulation of gene expression. In the nucleus, ligand-bound GR dimerises and binds to positive or negative GRE, leading to an increase or decrease in gene transcription (*trans*-activation or *cis*-repression), respectively. In contrast, activated GR, as a monomer, also suppresses inflammatory genes (*trans*-repression) via interaction with transcription factors (Barnes 2011).

1.7.3.1 Ligand binding and nuclear translocation

Lipophilic in nature, glucocorticoids exert their effects by freely diffusing across the plasma membrane of cells and binding to a ubiquitously expressed GR that is localised in the cytoplasm of target cells. In the absence of the hormone, GR is retained in the cytoplasm in an inactive state as part of a large multi-protein complex that includes the chaperone protein heat shock protein (hsp) 90. The association with hsp90 appears to maintain the receptor in a conformation that favours high-affinity hormone binding. Binding of the hormone induces a conformational change in the receptor, resulting in dissociation of hsp90 and exposure of the nuclear localisation signals. This allows nuclear translocation of the activated glucocorticoid-GR complex utilising selective importins (Bhavsar and Adcock 2008).

1.7.3.2 Transactivation

One of the major ways in which the GR elicits its genomic effects is by direct binding to DNA. Within the nucleus, GR homodimerises and binds to GRE in the regulatory regions of glucocorticoid-responsive genes. The consensus GRE sequence GGTACAnnnTGTTCT consists of two palindromic half-sites separated by a three-base-pair spacer and is most often found in the promoter regions of target genes. The GR homodimer binds the GRE and undergoes additional conformational changes, resulting in the recruitment of coactivators such as SRC, CBP and pCAF. These coactivators induce remodelling of chromatin structure and unwinding of DNA by their intrinsic histone acetyltransferase and methyltransferase activities. RNA

polymerase II and the basal transcription machinery are then recruited to the newly accessible promoter to stimulate transcription of the linked gene (Fig. 1.14).

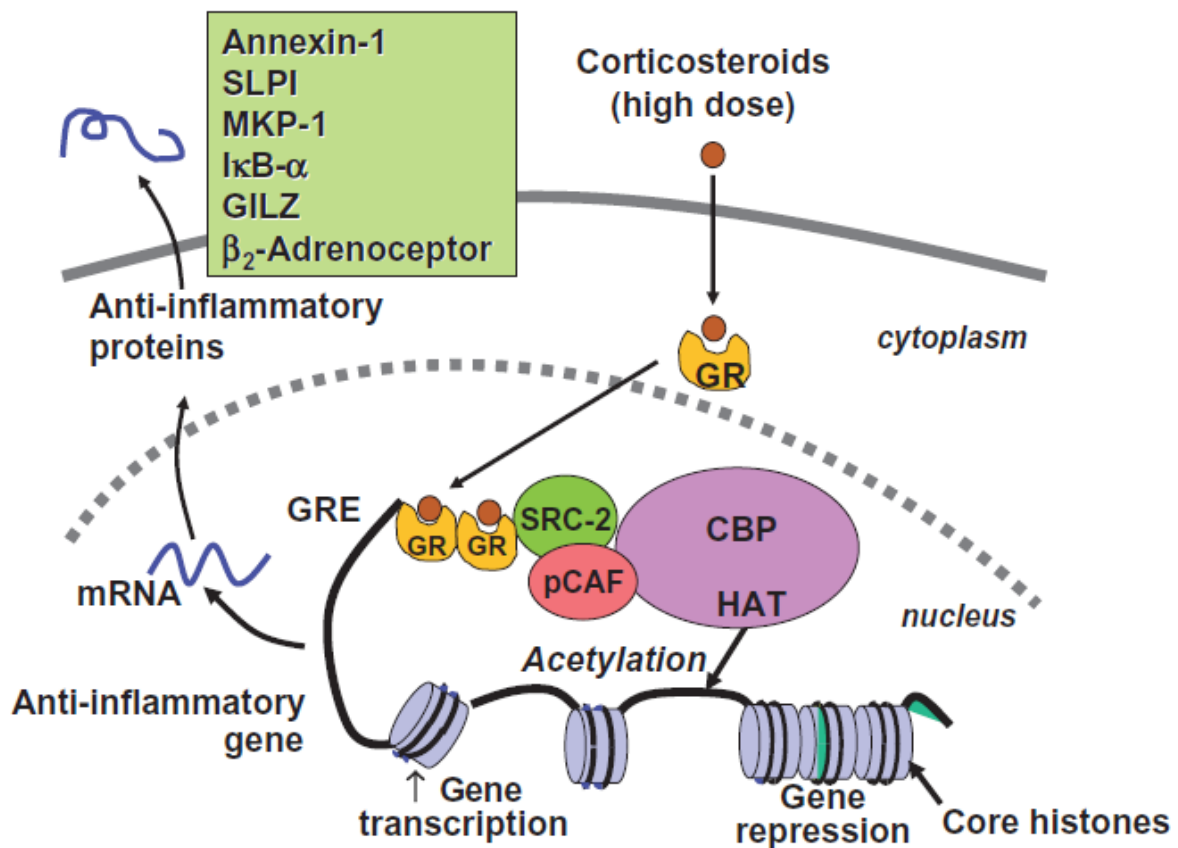


Figure 1.14. Corticosteroid activation of anti-inflammatory gene expression (transactivation). Corticosteroids bind to cytoplasmic GR that translocate into the nucleus, where they bind to GRE in the promoter region of steroid-sensitive genes and also directly or indirectly to coactivator molecules such as CBP, pCAF or SRC, which have intrinsic HAT activity, causing acetylation of lysines on histone H4, which leads to activation of genes encoding anti-inflammatory proteins (Barnes 2011).

1.7.3.3 Transrepression

The activated GR can also regulate gene expression, as a monomer, physically associating with other transcription factors and modulating their activity on responsive genes. For example, GR interacts with the c-jun and c-fos components of AP-1 and represses its responsive genes. Similarly, the receptor inhibits the transcriptional activity of NF- κ B by associating with the p65 subunit, which is mediated by the second zinc-finger in the DNA-binding domain of GR. GR may either prevent these transcription factors from binding to their cognate response element or tether to the DNA-bound proteins and interfere with chromatin remodelling and/or recruitment of basal transcription machinery (Bhavsar and Adcock 2008) (Fig. 1.15).

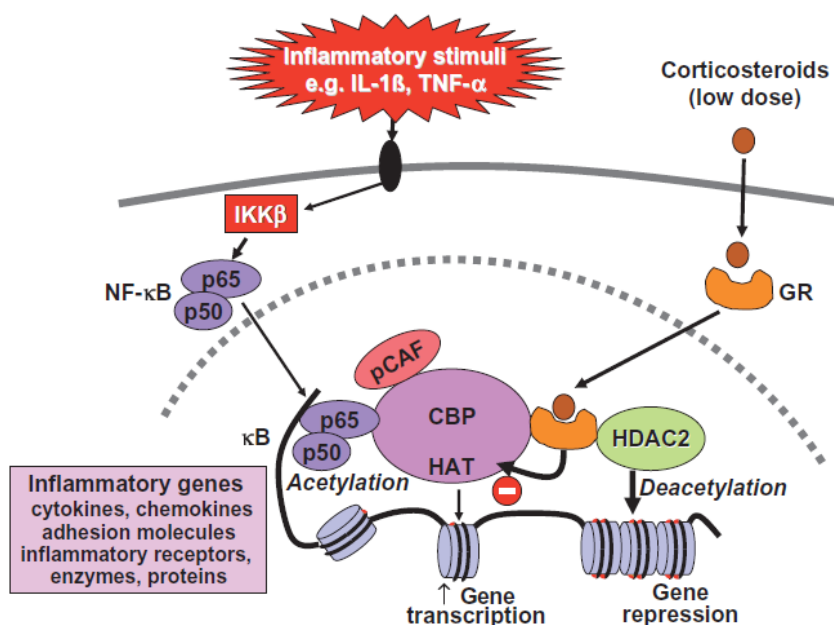


Figure 1.15. Corticosteroid suppression of activated inflammatory genes expression (transrepression). NF- κ B (p65-p50) activated by pro-inflammatory stimuli translocates into the nucleus, binding to specific κ B recognition sites and to coactivators which have intrinsic HAT activities. This results in acetylation of core histone H4, leading to increased expression of inflammatory genes. Activated GR translocates into the nucleus and binds to coactivators in order to inhibit HAT activities and recruit HDAC2, which reverses histone acetylation, leading to suppression of these induced inflammatory genes (Barnes 2011).

1.7.3.4 Destabilisation of inflammatory mRNA

Glucocorticoids also exert anti-inflammatory actions that do not depend on the receptor's ability to regulate transcription in the nucleus. Adenylate-uridylate-rich elements (ARE) in the three prime untranslated region (3'-UTR) of many inflammatory genes control the stability of mRNA. Binding of ARE-binding proteins to mRNA results in the formation of messenger ribonucleoprotein (mRNP) complexes which control mRNA decay. Several ARE-binding proteins have been reported and include tristetrapolin (TTP), which promotes mRNA decay, and HuR family members, which are associated with mRNA stability. Importantly, HuR binding to ARE is dependent upon p38 MAPK activation. Dexamethasone has been reported to regulate the levels of HuR and TTP, thereby reducing the levels of inflammatory gene mRNAs through a p38 MAPK-mediated pathway subsequent to induction of MAPK phosphatase (MKP)-1 (Barnes 2006) (Fig. 1.16).

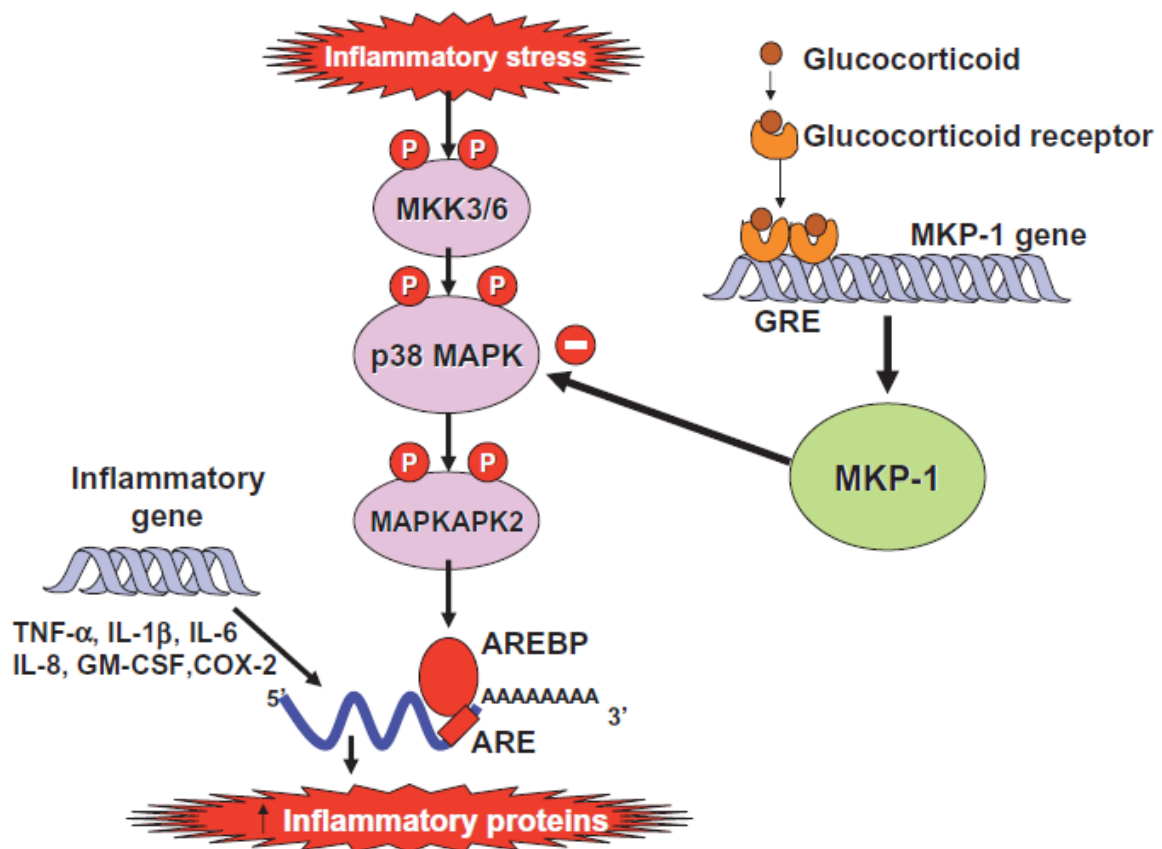


Figure 1.16. Destabilisation of inflammatory mRNA by corticosteroids through suppression of p38 MAPK by induction of MKP-1. The stability of mRNA coding for several inflammatory proteins is controlled by AU-rich element in the 3'-UTR. p38 MAPK activated by inflammatory stress phosphorylates MAPKAPK2, which activates ARE binding proteins (AREBP), thus stabilising the mRNA by binding to ARE. Corticosteroid induces expression of MKP-1, which inhibits p38 and thus prevents stabilisation of multiple inflammatory proteins (Barnes 2011).

1.7.3.5 Post-translational modification of GR: focus on phosphorylation

GR is also a substrate for several types of post-translational modifications that regulate corticosteroid responsiveness by modulating the levels or transcriptional activity of the receptor. These modifications include phosphorylation, acetylation, nitrosylation, redox regulation, ubiquitination and SUMOylation (Fig.1.17).

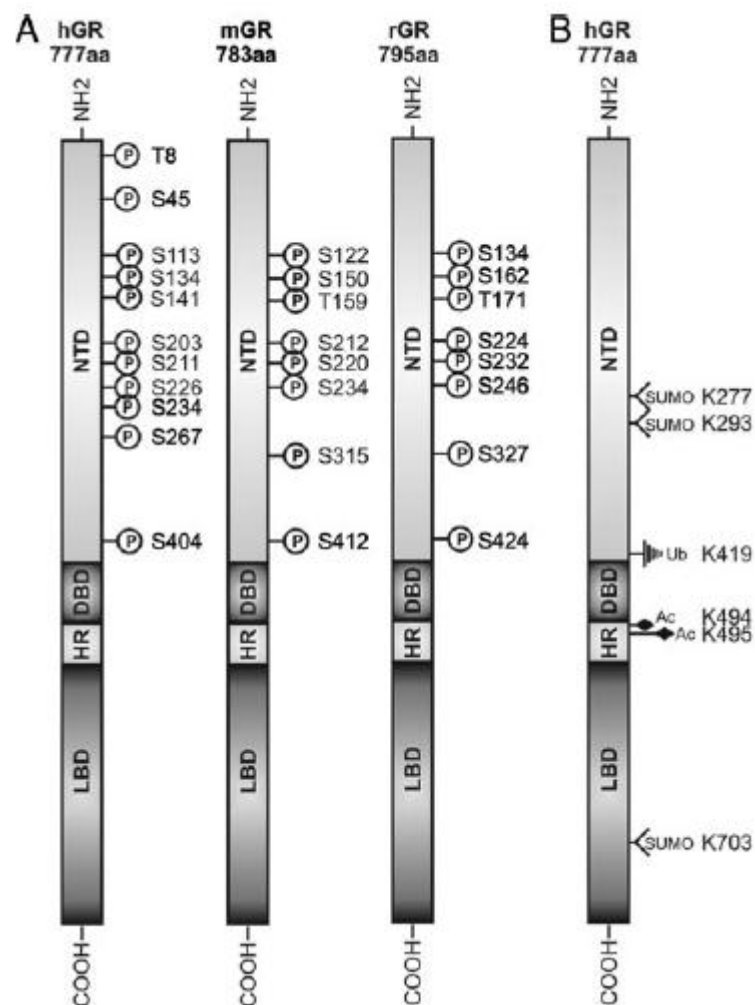


Figure 1.17. Structure and post-translational modifications of GR. (A) Phosphorylation sites for human (h), murine (m) and rat (r) GR. (B) Post-translation modifications of hGR, except from the phosphorylation sites. DBD: DNA-binding domain; LBD: ligand-binding domain; NTD: N-terminal domain; HR: hinge region; aa: amino acids; P: phosphorylation; SUMO: SUMOylation; Ub: ubiquitination; Ac: acetylation (Beck, Vanden Berghe et al. 2009).

GR is a phosphoprotein that becomes hyper-phosphorylated typically after binding glucocorticoids (Bodwell, Orti et al. 1991). At least six different serine residues are identified as sites of phosphorylation in the AF-1 region of its N-terminal domain: S113, S141, S203, S211, S226 and S404 (Oakley and Cidlowski 2011). The receptor displays a basal level of phosphorylation and becomes hyper-phosphorylated upon binding of glucocorticoids, with the extent of phosphorylation dependent on the nature of the bound ligand. Altered GR phosphorylation status can affect the binding of GR and its ligand, hsp90 interactions, subcellular localisation, and nuclear-cytoplasmic shuttling. Phosphorylation can also enhance the transcriptional activity of GR in a promoter-specific fashion and regulates both the basal and glucocorticoid-induced turnover of the receptor (Nicolaidis, Galata et al. 2010). The major kinases that phosphorylate GR include MAPK, cyclin-dependent kinases (CDKs) and glycogen synthase kinase-3 β (GSK-3 β) (Oakley and Cidlowski 2011).

Of the six human phosphorylated serine residues of GR, S203, S211 and S226 have been more extensively investigated (Table 1.2). It has been proposed that phosphorylation of GR at S211 is correlated with its transcriptional activity (Oakley and Cidlowski 2011). In the absence of the ligand, basally phosphorylated GRs at S203 and S211 are both in the cytoplasm, while dexamethasone-induced S211 phosphorylated GR is mainly nuclear, while S203 phosphorylated GR preferentially resides in the cytoplasm. In addition, although dexamethasone induces phosphorylation of GR at both S203 and S211, the antagonist RU486 reverses

phosphorylation only of the latter (Wang, Frederick et al. 2002). On the contrary, phosphorylation of GR at S226 impairs its signalling capability (Oakley and Cidlowski 2011), and GR transactivation function is found to be at its peak when the relative phosphorylation of S211 surpasses that of S226 (Chen, Dang et al. 2008). Furthermore, in lung epithelial cells, the degree of S211 phosphorylation correlates with ligand bind, nuclear translocation and GR transactivation (Adcock and Barnes 2008).

Phosphorylation site	Kinase(s)	Effect
Human Serine 203 (Rat Ser 224) (Mouse Ser 212)	Cyclin A-CDK2 Cyclin A-CDC2 Cyclin B-CDK2 Cyclin B-CDC2 Cyclin E- CDK2 ERK MAPK	pSer203 GR is contained within the cytoplasmic fraction of the cell and fails to bind GRE-containing promoters, suggesting that pSer203 is a transcriptionally inactive form of the GR.
Human Serine 211 (Rat Ser 232) (Mouse Ser 220)	Cyclin A-CDK2 Cyclin A-CDC2 Cyclin B-CDK2 Cyclin B-CDC2 Cyclin E- CDK2 ERK MAPK CDK5 p38 MAPK	The GR is transcriptionally more active when phosphorylated on Ser211 in part due to a conformational change and increased recruitment to GRE-containing promoters, leading to increased apoptosis in several cell types. However, in neuronal cells, pSer211-GR is less active than unphosphorylated receptor.
Human Serine 226 (Rat Ser 246) (Mouse Ser 234)	JNK CDK5	Although pSer226-GR can associate with GRE-containing promoters, the phosphorylation of Ser226 leads to blunted hormone signaling because of due to enhanced nuclear export of the GR.
Human Serine 404 (Rat Ser 424) (Mouse Ser 412)	GSK-3	Phosphorylation of Ser404 leads to a conformational change within the GR, resulting in altered co-factor recruitment and a re-direction of the transcriptional response of GR, attenuating GC signaling.

Table 1.2. Summary of effects of GR phosphorylation at S201, S211 and S226 (Galliher-Beckley and Cidlowski 2009).

1.7.4 Corticosteroid insensitivity in severe asthma

Recently, endobronchial biopsy performed in patients with severe asthma has become feasible and provides an important contribution to the field, by enabling studies to investigate the involvement of more distal parts of the lung. Distinct inflammatory and structural features are demonstrated in the airways of patients with severe asthma when compared to patients with milder forms of the disease (Pepe, Foley et al. 2005). In addition, pathological studies have identified different patterns of inflammation in the airways of patients with severe asthma, demonstrating that different phenotypes of severe asthma exist (Wenzel, Schwartz et al. 1999).

It has been demonstrated that the peripheral blood mononuclear cells (PBMC) (Hew, Bhavsar et al. 2006) and alveolar macrophages (Bhavsar, Hew et al. 2008) from patients with severe asthma show impaired response to dexamethasone in terms of suppressing LPS-induced inflammatory cytokines, compared with subjects with non-severe asthma. In addition, alveolar macrophages of patients with severe asthma release decreased lipoxin A4, an anti-inflammatory lipid product, but increased leukotriene B4, a pro-inflammatory 5-lipoxygenase product, compared with non-severe asthmatic subjects. Similarly, there is impaired corticosteroid sensitivity to LPS-induced leukotriene B4 but not lipoxin A4 in macrophages of patients with severe asthma (Bhavsar, Levy et al. 2010). It is still uncertain whether airway structural cells, such as ASMC, display differential corticosteroid sensitivity in a similar manner.

1.8 Hypothesis and aims

Rationale:

The clinical feature of severe asthma is relatively decreased therapeutic effect of corticosteroids to control the disease, suggesting the presence of corticosteroid insensitivity in severe asthma. Impaired effect of corticosteroid in terms of suppressing inflammatory cytokines have been reported in PMBC and alveolar macrophages of patients with severe asthma compared with non-severe asthmatic subjects. It is still unclear whether ASMC display in a similar manner.

Therefore I hypothesise that *relative corticosteroid insensitivity exists in ASMC of patients with severe asthma.*

To test this hypothesis, I aim to investigate and compare:

1. cytokine-induced chemokine release and mRNA expression, and
2. effect of dexamethasone on regulation of induced chemokines

in ASMC of healthy subjects and patients with non-severe or severe asthma.

Then the possible mechanisms are explored, focusing on expression and activation of MAPK, NF- κ B and GR pathways.

Chapter 2.

Materials and Methods

Chapter 2. Materials and Methods

2.1 Materials

2.1.1 General reagents

General laboratory reagents were purchased from Sigma-Aldrich (Dorset, UK) unless otherwise stated.

2.1.2 Cell culture

Dulbecco's Modified Eagle's Medium (DMEM), Hank's Balanced Salt Solution (HBSS), cell culture grade bovine serum albumin (BSA), non-essential amino-acid solution, L-glutamine, penicillin/streptomycin solution, trypsin-EDTA solution, amphotericin B, and cell freezing medium were purchased from Sigma-Aldrich (Dorset, UK). Foetal calf serum (FCS) was purchased from Biosera (East Sussex, UK). The details for different cell treatments used in this thesis are shown in table 2.1.

Treatment	Source	Stock solution
TNF- α	R&D Systems	10 μ g/mL in 0.1% BSA
IFN- γ	R&D Systems	50 μ g/mL in 0.1% BSA
Dexamethasone	Sigma-Aldrich	10 ⁻³ M in water
GW-856553	GlaxoSmithKline	10 ⁻² M in DMSO
SP600125	Sigma-Aldrich	10 ⁻² M in DMSO
U0126	Sigma-Aldrich	10 ⁻² M in DMSO

Table 2.1. Cytokines and inhibitors used in the project.

2.1.3 Cell viability assay

Methylthiazolyldiphenyl-tetrazolium bromide (MTT) and dimethyl sulphoxide (DMSO) were purchased from Sigma-Aldrich (Dorset, UK).

2.1.4 Enzyme linked immunosorbent assays (ELISA)

DuoSet ELISA kits for CCL11, CXCL8, CXCL10 and CX3CL1 were purchased from R&D Systems (Abingdon, UK). Nunc MaxiSorp flat-bottom 96 well plates were purchased from Fisher Scientific (Leicestershire, UK). SpectraMax Plus absorbance microplate reader was manufactured by Molecular Devices (California, USA).

2.1.5 RNA extraction, cDNA preparation and real-time quantitative PCR

The RNeasy Mini Kit, QIAshredder spin columns, DNase enzyme and SYBR Green PCR Master Mix Reagent were purchased from Qiagen (West Sussex, UK). The NanoDrop 1000 spectrophotometer was from Thermo Scientific (Massachusetts, USA). The dNTPs and nuclease-free water were from Bioline (London, UK). Avian myeloblastosis virus (AMV) reverse transcriptase reaction buffer and enzyme, random primers and recombinant RNasin ribonuclease inhibitor were from Promega (Southampton, UK). The MBS 0.5G thermal cycler was from Thermo Hybaid (Middlesex, UK). The Rotor Gene 6000 real-time cycler, capillary tubes, and the Rotor Gene 6000 Series software (version 1.7) were from Corbett Research (Cambridge, UK).

2.1.6 Whole cell/cytoplasmic/nuclear protein extraction and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE)/Western blotting

Hypotonic buffer (10X) and detergent were purchased from Active Motif (Rixensart, Belgium). Radioimmunoprecipitation assay (RIPA) lysis buffer was from Santa Cruz (Heidelberg, Germany). The complete Mini protease inhibitor cocktail was from Roche Applied Science (West Sussex, UK) and a 25X stock solution was prepared

in tissue culture grade water. Phenylmethylsulphonyl fluoride (PMSF) was from Sigma-Aldrich (Dorset, UK); a 200 mM stock solution was prepared in isopropanol. Bicinchoninic acid (BCA) assay was from Pierce Biotechnology (Illinois, USA). 4-12% Bis-Tris Novex precast mini gels, XCell SureLock electrophoresis cells, PowerEase 500 power supply, NuPAGE MOPS SDS running buffer (20X) and iBlot transfer system were from Invitrogen (Paisley, UK). The full-range rainbow molecular weight markers, enhanced chemiluminescence (ECL) and ECL Plus solution were from GE Healthcare Life Sciences (Buckinghamshire, UK). FUJIFILM medical X-ray film was from Genetic Research Instrumentation Ltd (Braintree, UK). Re-Blot Plus mild antibody stripping solutions (10X) were from Millipore (Watford, UK). The Gel Doc-It imaging system was from Ultra-Violet Products (California, USA).

2.2 Methods

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

12 healthy subjects and 10 each patients with non-severe or severe asthma, respectively, were recruited in this research. All healthy subjects were normal volunteers without any disease, aged between 18 and 65 years, and had a negative PC₂₀ to methacholine, normal spirometry, physical examination and no history of significant medical problems. Severe asthma patients were defined as needing either continuous or near-continuous oral corticosteroids, or high-dose inhaled corticosteroids, or both to achieve a level of mild-to-moderate persistent asthma and by 2 or more minor criteria (ATS. 2000). Patients with non-severe asthma used inhaled beclomethasone (0-1000 µg/day or equivalent) with perfect control of their asthma. Current and ex-smokers of greater than 5 pack-years were excluded. All patients gave informed consent to participate in this study which was approved by

the local Ethics Committee. All the subjects were free from upper respiratory infections and acute exacerbations within three months preceding the bronchoscopy.

Compared with those with non-severe asthma, the patients with severe asthma suffer from longer duration of the disease and use higher doses of inhaled corticosteroids, and half receive oral prednisolones. These patients also have worse pulmonary functions and greater airway hyper-responsiveness (Table 2.2).

	Non-asthma	Non-severe Asthma	Severe Asthma
Numbers	12	10	10
Age, years	46.1 ± 12.2	42.3 ± 17.2	46.2 ± 14.4
Gender, F/M	7/5	4/6	5/5
Duration of asthma, years	N/A	23.9 ± 12.3	35.6 ± 12.0 *
Inhaled corticosteroid dose, µg BDP equivalent	0	740 ± 353	1563 ± 272 ***
Atopy (n) ^{\$}	3	7	5
Receiving oral corticosteroids (n)	0	0	5 *
FEV ₁ , L	3.35 ± 0.81	2.88 ± 0.41	2.24 ± 0.36 **
FEV ₁ , % predicted	96.8.0 ± 12.4	88.6 ± 11.1	74.9 ± 12.4 *
FEV ₁ /FVC, %	80.3 ± 12.0	77.9 ± 4.6	67.7 ± 10.0 **
β-agonist reversibility [#] , %	N/A	15.7 ± 5.7	22.8 ± 10.6
PC ₂₀ , mg/mL	> 16	2.91 ± 2.88	0.91 ± 0.72 *

Table 2.2. Characteristics of studied subjects. BDP, beclomethasone dipropionate; FEV₁, forced expiratory volume in 1s; FVC, forced vital capacity; PC₂₀, provocative concentration of methacholine causing a 20% fall in FEV₁; N/A: not available. \$ Defined as positive skin prick tests to one or more common aeroallergens. # Measured as percent increase in FEV₁ after 400 µg salbutamol. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs non-severe asthma. Data shown as mean ± SEM.

2.2.2 Human ASMC isolation and culture

The ASMC from healthy subjects and patients with non-severe and severe asthma were obtained from the biopsies of the right lower bronchus, taken by the bronchoscopists. Biopsies were separated by aseptic needles into small pieces with the size less than 1 mm². Each piece of tissue was transferred to a 6-well culture plate for attachment and growth. When confluent, cells were harvested and split into 25-cm² and then 75-cm² flasks at the next passage. Subsequently, they were split into 150-cm² flasks in preparation for experiments or stored at -80 °C in the liquid nitrogen.

The cells were cultured in DMEM medium supplemented with 10% FCS, 4 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL amphotericin B. The presence of ASMC was confirmed by identifying the characteristic “hill and valley” morphology, and immunofluorescence techniques for calponin, smooth muscle α-actin and myosin heavy chain revealed that more than 95% of the cells displayed the characteristics of smooth muscle cells in culture (Oltmanns, Walters et al. 2008). Cell stocks were kept in 150-cm² flasks at 37°C, 5% CO₂, and humidified atmosphere. Cells at passage 4 or 5 were used for experiments. At 90% confluence, the cells were serum deprived for 24 hours in DMEM medium supplemented with 4 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 2.5 µg/mL amphotericin B, 1% non-essential amino acids, and 0.1% BSA.

2.2.3 Cell viability assay

ASMC were seeded in 48-well tissue culture plates and stimulated as indicated. At the end of the period of stimulation the supernatant was removed, and cells were incubated with 150 μ L of 1 mg/ml MTT solution in serum-free DMEM at 37°C in 5% CO₂/95% air (vol/vol) for 15 minutes. Staining of the cells was confirmed by observation under a light microscope, the MTT solution was removed, and the plate dried by blotting on clean paper towels. 300 μ L of DMSO was then added to each well and thoroughly mixed. The resulting solution was then transferred into 96-well microtitre plates and the absorbance at 550 nm was measured with the absorbance microplate reader. The effect of a treatment on cell viability was determined as a fold change in absorbance compared to the unstimulated control.

2.2.4 ELISA

Chemokine release was measured in supernatants from 90% confluent cells in 24-well (mostly) or 6-well (when RNA was also required) cell culture plates. At the end of the stimulation period supernatants were collected and stored at -20°C or assayed directly. Chemokine concentrations in the supernatants were determined by using specific DuoSet ELISA kits according to the manufacturer's instructions. The antibody working concentrations, standard concentrations and supernatant dilutions for each assay are summarised in Table 2.3.

Chemokine	Capture Antibody (working concentration)	Detection Antibody (working concentration)	Standard concentration range
CCL11	Mouse anti-human (2 ug/mL)	Biotinylated goat anti-human (100 ng/mL)	15.63-1000 pg/mL
CXCL8	Mouse anti-human (4 ug/mL)	Biotinylated goat anti-human (20 ng/mL)	31.25-2000 pg/mL
CXCL10	Mouse anti-human (2 ug/mL)	Biotinylated goat anti-human (50 ng/mL)	31.25-2000 pg/mL
CX3CL1	Mouse anti-human (4 ug/mL)	Biotinylated mouse anti-human (250 ng/mL)	31.25-2000 pg/mL

Table 2.3. Conditions used for ELISA.

Briefly, 96-well microtitre plates were coated by dispensing 100 µL of capture antibody solution at the working concentration in PBS and incubated overnight at room temperature. The capture antibody solution was aspirated, and the plate washed three times with 300 µL of wash buffer using an ELISA plate washer

(Skatron, Norway) and dried by blotting against paper towels. The plate was then blocked by dispensing 300 μL of Block Buffer (1% BSA and 0.05% NaN_3 in PBS) into each well and incubated for one hour at room temperature, followed by another wash step as described above. Seven recombinant protein standards were then prepared by carrying out 2-fold serial dilutions of the stock protein standard in the reagent diluent (1% BSA in PBS). 100 μL of the standards and diluted supernatants were then dispensed in the plate and incubated for 2 hrs at room temperature. The plate was then washed, and 100 μL of detection antibody diluted to the working concentration in the reagent diluent and dispensed into each well and incubated for 2 hrs at room temperature. The plate was then washed and 100 μL of streptavidin-HRP solution, diluted in 1/200 in Reagent Diluent, were dispensed in each well and incubated for 20 mins at room temperature protected from light. After the final wash step, 50 μL of the substrate solution, prepared by mixing equal volumes of Colour Reagents A (H_2O_2) and B (tetramethylbenzidine), were dispensed into each well and incubated until a change of colour was observed. The substrate reaction was then stopped by adding 50 μL of Stop Solution (2 N H_2SO_4). The absorbance of each well was then measured at 450 nm with a 540 nm correction with the absorbance microplate reader, and a four-parameter logistic standard curve was automatically generated using the microplate reader software. The chemokine concentration of each sample was determined from the standard curve and corrected for the dilution by multiplying by the dilution factor.

2.2.5 Determination of mRNA expression by RT-qPCR

2.2.5.1 RNA extraction

ASMC were seeded in 6-well culture plates at an initial density of 80,000 cells/well. After treatment as indicated, cells were washed with 1 ml HBSS and then lysed by adding 350 μ L/well of RLT buffer containing 1% β -mercaptoethanol for 5 minutes at room temperature. The lysates were stored at -80 °C or further processed for RNA extraction with the RNeasy Mini Kit according to the manufacturer's instructions. Briefly, cell lysates were homogenised by centrifugation through QIAshredder spin columns in the MIKRO 22R microcentrifuge, with radius of 87 mm, (Hettich, Germany) at 12,000 rpm for 2 minutes. An equal amount (350 μ L) of 70% ethanol was then mixed with the lysate, and the resulting solution was applied to the RNeasy Mini spin column by centrifugation at 10,000 rpm for 15 seconds, in order for total RNA to be retained on the silica-based membrane. The membrane bound RNA was washed using suitable buffers to remove contaminants and incubated with a DNase enzyme for 15 minutes at room temperature to remove DNA contamination. RNA was eluted from the column by applying 30 μ L H₂O and centrifuging at 10,000 rpm for one minute. The concentration of RNA was measured by the spectrophotometer. RNA purity was determined by determining the ratio of absorbance at 260 nm over the absorbance at 280 nm (A₂₆₀/A₂₈₀).

2.2.5.2 Reverse transcription

0.5 μ g of total RNA in a final volume of 10 μ L was incubated for 5 min at 70°C on the thermal cycler in order to denature the RNA strands. At the end of the denaturation step, 10 μ L of reverse-transcription reaction mix was added, with total volume of 20

μL containing 1mM dNTPs, 1X AMV reverse transcriptase reaction buffer, 25 ng/mL random primers, 40 U recombinant RNasin ribonuclease inhibitor and 10 U AMV reverse transcriptase. The mixture was incubated at 42°C for 60 minutes followed by incubation at 90°C for 4 min to inactivate the reverse transcriptase enzyme. The final cDNA product was then diluted 4-fold in nuclease-free water.

2.2.5.3 Real-time PCR

5 μL of cDNA was added to capillary tubes containing 15 μL of reaction mixture, which consists of SYBR Green PCR Master Mix Reagent and gene specific primers. The PCR reaction was carried out in the real-time cycler, where the cycling conditions were 15 minutes at 95°C (enzyme activation) followed by 35-60 cycles of 20 seconds at 94°C (denaturing step), 20 seconds at 60°C (annealing step) and 20 seconds at 72°C (elongation step). Fluorescence emitted from DNA-bound SYBR Green was detected at 510 nm after excitation at 470 nm during the extension step. Data from the reaction were analysed using the Rotor-Gene 6000 Series Software (version 1.7). DNA was quantified by the standard curve method of relative quantification, created by serial dilution of a suitable sample. Relative quantitation of gene expression was normalized to 18S rRNA expression. Specific primers for CCL11, CXCL8, CX3CL1 and 18S (Table 2.4) were designed according to their published sequences using the GenScript online primer design software and synthesised by Sigma-Genosys (Haverhill, Suffolk, UK). Primer specificity was measured by using the online sequence analysis software BLAST (www.ncbi.nlm.nih.gov/BLAST/). Melting curve analysis and agarose gel electrophoresis were carried out in order to ensure the presence of one specific PCR product.

Gene	Primer sequence
CCL11	Forward: 5'-AAG CTT ACG CCA AAG CAC ACA CCT-3'
	Reverse: 5'-GAA TCC TGG CTT TGG AGT TGG AGA T-3'
CXCL8	Forward: 5'-GCC AAC ACA GAA ATT ATT GTA AAG CTT-3'
	Reverse: 5'-CCT CTG CAC CCA GTT TTC CTT-3'
CX3CL1	Forward: 5'-CCT GTA GCT TTG CTC ATC CAC TCT C-3'
	Reverse: 5'-TCC AAG ATG ATT GCG CGT T-3'
18S	Forward: 5'-CTT AGA GGG ACA AGT GGC G-3'
	Reverse: 5'-AGC CTG AGC CAG TCA GTG TA-3'

Table 2.4. Primers used for RT-qPCR.

2.2.6 Chromatin immunoprecipitation (ChIP) assay

2.2.6.1 Optimisation of sonication

ChIP assays were performed using the ChIP assay kit supplied by Millipore and the protocol therein. Briefly, ASMC were seeded in 75-cm² flasks; at 90% confluency (estimated 2×10^6 cells), they were serum deprived for 24 hours. After treatment the cells were fixed in 1% formaldehyde at room temperature for 10 minutes. After neutralization with 0.125 M of glycine, cells were scraped, pelleted, and lysed in 400 μ L of 1% SDS lysis buffer. The DNA was fragmented by sonication with Vibra Cell high intensity ultrasonic liquid processor (Sonics, USA), the conditions for which were determined by optimisation over a range of 15-second pulses (1-9 pulses). The sonicated samples were de-crosslinked, and DNA recovered as described below. The purified DNA was dissolved in 50 μ L of nuclear free water, and 5 μ L of samples (2×10^5 cell equivalent) was loaded in a 2% agarose gel in TBE (Tris/Borate/EDTA) buffer. Electrophoresis was performed at 100 V for 30 minutes; then the gel was soaked in 0.5 μ L/mL of ethidium bromide for 10 minutes. After washing with TBE buffer at room temperature for 30 minutes, the gel was exposed under UV light and

scanned by GelDoc-It imaging system (UVP Ltd, UK). Five 15-second pulses of sonication were shown to be optimal for fragmenting DNA with the range 200 to 1000 bp (Fig.2.1).

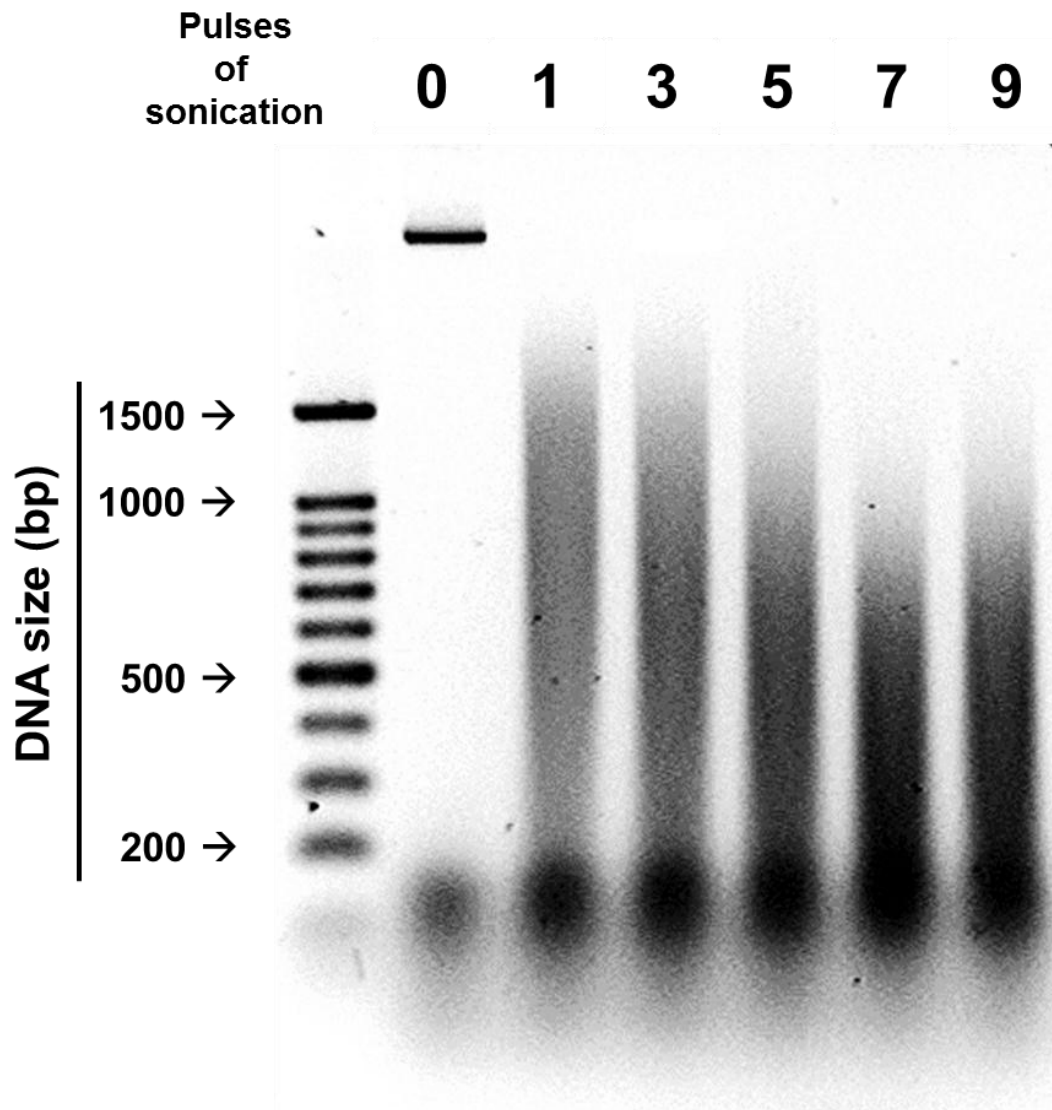


Figure 2.1. Optimisation of sonication for ChIP assay. Following fixing with 1% formaldehyde, 2×10^6 ASMC lyzed in SDS lysis buffer were sonicated under 40% maximal power, 15 seconds per pulse. After reverse crosslinking and DNA purification, 2×10^5 cell equivalent was loaded into a 2% agarose gel for electrophoresis. The gel was fluorescent-tagged with ethidium bromide and exposed to UV light.

2.2.6.2 Chromatin immunoprecipitation

After sonication, the supernatant was collected following centrifuge at 13,000 rpm at 8°C for 10 minutes. After addition of 1.6 mL of ChIP dilution buffer, 100 µL of sample was saved as input. After the protein-chromatin complex solution was pre-cleared with 80 µL of protein A agarose/salmon sperm DNA (provided as a 50% gel slurry in 10mM Tris-HCl, 1mM EDTA, pH 8.0 containing 0.05% sodium azide), 4 µg of anti-p65 antibody was added, and the sample incubated at 4°C overnight. Antibody/DNA complexes were captured with protein 60 µL of protein A agarose/salmon sperm DNA, washed with 1mL of low salt (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 150mM NaCl), high salt (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 500mM NaCl), lithium chloride immune complex wash buffer (0.25M LiCl, 1% IGEPAL CA630, 1% deoxycholic acid (sodium salt), 1mM EDTA, 10mM Tris, pH 8.1) once and TE buffer (10mM Tris-HCl, pH 8.0, 1mM EDTA) twice, respectively, eluted with 250 µL of freshly prepared 1% SDS containing 0.1M NaHCO₃, and reverse cross-linked by adding 0.2 M NaCl and incubation at 65 °C overnight. Both the DNA and input fractions were purified by phenol/chloroform wash and ethanol precipitation. The precipitated DNA out of 2 x 10⁶ cell equivalent was resuspended in 50 µL of nuclease-free water, and qPCR (as described in 2.2.5.3) was performed on 5 µL of sample. Sample DNA was normalized to input DNA. The primer sequences (Chang, Bhavsar et al. 2012) were listed in Table 2.5.

Gene	Primer sequence
CCL11	Forward: 5'-GCA AAT CAG GAA TCC CTT CAT CGT G-3'
	Reverse: 5'-GCT CCT CTG GCC CAT CTG CCY GCC-3'
CXCL8	Forward: 5'-AAC AGT GGC TGA ACC AGA G-3'
	Reverse: 5'-AGG AGG GCT TCA ATA GAG G-3'
CX3CL1	Forward: 5'-GGC ATG TTC CCA GCT TGT GGC AGG-3'
	Reverse: 5'-GTT GCC AAG GAA CCA AGC CGG C-3'

Table 2.5. Primer sequences for ChIP assays.

2.2.7 Determination of protein expression by Western blotting

2.2.7.1 Whole cell extraction

ASMC were seeded in 75-cm² flasks. After treatment, cells were washed once with ice cold PBS. In experiments where the expression of phosphorylated proteins was to be determined, PBS contained phosphatase inhibitor cocktail. After scraping, the cell suspension was transferred into pre-chilled tubes and pelleted at 7,000 rpm at 4°C for 5 minutes. The supernatant was then removed and the cell pellet was resuspended in 60 µL of RIPA buffer containing 1 mM PMSF and 1X complete mini protease inhibitor cocktail, vortexed for 10 seconds and incubated on the ice for 30 minutes. The lysates were then centrifuged at 13,000 rpm for 15 minutes, and the supernatant transferred in new pre-chilled tubes and stored at -80°C. To prepare a standard protein to compare the abundance of target proteins between healthy and diseased groups between different Western Blots, 12 flasks of confluent ASMC were trypsinised, followed by centrifuge and resuspension with RIPA buffer in the same way, and finally were aliquoted and stored at -80°C.

2.2.7.2 Cytoplasmic and nuclear extraction

ASMC were seeded in 75-cm² flasks. After treatment, cells were washed once with ice cold PBS. After scraping, the cell suspension was transferred into pre-chilled tubes and pelleted at 7,000 rpm at 4°C for 5 minutes. The supernatant was then removed and the cell pellet resuspended in 80 µL hypotonic buffer containing complete mini protease inhibitor cocktail and incubated in the ice for 15 minutes. 4 µL of detergent was added, and each sample was vortexed for 10 second. After centrifuge at 10,000 rpm at 4°C for 1 minute, the supernatant was collected (cytoplasmic extract).

The pellet was then washed by resuspension with 40 μ L hypotonic buffer and centrifuge at 10,000 rpm for 1 minute at 4°C. The supernatant was then removed, and the cell pellet resuspended in 40 μ L RIPA buffer containing 1 mM PMSF and 1X complete mini protease inhibitor cocktail, vortexed for 10 seconds and incubated in the ice for 30 minutes. The lysates were then centrifuged at 13,000 rpm for 15 minutes, and the supernatant (nuclear extract) was transferred in new pre-chilled tubes and stored at -80°C.

2.2.7.3 Determination of protein concentration

Protein concentration was determined by performing BCA assay in a 96-well plate according to the manufacturer's instructions. Briefly, 2 μ L of protein extract was diluted 5-fold by addition of 8 μ L tissue culture grade water. BSA standards (0.125, 0.25, 0.5, 1 and 2 mg/mL) were prepared by serial 2-fold dilutions with equal amount of cell culture grade water. 2 μ L of hypotonic buffer (for cytoplasmic extracts) and RIPA buffer (for nuclear extracts or whole cell lysates) was then added into 8 μ L of BSA standards to make final concentrations of 0.1, 0.2, 0.4, 0.8 and 1mg/mL, respectively. The BCA protein assay reaction mixture was then prepared by mixing reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide) and B (4% cupric sulphate) in a ratio of 50:1. 200 μ L of the mixture was added to the protein extracts and standards, mixed for 30 seconds, and the reaction incubated at 37°C for 30 minutes. The absorbance of the product was measured at 562 nm by absorbance microplate reader (Molecular Devices, UK) and the protein concentrations were determined by comparison to the standard curve and corrected by the dilution factor.

2.2.7.4 Western blotting

40 µg of protein extracts were aliquoted into tubes and the total volume was adjusted to 21 µL by adding hypotonic buffer (for cytoplasmic extracts) or RIPA buffer (for nuclear extracts or whole cell lysates). 7 µL of 4X NuPAGE LDS sample buffer (glycerol 10%, Tris base 141 mM, Tris HCl 106 mM, LDS 2%, EDTA 0.51 mM, SERVA Blue G250 0.22 mM, phenol red 0.175 mM, pH 8.5) containing 3.8% β-mercaptoethanol were added to the protein extracts, and the samples were heated at 70°C for 10 minutes to denature the proteins. The samples were then loaded onto a 4-12% Bis-Tris Novex pre-cast mini-gel. The gels were placed in the electrophoresis cell connected to a power supply and were electrophoresed at 200 V for 50 minutes in NuPAGE MOPS SDS running buffer (MOPS 50 mM, Tris base 50 mM, SDS 0.1%, EDTA 1 mM, pH 7.7). The gel cassette was opened and the gel was trimmed to an appropriate size with a gel knife, proteins were transferred from the gel to a nitrocellulose membrane using the iBlot transfer system at 20 V for 7 minutes. Visualisation of the proteins on the membrane was performed by incubation with Ponceau Red (0.2% Ponceau S in 3% trichloroacetic acid and 3% sulfosalicylic acid) for 5 minutes to confirm protein transfer.

After Ponceau Red was washed off with TBS-Tween (0.1% Tween), the membrane was blocked by incubating into 5% BSA (for phosphorylated proteins) or 5% skim milk (for unphosphorylated proteins) in TBS-Tween at room temperature for 30 minutes. The membrane was then incubated with a suitable dilution of the primary antibody (Table 2.6), at 4°C overnight. The membrane was then washed three times with TBS-Tween for 5 minutes and then incubated with a suitable secondary antibody (Table 2.6) for 45 minutes at room temperature. The membrane was

washed four times with TBS-Tween for 5 minutes and then covered with 2 mL of ECL or ECL Plus solution for 1 minute or 5 minutes, respectively, at room temperature. Excess solution was removed, and the membrane wrapped in plastic film and exposed to a medical X-ray film in a cassette. After the exposed films were developed, the relevant bands were scanned by GelDoc-It imaging system (UVP Ltd, UK), and the band intensities were quantified by Launch VisionWorksLS acquisition and analysis system (version 6.8).

In the experiments for which phosphorylated proteins were to be probed, after the bands were quantified, the membrane was stripped by incubation in 1X ReBlot Plus mild antibody stripping solution for 15 minutes. Complete stripping was confirmed by showing the disappearance of the bands after re-incubation with ECL/ECL Plus solution and the X-ray film was exposed. After washing and incubation with 5% skim milk in TBS-Tween, the total proteins on the membrane were probed with a suitable dilution of the primary antibody at 4°C on a shaker overnight. The following procedures were the same as described above.

2.2.8 Statistical analysis

GraphPad Prism Version 5.03 was the software for statistical analysis. Repeated measures analysis of variance (ANOVA) with Dunnet multiple comparison test was used for intra-group analysis of i) the effect of cytokine compared to unstimulation and ii) the effect of IFN- γ , dexamethasone, or MAPK inhibitor compared to TNF- α alone. Kruskal-Wallis test with Dunn's multiple comparison was used to compare results between the three groups. $p < 0.05$ was taken as significant.

Antibody	Type	Source	Dilution
Primary antibodies			
Phospho-p38	Rabbit polyclonal	Cell Signalling (#9211)	1:1,000
Total p38	Rabbit polyclonal	Cell Signalling (#9212)	1:1,000
Phospho-JNK	Rabbit polyclonal	Cell Signalling (#9251)	1:2,000
Total JNK	Rabbit polyclonal	Cell Signalling (#9252)	1:2,000
Phospho-ERK	Rabbit polyclonal	Cell Signalling (#4370)	1:50,000
Total ERK	Rabbit polyclonal	Cell Signalling (#9102)	1:2,000
Phospho-GR (S211)	Rabbit polyclonal	Cell Signalling (#4161)	1:2,000
Total GR (E-20)	Rabbit polyclonal	Santa Cruz (sc-1003)	1:1,000 (whole cell) 1:2,000 (nuclear)
p65 (C-20)	Rabbit polyclonal	Santa Cruz (sc-372)	1:5,000
β -actin	Mouse polyclonal	Santa Cruz (sc-130065)	1:20,000
TBP	Mouse mclonal	Abcam (ab51841)	1:5,000
α -tubulin (B-7)	Mouse polyclonal	Santa Cruz (sc-5286)	1:5,000
Secondary antibodies			
HRP-conjugated anti-rabbit IgG	Goat polyclonal	DAKO (P0448)	1:2,000
HRP-conjugated anti-mouse IgG	Rabbit polyclonal	DAKO (P0447)	1:2,000

Table 2.6. Antibodies used for Western blot.

Chapter 3.

Regulation of chemokines in ASMC:

effect of TNF- α and IFN- γ

Chapter 3. Regulation of chemokines in ASMC: effect of TNF- α and IFN- γ

3.1 Introduction

Although both TNF- α and IFN- γ are Th1 cytokines, they are involved in pathogenesis of asthma (Barnes 2008). TNF- α is one of the cytokines that amplify the inflammatory response in asthma, while IFN- γ has important immunomodulating effects.

The modulating effect of IFN- γ on TNF α -mediated chemokine expression is variable: depending on the targeted gene, IFN- γ may either suppress or potentiate TNF α -induced inflammatory chemokines. Some chemokines, such as CX3CL1, are only induced, synergistically, by the combined cytokines.

NF- κ B activity is critically involved in regulation of the inflammation in asthma. Expression of CCL11, CXCL8, CXCL10 and CX3CL1 is dependent on the NF- κ B pathway (Edwards, Bartlett et al. 2009). NF- κ B binding sites in the promoters of these genes have been identified (Mukaida, Okamoto et al. 1994; Matsukura, Stellato et al. 1999). Importantly, recruitment of the p65 component of NF- κ B to the promoters of CCL11 (Nie, Knox et al. 2005) and CXCL8 (John, Zhu et al. 2009) in ASMC and CX3CL1 (Bhavsar, Sukkar et al. 2008) in airway epithelial cells has been investigated.

Despite observations of elevated expression of CCL11 (Chan, Burgess et al. 2006), CXCL8 (John, Zhu et al. 2009), CXCL10 (Brightling, Ammit et al. 2005) and CX3CL1 (El-Shazly, Berger et al. 2006) in ASMC of patients with asthma, the difference in

chemokine release and mRNA expression in ASMC between patients with non-severe and severe asthma has not yet be investigated.

In this chapter, the effects of TNF- α , IFN- γ , or the combination, on chemokine release in ASMC of healthy subjects are examined. The modulating effects of IFN- γ on TNF α -mediated chemokine expression are extensively investigated, in terms of protein release, mRNA expression and recruitment of the p65 component of NF- κ B to the gene promoters. Cytokine-induced chemokines are compared between ASMC of healthy subjects and patients with non-severe and severe asthma. Finally, regulation of p65, in terms of expression, nuclear translocation, and recruitment to the gene promoters, is investigated and compared between groups.

3.2 Results

3.2.1 Effect of TNF- α and IFN- γ on chemokine expression in ASMC

3.2.1.1 Effect of TNF- α and IFN- γ on CCL11 release

To investigate the regulatory effect of TNF- α and IFN- γ on CCL11 release, ASMC of healthy subjects were stimulated with TNF- α (1, 3, 10, 30, 100 ng/mL) or IFN- γ (1, 10, 100 ng/mL) alone or in combination. Supernatants were collected 24 hours later, and CCL11 measured by ELISA. ASMC constitutively released CCL11 (266 ± 89 pg/mL); this was further induced by TNF- α in a concentration-dependent manner, with maximal induction of 2860 ± 542 pg/mL by TNF- α at 100 ng/mL (Fig. 3.1A; $p < 0.001$ vs unstimulated). In contrast, CCL11 release was induced to a lesser extent (548 ± 144 pg/mL, $p < 0.05$ vs unstimulated) by IFN- γ at 1 ng/mL, which was maintained at higher concentrations (Fig. 3.1B).

The influence of IFN- γ on TNF- α induced CCL11 release was also investigated. The stimulatory effect of TNF- α was suppressed by IFN- γ in a concentration-dependent manner, with maximal inhibition of approximately 50% achieved by IFN- γ at 10 ng/mL ($p < 0.05$ at all concentrations of TNF- α) and maintained at 100 ng/mL (Fig. 3.1C).

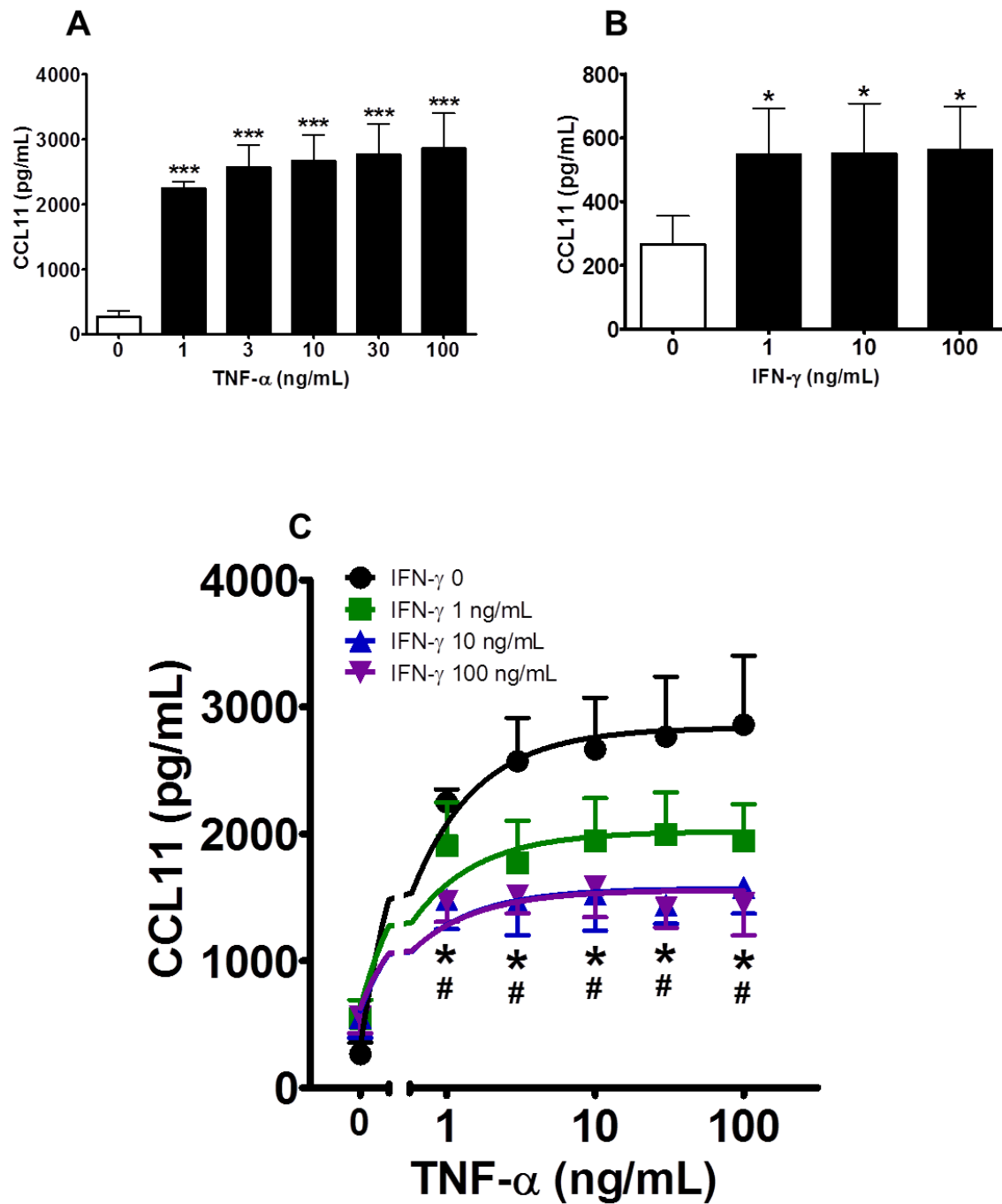


Figure 3.1. Effect of TNF- α and IFN- γ on CCL11 release in ASMC. ASMC were stimulated with (A) TNF- α (1-100 ng/mL), (B) IFN- γ (1-100 ng/mL), or (C) both, for 24 hours. CCL11 release was measured by ELISA. Bars and points represent mean $\pm$ SEM of ASMC from 3 healthy subjects. (A, B) * $p < 0.05$, ** $p < 0.01$ vs unstimulated. (C) * $p < 0.05$ IFN- γ 100 ng/mL vs TNF- α alone, # $p < 0.05$ IFN- γ 10 ng/mL vs TNF- α alone.

3.2.1.2 Effect of TNF- α and IFN- γ on CXCL8 release

To investigate the regulatory effect of TNF- α and IFN- γ on CXCL8 release, ASMC of healthy subjects were stimulated with TNF- α (1, 3, 10, 30, 100 ng/mL) or IFN- γ (1, 10, 100 ng/mL) alone or in combination. Supernatants were collected 24 hours later, and CXCL8 measured by ELISA. Baseline CXCL8 release was below the limit of detection of ELISA (< 31.25 pg/mL), while it was induced by TNF- α in a concentration-dependent manner, with maximal induction of 4502 ± 743 pg/mL by TNF- α at 100 ng/mL (Fig. 3.2A; $p < 0.01$). In contrast, treatment with IFN- γ did not induce CXCL8 release (Fig. 3.2B).

The influence of IFN- γ on TNF- α induced CXCL8 release was also investigated. The stimulatory effect of TNF- α was suppressed by IFN- γ in a concentration-dependent manner, with maximal inhibition of approximately 30% achieved with IFN- γ at 100 ng/mL (Fig. 3.2C; $p < 0.01$ vs TNF- α alone).

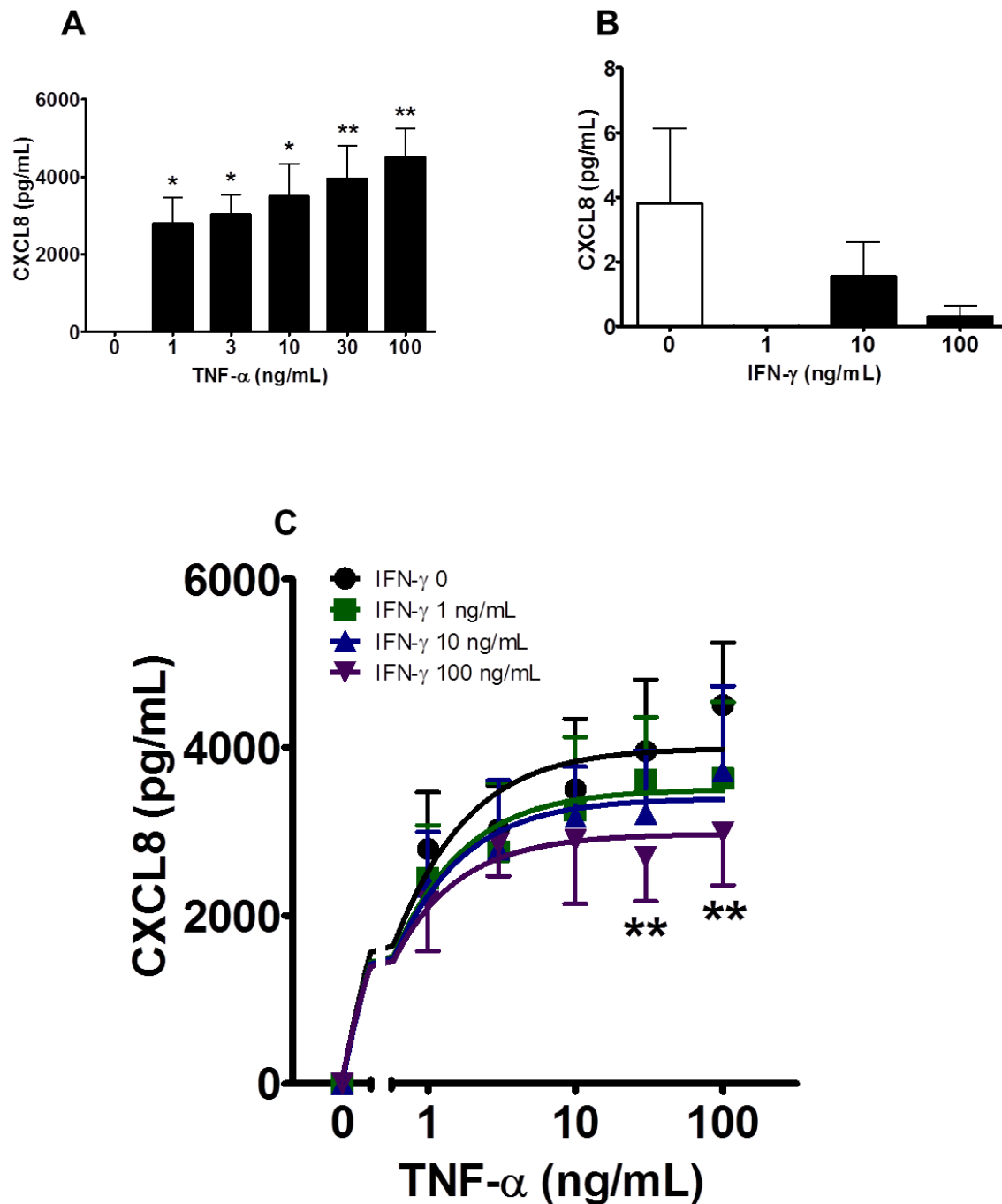


Figure 3.2. Effect of TNF- α and IFN- γ on CXCL8 release in ASMC. ASMC were stimulated with (A) TNF- α (1-100 ng/mL), (B) IFN- γ (1-100 ng/mL), or (C) both, for 24 hours. CXCL8 release was measured by ELISA. Bars and points represent mean $\pm$ SEM of ASMC from 3 healthy subjects. (A) * $p < 0.05$, ** $p < 0.01$ vs unstimulated. (C) ** $p < 0.01$ vs TNF- α alone.

3.2.1.3 Effect of TNF- α and IFN- γ on CXCL10 release

To investigate the regulatory effect of TNF- α and IFN- γ on CXCL10 release, ASMC of healthy subjects were stimulated with TNF- α (1, 3, 10, 30, 100 ng/mL) or IFN- γ (1, 10, 100 ng/mL) alone or in combination. Supernatants were collected after 24 hours, and CXCL10 was measured by ELISA. CXCL10 release was below the limit of detection (<31.25 pg/mL) at baseline but was induced by TNF- α in a concentration-dependent manner, with maximal release reached at 10 ng/mL (6.3 ± 1.5 ng/mL; $p < 0.001$ vs unstimulated) and maintained at higher concentrations (Fig. 3.3A). In contrast, CXCL10 was induced to a lesser extent by IFN- γ in a concentration-dependent manner, maximally 2.0 ± 0.7 ng/mL with IFN- γ at 100 ng/mL (Fig. 3.3B; $p < 0.05$ vs unstimulated).

The influence of IFN- γ on TNF α -induced CXCL10 release was also investigated. In contrast to the effect seen in terms of regulating CCL11 and CXCL8 release, the stimulatory effect of TNF- α on CXCL10 was potentiated by IFN- γ in a concentration-dependent manner, with approximately 18-fold potentiation by IFN- γ at 100 ng/mL (Fig. 3.3C).

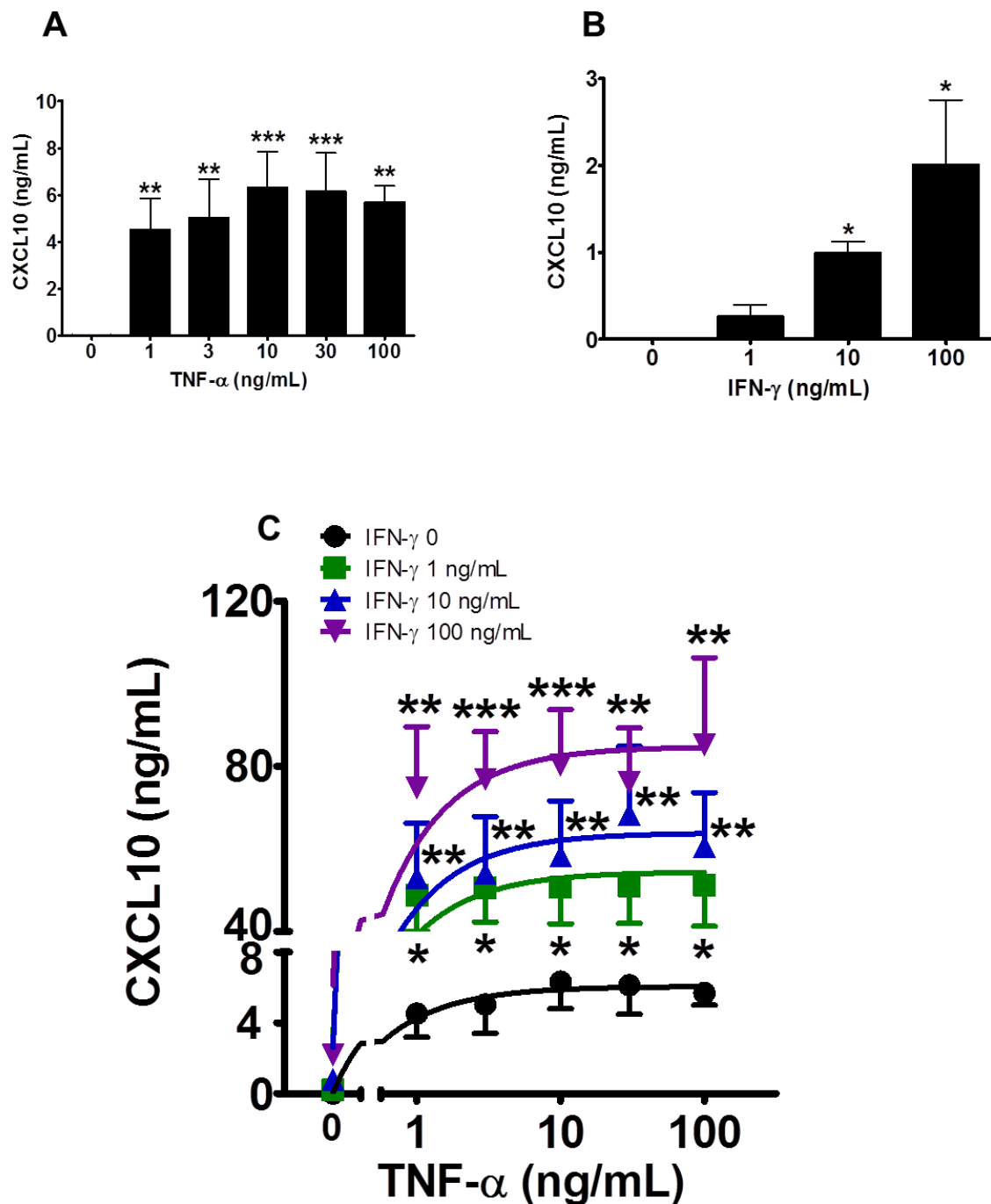


Figure 3.3. Effect of TNF- α and IFN- γ on CXCL10 release in ASMC. ASMC were stimulated with (A) TNF- α (1-100 ng/mL), (B) IFN- γ (1-100 ng/mL), or (C) both, for 24 hours. CXCL10 release was measured by ELISA. Bars and points represent mean $\pm$ SEM of ASMC from 3 healthy subjects. (A, B) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs unstimulated. (C) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs TNF- α alone.

3.2.1.4 Effect of TNF- α and IFN- γ on CX3CL1 release

To investigate the regulatory effect of TNF- α and IFN- γ on CX3CL1 release, ASMC of healthy subjects were stimulated with TNF- α (1, 3, 10, 30, 100 ng/mL) or IFN- γ (1, 10, 100 ng/mL) alone or in combination. Supernatants were collected after 24 hours, and CX3CL1 measured by ELISA. In contrast to the effect seen in terms of regulation of CCL11, CXCL8 and CXCL10 release, neither TNF- α nor IFN- γ induced CX3CL1 (Fig. 3.4A and B). However, under combination of TNF- α and IFN- γ there was a concentration-dependent increase in CX3CL1 release, with a maximum of 1508 ± 357 pg/mL induced by TNF- α and IFN- γ at 10 ng/mL each (Fig. 3.4C; $p < 0.01$ vs unstimulated), suggesting that TNF- α and IFN- γ synergistically induce CX3CL1 release.

This synergy was better displayed by a concentration response curve. CX3CL1 was not induced by TNF- α alone. In the presence of IFN- γ , TNF- α induced CX3CL1 release in a concentration-dependent manner; the synergistic effect of IFN- γ was also concentration-dependent (Fig. 3.4D; $p < 0.05$ and $p < 0.01$ at IFN- γ 10 ng/mL and 100 ng/mL, respectively).

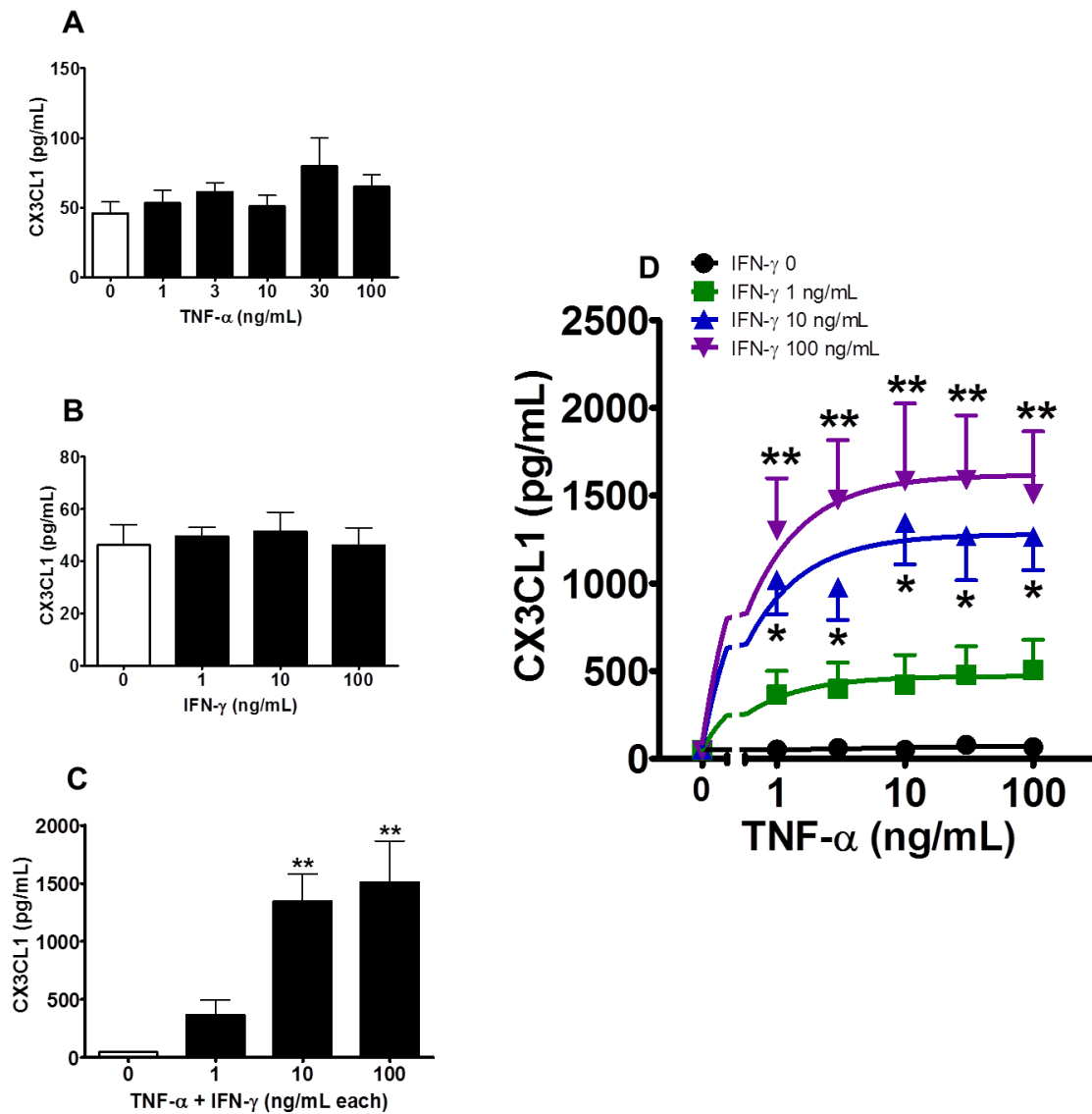


Figure 3.4. Effect of TNF-α and IFN-γ on CX3CL1 release in ASMC. Cells were stimulated with (A) TNF-α (1-100 ng/mL), (B) IFN-γ (1-100 ng/mL), or (C, D) both, for 24 hours. CX3CL1 release was measured by ELISA. Bars and points represent mean $\pm$ SEM of ASMC from 3 healthy subjects. (C) ** $p < 0.01$ vs unstimulated. (D) * $p < 0.05$, ** $p < 0.01$ vs TNF-α alone.

3.2.1.5 Effect of IFN- γ on TNF α -induced CCL11 in ASMC of healthy subjects and patients with non-severe and severe asthma

Having shown that IFN- γ inhibits TNF α -induced CCL11 (3.3.1.1), an investigation was undertaken to determine whether this suppressive effect occurs at the level of mRNA and in ASMC of patients with asthma. ASMC were stimulated with TNF- α (10 ng/mL) or IFN- γ (10 ng/mL) alone or in combination for 24 hours. CCL11 release in supernatants and mRNA extracted from cell lysates were measured by ELISA and RT-qPCR, respectively. TNF α induced CCL11 release was suppressed by over 50% by IFN- γ . This effect was observed in ASMC of healthy subjects (Fig. 3.5A) as well as patients with non-severe (Fig. 3.5B) and severe asthma (Fig. 3.5C). RT-qPCR revealed a more pronounced suppressive effect of IFN- γ on TNF α -induced CCL11 mRNA, in ASMC, by over 80%, of healthy subjects (Fig. 3.6A) and patients with non-severe (Fig. 3.6B) and severe asthma (Fig. 3.6C).

3.2.1.6 Effect of IFN- γ on TNF α -induced CXCL8 in ASMC of healthy subjects and patients with non-severe and severe asthma

ASMC were stimulated with TNF- α (10 ng/mL) or IFN- γ (10 ng/mL) alone or in combination for 24 hours. CXCL8 release in supernatants and mRNA extracted from cell lysates were measured by ELISA and RT-qPCR, respectively. CXCL8 release induced by TNF- α was not altered by IFN- γ at 10 ng/mL. This effect was consistent in ASMC of the healthy (Fig. 3.7A) and patients with non-severe (Fig. 3.7B) and severe asthma (Fig. 3.7C). RT-qPCR also revealed that induced CXCL8 mRNA was not influenced by IFN- γ in ASMC of healthy subjects (Fig. 3.8A) and patients with non-severe (Fig. 3.8B) and severe asthma (Fig. 3.8C).

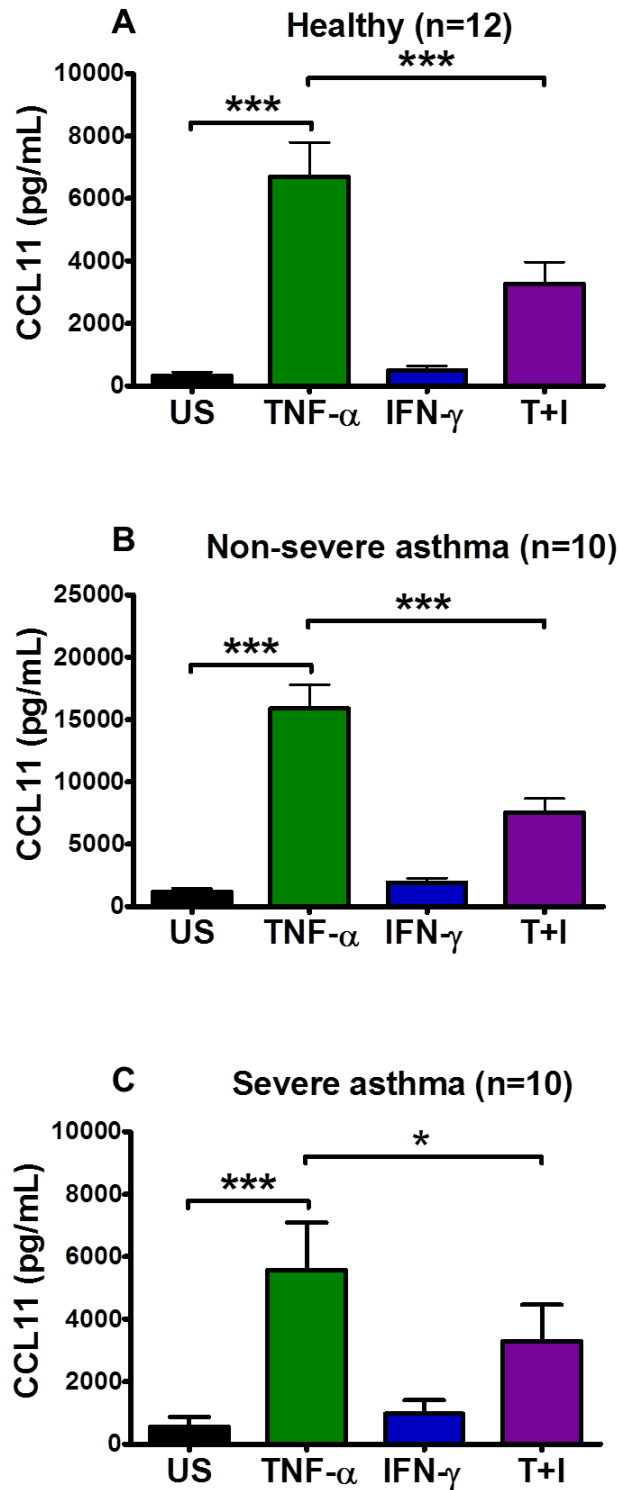


Figure 3.5. Effect of IFN- γ on TNF α -induced CCL11 release in ASMC of healthy subjects and patients with non-severe and severe asthma. Cells were stimulated with TNF- α (10 ng/mL) and/or IFN- γ (10 ng/mL) for 24 hours. CCL11 release from ASMC of the (A) healthy and patients with (B) non-severe and (C) severe asthma was measured by ELISA. Bars represent mean $\pm$ SEM. * $p < 0.05$, *** $p < 0.001$.

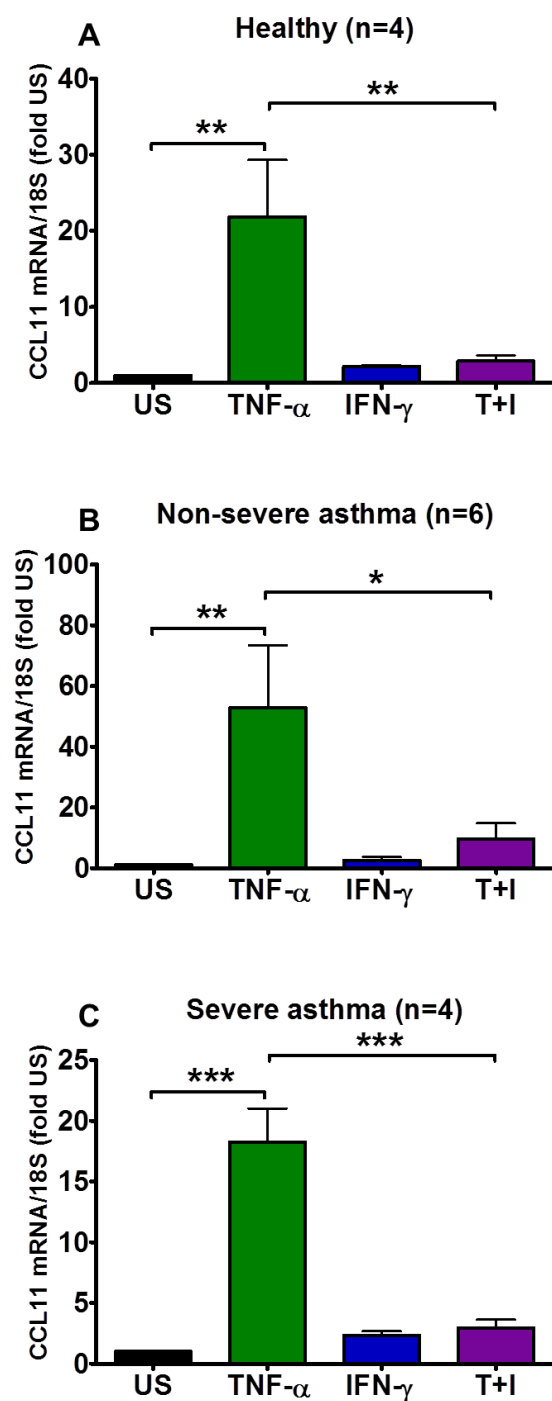


Figure 3.6. Effect of IFN- γ on TNF α -induced CCL11 mRNA expression in ASMC of healthy subjects and patients with non-severe and severe asthma. Cells were stimulated with TNF- α (10 ng/mL) and/or IFN- γ (10 ng/mL) for 24 hours. CCL11 mRNA and 18S from ASMC of the (A) healthy subjects and patients with (B) non-severe and (C) severe asthma were measured by RT-qPCR. Bars represent mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

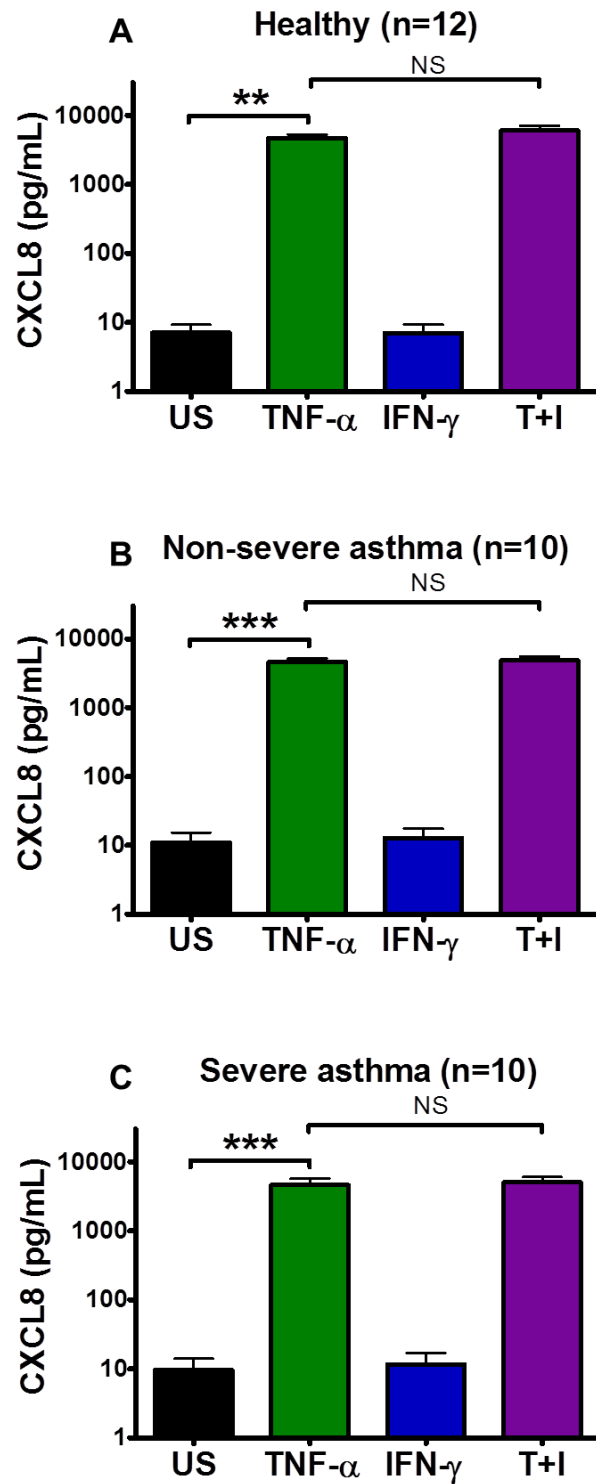


Figure 3.7. Effect of IFN- γ on TNF α -induced CXCL8 release in ASMC of healthy subjects and patients with non-severe and severe asthma. Cells were stimulated with TNF- α (10 ng/mL) and/or IFN- γ (10 ng/mL) for 24 hours. CXCL8 release from ASMC of the (A) healthy and patients with (B) non-severe and (C) severe asthma was measured by ELISA. Bars represent mean $\pm$ SEM. ** $p < 0.01$, *** $p < 0.001$.

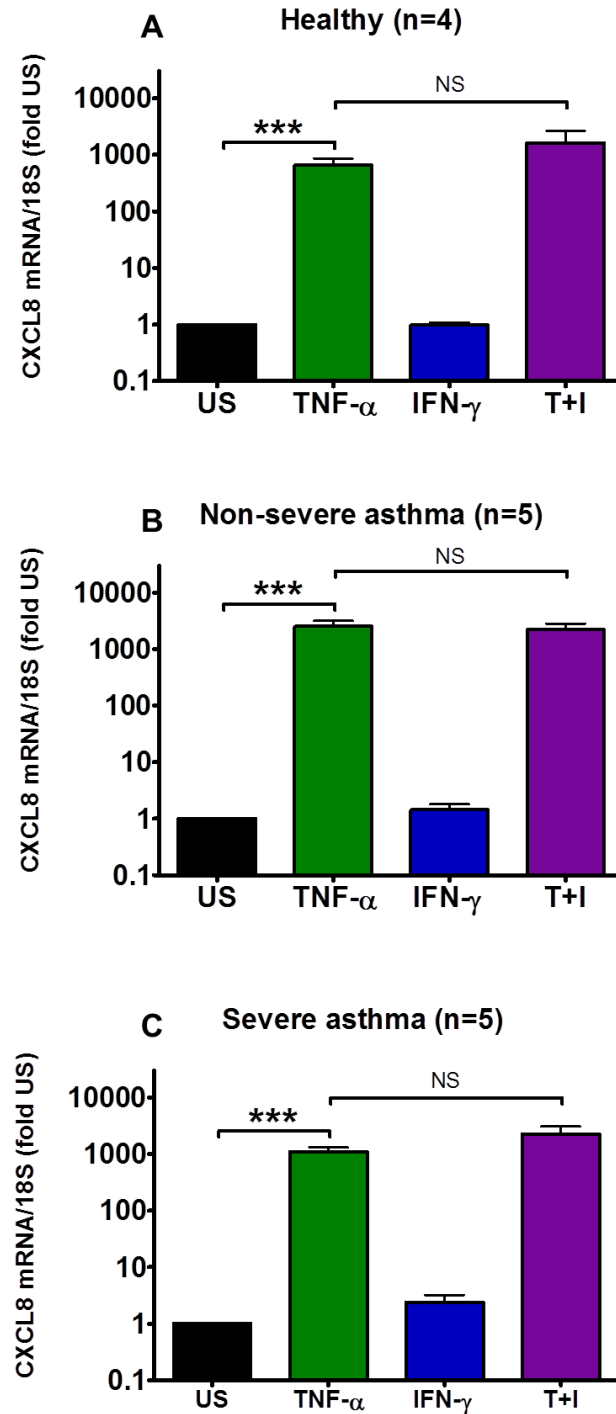


Figure 3.8. Effect of IFN- γ on TNF α -induced CXCL8 mRNA expression in ASMC of healthy subjects and patients with non-severe and severe asthma. Cells were stimulated with TNF- α (10 ng/mL) and/or IFN- γ (10 ng/mL) for 24 hours. CXCL8 mRNA and 18S from ASMC of the (A) healthy and patients with (B) non-severe and (C) severe asthma were measured by RT-qPCR. Bars represent mean $\pm$ SEM. *** $p < 0.001$.

3.2.1.7 Effect of IFN- γ on TNF α -induced CXCL10 in ASMC of the healthy subjects and patients with non-severe and severe asthma

Having shown that either TNF- α or IFN- γ alone induces CXCL10 release in ASMC of healthy subjects, and that the combination has a synergistic effect (3.3.1.3), an investigation was undertaken to determine whether this effect is present in ASMC of patients with asthma. Cells were stimulated with TNF- α (10 ng/mL) or IFN- γ (10 ng/mL) alone or in combination. Supernatants were collected after 24 hours, and CXCL10 measured by ELISA. TNF- α and IFN- γ , to a lesser extent, induced CXCL10 release, while in combination there was nearly 10-fold greater induction compared to TNF- α alone. This effect was observed in ASMC of healthy subjects (Fig. 3.9A) and patients with non-severe (Fig. 3.9B) and severe asthma (Fig. 3.9C).

3.2.1.8 Effect of TNF- α and IFN- γ on CX3CL1 in ASMC of the healthy subjects and patients with non-severe and severe asthma

Having shown that TNF- α and IFN- γ synergistically induce CX3CL1 release in ASMC of healthy subjects, an investigation was undertaken to determine whether this effect also occurs at the mRNA level and in ASMC of asthmatic subjects. Cells were stimulated with TNF- α (10 ng/mL) or IFN- γ (10 ng/mL) alone or in combination for 24 hours. CX3CL1 release in supernatants and mRNA extracted from cell lysates were measured by ELISA and RT-qPCR, respectively. Neither TNF- α nor IFN- γ alone significantly induced CX3CL1 release. The induction was only seen with a combination of these cytokines. This effect was consistent in ASMC of healthy subjects (Fig. 3.10A) and patients with non-severe (Fig. 3.10B) and severe asthma (Fig. 3.10C). RT-qPCR revealed a similar effect in ASMC of healthy subjects (Fig. 3.11A) and patients with non-severe (Fig. 3.11B) and severe asthma (Fig. 3.11C).

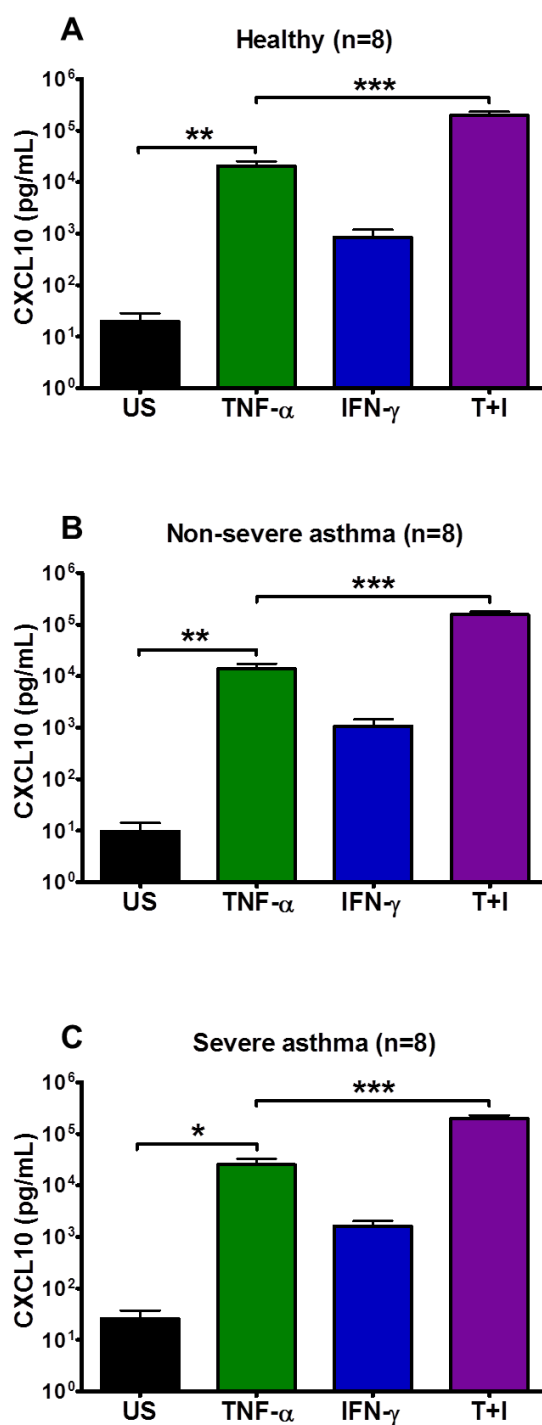


Figure 3.9. Effect of IFN- γ on TNF α -induced CXCL10 release in ASMC of healthy subjects and patients with non-severe and severe asthma. Cells were stimulated with TNF- α (10 ng/mL) and/or IFN- γ (10 ng/mL) for 24 hours. CXCL10 release from ASMC of the (A) healthy and patients with (B) non-severe and (C) severe asthma was measured by ELISA. Bars represent mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$ vs unstimulated.

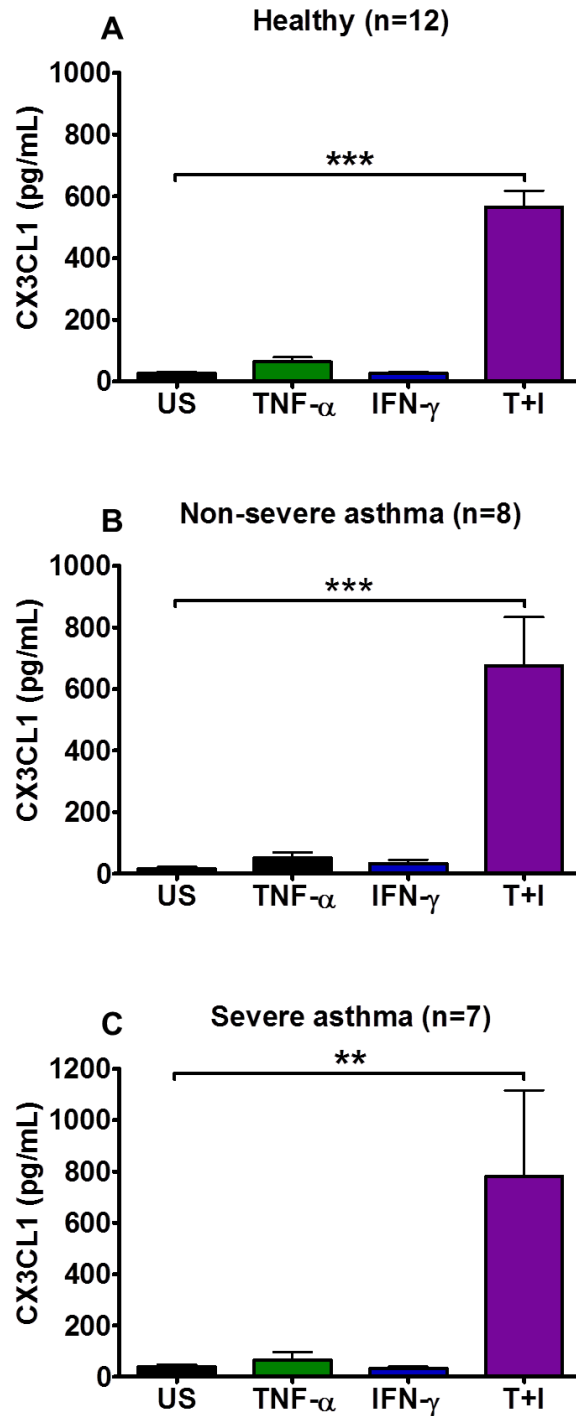


Figure 3.10. Effect of TNF- α and IFN- γ on CX3CL1 release in ASMC of healthy subjects and patients with non-severe and severe asthma. Cells were stimulated with TNF- α (10 ng/mL) and/or IFN- γ (10 ng/mL) for 24 hours. CX3CL1 release from ASMC of the (A) healthy and patients with (B) non-severe and (C) severe asthma was measured by ELISA. Bars represent mean $\pm$ SEM. ** $p < 0.01$, *** $p < 0.001$.

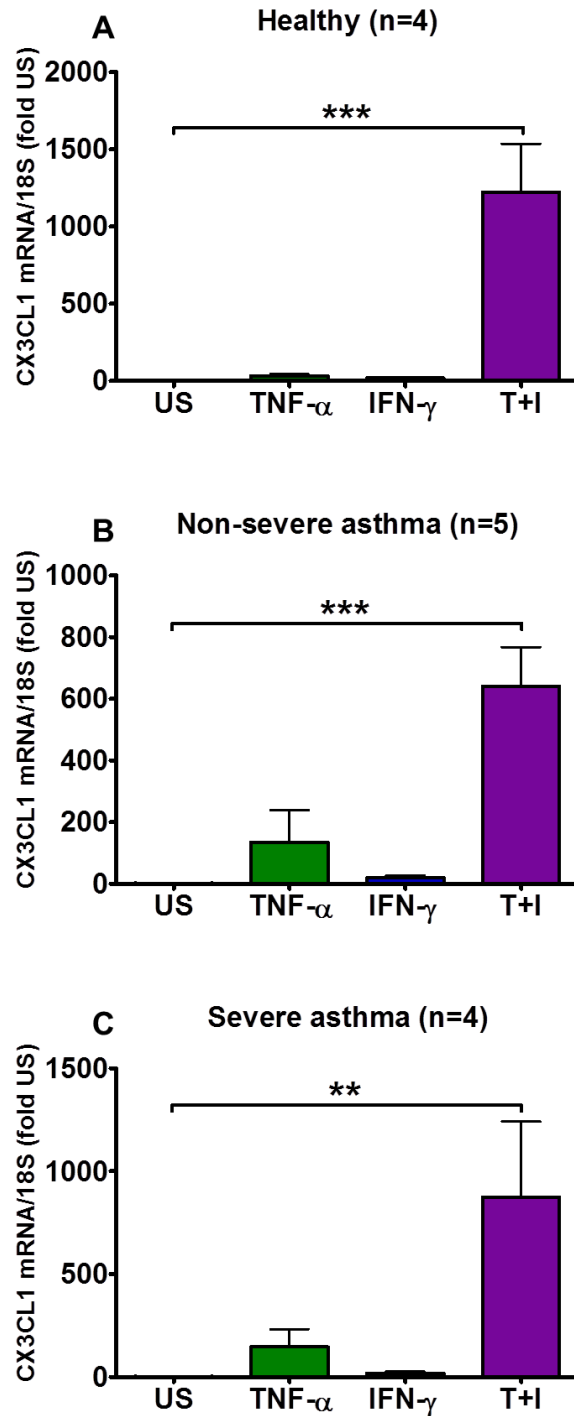


Figure 3.11. Effect of TNF- α and IFN- γ on CX3CL1 mRNA expression in ASMC of healthy subjects and patients with non-severe and severe asthma. Cells were stimulated with TNF- α (10 ng/mL) and/or IFN- γ (10 ng/mL) for 24 hours. CX3CL1 mRNA and 18S from ASMC of the (A) healthy (A) and patients with (B) non-severe and (C) severe asthma were measured by RT-qPCR. Bars represent mean $\pm$ SEM. ** $p < 0.01$, *** $p < 0.001$.

3.2.1.9 Effect of TNF- α on recruitment of NF- κ B (p65) to the promoter of CCL11 in ASMC

As TNF α -induced CCL11 expression is NF κ B-dependent, which includes recruitment of the p65 component of NF- κ B to the gene promoter (described in 3.1), a time-course effect of TNF- α on p65 recruitment was examined before comparison between groups was undertaken. ASMC of healthy subjects were stimulated with TNF- α (10 ng/mL) for 30-240 minutes. p65 binding to the promoter of CCL11 was measured by ChIP assay. TNF- α induced recruitment of p65 to the CCL11 promoter in a time-dependent manner, with a maximal 13-fold increase over baseline occurring at 60 minutes, which then decreased over time (Fig. 3.12).

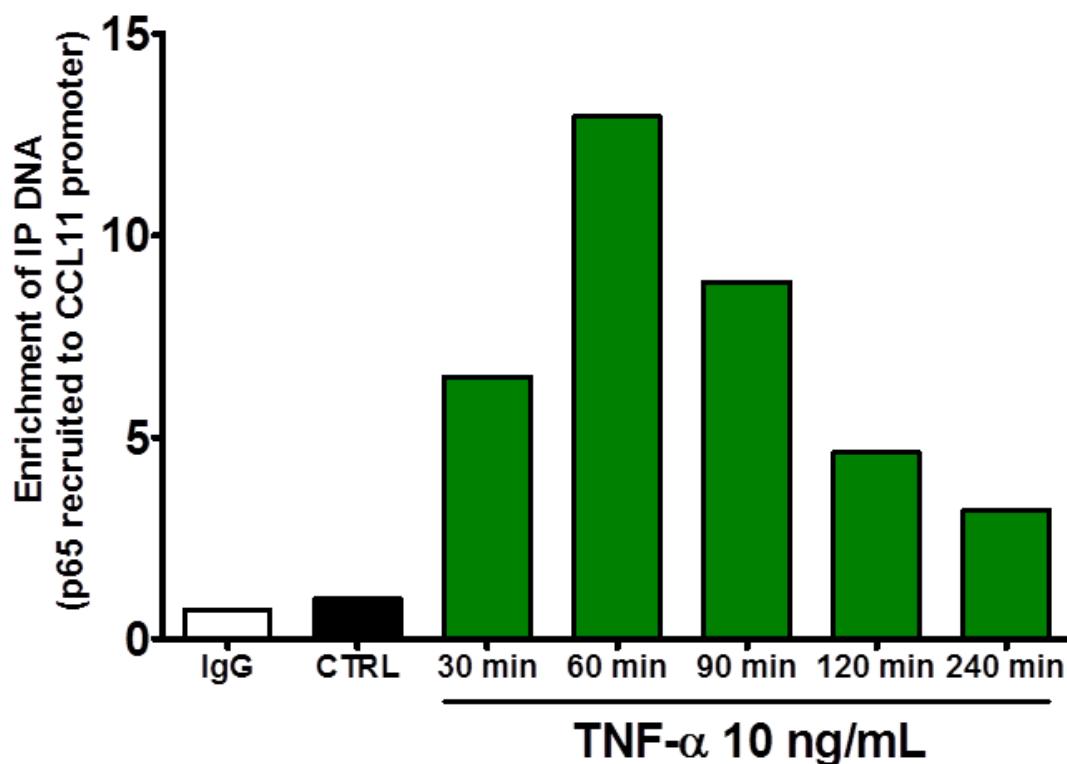


Figure 3.12. Effect TNF- α on p65 NF- κ B recruitment to the CCL11 promoter in ASMC. Cells were stimulated with TNF- α (10 ng/mL) for time indicated. p65 recruitment to CCL11 promoter was measured by ChIP assay. Bars represent mean of ASMC from 2 healthy subjects.

3.2.1.10 Effect of TNF- α on recruitment of NF- κ B (p65) to the promoter of CXCL8 in ASMC

As TNF α -induced CXCL8 expression is NF κ B-dependent through recruitment of the p65 to the gene promoter (described in 3.1), a time-course effect of TNF- α on p65 recruitment to the CXCL8 promoter was examined before comparison between groups was undertaken. ASMC of healthy subjects were stimulated with TNF- α (10 ng/mL) for 30-240 minutes. p65 binding to the promoter of CXCL8 was measured by ChIP assay. TNF- α induced p65 recruitment to the CXCL8 promoter in a time-dependent manner, with a maximal 6.2-fold increase over baseline occurring at 60 minutes, which then decreased over time (Fig. 3.13).

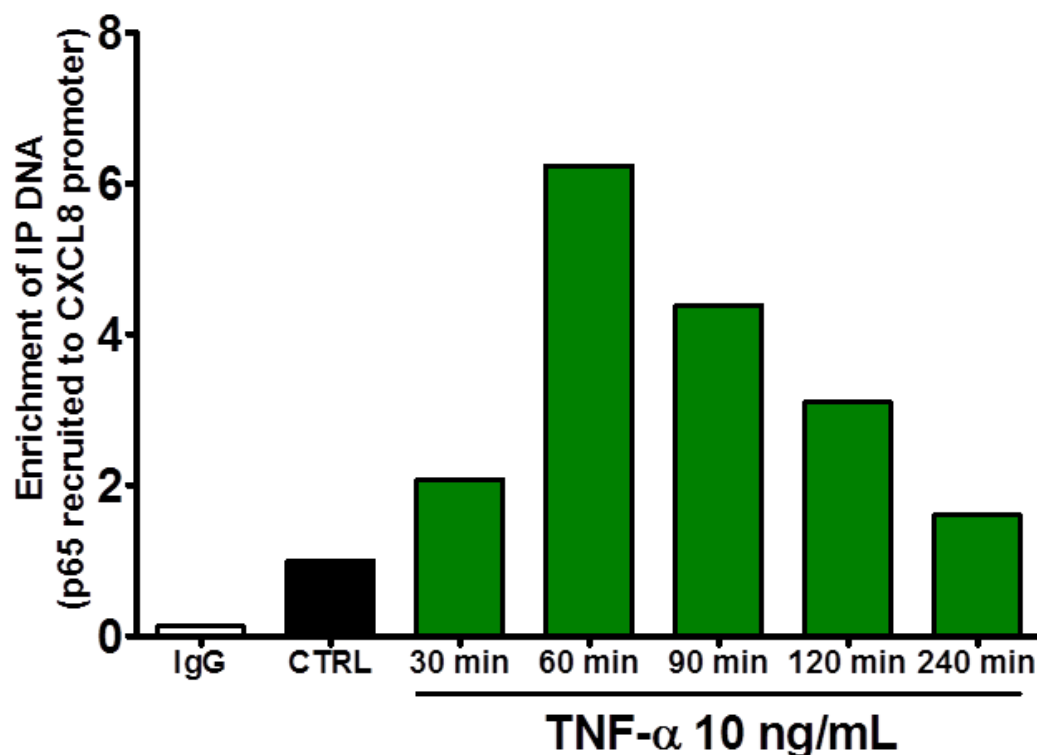


Figure 3.13. Effect TNF- α on p65 NF- κ B recruitment to the CXCL8 promoter in ASMC. Cells were stimulated with TNF- α (10 ng/mL) for time indicated. p65 recruitment to CXCL8 promoter was measured by ChIP assay. Bars represent mean of ASMC from 2 healthy subjects.

3.2.1.11 Effect of TNF- α and IFN- γ on recruitment of NF- κ B (p65) to the promoter of CX3CL1 in ASMC

To investigate the effect of TNF- α and IFN- γ on recruitment of p65 to the CX3CL1 promoter, ASMC of healthy subjects were stimulated with TNF- α alone or in combination with IFN- γ (10 ng/mL each) for 30-120 minutes. p65 binding to the promoters of CX3CL1 was measured by ChIP assay. TNF- α induced p65 recruitment to the CX3CL1 promoter in a time-dependent manner, with a maximal increase of 3.4-fold increase over baseline occurring at 60 minutes, which then decreased over time. IFN- γ did not modulate p65 recruitment induced by TNF- α (Fig. 3.14).

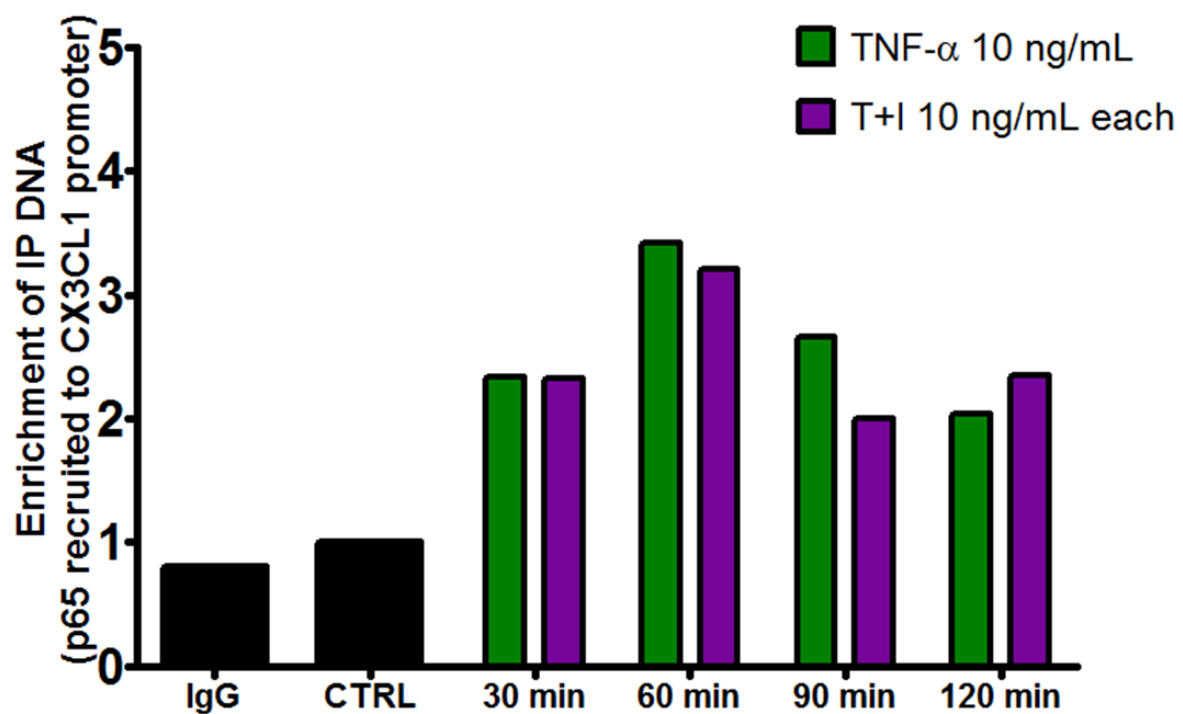


Figure 3.14. Effect TNF- α and IFN- γ on p65 NF- κ B recruitment to the CX3CL1 promoter in ASMC. Cells were stimulated with TNF- α (10 ng/mL) or combined TNF- α and IFN- γ (10 ng/mL each) for indicated time. p65 recruitment to the CX3CL1 promoter was measured by ChIP assay. Bars represent mean of ASMC from 2 healthy subjects.

3.2.1.12 Effect of IFN- γ on TNF α -induced p65 recruitment to the CCL11 promoter in ASMC of healthy subjects and patients and non-severe and severe asthma

Given the observation that IFN- γ inhibited CCL11 release and mRNA expression induced by TNF- α (3.3.1.1 and 3.3.1.5), an investigation into the effect of IFN- γ on TNF α -induced p65 recruitment to the CCL11 promoter was undertaken. ASMC were stimulated with TNF- α (10 ng/mL) or IFN- γ (10 ng/mL) alone or in combination for one hour. p65 recruitment to the CCL11 promoter was measured by ChIP assay. TNF- α , but not IFN- γ , induced p65 recruitment to the CCL11 promoter, and this induction was partially suppressed by IFN- γ . These effects were consistent in ASMC of the healthy (Fig. 3.15A) as well as patients with non-severe (Fig. 3.15B) and severe asthma (Fig. 3.15C).

3.2.1.13 Effect of IFN- γ on TNF α -induced p65 recruitment to the CXCL8 promoter in ASMC of healthy subjects and patients and non-severe and severe asthma

Given the observation that IFN- γ inhibited CXCL8 release induced by TNF- α (3.3.1.2), the effect of IFN- γ on TNF α -induced p65 recruitment to the CXCL8 promoter was investigated. ASMC were stimulated with TNF- α (10 ng/mL) or IFN- γ (10 ng/mL) alone or in combination for one hour. p65 recruitment to the CXCL8 promoter was measured by ChIP assay. TNF- α but not IFN- γ induced p65 recruitment to the CXCL8 promoter, and this induction was partially inhibited by IFN- γ . These effects were consistent in ASMC of the healthy (Fig. 3.16A) as well as patients with non-severe (Fig. 3.16B) and severe asthma (Fig. 3.16C).

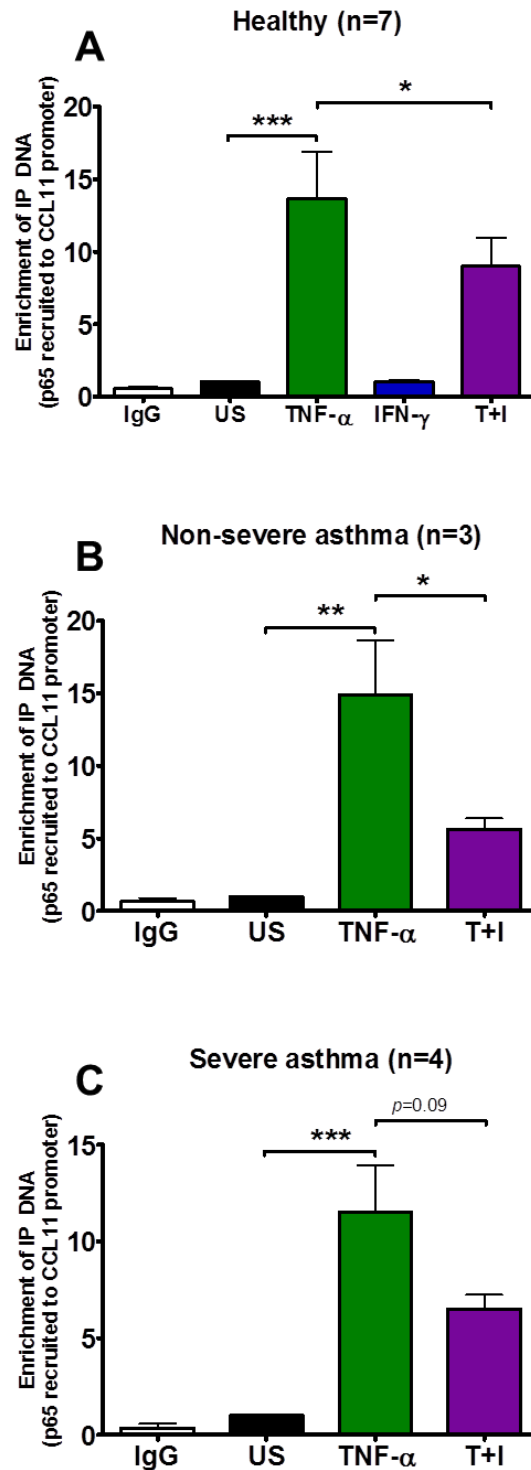


Figure 3.15. Effect of IFN- γ on TNF α -induced p65 recruitment to the CCL11 promoter in ASMC. Cells were stimulated with TNF- α (10 ng/mL), IFN- γ (10 ng/mL), or both for one hour. p65 recruitment to CCL11 promoter in ASMC of the (A) healthy and patient with (B) non-severe and (C) severe asthma was measured by ChIP assay. Bars represent mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

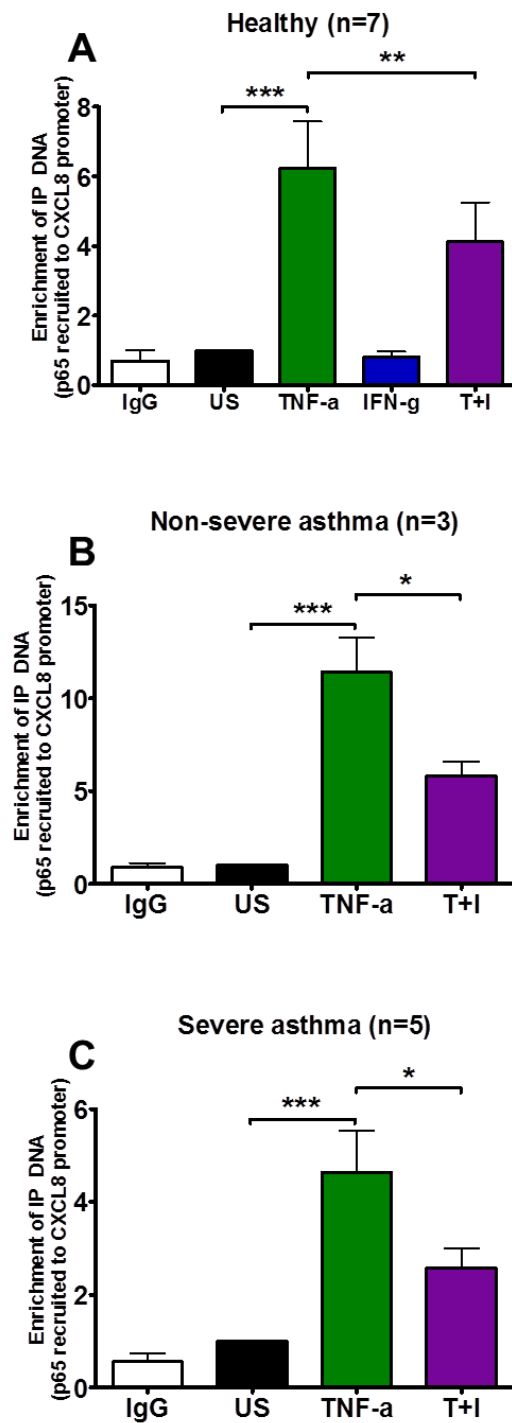


Figure 3.16. Effect of IFN- γ on TNF α -induced p65 recruitment to the CXCL8 promoter in ASMC. Cells were stimulated with TNF- α (10 ng/mL), IFN- γ (10 ng/mL), or both for one hour. p65 recruitment to the CXCL8 promoter in ASMC of the (A) healthy and patients with (B) non-severe and (C) severe asthma was measured by ChIP assay. Bars represent mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.2.1.14 Effect of IFN- γ on TNF α -induced p65 recruitment to CX3CL1 promoter in ASMC of healthy subjects and patients and non-severe and severe asthma

Given the observation that TNF- α and IFN- γ synergistically induced CX3CL1 release and mRNA expression, the effect of IFN- γ on TNF α -induced p65 recruitment to the CX3CL1 promoter was investigated. ASMC were stimulated with TNF- α (10 ng/mL), IFN- γ (10 ng/mL), alone or in combination, for one hour. p65 recruitment to the CX3CL1 promoter was measured by ChIP assay. TNF- α , but not IFN- γ , induced p65 recruitment to the CX3CL1 promoter, and IFN- γ did not modulate TNF α -induced p65 recruitment. These effects were consistent in ASMC of the healthy (Fig. 3.17A) as well as patients with non-severe (Fig. 3.17B) and severe asthma (Fig. 3.17C).

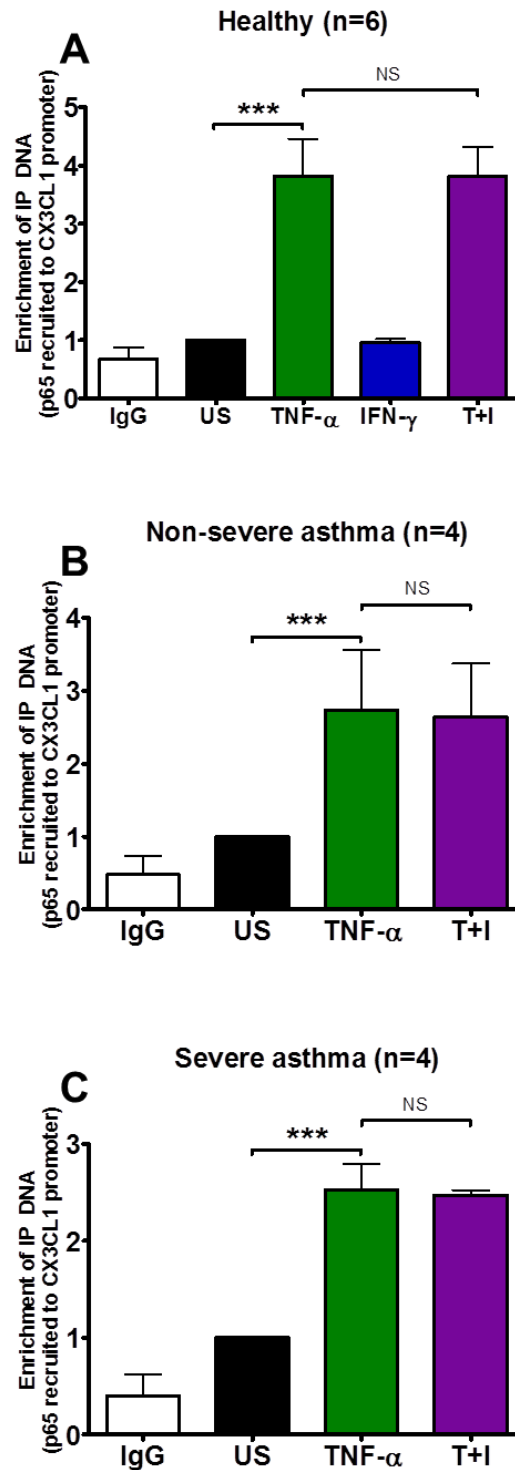


Figure 3.17. Effect of IFN- γ on TNF α -induced p65 recruitment to the CX3CL1 promoter in ASMC. Cells were stimulated with TNF- α (10 ng/mL), IFN- γ (10 ng/mL), or both for one hour. p65 recruitment to the CX3CL1 promoter in ASMC of the healthy (A) and patients with non-severe (B) and severe asthma (C) was measured by ChIP assay. Bars represent mean $\pm$ SEM. *** $p < 0.001$.

3.2.2 Comparison of cytokine-induced chemokine release and mRNA expression in ASMC of healthy subjects and patients with non-severe and severe asthma

3.2.2.1 Comparison of TNF α -induced CCL11 in ASMC of healthy subjects and patients with non-severe and severe asthma

Baseline and induced CCL11 release and mRNA expression from ASMC of the healthy and patients with non-severe and severe asthma were compared. Cells were stimulated with TNF- α (10 ng/mL) for 24 hours. CCL11 release and mRNA expression was measured by ELISA and RT-qPCR, respectively. Baseline CCL11 release from ASMC of patients with non-severe asthma was 4.4-fold greater than that of healthy subjects ($p<0.01$) and 6.6-fold greater than that of patients with severe asthma ($p<0.01$). In addition, TNF α -induced CCL11 release from ASMC of patients with non-severe asthma was 2.5-fold greater than that of healthy subjects ($p<0.01$) and 3.1-fold greater than that of patients with severe asthma. There was no significant difference in either baseline or induced CCL11 release between the healthy and severe asthmatics (Fig.18A).

RT-qPCR revealed a similar effect: both baseline and induced CCL11 mRNA expression from ASMC of non-severe asthma was greater than that of healthy subjects and patients with severe asthma. No significant difference in either baseline or induced CCL11 mRNA was observed between the healthy and patients with severe asthma (Fig.18B).

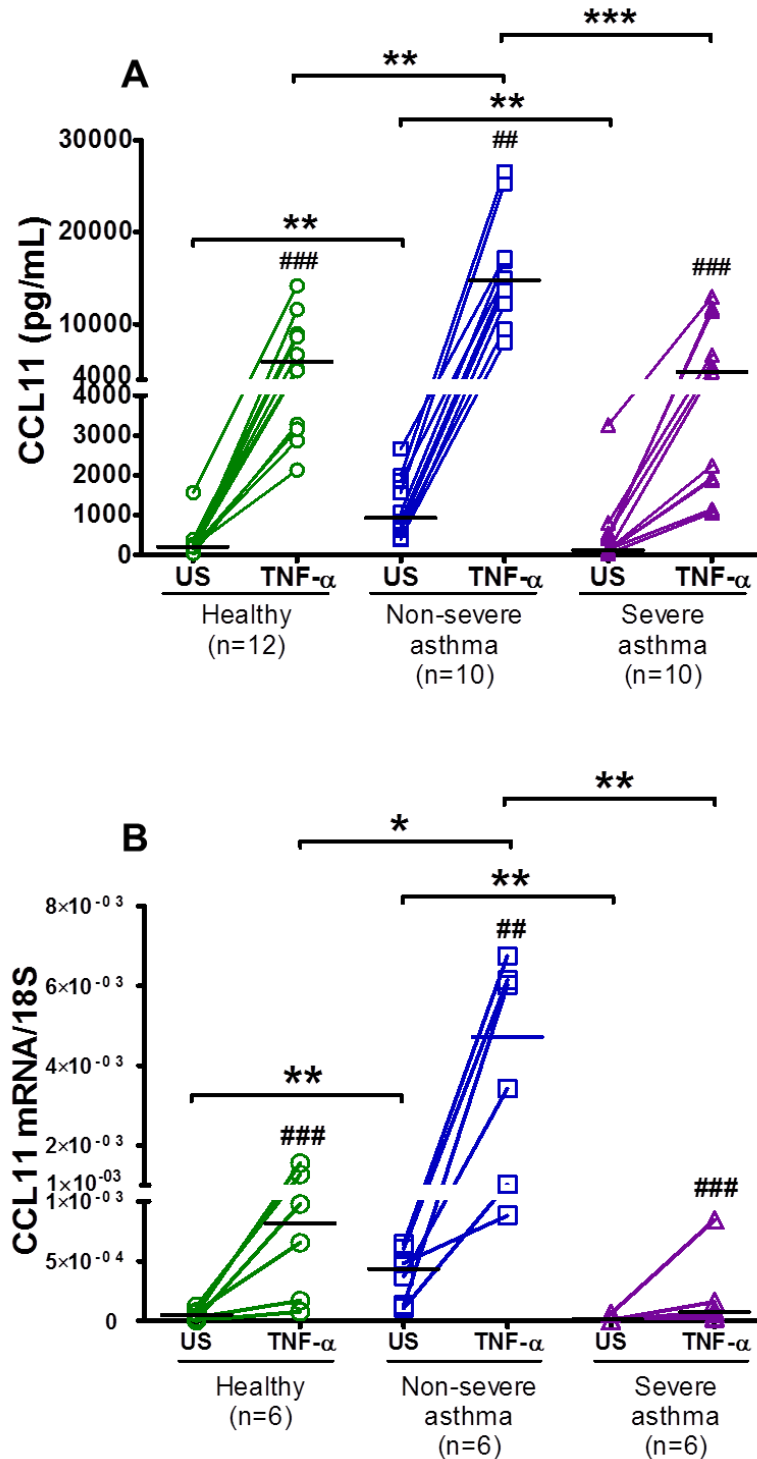


Figure 3.18. Comparison of TNF α -induced CCL11 in ASMC of healthy subjects and patients with non-severe and severe asthma. ASMC were stimulated with TNF- α (10 ng/mL) for 24 hours. CCL11 (A) release and (B) mRNA expression were measured by ELISA and RT-qPCR, respectively. Horizontal lines represent median. ## $p < 0.01$, ### $p < 0.001$ vs unstimulated (US). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.01$.

3.2.2.2 Comparison of TNF α -induced CXCL8 in ASMC of healthy subjects and patients with non-severe and severe asthma

Baseline and induced CXCL8 release and mRNA expression from ASMC of the healthy and patients with non-severe and severe asthma were compared. Cells were stimulated with TNF- α (10 ng/mL) for 24 hours. CXCL8 release and mRNA expression was measured by ELISA and RT-qPCR, respectively. While CXCL8 was induced by TNF- α in ASMC, neither baseline nor induced CXCL8 release differed between three groups (Fig. 3.19A). RT-qPCR revealed a similar effect, with no significant difference of baseline or induced CXCL8 mRNA between all groups (Fig. 3.18B).

3.2.2.3 Comparison of cytokine-induced CXCL10 release in ASMC of healthy subjects and patients with non-severe and severe asthma

Baseline and cytokine-induced CXCL10 release and mRNA expression from ASMC of the healthy and patients with non-severe and severe asthma were compared. Cells were stimulated with TNF- α (10 ng/mL) or IFN- γ (10 ng/mL) alone or the combination (10 ng/mL) for 24 hours. CXCL10 release was measured by ELISA. The levels of baseline CXCL10 release were not significantly different between the groups. While CXCL10 was induced by either cytokine alone or in combination in all the three groups, IFN γ -induced CXCL10 was almost two-fold greater in ASMC of patients with severe asthma (1135.0 pg/mL) than those of both healthy subjects (556.3 pg/mL) and non-severe asthma (592.6 pg/mL) ($p < 0.05$, respectively) (Fig. 3.20B). In contrast, there was no difference of CXCL10 release induced by TNF- α alone or in combination with IFN- γ in ASMC between the groups (Fig. 3.20 A and C).

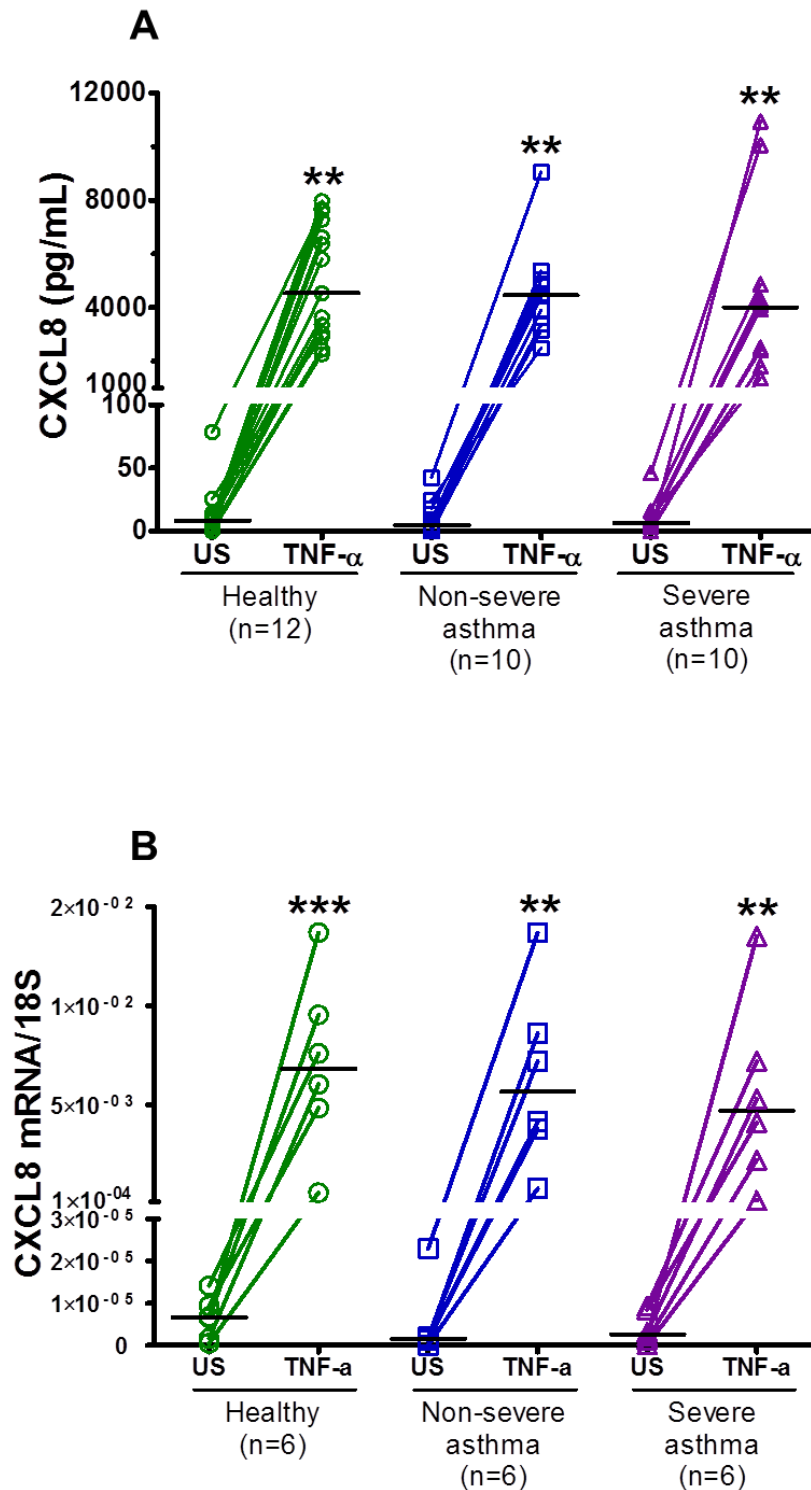


Figure 3.19. Comparison of TNF α -induced CXCL8 in ASMC of healthy subjects and patients with non-severe and severe asthma. ASMC were stimulated with TNF- α (10 ng/mL) for 24 hours. CXCL8 (A) release and (B) mRNA expression were measured by ELISA and RT-qPCR, respectively. Horizontal lines represent median. ** $p < 0.01$, *** $p < 0.001$ vs unstimulated.

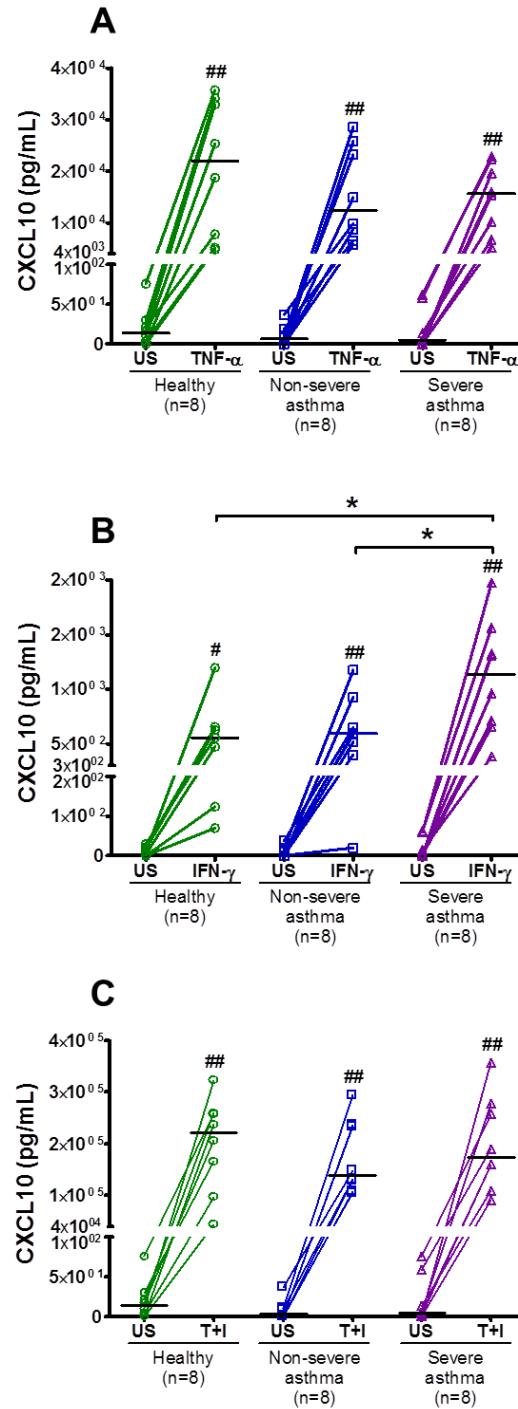


Figure 3.20. Comparison of cytokine-induced CXCL10 in ASMC of healthy subjects and patients with non-severe and severe asthma. ASMC were stimulated with (A) TNF- α (10 ng/mL), (B) IFN- γ (10 ng/mL) alone or (C) in combination for 24 hours. CXCL10 release was measured by ELISA. Horizontal lines represent median. # $p < 0.05$ ## $p < 0.01$ vs unstimulated. * $p < 0.05$.

3.2.2.4 Comparison of TNF α and IFN γ -induced CX3CL1 in ASMC of healthy subjects and patients with non-severe and severe asthma

Baseline and induced CX3CL1 release and mRNA expression from ASMC of the healthy and patients with non-severe and severe asthma were compared. Cells were stimulated with TNF- α and IFN- γ (10 ng/mL each) for 24 hours. CX3CL1 release and mRNA expression was measured by ELISA and RT-qPCR, respectively. While CX3CL1 was induced by TNF- α and IFN- γ in combination, neither baseline nor induced CX3CL1 release differed between three groups (Fig. 3.21A). q-RT PCR revealed a similar effect, with no significant difference of baseline or induced CX3CL1 mRNA between all groups (Fig. 3.21B).

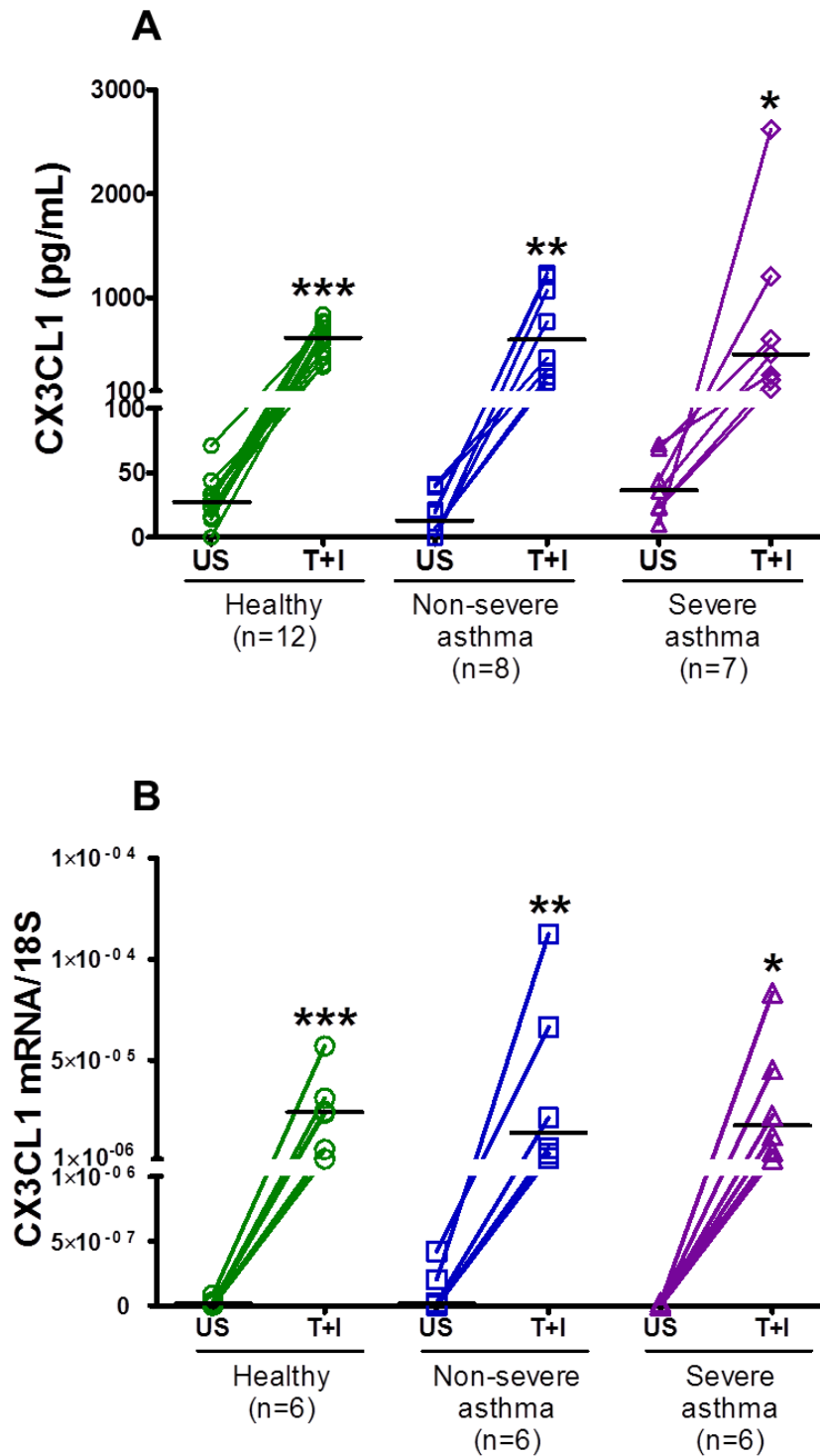


Figure 3.21. Comparison of TNF α and IFN γ -induced CX3CL1 in ASMC of healthy subjects and patients with non-severe and severe asthma. ASMC were stimulated with TNF- α and IFN- γ (10 ng/mL each) for 24 hours. CX3CL1 (A) release and (B) mRNA expression were measured by ELISA and RT-qPCR, respectively. Horizontal lines represent median. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs unstimulated.

3.2.3 Investigation and comparison of NF- κ B (p65) regulation in ASMC

3.2.3.1 Comparison of TNF α -induced p65 protein and mRNA expression in ASMC of the healthy subjects and patients with non-severe and severe asthma

To compare p65 expression in response to TNF- α , ASMC of healthy subjects and patients with non-severe and severe asthma were stimulated with TNF- α (10 ng/mL) for 24 hours. p65 protein in whole cell lysates and mRNA were measured by Western Blot and RT-qPCR, respectively. In ASMC of healthy subjects, TNF- α slightly, but consistently, induced p65 protein expression by approximately 1.2-fold, and there was a trend towards greater p65 induction in cells of patients with severe asthma (Fig. 3.22A). This difference was greater at the level of mRNA abundance: in ASMC of healthy subjects, p65 mRNA was induced by TNF- α by 2.3-fold, while there was a 5.6-fold induction in cells of patients with severe asthma (Fig. 3.22B; $p < 0.05$).

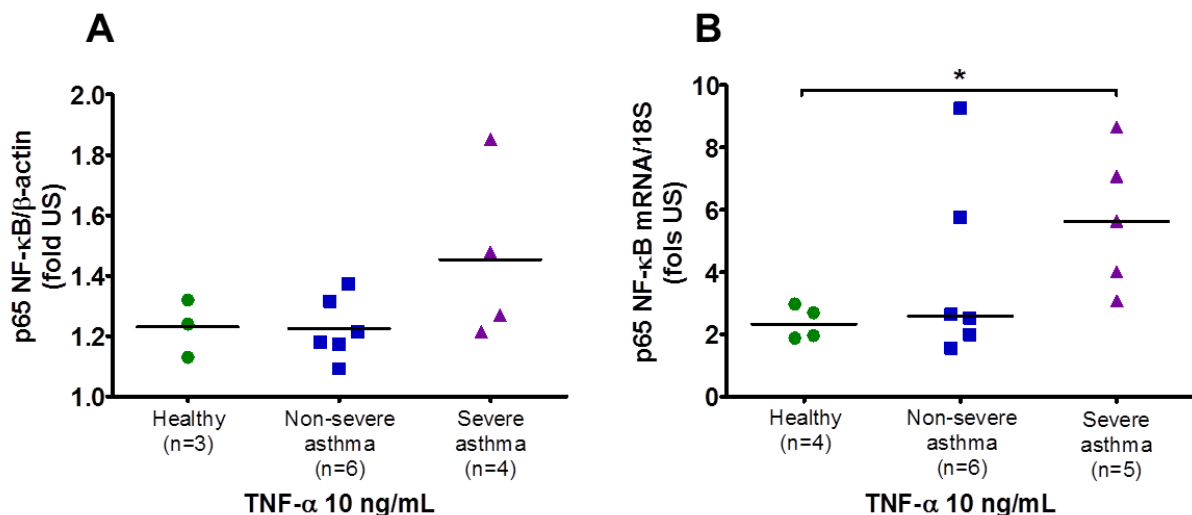


Figure 3.22. Comparison of induced p65 protein and mRNA expression in ASMC of healthy subjects and patients with non-severe and severe asthma. Cells were stimulated with TNF- α (10 ng/mL) for 24 hours. p65 (A) protein and (B) mRNA expression was measured by Western Blot and RT-qPCR, respectively. Horizontal lines represent median. * $p < 0.05$.

3.2.3.2 Effect of TNF- α on nuclear translocation of p65 in ASMC

To determine the effect of TNF- α on p65 nuclear translocation in ASMC, cells of healthy subjects were stimulated with TNF- α (10 ng/mL) over a time course (30 minutes to 2 hours). p65, TBP (TATA box-binding protein) and α -tubulin in the both nuclear and cytoplasmic extracts were measured by Western Blot. TNF- α increased nuclear abundance of p65 at 30 minutes, which was maintained at one hour and decreased slightly at 2 hour post-stimulation (Fig. 3.23A). In contrast, cytoplasmic p65 revealed a reciprocal change (Fig. 3.23B). These results confirm that TNF- α induces p65 nuclear translocation in ASMC.

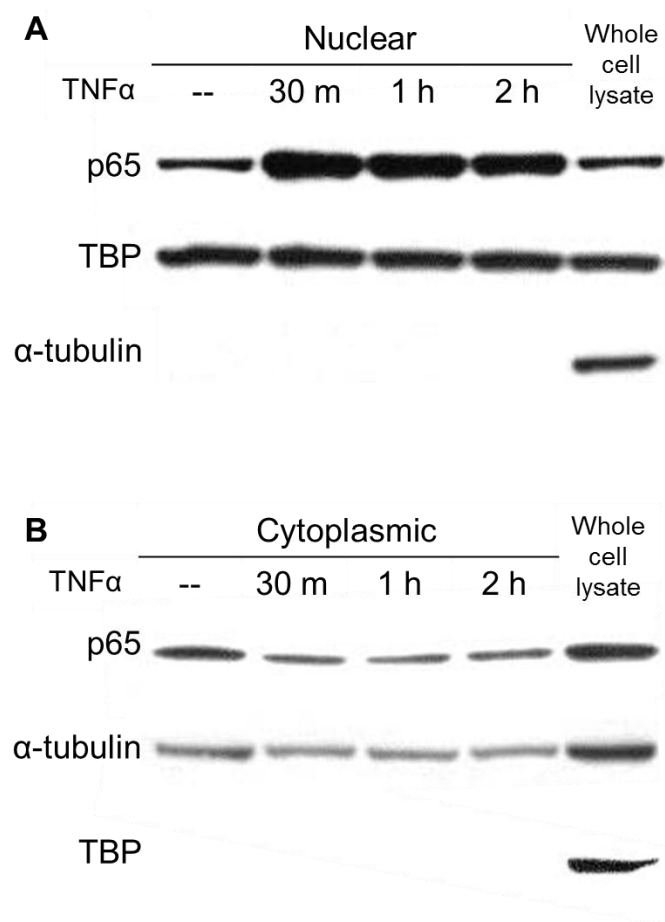


Figure 3.23. Effect of TNF- α on p65 NF- κ B nuclear translocation in ASMC. Cells were stimulated with TNF- α (10 ng/mL) for time indicated. p65 and TBP in (A) nuclear and (B) cytoplasmic extracts were measured by Western Blot. A representative blot of ASMC from 2 healthy subjects is shown.

3.2.3.3 Comparison of baseline nuclear p65 abundance in ASMC of healthy subjects and patients with non-severe and severe asthma

Baseline nuclear abundance of p65 in ASMC between the three groups was compared. After serum starvation for 24 hours, nuclear extracts were collected. p65 and TBP were measured by Western Blot and normalised to the standard protein (described in 2.2.7.1). The quantity of nuclear p65 was consistent between three groups (Fig. 3.24).

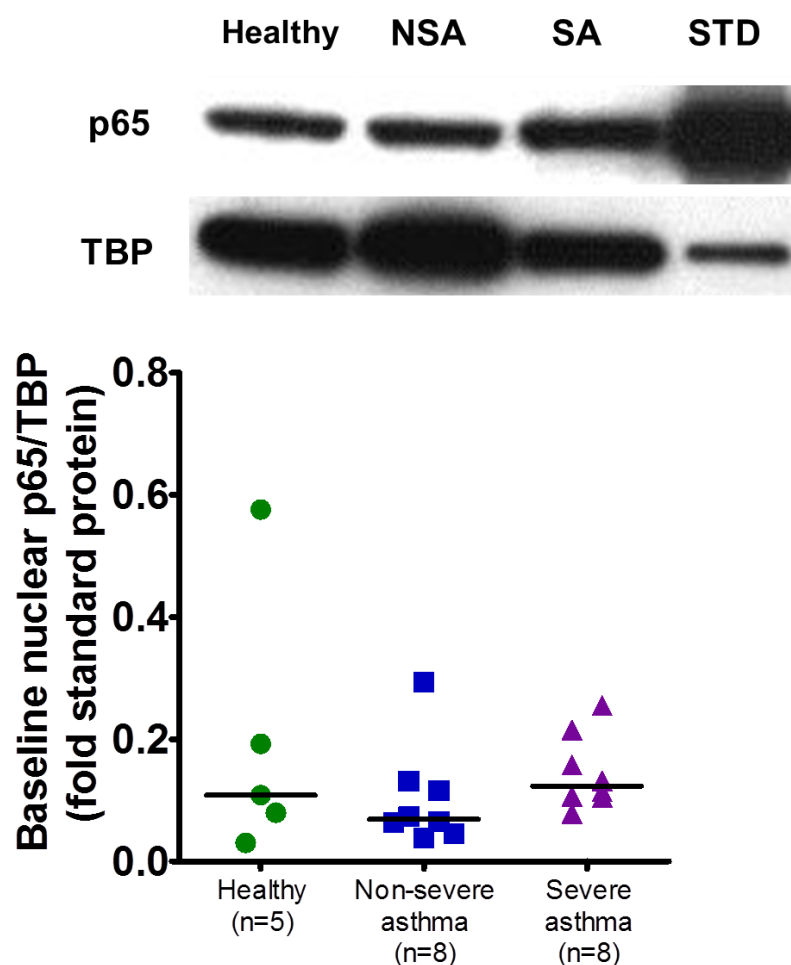


Figure 3.24. Comparison of baseline abundance of nuclear p65 in ASMC of healthy subjects and patients with non-severe (NSA) and severe asthma (SA). Cells were serum starved for 24 hours. p65 and TBP in nuclear extracts were measured by Western Blot. A representative is shown. Horizontal lines represent median. STD: standard protein.

3.2.3.4 Comparison of nuclear translocation of p65 induced by TNF- α in ASMC of healthy subjects and patients with non-severe and severe asthma

To compare nuclear translocation of p65 induced by TNF- α in ASMC, cells were stimulated by TNF- α (10 ng/mL) over a time course (30 minutes to 2 hours). p65 and TBP in the nuclear extracts were measured by Western Blot. In ASMC of healthy subjects, nuclear abundance of p65 was increased by TNF- α compared to baseline by approximately 2.5-fold after 30 minutes, sustained at one hour, and decreased slightly at 2 hours post-stimulation. This effect was similar in ASMC of patients with non-severe and severe asthma (Fig. 3.25).

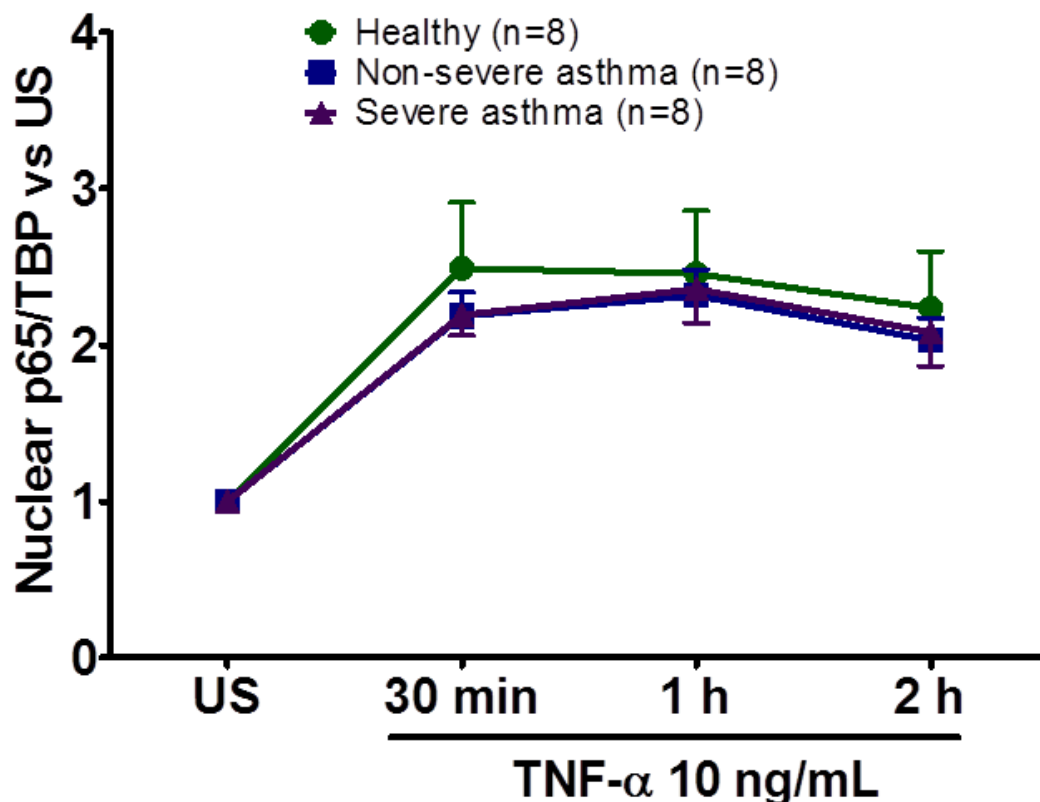


Figure 3.25. Comparison of TNF α -induced p65 nuclear translocation in ASMC of healthy subjects and patients with non-severe and severe asthma. Cells were stimulated with TNF- α (10 ng/mL) for time indicated. p65 and TBP in the nuclear extracts were measured by Western Blot. Points represent mean $\pm$ SEM.

3.2.3.5 Comparison of cytokine-induced p65 recruitment to chemokine promoters in ASMC of healthy subjects and patients with non-severe and severe asthma

Given the observation of differential CCL11 expression in ASMC of patients with non-severe asthma compared to those of healthy subjects and patients with severe asthma (3.3.2.1), p65 recruitment to chemokine promoters was compared. Cells were stimulated with either TNF- α (10 ng/mL) for CCL11 and CXCL8 or combined TNF- α and IFN- γ (10 ng/mL each) for CX3CL1 for one hour. p65 recruitment to chemokine promoters was measured by ChIP assay. Recruitment of p65 to promoters of CCL11, CXCL8, or CX3CL1 was not different between three groups (Fig. 3.26).

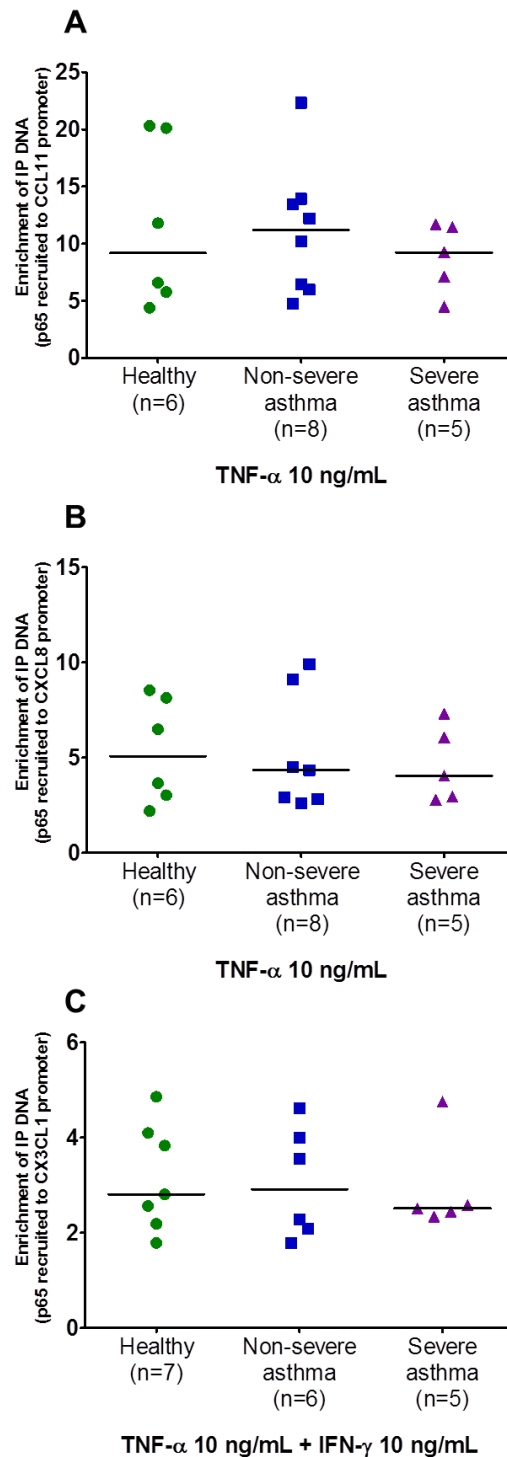


Figure 3.26. Comparison of cytokine-induced p65 NF- κ B recruitment to chemokine promoters in ASMC of healthy subjects and patients with non-severe and severe asthma. ASMC were stimulated with TNF- α (10 ng/mL) for CCL11 (A) and CXCL8 (B) or combined TNF- α and IFN- γ (10 ng/mL each) for CX3CL1 (C) for one hour. Recruitment of p65 to promoters of chemokines were measured by ChIP assay. Horizontal lines represent median.

3.3 Discussion

Summary of results:

The release of CCL11, CXCL8 and CX3CL10 was induced by TNF- α . IFN- γ suppressed the former two TNF α -induced chemokines but potentiated the latter one. In contrast, while CX3CL1 was induced by neither TNF- α or IFN- γ alone, it was synergistically induced by combination of the two cytokines. These suppressing effects of IFN- γ were associated with inhibition of the p65 component of NF- κ B which is recruited to the targeted chemokine gene promoters. Additionally, both baseline and TNF α -induced CCL11 was greater in ASMC of patients with non-severe asthma, while CXCL10 induced by IFN- γ , rather than TNF- α alone or in combination with IFN- γ , was heightened in those of severe asthma. The p65 component of NF- κ B expression was heightened in ASMC of patients with severe asthma, while its nuclear translocation and recruitment to gene promoters were not different between groups.

3.3.1 Modulating effect of IFN- γ on TNF α -induced inflammation

The observations of suppression of TNF α -induced CCL11 and CXCL8 by IFN- γ in ASMC (3.3.1.1 and 3.3.1.2) are consistent with literature (Keszeczy, Tliba et al. 2007). This consistent effect is further confirmed in ASMC of patients with non-severe and severe asthma (3.3.1.5). Other reported TNF α -induced inflammatory genes suppressed by IFN- γ in ASMC include IL-6 (Keszeczy, Tliba et al. 2007), IL-17 receptor (Lajoie-Kadoch, Joubert et al. 2006), and TLR3 (Sukkar, Xie et al. 2006).

It is reported that IFN- γ is an inhibitor of TNF α -induced NF- κ B transcriptional activity in ASMC: IFN- γ suppresses TNF α -induced NF- κ B reported activity, and increased acetylation of the p65 subunit of NF- κ B after TNF- α exposure is attenuated by IFN- γ . In addition, trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor, partially reverses the inhibitory effect of IFN- γ on TNF α -induced genes which are NF- κ B dependent. (Keslacy, Tliba et al. 2007). As p65 acetylation contributes to NF- κ B activity (Ishinaga, Jono et al. 2007; Barroso, Eyre et al. 2011), these findings suggest that IFN- γ suppresses the expression of some of the TNF α -induced pro-inflammatory genes by interfering with NF- κ B transcriptional activity, possibly through reducing acetylation levels of key regulatory proteins.

TNF- α induces p65 recruitment to the promoters of CCL11 and CXCL8; in addition, these recruitments are suppressed by IFN- γ (3.3.1.12 and 3.3.1.13). These observations suggest that the suppressive effect of IFN- γ on TNF α -induced inflammatory gene expression is at least partially through inhibition of NF- κ B recruitment to the gene promoters. Of interest, it is reported that TNF α -induced p65 recruitment of CCL11 in ASMC is also inhibited by fluticasone in association with reduced recruitment of acetylated histone H4 (Nie, Knox et al. 2005). Given that the anti-inflammatory effect exerted by glucocorticoids is through reversal of histone acetylation mediated by HDAC (Barnes 2006), and that the suppressive of IFN- γ on TNF α -induced, NF- κ B dependent genes is prevented by the HDAC inhibitor (Keslacy, Tliba et al. 2007), it is possible that the suppressive effect of IFN- γ and glucocorticoid on TNF α -induced CCL11 is through similar mechanisms, such as induction of HDAC activity.

TNF α -induced CXCL8 release and mRNA expression are not suppressed by IFN- γ at 10 ng/mL (3.3.1.6) as the concentration optimisation experiment shows the inhibition occurs only with IFN- γ at 100 ng/mL (3.3.1.2). Intriguingly, IFN- γ at 10 ng/mL still partially inhibits TNF α -induced p65 recruitment to the CXCL8 promoter (3.3.1.13). Perhaps this partial suppressive effect of p65 recruitment by IFN- γ at this concentration is not sufficient to cause a change at the level of gene transcription.

The synergistic effects of TNF- α and IFN- γ on induction of CXCL10 and CX3CL1 (3.3.1.3 and 3.3.1.4) in ASMC are consistent with literature (Hardaker, Bacon et al. 2004; Sukkar, Issa et al. 2004). These effects are further confirmed, extending from ASMC of healthy subjects to those of non-severe and severe asthma (3.3.1.7 and 3.3.1.8). Other pro-inflammatory genes reported which are synergistically induced by TNF- α and IFN- γ in ASMC include CCL5 (John, Hirst et al. 1997), CD38 (Tliba, Panettieri et al. 2004), or TLR2 and TLR3 (Sukkar, Xie et al. 2006).

The synergistic effect of combined TNF- α and IFN- γ treatment on CXCL10 in ASMC is not regulated at the level of NF- κ B activation, STAT-1 phosphorylation or *in vivo* recruitment of these transcription factors to the gene promoter. Instead, the synergy results from increased recruitment of CBP to the CXCL10 promoter, which is associated with increased RNA polymerase II recruitment (Clarke, Clifford et al. 2010). In contrast, the mechanism of the synergy on CX3CL1 is still not clear and need further investigation.

In other cell types, TNF- α and IFN- γ collaborate at the gene level by increasing promoter activation through a synergistic interaction between NF- κ B and STATs activated by TNF- α and IFN- γ , respectively. A functional cooperation has been demonstrated between NF- κ B and STAT1 in the regulation of genes activated by these cytokines, including ICAM-1 (Jahnke and Johnson 1995), CCL5 (Ohmori, Schreiber et al. 1997), and caspase 11 (Schauvliege, Vanrobaeys et al. 2002). Other mechanisms which may account for the synergistic effect of TNF- α and IFN- γ include IFN γ -induced up-regulation of TNF- α receptors (Ruggiero, Tavernier et al. 1986; Tsujimoto and Vilcek 1986; Kost, Mutch et al. 1999) or vice versa (Sanceau, Merlin et al. 1992; Krakauer and Oppenheim 1993). Intriguingly, in ASMC, whereas TNF- α induces p65 recruitment to the CX3CL1 promoter, this recruitment is not modulated by IFN- γ (3.3.1.11 and 3.3.1.14). This suggests that the synergistic induction of CX3CL1 by TNF- α and IFN- γ in ASMC is through mechanism(s) other than the NF- κ B pathway.

In summary, depending on the inflammatory milieu, IFN- γ can antagonize or synergize TNF α -induced inflammatory actions in ASMC. The suppressive effect of IFN- γ is through inhibition of TNF α -induced p65 to the promoters of specific inflammatory genes. Further studies investigating whether STAT1 or CBP plays a role in the synergy between these two cytokines will provide further mechanistic insights.

3.3.2 Differential expression of inflammatory chemokines in ASMC

CCL11 release and mRNA expression both at baseline and post-stimulation by TNF- α are elevated in ASMC of patients with non-severe asthma (3.3.2.1). Similarly, increased expression of CCL11 induced by IL-1 β and IL-13 in ASMC of patients with asthma is reported, which is in part mediated by enriched extracellular matrix substrates such as fibronectin (Chan, Burgess et al. 2006). Intriguingly, the greater CCL11 expression exists in ASMC of patients with non-severe but not severe asthma (3.3.2.1). CCL11 is a potent chemoattractant for eosinophils and hence contributes to eosinophilic inflammation in diseased tissue. Investigation into differential inflammatory phenotypes of the airways of severe asthma has been inconclusive. One study reports a higher concentration of eosinophils in BAL fluid in the patients with moderate asthma, while there is very little difference between normal controls and patients with severe asthma (Wenzel, Szeffler et al. 1997). My results suggest a possible mechanism accounting for this differential eosinophil count between groups.

The results showing no difference in CXCL8 expression in any of the groups (3.2.2.2) are not consistent with the literature, which demonstrates elevated CXCL8 expression in ASMC of patients with asthma compared to those of healthy subjects (John, Zhu et al. 2009). The induced CXCL8 release from healthy and asthmatic subjects described by John *et al* is 10,000 and 40,000 pg/mL, respectively, which is 2.5-10 fold greater levels compared to my results when the cells are treated with TNF- α at the same concentration and duration. Possible explanations include different manipulation or different passages of the cells for experiment.

It has been recognised that neutrophilic inflammation in the airway is a feature of severe asthma (Wenzel, Szefer et al. 1997; Shannon, Ernst et al. 2008) although the neutrophil count is not different in the bronchial biopsies of patients with non-severe or severe asthma (Macedo, Hew et al. 2009). Given that CXCL8 is a potent chemoattractant for neutrophils, it is rational to suggest that this chemokine release may be increased from ASMC of patients with severe asthma. However, the results reveal no difference in CXCL8 release or mRNA expression in ASMC between the groups (3.2.2.2). This indicates that in contrast to epithelial and subepithelial cells, where higher CXCL8 is expressed (Shannon, Ernst et al. 2008), ASMC may not be the cells which contribute to neutrophilic inflammation in severe asthma.

Comparison of cytokine-induced CXCL10 in ASMC of healthy subjects and patients with asthma in literature is inconsistent: while increased CXCL10 release induced by Cytomix (combination of TNF- α , IL-1 β and IFN- γ) is displayed in ASMC of patients with asthma (Brightling, Ammit et al. 2005), conflicting results of release and mRNA expression of CXCL10 induced by each of these cytokines individually are reported (Alrashdan, Alkhouri et al. 2012). My results reveal ASMC of patients with severe asthma release greater CXCL10 after exposure to IFN- γ , rather than TNF- α alone or in combination with IFN- γ , compared to those of healthy subjects and non-severe asthma (3.2.2.3). Increased serum CXCL10 levels are predictive of virus-induced asthma and strongly associated with more severe airway obstruction (Wark, Bucchieri et al. 2007), and the CXCL10/CXCR3 axis mediates mast cell migration to ASMC of patients with asthma (Brightling, Ammit et al. 2005), which is a major

determinant of asthma. Hence, in severe asthma, perhaps especially in response to viral infection, the interaction between ASMC-derived CXCL10 and mast cell-expressing CXCR3 may be an important pathway facilitating the migration of mast cells into the airway smooth muscle bundles.

Although immunohistochemistry in airways in literature reveals increased staining of CX3CL1 in airway smooth muscle of asthma patients compared to that of healthy subjects (El-Shazly, Berger et al. 2006), there is no difference in TNF α and IFN γ - induced CX3CL1 release from ASMC between healthy subjects and patients with non-severe or severe asthma. This suggests the greater expression of membrane-bound CX3CL1 does not result in increased release of cytokine-induced of this chemokine in the soluble form.

3.3.3 Regulation and differential expression of NF- κ B (p65) in ASMC

There have been several lines of evidence to indicate enhanced NF- κ B signalling in tissues of asthma, especially of severe asthma (Edwards, Bartlett et al. 2009). For example, PBMC of adult severe uncontrolled asthma display higher expression of p65 and IKK- β proteins and greater p65-DNA binding affinity than normal individuals (Gagliardo, Chanez et al. 2003). Nevertheless, expression of each NF κ B-mediated gene is differentially regulated, depending on the intracellular milieu and related signalling pathways, and may not be influenced equally by overall heightened NF- κ B activity in diseased cells or tissue. Hence, investigation of targeted inflammatory genes individually, by techniques such as ChIP assay, which examines the recruitment of transcription factors to the specific gene promoter, is necessary.

The observations of increased p65 protein and mRNA in ASMC of patients with severe asthma (3.2.3.1) are an extension of increased NF- κ B expression in other human cells, such as PBMC (Gagliardo, Chanez et al. 2003) and peripheral (Abdulmir, Kadhim et al. 2009) and BAL lymphocytes (Abdulmir, Hafidh et al. 2009). Whereas CXCL10 release induced by IFN- γ is increased in ASMC of severe asthma (3.2.2.3), this is less likely to be attributed to greater p65 expression as IFN- γ only causes transient nuclear translocation of p65 and does not induce p65 recruitment to the CXCL10 promoter (Clarke, Clifford et al. 2010). In addition, despite increased p65 expression in severe asthma, the nuclear abundance of this protein was not different between groups (3.2.3.3) and TNF- α induced p65 nuclear translocation was similar in ASMC of healthy subjects and patients with non-severe and severe asthma (3.2.3.4). This is in contrast to the findings of increased or rapid nuclear translocation of p65 in human PBMC (Tomari, Matsuse et al. 2003) or murine bronchial epithelium of asthma (Poynter, Irvin et al. 2002), respectively. Therefore, the differential regulation of NF- κ B signalling pathway in healthy and asthmatic subjects may be organism or cell –specific.

Although the release and mRNA expression of CCL11 is increased in ASMC of patients with non-severe asthma (3.2.2.1), there is no difference in p65 recruitment to the CCL11 promoter between the groups. Therefore, the mechanism which underlies differential expression of CCL11 is uncertain and needs further investigation. Post-translational modification of transcription factors, by phosphorylation, methylation or acetylation may affect catalytic activity, stability,

trafficking and protein-protein interactions such as co-factor recruitment (Shen, Kirmani et al. 2011). For example, serine 276 on p65 is a substrate for p38 α (Ji, Liu et al. 2010), and phosphorylation of serine 276, while not interfering with translocation or DNA-binding affinity, may be essential for transcriptional activity (Okazaki, Sakon et al. 2003). Thus, it could be the phosphorylation status of p65 bound to each promoter that influences the ability of this transcription factor to induce gene transcription.

In summary, depending on the inflammatory milieu, there is differential expression of inflammatory chemokines in ASMC of patients with asthma. This is not attributed to differential expression of p65 or altered recruitment to the gene promoters. Further investigation is required to determine the underlying mechanism.

Chapter 4.

**Corticosteroid responsiveness of ASMC of
healthy subjects and patients with non-severe
and severe asthma**

Chapter 4. Corticosteroid responsiveness of ASMC of healthy subjects and patients with non-severe and severe asthma

4.1 Introduction

Corticosteroids are the mainstay treatment of asthma. They are effective in improving asthma symptoms, pulmonary function and result in reduction of the need for short-acting β_2 -agonist as well as airway hyper-responsiveness (Barnes 2006). However, patients with severe asthma are either barely maintained or even not well controlled despite administration of high-dose corticosteroids (Bousquet, Mantzouranis et al. 2010). The clinical feature of these patients is relatively reduced effect of corticosteroids to control the symptoms of their asthma, suggestive of the presence of corticosteroid insensitivity in severe asthma.

In PBMC (Hew, Bhavsar et al. 2006) and alveolar macrophages (Bhavsar, Hew et al. 2008), the effect of corticosteroids is impaired in terms of suppressing induced cytokines in patients with severe asthma compared to those with non-severe asthma. It is unclear whether ASMC display a similar phenotype. It is also unknown if the response to corticosteroids is different in ASMC between healthy subjects and patients with asthma in terms of suppressing or potentiating inflammatory chemokines.

IFN- γ acts a pleiotropic cytokine in ASMC. In addition to its ability to induce chemokines such as CXCL10, IFN- γ modulates, in terms of either suppression or potentiation, the cytokine-induced chemokines, as shown in the previous chapter.

Furthermore, IFN- γ influences the effect of corticosteroids. In ASMC, IFN- γ impairs the suppressive effect of corticosteroids on TNF α -induced pro-asthmatic genes, such as CD38 (Tliba, Cidlowski et al. 2006), CCL5 and CXCL10 (Banerjee, Damera et al. 2008). It is uncertain how this cytokine impacts on other asthma-related chemokines, such as CCL11 and CXCL8, and whether there is any difference in the modulating effects of IFN- γ in ASMC between healthy and asthmatic subjects.

In this chapter, the effects of dexamethasone on suppression (CCL11, CXCL8 and CXCL10) or potentiation (CX3CL1) of chemokine expression are compared between ASMC of healthy subjects and patients with non-severe and severe asthma, at the level of both protein release and mRNA expression. The effects of IFN- γ on the suppressive effect of dexamethasone on the former chemokines are investigated, followed by comparison of the effects between groups.

4.2 Results

4.2.1 Effect of dexamethasone on cell number and viability of ASMC

To investigate whether the effect of dexamethasone on chemokine modulation in ASMC is confounded by altered cell numbers, cells were pretreated with dexamethasone at 10^{-10} - 10^{-6} M for 2 hours and subsequently stimulated with TNF- α and/or IFN- γ (10 ng/mL each) for 24 hours. Cell numbers were unchanged with exposure to different concentrations of dexamethasone (10^{-8} or 10^{-6} M) in the absence or presence of TNF- α and IFN- γ (Fig. 4.1A).

Cell viability was also determined by MTT assay. Compared to its absence, dexamethasone at different concentrations (10^{-10} - 10^{-6} M) did not significantly influence the MTT signal, regardless of treatment with TNF- α 10 ng/mL or combined TNF- α and IFN- γ at 10 ng/mL each (Fig. 4.1B).

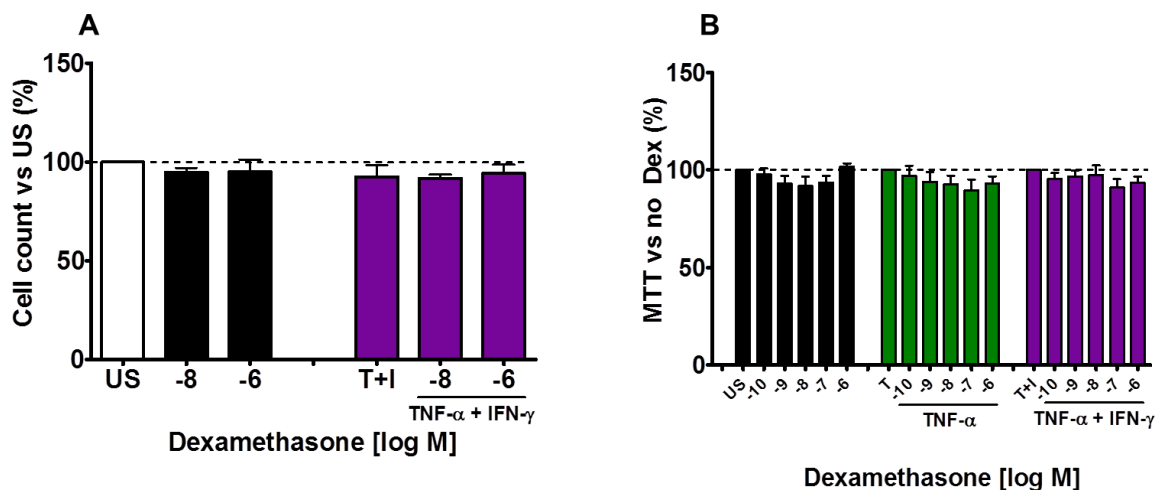


Figure 4.1. Effect of dexamethasone, TNF- α , and IFN- γ on cell number and viability of ASMC. (A) ASMC were pretreated with dexamethasone (10^{-8} or 10^{-6} M) for 2 hrs and stimulated with combined TNF- α and IFN- γ (10 ng/mL each) for 24 hrs. (B) ASMC were pretreated with dexamethasone (10^{-10} - 10^{-6} M) for 2 hrs and stimulated with TNF- α (10 ng/mL) or combined TNF- α and IFN- γ (T+I; 10 ng/mL each) for 24 hrs. Bars represent mean $\pm$ SEM of ASMC from 3 healthy subjects.

4.2.2 Comparison of the corticosteroid response in ASMC of healthy subjects and patients with non-severe and severe asthma

4.2.2.1 Comparison of the suppressive effect of dexamethasone on CCL11 release

To compare the effect of corticosteroid in terms of suppressing CCL11 release, ASMC were pretreated with dexamethasone (10^{-10} - 10^{-6} M) for 2 hours, followed by stimulation with TNF- α (10 ng/mL) for 24 hours. CCL11 was measured by ELISA. In ASMC of the healthy and patients with non-severe asthma, dexamethasone inhibited induced CCL11 release in a concentration-dependent manner, with maximal dexamethasone mediated-suppression of CCL11 at 10^{-6} M by 45.2% and 49.56%, respectively. In contrast, the concentration response curve for dexamethasone in ASMC of patients with severe asthma was less marked, where dexamethasone at 10^{-6} M suppressed induced CCL11 release by merely 20.31%. The suppressive effect of dexamethasone at 10^{-7} and 10^{-6} M in patients with severe asthma was significantly less than observed in the healthy and patients with non-severe asthmatics (Fig. 4.2; $p < 0.05$, respectively).

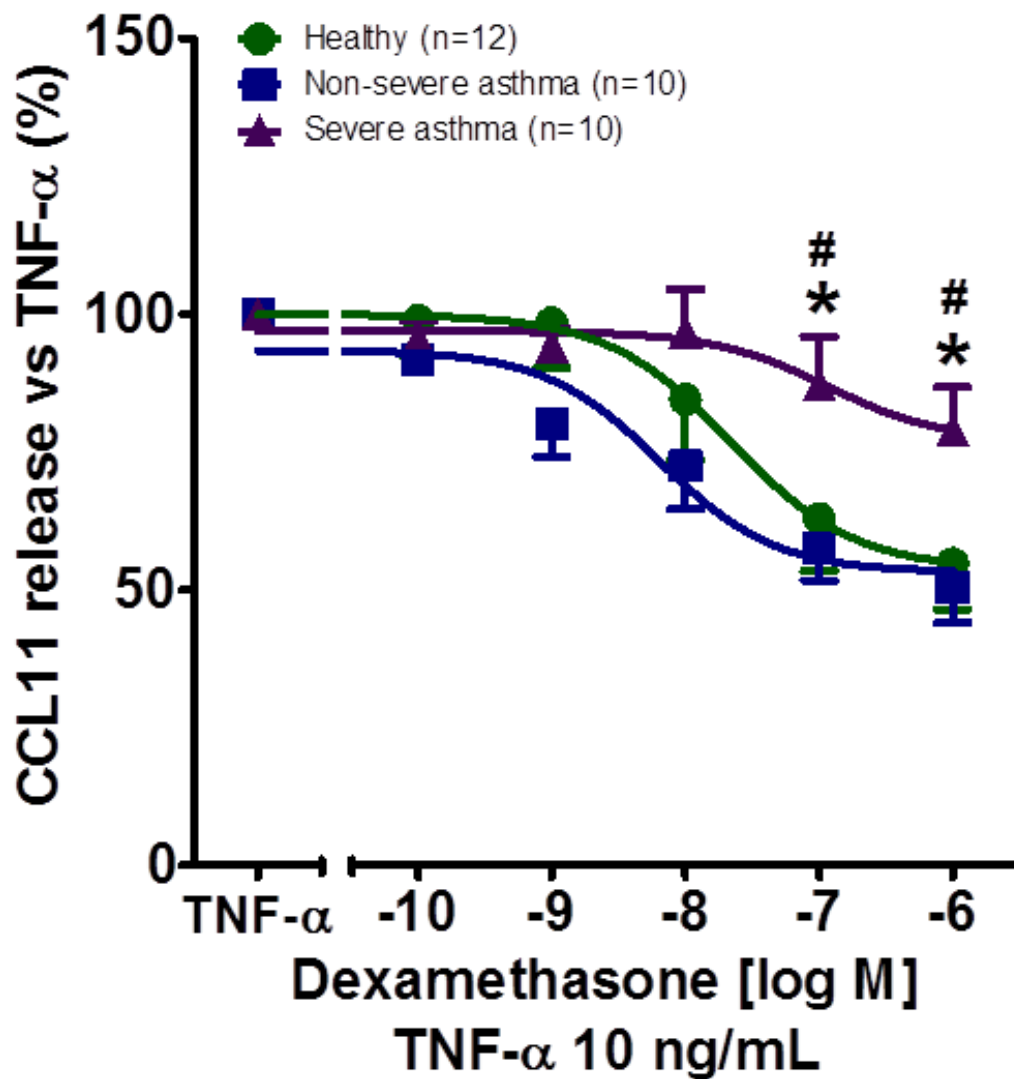


Figure 4.2. Comparison of the suppressive effect of dexamethasone on CCL11 release in ASMC of healthy subjects and patients with non-severe and severe asthma. ASMC were pretreated with dexamethasone (10⁻¹⁰-10⁻⁶ M) for 2 hours and stimulated with TNF-α (10 ng/mL) for 24 hours. CCL11 release was measured by ELISA. Points represent mean ± SEM. * *p*<0.05 vs healthy subjects. # *p*<0.05 vs patients with non-severe asthma.

4.2.2.2 Comparison of the suppressive effect of dexamethasone on CXCL8 release

To compare the effect of corticosteroid in terms of suppressing CXCL8 release, ASMC were pretreated with dexamethasone (10^{-10} - 10^{-6} M) for 2 hours, followed by stimulation with TNF- α (10 ng/mL) for 24 hours. CXCL8 was measured by ELISA. In ASMC of healthy subjects, dexamethasone at 10^{-6} M suppressed TNF α -induced CXCL8 release by 49.8%. At this concentration, dexamethasone inhibited induced CXCL8 release in ASMC of patients with non-severe and severe asthma by, to a lesser extent, 39.9% and 25.7%, respectively. The difference in dexamethasone response between the cells of the healthy subjects and patients with severe asthma was statistically significant over a range of dexamethasone concentrations (Fig. 4.3; $p < 0.05$ at 10^{-8} and 10^{-7} M, $p < 0.01$ at 10^{-6} M, respectively).

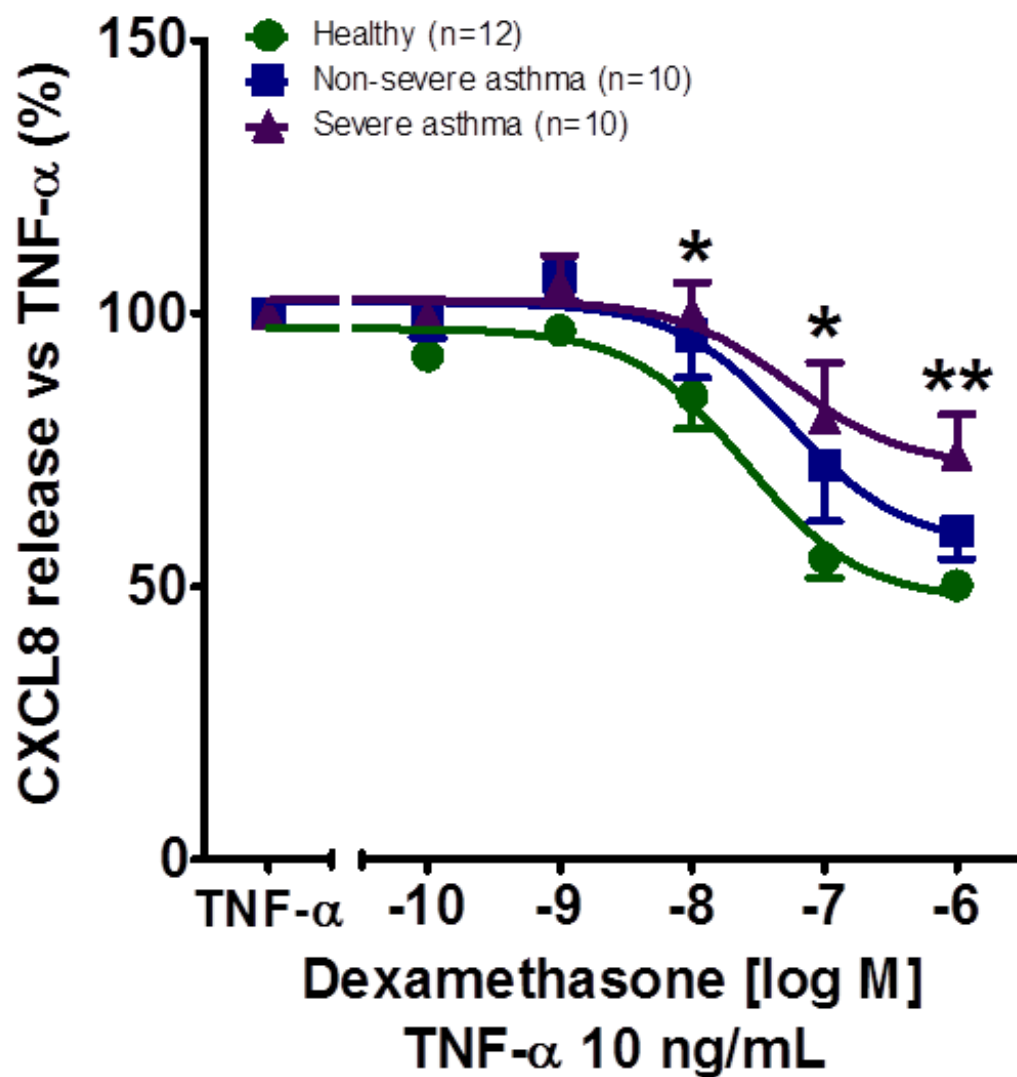


Figure 4.3. Comparison of the suppressive effect of dexamethasone on CXCL8 release in ASMC of healthy subjects and patients with non-severe and severe asthma. ASMC were pretreated with dexamethasone (10^{-10} - 10^{-6} M) for 2 hours and stimulated with TNF- α (10 ng/mL) for 24 hours. CXCL8 release was measured by ELISA. Points represent mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ vs healthy subjects.

4.2.2.3 Comparison of the suppressive effect of dexamethasone on CXCL10 release

To compare the effect of corticosteroid in terms of suppressing CXCL10 release, ASMC were pretreated with dexamethasone (10^{-10} - 10^{-6} M) for 2 hours, followed by stimulation with TNF- α (10 ng/mL) for 24 hours. CXCL10 was measured by ELISA. CXCL10 release induced by TNF- α was suppressed by dexamethasone in a concentration-dependent manner, with statistical significance achieved at 10^{-9} - 10^{-6} M. In contrast to CCL11 and CXCL8 release, the suppression of which by dexamethasone was impaired in ASMC of patients with severe asthma, the induced CXCL10 was not dissimilar in those of healthy subjects and patients with non-severe and severe asthma (Fig. 4.4).

4.2.2.4 Comparison of the potentiating effect of dexamethasone on CX3CL1 release

To compare the effect of corticosteroid in terms of potentiating CX3CL1 release, ASMC were pretreated with dexamethasone (10^{-10} - 10^{-6} M) for 2 hours, followed by stimulation with combined TNF- α and IFN- γ (10 ng/mL each) for 24 hours. CX3CL1 was measured by ELISA. CX3CL1 release induced by TNF- α and IFN- γ was potentiated, rather than suppressed, by dexamethasone in a concentration-dependent manner, with statistical significance reached at 10^{-9} - 10^{-6} M. There was no difference in the potentiating effect of dexamethasone on induced CX3CL1 release in ASMC of the healthy and patients with non-severe and severe asthma (Fig. 4.5).

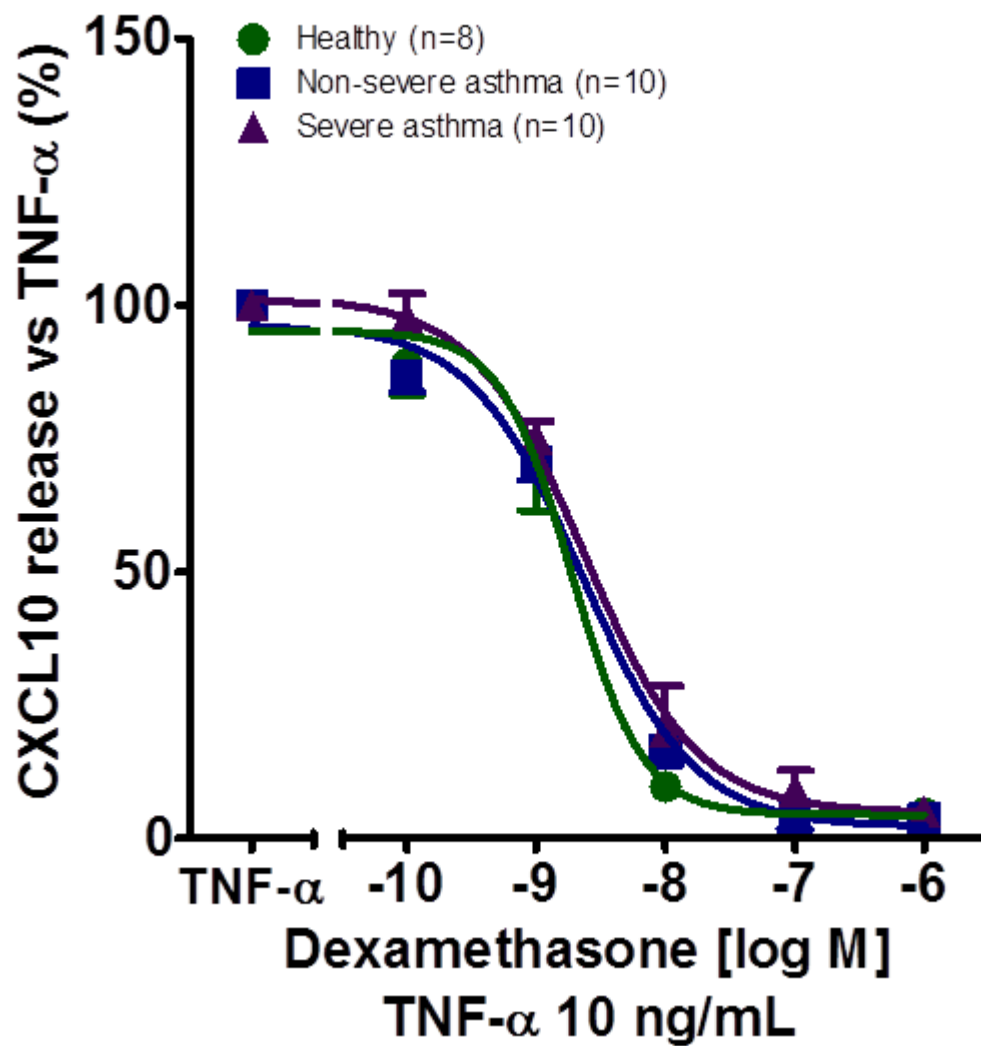


Figure 4.4. Comparison of the suppressive effect of dexamethasone on CXCL10 release in ASM cells of healthy subjects and patients with non-severe and severe asthma. ASM cells were pretreated with dexamethasone (10^{-10} - 10^{-6} M) for 2 hours and stimulated with TNF- α (10 ng/mL) for 24 hours. CXCL10 release was measured by ELISA. Points represent mean $\pm$ SEM.

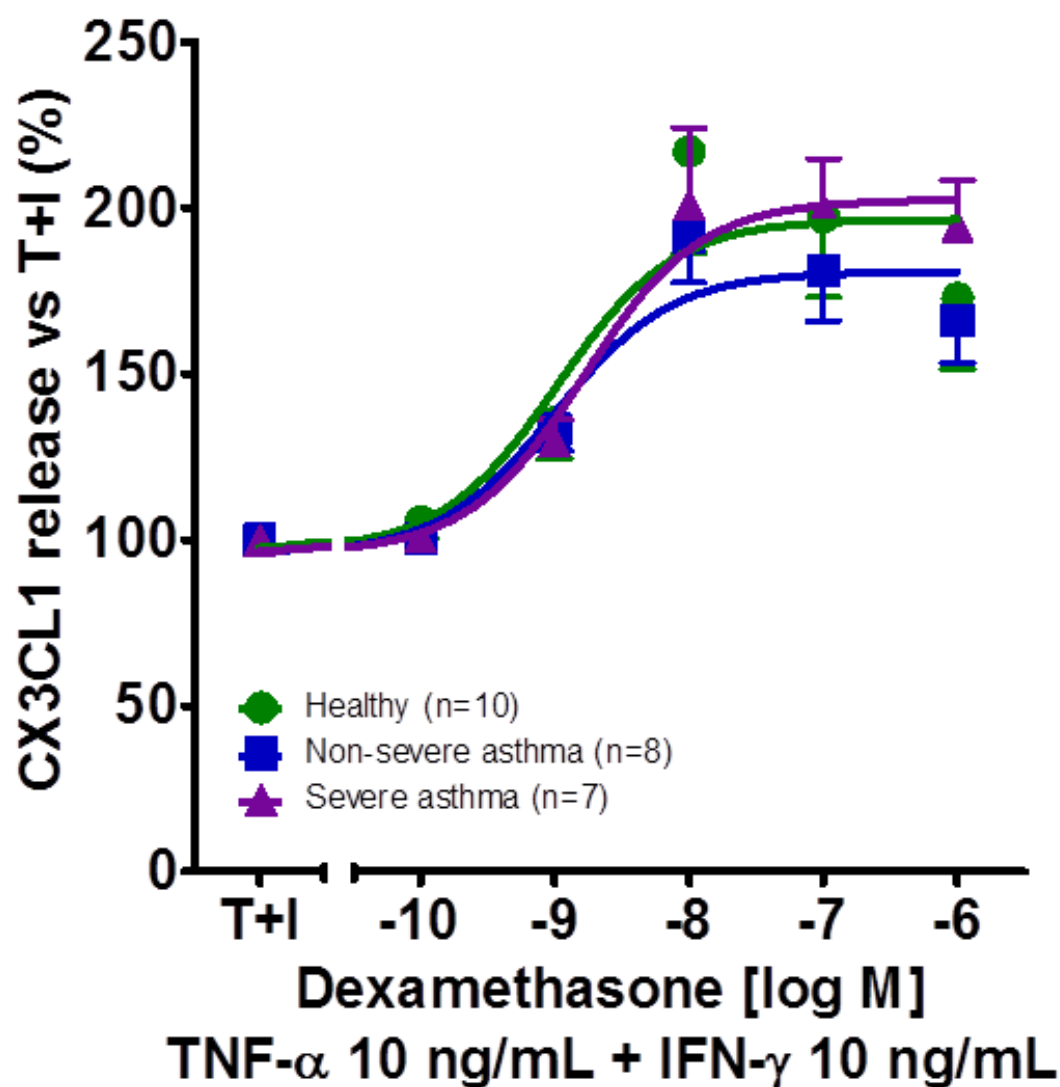


Figure 4.5. Comparison of the potentiating effect of dexamethasone on CX3CL1 release in ASMC of healthy subjects and patients with non-severe and severe asthma. ASMC were pretreated with dexamethasone (10^{-10} - 10^{-6} M) for 2 hours and stimulated with TNF- α and IFN- γ (10 ng/mL each) for 24 hours. CX3CL1 release was measured by ELISA. Points represent mean $\pm$ SEM.

4.2.2.5 Comparison of the suppressive effect of dexamethasone on CCL11 mRNA expression

To determine the optimal concentration of dexamethasone in ASMC for investigation of the suppressive effect on induced CCL11 mRNA, cells of healthy subjects were pretreated with dexamethasone at 10^{-10} - 10^{-6} M for 2 hours, followed by stimulation with TNF- α at 10 ng/mL for 24 hours. CCL11 mRNA and 18S rRNA were measured by RT-qPCR. TNF α -induced CCL11 mRNA was inhibited by dexamethasone in a concentration dependent manner, with maximal suppression of 51.6% at 10^{-7} M ($p<0.01$); this was maintained at a higher concentration (Fig. 4.6).

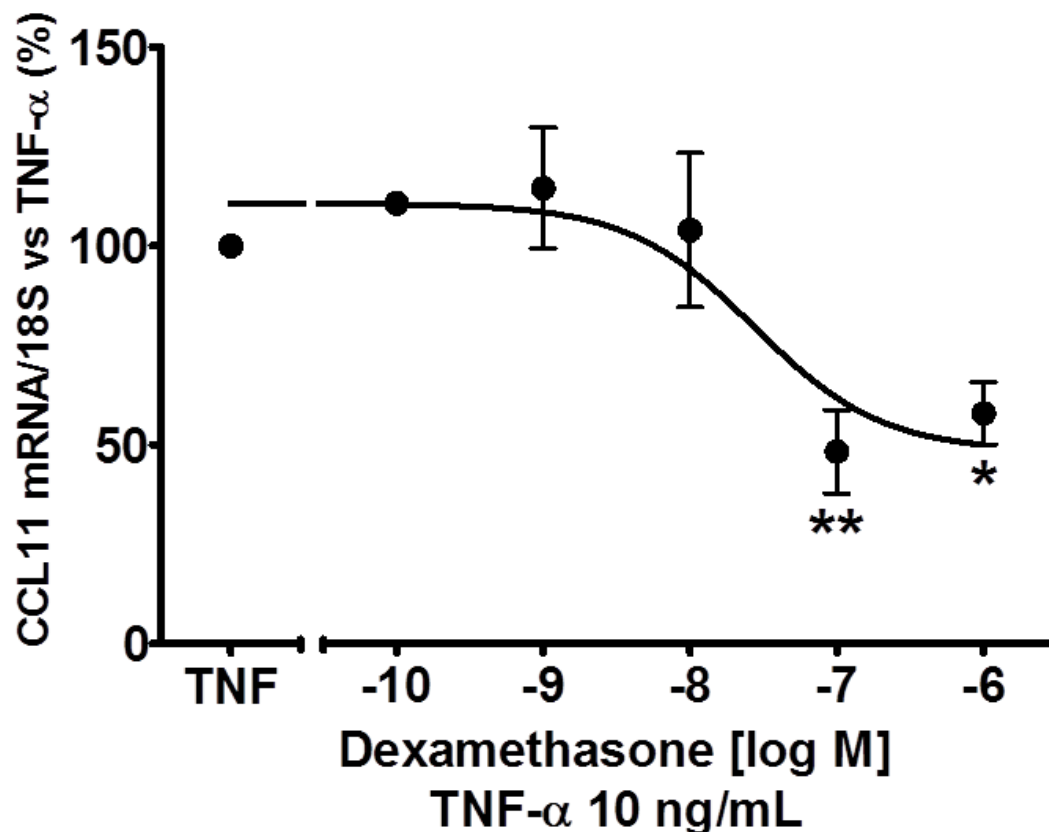


Figure 4.6. Effect of dexamethasone on induced CCL11 mRNA expression in ASMC. Cells were pretreated with dexamethasone (10^{-10} - 10^{-6} M) for 2 hours and stimulated with TNF- α (10 ng/mL) for 24 hours. CCL11 mRNA and 18S rRNA were measured by RT-qPCR. Bars and points represent mean $\pm$ SEM of ASMC from 3 healthy subjects. * $p<0.05$, ** $p<0.01$ vs TNF- α alone.

To compare the suppressive effect of dexamethasone on induced CCL11 mRNA expression in ASMC between different groups, cells were pretreated with dexamethasone (10^{-7} M) for 2 hours and then stimulated with TNF- α (10 ng/mL) for 24 hours. In ASMC of healthy subjects and patients with non-severe asthma, TNF α -induced CCL11 mRNA was inhibited by dexamethasone by 60.5% (Fig. 4.7A; $p<0.001$) and 72.1% (Fig. 7B; $p<0.001$), respectively. In contrast, in those of severe asthma, there was no dexamethasone-mediated suppression of CCL11 mRNA (Fig. 4.7C).

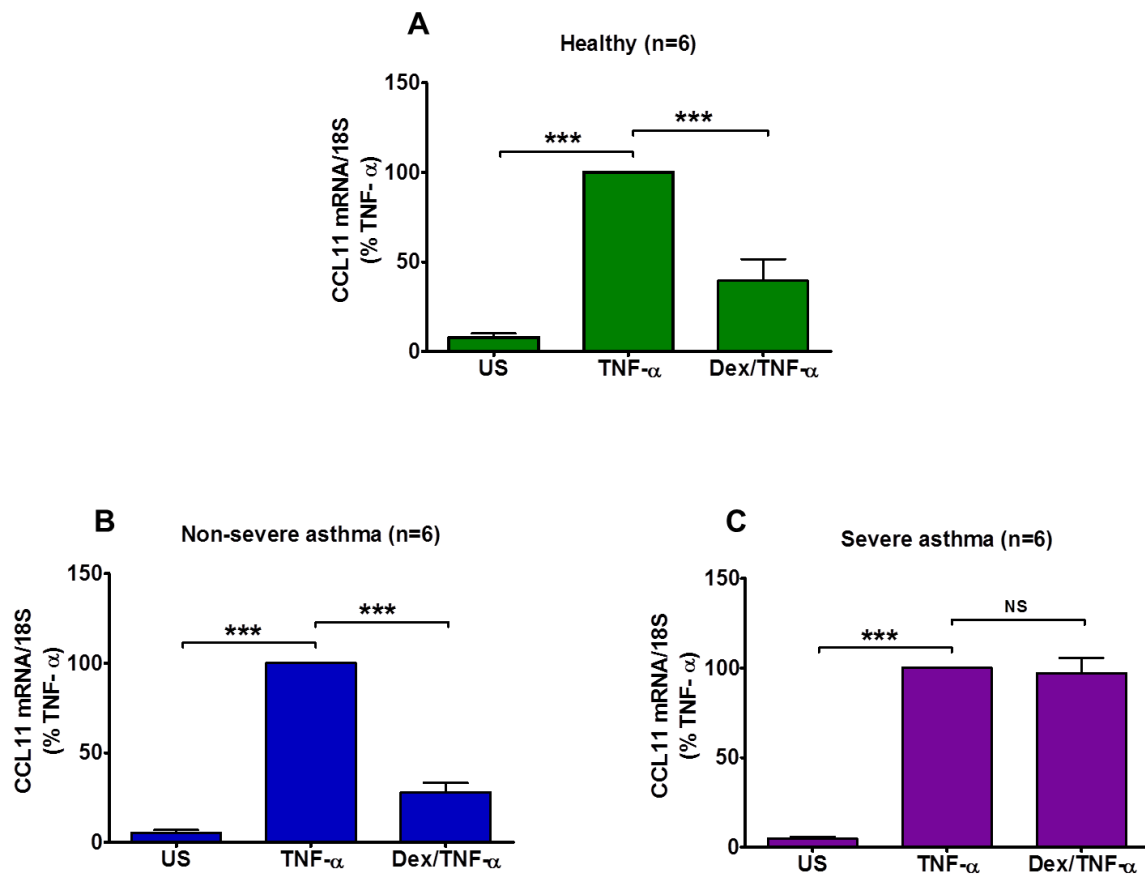


Figure 4.7. Comparison of the suppressive effect of dexamethasone on CCL11 mRNA expression. ASMC were pretreated with dexamethasone (10^{-7} M) for 2 hours and stimulated with TNF- α (10 ng/mL) for 24 hours. CCL11 mRNA and 18S rRNA from ASMC of (A) healthy subjects and patients with (B) non-severe and (A) severe asthma (C) were measured by RT-qPCR. Bars represent mean $\pm$ SEM. *** $p<0.01$.

4.2.2.6 Comparison of the suppressive effect of dexamethasone on CXCL8 mRNA expression

To determine the optimal concentration of dexamethasone in ASMC to investigate its suppressive effect on induced CXCL8 mRNA, cells of healthy subjects were pretreated with dexamethasone at 10^{-10} - 10^{-6} M for 2 hours, followed by stimulation with TNF- α at 10 ng/mL for 24 hours. CXCL8 mRNA and 18S rRNA were measured by RT-qPCR. TNF α -induced CXCL8 mRNA was inhibited by dexamethasone in a concentration dependent manner, with maximal suppression of 43.5% at 10^{-7} M (Fig.4.8; $p<0.05$).

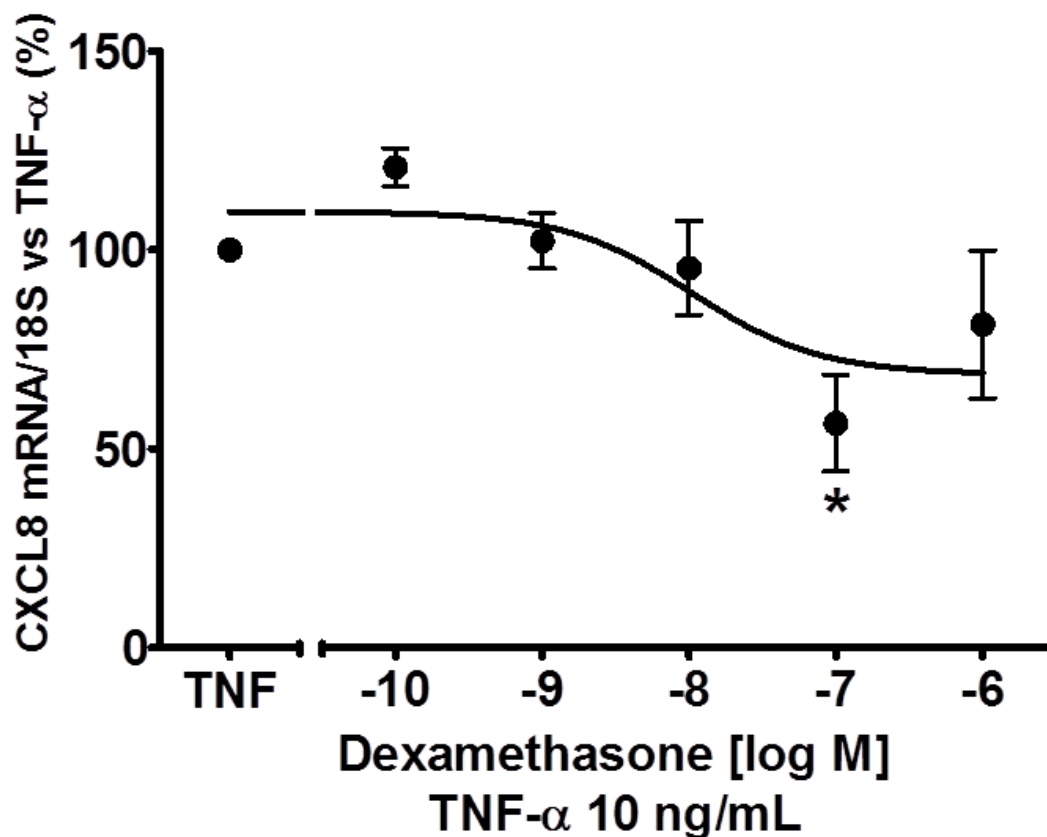


Figure 4.8. Effect of dexamethasone on induced CXCL8 mRNA expression in ASMC. Cells were pretreated with dexamethasone (10^{-10} - 10^{-6} M) for 2 hours and stimulated with TNF- α (10 ng/mL) for 24 hours. CXCL8 mRNA and 18S rRNA were measured by RT-qPCR. Bars and points represent mean $\pm$ SEM of ASMC from 4 healthy subjects. * $p<0.05$ vs TNF- α alone.

To compare the suppressive effect of dexamethasone on induced CXCL8 mRNA expression in ASMC between different groups, cells were pretreated with dexamethasone (10^{-7} M) for 2 hours and then stimulated with TNF- α (10 ng/mL) for 24 hours. In ASMC of healthy subjects and patients with non-severe asthma, TNF α -induced CXCL8 mRNA was inhibited by dexamethasone by 58.8% (Fig. 4.9A; $p<0.001$) and 42.2% (Fig. 4.9B; $p<0.05$), respectively. In contrast, in cells of patients with severe asthma, there was no dexamethasone-mediated suppression of CCL11 mRNA (Fig. 4.9C).

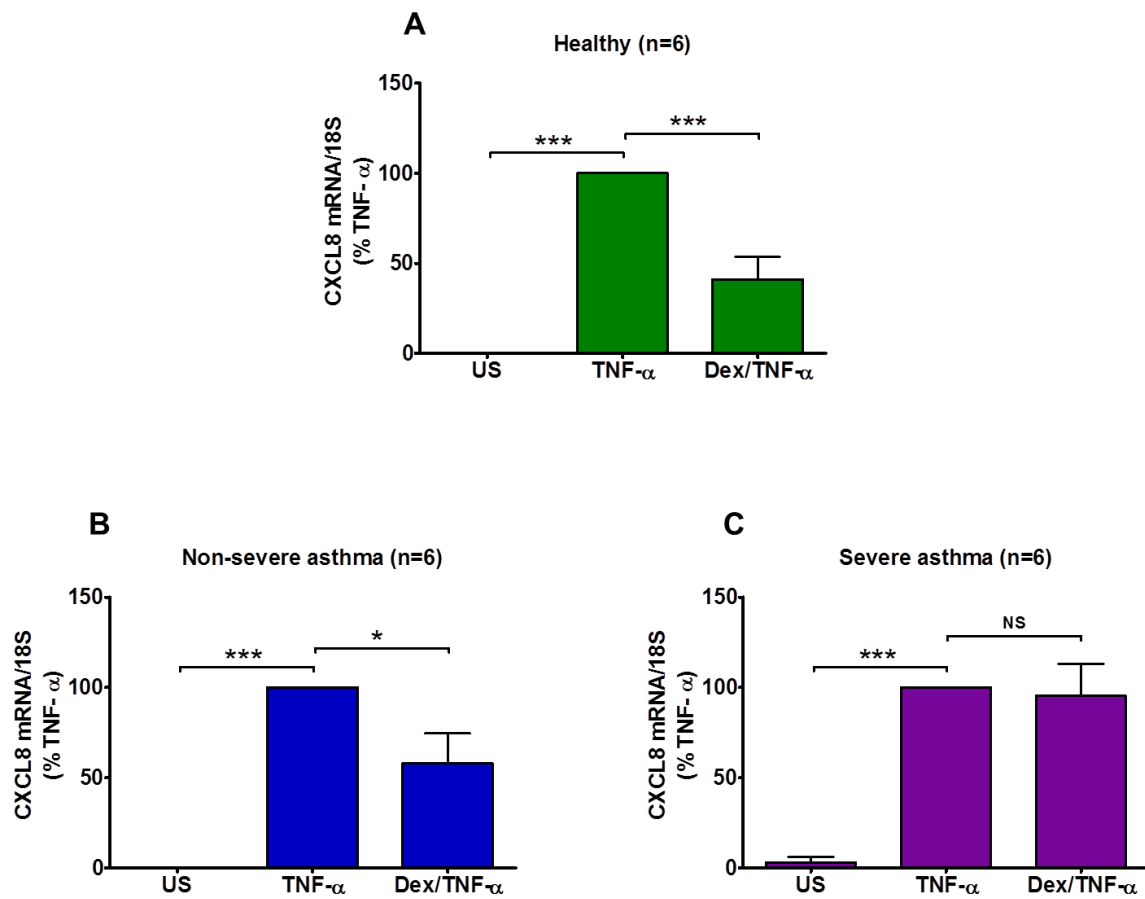


Figure 4.9. Comparison of the suppressive effect of dexamethasone on CXCL8 mRNA expression. ASMC were pretreated with dexamethasone (10^{-7} M) for 2 hours and stimulated with TNF- α (10 ng/mL) for 24 hours. CXCL8 mRNA and 18S rRNA from ASMC of (A) healthy subjects and patients with (B) non-severe and (C) severe asthma were measured by RT-qPCR. Bars represent mean $\pm$ SEM. * $p<0.01$, *** $p<0.001$.

4.2.2.7 Comparison of the potentiating effect of dexamethasone on CX3CL1 mRNA expression

To determine the optimal concentration of dexamethasone in ASMC to investigate its potentiating effect on induced CX3CL1 mRNA, cells of healthy subjects were pretreated with dexamethasone at 10^{-10} - 10^{-6} M for 2 hours, followed by stimulation with TNF- α and IFN- γ at 10 ng/mL each for 24 hours. CX3CL1 mRNA and 18S rRNA were measured by RT-qPCR. TNF α and IFN γ -induced CX3CL1 mRNA was potentiated by dexamethasone in a concentration-dependent manner, with a maximal 3.1-fold potentiation at 10^{-7} M, which was maintained at a higher concentration (Fig. 4.10).

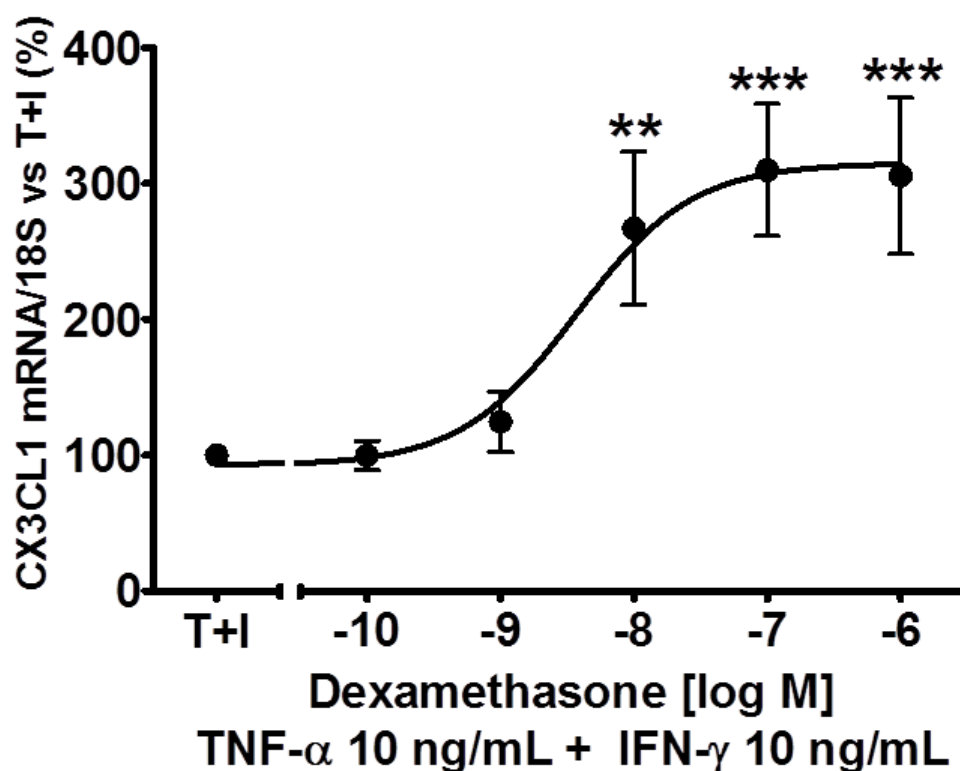


Figure 4.10. Effect of dexamethasone on induced CX3CL1 mRNA expression in ASMC. Cells were pretreated with dexamethasone (10^{-10} - 10^{-6} M) for 2 hours and stimulated with TNF- α and IFN- γ (T+I; 10 ng/mL each) for 24 hours. CX3CL1 mRNA and 18S rRNA were measured by RT-qPCR. Bars and points represent mean $\pm$ SEM of ASMC from 4 healthy subjects. ** $p < 0.01$, *** $p < 0.001$ vs T+I alone.

To compare the potentiating effect of dexamethasone on induced CX3CL1 mRNA expression in ASMC between different groups, cells were pretreated with dexamethasone (10^{-7} M) for 2 hours and then stimulated with TNF- α and IFN- γ (10 ng/mL each) for 24 hours. Dexamethasone potentiated induced CX3CL1 mRNA expression in to a similar degree (3.5-4.3 fold) in ASMC of healthy subjects (Fig. 4.11A; $p<0.01$) and patients with non-severe (Fig. 4.11B; $p<0.001$) and severe asthma (Fig. 4.11C; $p<0.001$).

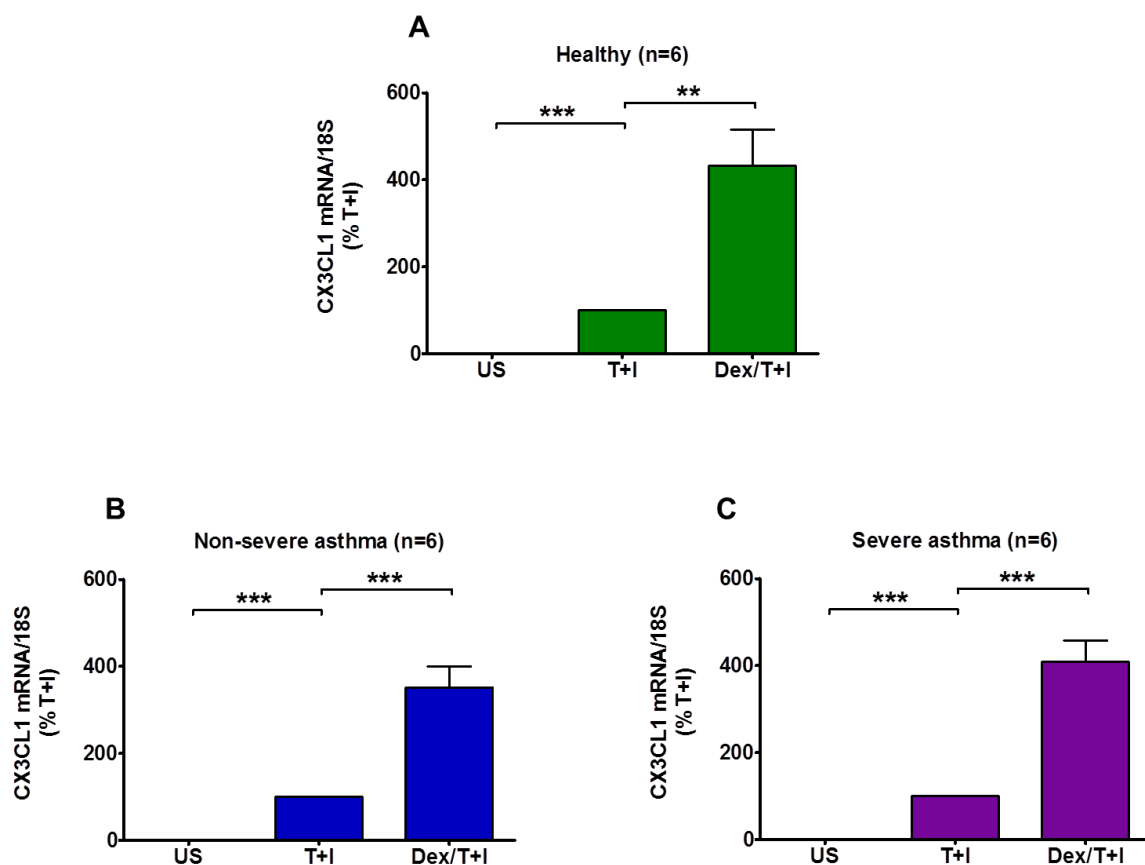


Figure 4.11. Comparison of the potentiating effect of dexamethasone on CX3CL1 mRNA expression. ASMC were pretreated with dexamethasone (10^{-7} M) for 2 hours and stimulated with TNF- α and IFN- γ (10 ng/mL each) for 24 hours. CX3CL1 mRNA and 18S rRNA from ASMC of (A) healthy subjects and patients with (B) non-severe and (C) severe asthma were measured by RT-qPCR. Bars represent mean $\pm$ SEM. ** $p<0.01$, *** $p<0.001$.

4.2.3 Effect of IFN- γ on the suppressive effect of dexamethasone in ASMC of healthy subjects and patients with non-severe and severe asthma

4.2.3.1 Effect of IFN- γ on suppressive effect of TNF α -induced CXCL10 release in ASMC

To investigate and compare the effect of IFN- γ on the suppressive effect of dexamethasone on induced CXCL10 release, ASMC were pretreated with dexamethasone (10^{-10} - 10^{-6} M) for 2 hours and subsequently stimulated with TNF- α (10 ng/mL) or IFN- γ (10 ng/mL) alone or in combination for 24 hours. CXCL10 release was measured by ELISA. In ASMC of healthy subjects, TNF α -induced CXCL10 was inhibited by dexamethasone in a concentration-dependent manner, with maximal suppression of 95% at 10^{-7} and 10^{-6} M (Fig. 4.12A; $p < 0.01$), respectively. In contrast, CXCL10 release was suppressed by dexamethasone at 10^{-6} M by 27% and minimally (1%) when induced by IFN- γ alone and in combination with TNF- α , respectively. The suppressive effects of dexamethasone on CXCL10 were significantly less on exposure to IFN- γ alone ($p < 0.01$) or combined with TNF- α ($p < 0.001$) than TNF- α alone, over a range of concentrations (10^{-8} - 10^{-6} M). The differences in dexamethasone inhibition of CXCL10 induced by IFN- γ alone and in combination with TNF- γ almost reached statistical significance (Fig. 4.14A; $p = 0.052$ - 0.058 at 10^{-8} - 10^{-6} M).

Similarly, in ASMC of patients with non-severe (Fig. 4.12B) and severe asthma (Fig. 4.12C), TNF α -induced CXCL10 release was nearly completely inhibited by dexamethasone at both 10^{-7} and 10^{-6} M, while this chemokine was not suppressed in the presence of IFN- γ . The suppressive effects of dexamethasone on CXCL10 induced by TNF- α alone and in combination with IFN- γ were significantly different over a range of concentrations ($p < 0.001$ at 10^{-9} - 10^{-6} M).

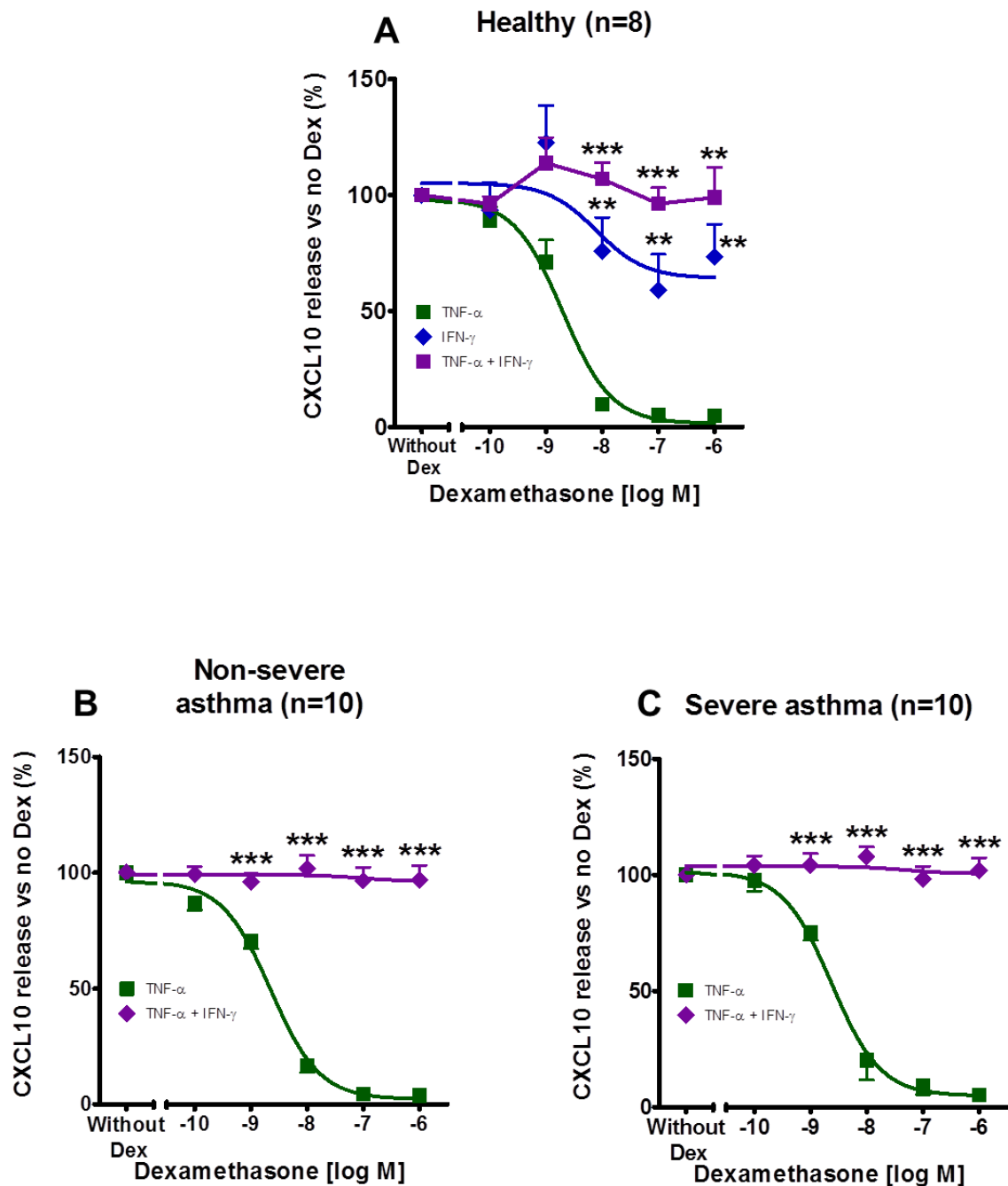


Figure 4.12. Effect of IFN- γ on suppression of TNF α -induced CXCL10 release by dexamethasone in ASMC. Cells were pretreated with dexamethasone (10^{-10} - 10^{-6} M) for 2 hours and stimulated with TNF- α (10 ng/mL), IFN- γ (10 ng/mL), or both, for 24 hours. CXCL10 release in ASMC of (A) healthy subjects and patients with (B) non-severe and (C) severe asthma was measured by ELISA. Points represent mean $\pm$ SEM. ** $p < 0.01$, *** $p < 0.001$ vs TNF- α .

4.2.3.2 Effect of IFN- γ on the suppressive effect of dexamethasone on TNF α -induced CCL11 release in ASMC

To investigate and compare the effect of IFN- γ on the suppressive effect of dexamethasone on induced CCL11 release, ASMC were pretreated with dexamethasone (10^{-10} - 10^{-6} M) for 2 hours and subsequently stimulated with TNF- α (10 ng/mL) or IFN- γ (10 ng/mL) alone or in combination for 24 hours. CCL11 release was measured by ELISA. In ASMC of healthy subjects, constitutive CCL11 release was inhibited by dexamethasone in a concentration-dependent manner, with maximal suppression of 65.5% at 10^{-7} M, which was maintained at higher concentration. In the presence of IFN- γ , dexamethasone at the same concentration suppressed CCL11 by only 41.7% (Fig. 4.13A; $p < 0.01$). The suppressive effects of dexamethasone on CCL11 at baseline and post-stimulation with IFN- γ were significantly different over a range of concentrations ($p < 0.05$ at 10^{-8} M and $p < 0.01$ at 10^{-7} and 10^{-6} M, respectively). This impairment in dexamethasone suppression by IFN- γ was more distinct when cells were exposed to TNF- α : suppression of TNF α -induced CCL11 release by maximally 62.9% was achieved with dexamethasone at 10^{-6} M. With addition of IFN- γ , dexamethasone at the same concentration only minimally suppressed induced CCL11 by 7.7% (Fig. 4.12B). The differences in suppressive effects of dexamethasone were significant over a range of concentrations ($p < 0.01$ at 10^{-8} M- 10^{-6} M).

In ASMC of patients with non-severe asthma, significant differences in the suppressive effect of dexamethasone were observed at 10^{-9} - 10^{-7} M. However, the slope of the concentration response curve was less distinct between cells stimulated with TNF- α alone and combined TNF- α and IFN- γ (Fig. 4.13C). Furthermore, in ASMC of patients with severe asthma, there was no modulation of the suppressive

effect of dexamethasone by IFN- γ : the concentration response curves of the two overlapped (Fig. 4.13D).

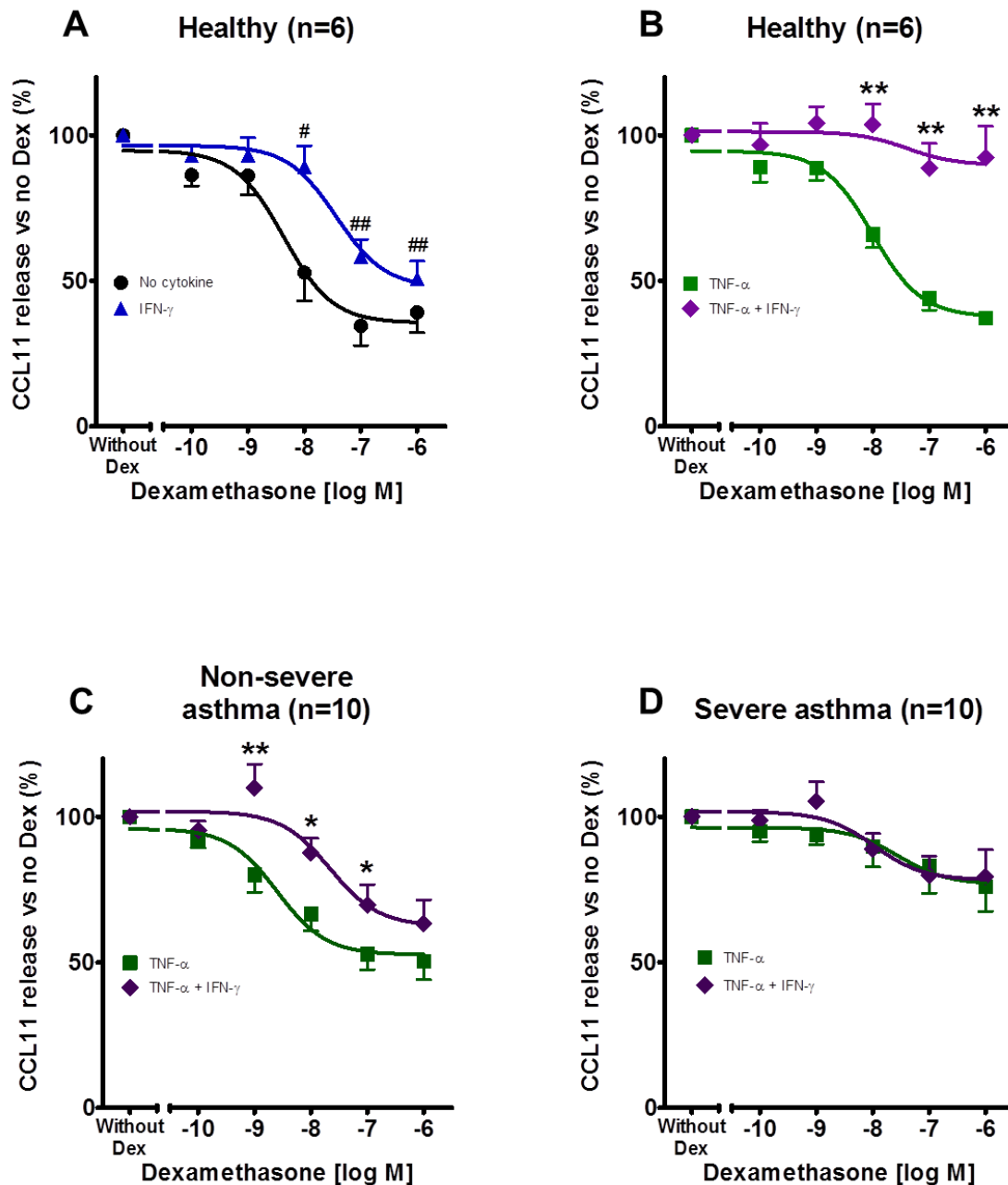


Figure 4.13. Effect of IFN- γ on the suppressive effect of TNF α -induced CCL11 release by dexamethasone in ASMC. ASMC were pretreated with dexamethasone (10^{-10} - 10^{-6} M) for 2 hours and stimulated with TNF- α (10 ng/mL) or IFN- γ (10 ng/mL each) alone or in combination for 24 hours. CCL11 release in ASMC of (A, B) healthy subjects and patients with (C) non-severe and (D) severe asthma was measured by ELISA. Points represent mean $\pm$ SEM. # $p < 0.05$, ## $p < 0.01$ vs no cytokine. * $p < 0.05$, ** $p < 0.01$ vs TNF- α .

4.2.3.3 Effect of IFN- γ on suppressive effect of TNF α -induced CXCL8 release in ASMC

To investigate and compare the effect of IFN- γ on the suppressive effect of dexamethasone on induced CXCL8 release, ASMC were pretreated with dexamethasone (10^{-10} - 10^{-6} M) for 2 hours and subsequently stimulated with TNF- α (10 ng/mL) in the presence or absence of IFN- γ (10 ng/mL) for 24 hours. CXCL8 release was measured by ELISA. In ASMC of healthy subjects, TNF α -induced CXCL8 was suppressed by dexamethasone in a concentration-dependent manner. With the addition of IFN- γ , there was a reduction in the suppressive effect of dexamethasone on induced CXCL8 release. Dexamethasone at 10^{-7} M suppressed TNF α -induced CXCL8 by 52.6%, while the suppression of TNF α and IFN γ -induced CXCL8 was reduced to 24.6% ($p<0.05$). At 10^{-8} M, dexamethasone suppressed TNF α -induced CXCL8 by 31.2% but did not suppress TNF α and IFN γ -induced CXCL8 at all. Intriguingly, TNF α and IFN γ -induced CXCL8 was paradoxically increased by 30.3% ($p<0.01$) by dexamethasone at 10^{-9} M (Fig. 4.14A).

Similarly, in cells of patients with non-severe asthma, with dexamethasone at 10^{-8} M, CXCL8 release was significantly higher in response to TNF- α and IFN- γ than to TNF- α alone ($p<0.05$). Again, TNF α and IFN γ -combined CXCL8 was potentiated by 25.4% ($p<0.01$) by dexamethasone at 10^{-9} M (Fig. 4.14B).

In ASMC of patients with severe asthma, as also shown in 4.2.2.2, the suppressive effect of dexamethasone was reduced in terms of inhibiting TNF α -induced CXCL8. In the presence of IFN- γ , the suppressive effect was further impaired. At 10^{-7} M, dexamethasone inhibited TNF α -induced CXCL8 by 19.2% but minimally (2.2%) following TNF- α and IFN- γ ($p<0.05$). In addition, dexamethasone potentiated TNF α

and IFN γ -induced CXCL8 release at 10^{-9} and 10^{-8} M by 43.1% ($p<0.01$) and 36.8% ($p<0.01$), respectively (Fig 4.14C).

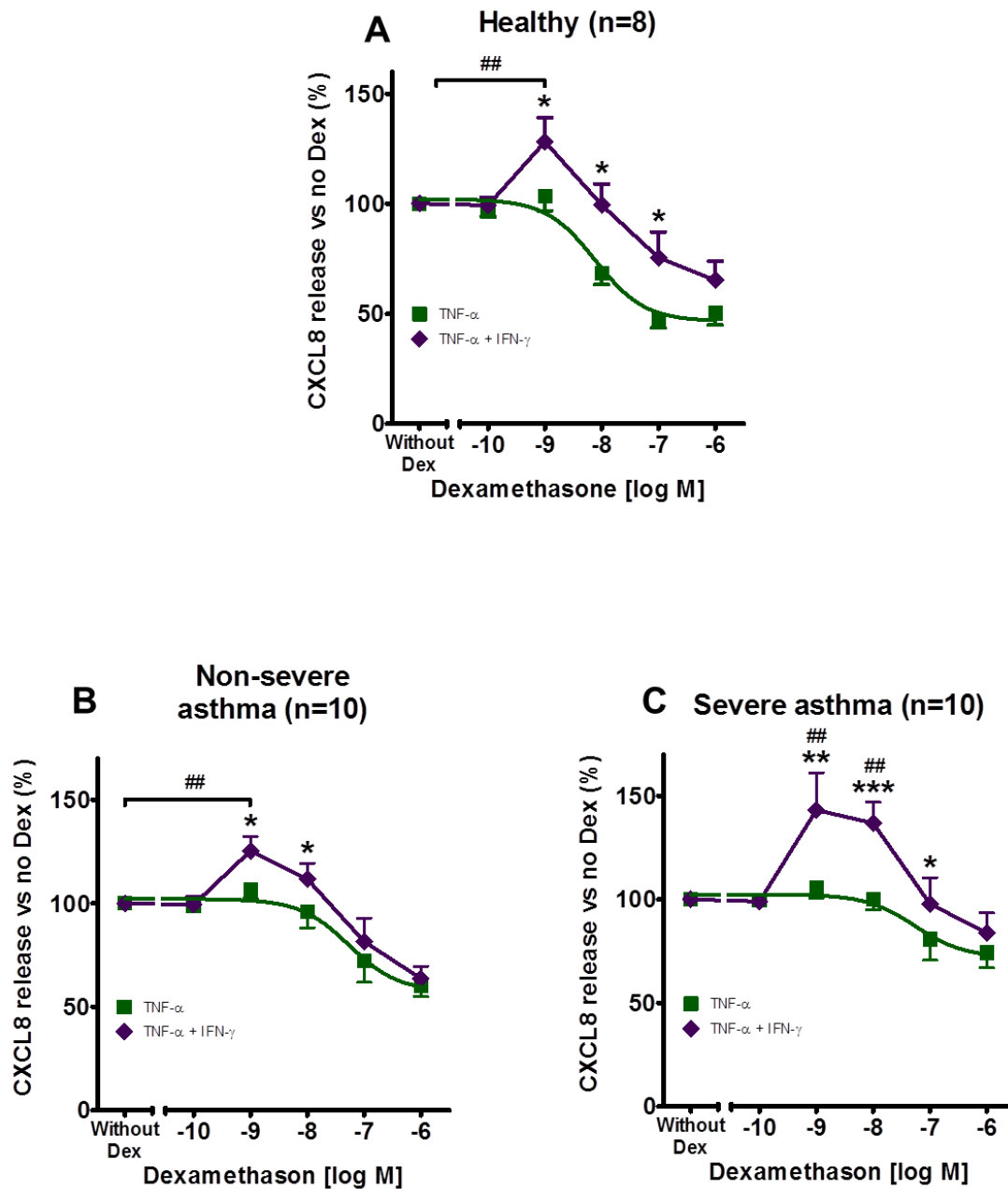


Figure 4.14. Effect of IFN- γ on the suppression of TNF α -induced CXCL8 release by dexamethasone in ASMC. ASMC were pretreated with dexamethasone (10^{-10} - 10^{-6} M) for 2 hours and stimulated with TNF- α (10 ng/mL) or combined TNF- α and IFN- γ (10 ng/mL each) for 24 hours. CXCL8 release in ASMC of (A) healthy subjects and patients with (B) non-severe and (C) severe asthma was measured by ELISA. Points represent mean $\pm$ SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs TNF- α . ## $p<0.01$ vs T+I alone.

4.3 Discussion

Summary of results:

The effects of dexamethasone in terms of suppressing release and mRNA expression of TNF α -induced CCL11 and CXCL8, but not CXCL10, were impaired in ASMC of patients with severe asthma, while the potentiating effect on TNF α and IFN γ -induced CX3CL1 was not different between groups. IFN- γ impaired the suppressive effects of dexamethasone on induced CXCL10, CCL11 and CXCL8 release in ASMC of healthy subjects and those of non-severe asthma. This modulating effect on dexamethasone suppression of CXCL10 was consistent in all groups but was minimal or absent on that of CCL11 and CXCL8 in cells of patients with severe asthma. Intriguingly, in the presence of TNF- α and IFN- γ , dexamethasone at lower concentration paradoxically increased induced CXCL8 release in ASMC of all groups.

4.3.1 Relative corticosteroid insensitivity in ASMC of patients with severe asthma

Whereas the pharmacological effects of corticosteroids have been largely investigated, the modulatory actions on inflammatory genes in ASMC remain complex and not fully characterised. Depending on the genes targeted, corticosteroids have either both anti-inflammatory or pro-inflammatory effect on chemokines. For instance, while corticosteroids antagonise cytokine-induced CCL11 (Pang and Knox 2001), CXCL8 (John, Au et al. 1998) and CXCL10 (Banerjee, Damera et al. 2008), they augment induced CX3CL1 (Sukkar, Issa et al. 2004; Banerjee, Damera et al. 2008).

The observation of reduced suppressive effect of dexamethasone on TNF α -induced release and mRNA of CCL11 (4.2.2.1 and 4.2.2.5) and CXCL8 (4.2.2.2 and 4.2.2.6) in ASMC of patients with severe asthma extend the impaired corticosteroid suppression of serum-induced ASMC proliferation in asthma (Roth, Johnson et al. 2004) as well as corticosteroid insensitivity in terms of suppressing induced cytokines in both PBMC (Hew, Bhavsar et al. 2006) and alveolar macrophages (Bhavsar, Hew et al. 2008) of patients with severe asthma. Noteworthy, the effects of dexamethasone on modulating cytokine-induced CXCL10 (4.2.2.3) and CX3CL1 (4.2.2.4 and 4.2.2.7) are not different in any of the groups, indicating that not all responses to corticosteroids are compromised in severe asthma. This is consistent with the findings in other cell types (Hew, Bhavsar et al. 2006; Bhavsar, Hew et al. 2008) of severe asthmatic subjects and supported by the clinical manifestation that patients with severe asthma suffer from side effects attributed to long term use of corticosteroids (Bousquet, Mantzouranis et al. 2010) and display different clinical features than subjects with generalized glucocorticoid resistance (Charmandari, Kino et al. 2008).

In ASMC, CX3CL1 induced by TNF- α and IFN- γ is potentiated by dexamethasone (4.2.2.5 and 4.2.2.6). Since CX3CL1 has pro-inflammatory properties, not all the effects of corticosteroids are considered to be anti-inflammatory. The potentiating effect of dexamethasone on CX3CL1 expression is likely through a transactivation mechanism with the binding of activated GR to its positive GRE site on the gene promoter (Garin, Pellet et al. 2002). Interestingly, this chemokine induced by the same cytokines is suppressed by dexamethasone in human airway epithelial cell line

A549 (Bhavsar, Sukkar et al. 2008). The mechanisms underlying the cell-specific opposite regulatory effects of corticosteroids on CX3CL1 expression are unknown and need further investigation. The fact that the potentiating effect of dexamethasone on CX3CL1 release and mRNA expression is similar in the three groups (4.2.2.5 and 4.2.2.6) implicates that there is likely to be no differential effects of dexamethasone on GRE binding.

4.3.2 Impaired suppressive effect of dexamethasone by IFN- γ in ASMC

Respiratory virus infection is the most common cause of asthma exacerbation, and IFN- γ is a major cytokine in response to viral infection (Wark and Gibson 2006). The IFN γ -mediated impairment of dexamethasone suppression of induced pro-asthmatic chemokines may explain why the current treatment of virus-induced exacerbation of asthma, i.e. high-dose inhaled or oral corticosteroids, is only partially effective (Message and Johnston 2001).

The suppressive effects of corticosteroid on inflammatory mediators in ASMC are stimuli-specific (Tliba and Amrani 2008). For example, while several steroids considerably inhibit IL1 β -induced GM-CSF, they only partially suppress this cytokine induced by thrombin (Tran, Fernandes et al. 2005). This may account for the observation of poorer dexamethasone suppression of CXCL10 release induced by IFN- γ than by TNF- α (4.2.3.1). In addition, the specific combination of TNF- α with IFN- γ , but not IL-1 β or IL-13, impairs corticosteroid suppression of several pro-asthmatic genes such as CCL5, CXCL10 (Banerjee, Damera et al. 2008) and CD38 (Tliba, Cidlowski et al. 2006). Such an effect of IFN- γ on corticosteroid suppression

of CXCL10 is further confirmed in ASMC of patients with non-severe and severe asthma (4.2.3.1). Similar modulating effects of IFN- γ are also demonstrated in regulation of TNF α -induced CCL11 (4.2.3.2) and CXCL8 (4.2.3.3) release in ASMC of healthy and non-severe asthmatic subjects.

In ASMC, the combination of TNF- α and IFN- γ inhibits GR α -DNA binding activity and GRE-dependent gene transcription through up-regulation of GR- β expression (Tliba, Cidlowski et al. 2006), which is increased in airways of patients with severe asthma (Bergeron, Fukakusa et al. 2006) and correlates with corticosteroid-resistant asthma (Leung, Hamid et al. 1997), perhaps through suppression of HDAC2 expression by inhibiting glucocorticoid response elements in its promoter (Li, Leung et al. 2010). Another possible mechanism contributing to IFN γ -induced corticosteroid resistance involves over-induction of interferon regulatory factor-1 (IRF-1). In ASMC, IFN- γ , but not TNF- α , induces recruitment of IRF-1 to the CD38 promoter, which makes this gene resistant to corticosteroid suppression. Exogenous expression of IRF-1 dose-dependently inhibits glucocorticoid-induced GRE reporter activity, and silencing IRF-1 expression consistently restores GRE reporter activity in TNF α - and IFN- γ treated cells (Tliba, Damera et al. 2008). Furthermore, a recent study suggests a role for serine/threonine protein phosphatase 5 in corticosteroid resistance induced by TNF- α and IFN- γ , possibly through down-regulation of phosphorylated GR at serine 211 (Bouazza, Krytska et al. 2012)

The chemokines CCL11 (4.2.2.1 and 4.2.2.5) and CXCL8 (4.2.2.2 and 4.2.2.6), which are less suppressed by dexamethasone in ASMC of patients with severe

asthma, are resistant to dexamethasone in the presence of IFN- γ (4.2.3.2 and 4.2.3.3), a cytokine which is highly expressed in the inflammatory cells of the airways of severe asthma (Shannon, Ernst et al. 2008). This implicates the possibility of IFN- γ being involved in the mechanisms underlying corticosteroid insensitivity in severe asthma. However, the fact that CXCL10, another chemokine which is resistant to corticosteroid inhibition in the presence of IFN- γ (4.2.3.1), is not insensitive to the suppressive effect of dexamethasone in ASMC of severe asthmatic subjects (4.2.2.3) argues against this deduction. Furthermore, while addition of IFN- γ impairs dexamethasone suppression of both baseline and TNF α -induced CCL11 release, combination of TNF- α and IFN- γ results in a poorer response to dexamethasone than IFN- γ alone (4.2.3.2). This suggests that not only IFN- γ but also TNF- α may have impact on the suppressive effect of corticosteroids.

Interestingly, whereas IFN- γ impairs the suppressive effects of dexamethasone on TNF α -induced CCL11, CXCL8 and CXCL10 in ASMC of healthy subjects, this cytokine differentially modulate the effects of dexamethasone in ASMC of patients with asthma, depending on the chemokine targeted. In contrast to a consistent IFN- γ -induced corticosteroid resistance in terms of suppressing CXCL10 in all groups (4.2.3.1), IFN- γ impairs the suppressive effect of dexamethasone on TNF α -induced CCL11 in ASMC of healthy subjects but has less influence in those of non-severe asthma. Furthermore, in ASMC of patients with severe asthma, where corticosteroid insensitivity exists, IFN- γ does not further modulate the response to dexamethasone in terms of suppressing CCL11 release (4.2.3.2). On the other hand, surprisingly, dexamethasone at lower concentration (i.e. 10^{-9} M) consistently increases TNF α and

IFN γ –induced CXCL8 release (4.2.3.3). This potentiating effect of dexamethasone at a specific concentration on induced CXCL8 is in contrast to the concentration-dependent potentiation of induced CX3CL1 release (4.2.2.4) and mRNA expression (4.2.2.7). The mechanism underlying the differential modulating effects of IFN- γ on corticosteroid response is unclear.

In summary, relative corticosteroid insensitivity exists in ASMC of patients with severe asthma in terms of suppressing CCL11 and CXCL8. IFN- γ induces corticosteroid resistance in terms of CCL11, CXCL8 and CXCL10 in ASMC of healthy patients but has differing modulating effects on corticosteroid, depending on the chemokine targeted. Further investigation is required to establish the role of IFN- γ in corticosteroid insensitivity in severe asthma.

Chapter 5.

Role of MAPK in corticosteroid insensitivity in ASMC of patients with severe asthma

Chapter 5. Role of MAPK in corticosteroid insensitivity in ASMC of patients with severe asthma

5.1 Introduction

Several distinct molecular mechanisms (Table 5.1) contributing to decreased anti-inflammatory effects of corticosteroids have been identified. There is heterogeneity of mechanisms even within a single disease; meanwhile, similar molecular mechanisms are also identified in different inflammatory diseases (Barnes 2010).

• Familial glucocorticoid resistance • Glucocorticoid receptor modification - Phosphorylation: decreased nuclear translocation - p38 MAP kinase due to IL-2 + IL-4 or IL-13 in severe asthma - p38 MAP kinase due to MIF in several inflammatory diseases - JNK due to proinflammatory cytokines - ERK due to microbial superantigens - Nitrosylation: ↑ NO from inducible NO synthase - Ubiquitination: ↑ degradation by proteasome • Increased GRβ expression • Increased proinflammatory transcription factors - Activator protein-1, JNK - STAT5, JAK3 • Defective histone acetylation - Decreased acetylation of lysine-5 on histone 4 - Decreased histone deacetylase-2 ↑ Oxidative stress ↑ Phosphoinositide-3-kinase-δ activation • Increased P-glycoprotein - Increased efflux of steroids

Table 5.1. Possible molecular mechanisms of corticosteroid insensitivity (Barnes 2010).

The MAPK p38, JNK and ERK have been implicated in mechanisms of corticosteroid insensitivity. For example, IL-2 and IL-4, which are increased in the airways of patients with steroid resistant asthma, impair the suppressive effect of corticosteroid in T-cells through induction of p38 MAPK activity (Irusen, Matthews et al. 2002). Elevated JNK activity is observed in dermal inflammation of patients with corticosteroid resistant asthma, and this activity is not suppressed by oral prednisolone (Sousa, Lane et al. 1999), and microbial superantigen induces corticosteroid resistance in T-cell through ERK activation (Li, Goleva et al. 2004). Notably, relative corticosteroid insensitivity in alveolar macrophages of patients with severe asthma is associated with elevated p38 MAPK activity, and the degree of p38 phosphorylation correlates inversely to the suppressive effects of dexamethasone on inflammatory chemokines (Bhavsar, Hew et al. 2008).

Increased activation of MAPK has been demonstrated in the airway cells of patients with asthma (Liu, Liang et al. 2008; Robins, Roussel et al. 2011). However, the role of MAPK in corticosteroid insensitivity in ASMC of severe asthma has not been investigated. In this study, activation of MAPK p38, JNK and ERK, as measured by phosphorylation of the kinases, is compared in ASMC of healthy subjects and patients with non-severe and severe asthma. Then the possible role for MAPK activation in corticosteroid responsiveness is investigated.

5.2 Results

5.2.1 Effect of TNF- α on MAPK activation in ASMC—Optimisation studies

To investigate the effect of TNF- α on MAPK activation in ASMC, cells of healthy subjects were stimulated with TNF- α (10 ng/mL) over a time course (5 minutes to 2 hours). Phosphorylated and total p38, JNK and ERK were measured by Western Blot. p38 phosphorylation was induced by TNF- α at 5 minutes, peaked at 15 minutes, and declined over time (Fig. 5.1A). In contrast, induced JNK and ERK phosphorylation peaked between 15-30 minutes and rapidly declined by one hour (Fig. 5.2B and C). 15 minute was chosen as the treatment duration for further experiments.

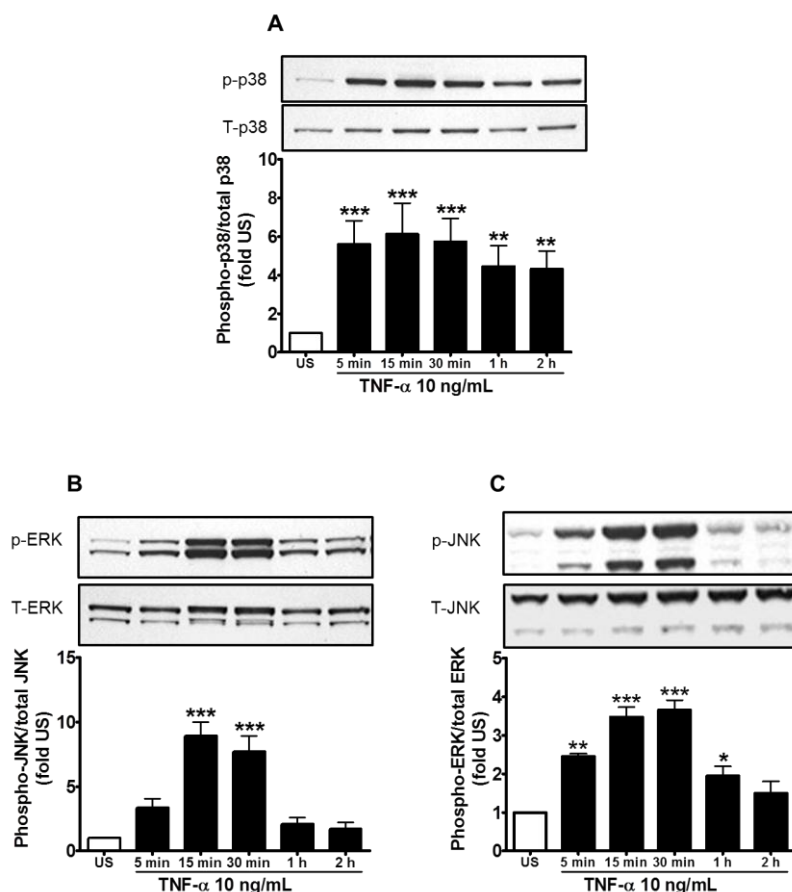


Figure 5.1. Regulation of MAPK in ASMC. Cells were stimulated with TNF- α (10 ng/mL) for indicated time. Phosphorylated and total (A) p38, (B) JNK and (C) ERK were measured by Western Blot. Bars represent mean $\pm$ SEM of ASMC from 3 healthy subjects. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs unstimulated.

5.2.2 Comparison of TNF α -induced p38 MAPK activation in ASMC of healthy subjects and patients with non-severe and severe asthma

To compare induced p38 activity between the three groups, ASMC were stimulated with TNF- α (10 ng/mL) for 15 minutes. Phosphorylated and total p38 expression was measured by Western Blot. TNF- α induced p38 phosphorylation by 2.2 and 2.5-fold, over baseline, in ASMC of the healthy and patients with non-severe asthma, respectively. In contrast, in ASMC of severe asthmatics, phosphorylation of p38 was induced by 10.0-fold, which is significantly greater than that observed in cells of healthy subjects ($p<0.01$) and patients with non-severe asthma ($p<0.05$) (Fig. 5.2).

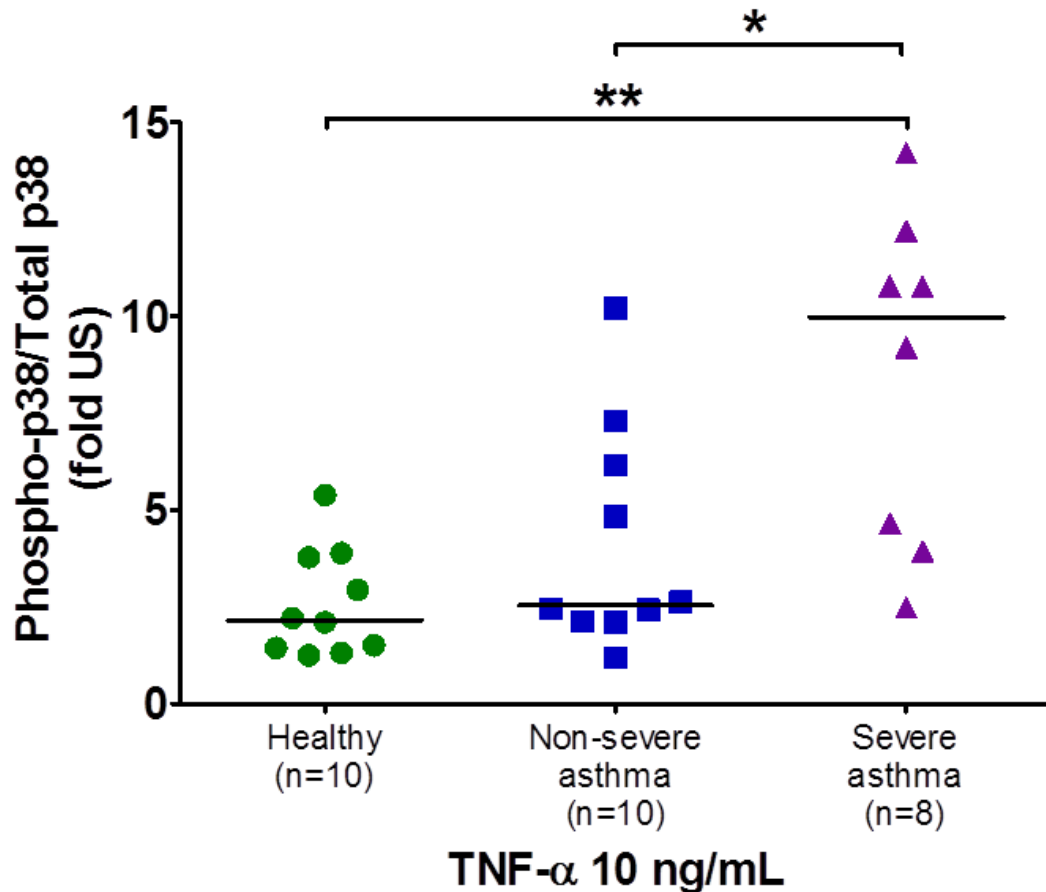


Figure 5.2. Comparison of induced p38 MAPK phosphorylation in ASMC of healthy subjects and patients with non-severe and severe asthma. ASMC were stimulated with TNF- α (10 ng/mL) for 15 minutes. Phosphorylated and total p38 were measured by Western Blot. Horizontal lines represent median. * $p<0.05$, ** $p<0.01$.

5.2.3 Comparison of TNF α -induced JNK activation in ASMC of healthy subjects and patients with non-severe and severe asthma

To compare induced JNK activity between the three groups, ASMC were stimulated with TNF- α (10 ng/mL) for 15 minutes. Phosphorylated and total JNK expression was measured by Western Blot. TNF- α induced a 6.2-fold increase over baseline in JNK phosphorylation in ASMC of the healthy. Phosphorylated JNK was induced, to a greater extent, in cells of non-severe (9.1 fold, $p<0.05$) and severe asthma (13.6 fold, $p<0.01$) than in healthy subjects, while there was no significant difference between non-severe and severe asthmatics (Fig. 5.3).

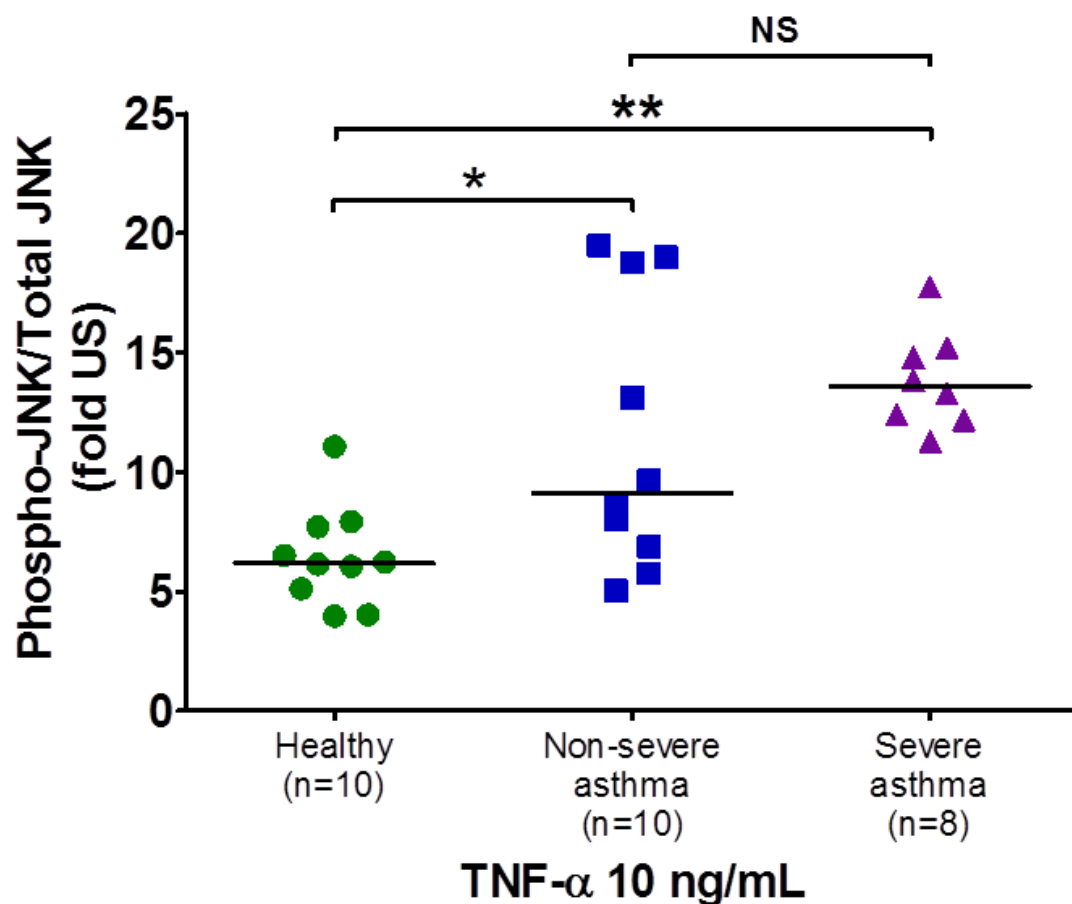


Figure 5.3. Comparison of induced JNK phosphorylation in ASMC of healthy non-severe and severe asthmatics. ASMC were stimulated with TNF- α (10 ng/mL) for 15 minutes. Phosphorylated and total JNK were measured by Western Blot. Horizontal lines represent median. * $p<0.05$, ** $p<0.01$.

5.2.4 Comparison of TNF α -induced ERK activation in ASMC of healthy subjects and patients with non-severe and severe asthma

To compare induced ERK activity in the three groups, ASMC were stimulated with TNF- α (10 ng/mL) for 15 minutes. Phosphorylated and total ERK expression was measured by Western Blot. TNF- α increased ERK phosphorylation by 3.5-fold, over baseline, in ASMC of healthy subjects and by 8.1-fold in those of asthma (Fig. 5.4A; $p<0.05$). However, there was no significant difference between non-severe and severe asthma (Fig. 5.4B; $p=0.63$).

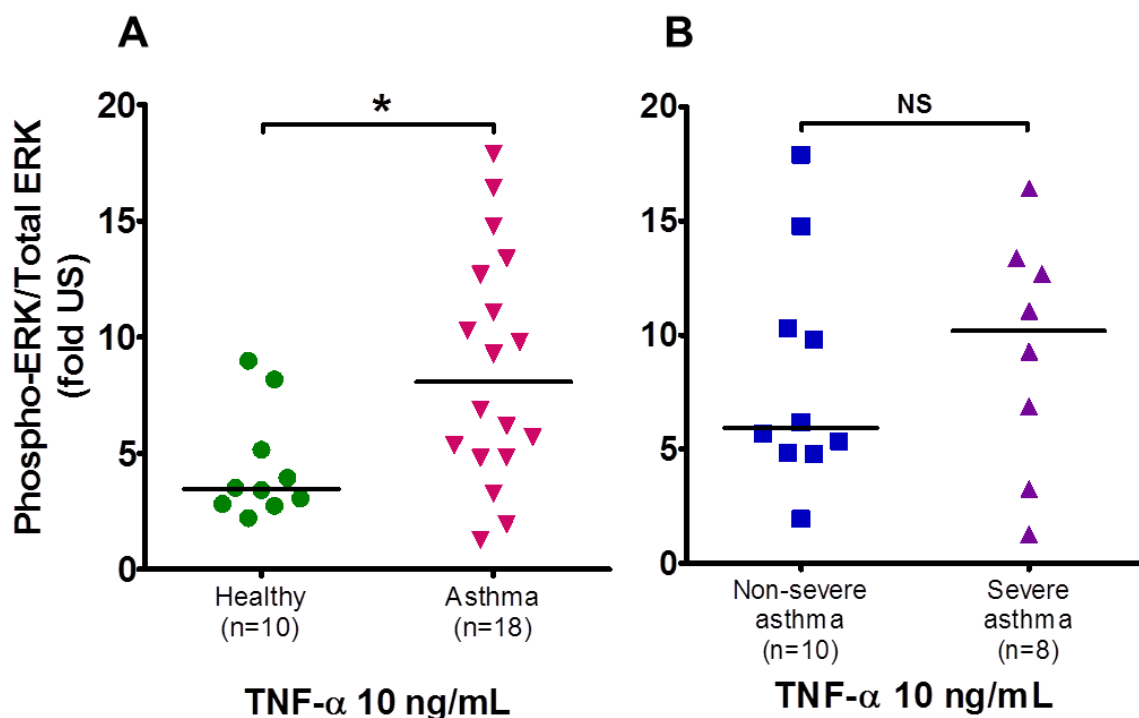


Figure 5.4. Comparison of induced ERK phosphorylation in ASMC of healthy subjects and patients with non-severe and severe asthma. ASMC were stimulated with TNF- α (10 ng/mL) for 15 minutes. Phosphorylated and total ERK expression in ASMC of (A) healthy subjects and patients with (A, B) non-severe and severe asthma were measured by Western Blot. Horizontal lines represent median. * $p<0.05$.

5.2.5 Correlation between induced MAPK phosphorylation and suppressive effect of dexamethasone in ASMC

It was observed that phosphorylation of all MAPK was increased in ASMC of patients with asthma, while only p38 phosphorylation was greater in ASMC of severe asthmatics compared to non-severe asthmatics (5.3.2-5.3.4). Data from healthy subjects and patients with non-severe and severe asthma were combined, and Spearman's rank-order correlation was performed to determine a correlation between MAPK activation and suppressive effect of dexamethasone on TNF α -induced release of CCL11 or CXCL8. Induced p38 phosphorylation correlated inversely to the degree of suppression of both induced CCL11 ($r=-0.50$, $p=0.007$; Fig. 5.5A) and CXCL8 ($r=-0.45$, $p=0.018$; Fig. 5.5B). In contrast, there was no significant correlation between either induced JNK (Fig. 5.6) or ERK (Fig. 5.7) phosphorylation and suppressive effect of dexamethasone on induced chemokines.

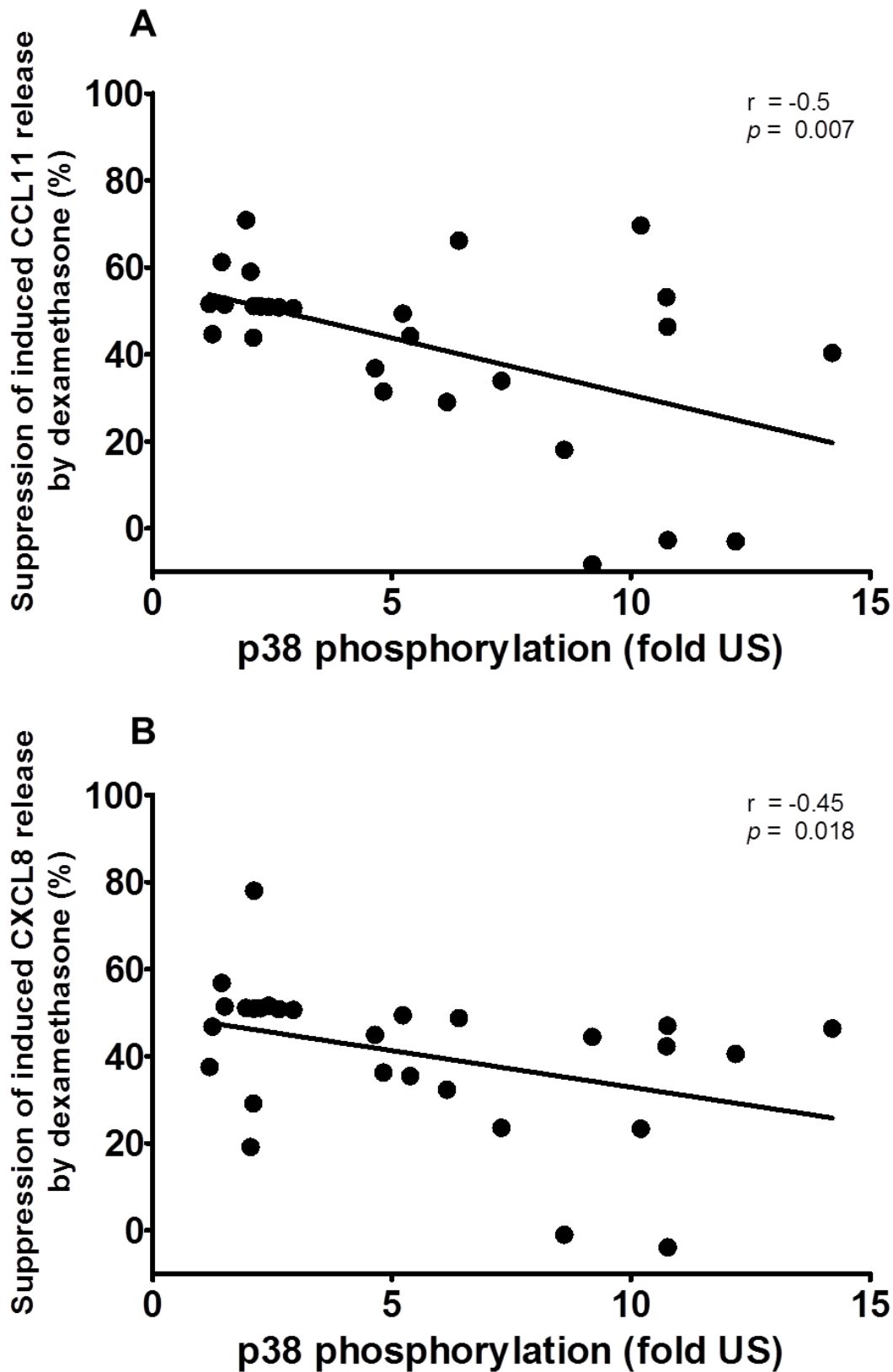


Figure 5.5. Correlation between suppression of induced (A) CCL11 and (B) CXCL8 release by dexamethasone and phosphorylation of p38 MAPK induced by TNF- α in ASMC.

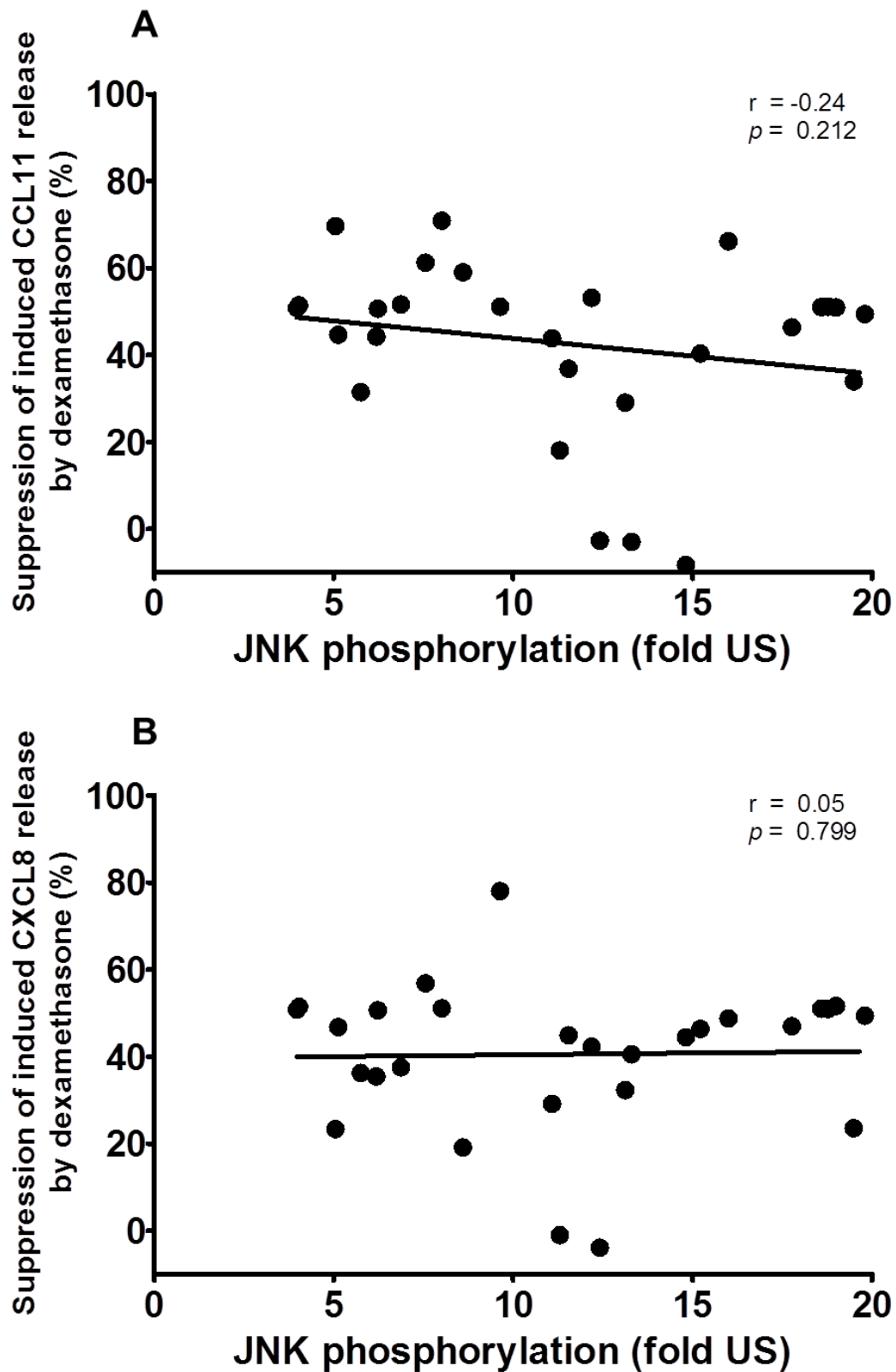


Figure 5.6. Correlation between suppression of induced (A) CCL11 and (B) CXCL8 release by dexamethasone and phosphorylation of JNK induced by TNF- α in ASMC.

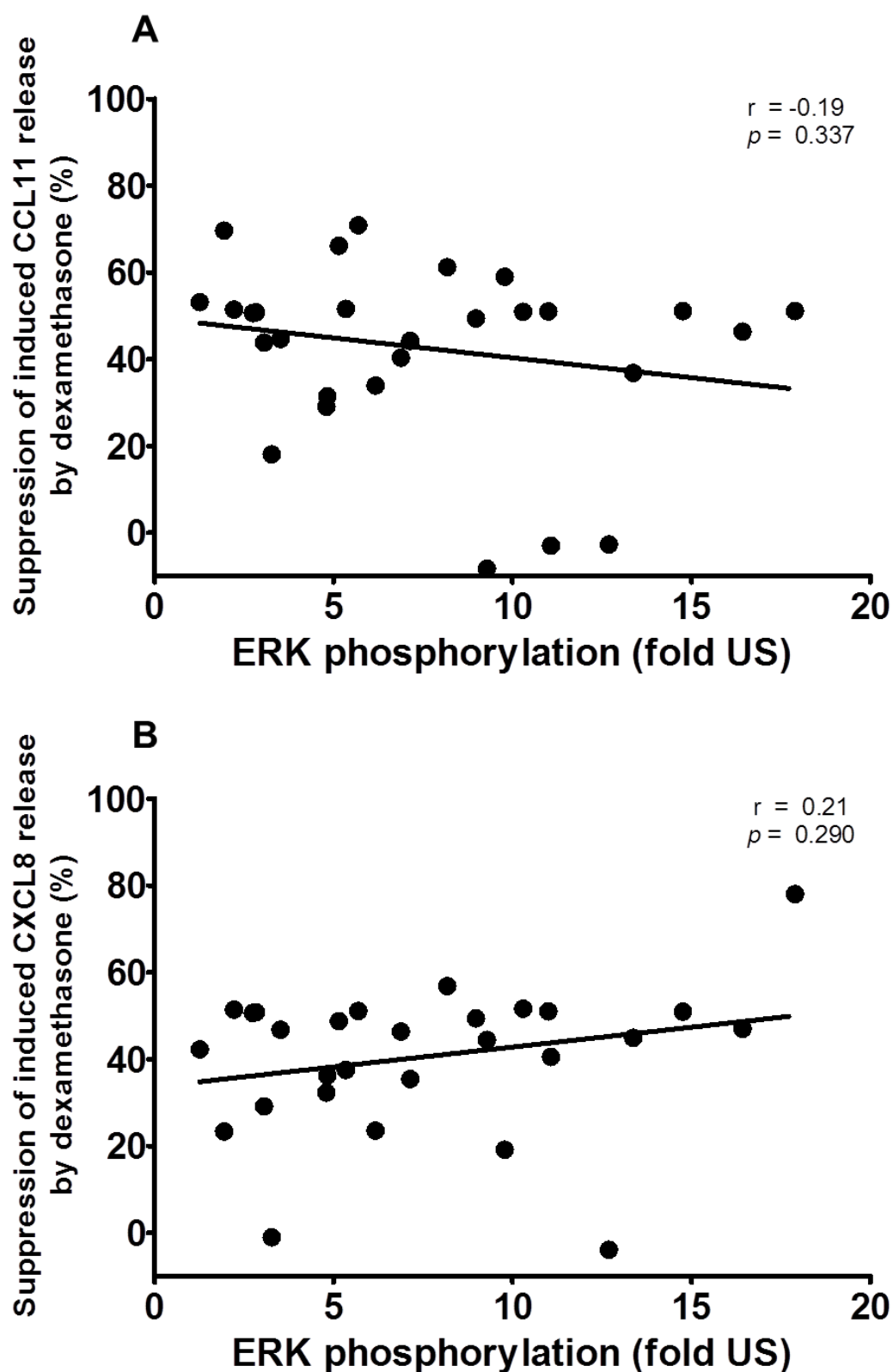


Figure 5.7. Correlation between suppression of induced (A) CCL11 and (B) CXCL8 release by dexamethasone and phosphorylation of ERK induced by TNF- α in ASMC.

5.2.6 Effect of p38 inhibition on chemokine release and suppressive effect of dexamethasone in ASMC of patients with severe asthma

A selective p38 α / β inhibitor, GW-856553, was used to investigate a role for p38 MAPK activity in chemokine release and dexamethasone-mediated suppression. To determine the specificity of this inhibitor for p38 MAPK activity, ASMC of healthy subjects were pretreated by GW-856553 (10^{-10} - 10^{-6} M) for 2 hours and stimulated with TNF- α (10 ng/mL) for 15 minutes. Phosphorylated and total p38, JNK and ERK were measured by Western Blot. TNF- α induced a 2-fold increase in p38 phosphorylation, which was suppressed by GW-856553 in a concentration-dependent manner. Phosphorylation of p38 was reduced to its baseline level by the inhibitor at 10^{-6} M (Fig. 5.8A). In contrast, TNF α -induced JNK and ERK phosphorylation was not modulated by GW-856553 (Fig. 5.8 B and C).

To investigate the role of p38 MAPK activation in chemokine release from ASMC of patients with severe asthma, cells were pretreated with GW-853556 (10^{-10} - 10^{-6} M) for 2 hours and stimulated with TNF- α (10 ng/mL) for 24 hours. CCL11 and CXCL8 release was measured by ELISA. TNF α -induced CCL11 was not significantly influenced by GW-856553 (Fig. 5.9A). In contrast, induced CXCL8 was partially inhibited in a concentration-dependent manner by GW-856553, with maximal suppression by 32% at 10^{-6} M (Fig. 5.9B; $p<0.01$).

To determine a role for p38 MAPK activation in corticosteroid insensitivity in severe asthma, ASMC of severe asthmatics, were pretreated with GW-856553 (10^{-8} or 10^{-6} M) and/or dexamethasone (10^{-9} - 10^{-6} M) for 2 hours and stimulated with TNF- α (10 ng/mL) for 24 hours. In the absence of the inhibitor, dexamethasone at 10^{-6} M suppressed induced CCL11 release by 11.9%, with GW-856553 at 10^{-6} M,

dexamethasone at the same concentration inhibited induced CCL11 by up to 55.0% (Fig. 5.10A; $p < 0.05$ vs vehicle control). Similarly, with GW-856553 at 10^{-6} M, dexamethasone (10^{-6} M) suppression of induced CXCL8 release was improved from 32.5% to 65.2% (Fig. 5.10B; $p < 0.01$).

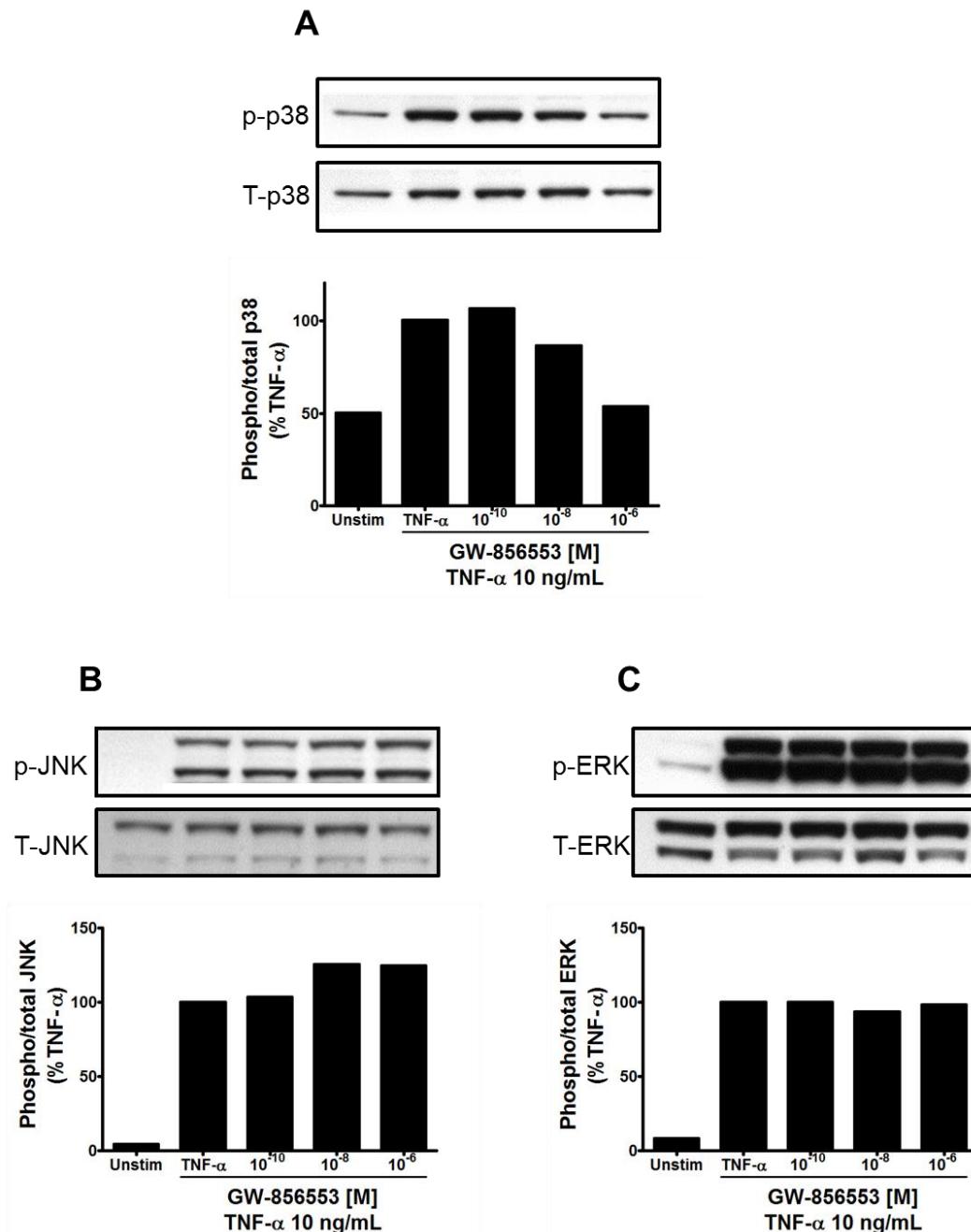


Figure 5.8. Effect of GW-856553 on MAPK activities in ASMC. Cells were pretreated with GW-856553 (10^{-10} - 10^{-6} M) for 2 hours and stimulated with TNF- α (10 ng/mL) for 15 minutes. Phosphorylated and total (A) p38, (B) JNK and (C) ERK were measured by Western Blot. Bars represent mean of 2 ASMC from healthy subjects.

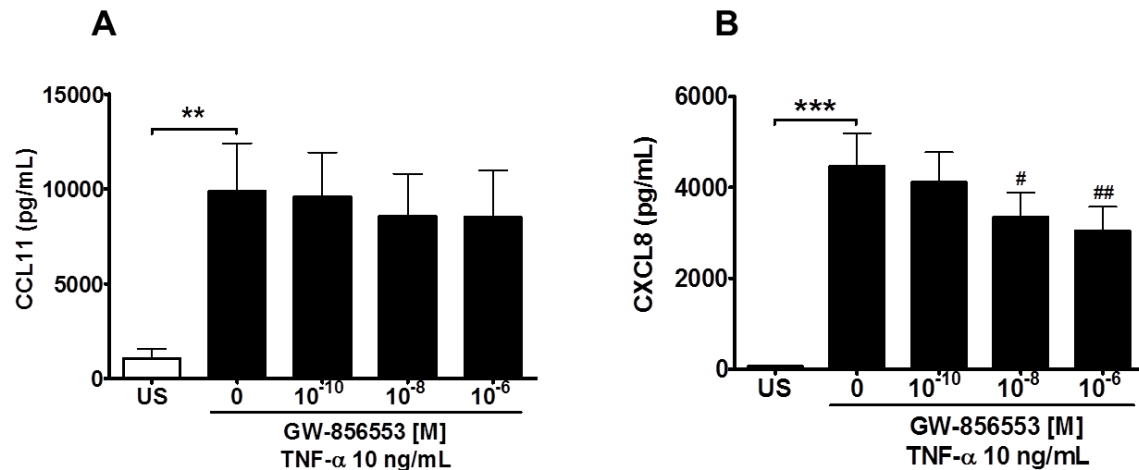


Figure 5.9. Effect of p38 MAPK inhibitor on chemokine release in ASMC of patients with severe asthma. Cells were pretreated with GW-856553 (10^{-10} - 10^{-6} M) for 2 hours and stimulated with TNF- α (10 ng/mL) for 24 hours. (A) CCL11 and (B) CXCL8 release was measured by ELISA. Bars represent mean $\pm$ SEM of ASMC from 6 patients with asthma. # $p < 0.05$, ## $p < 0.01$ vs TNF- α alone. ** $p < 0.01$, *** $p < 0.001$.

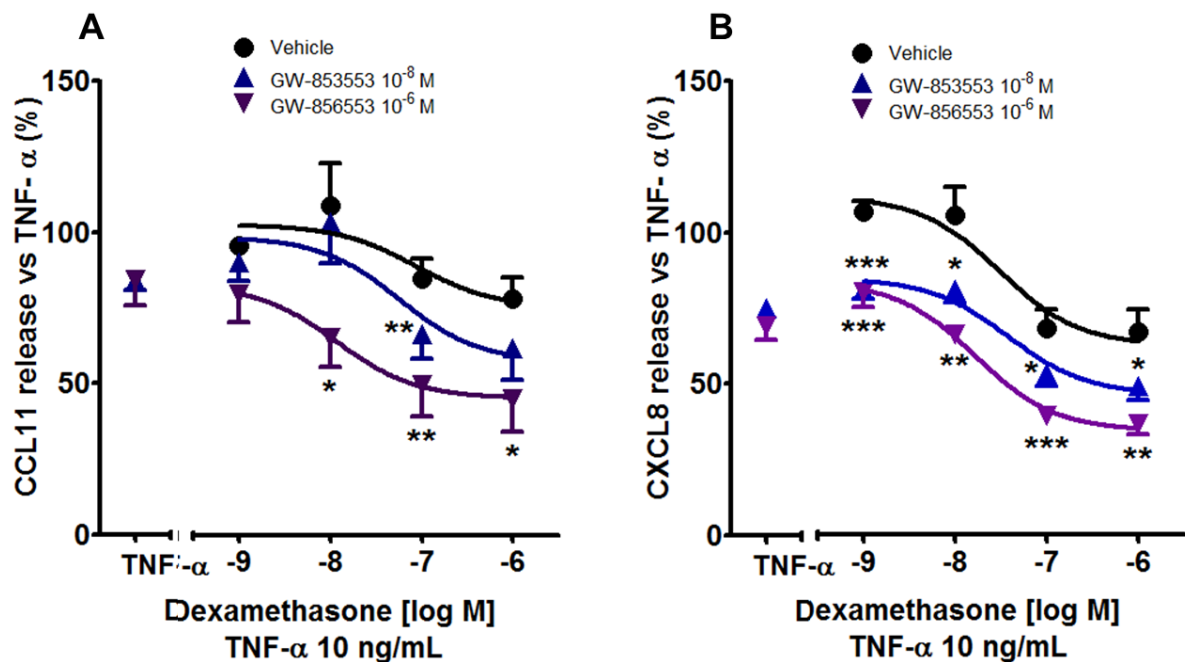


Figure 5.10. Effect of p38 MAPK inhibition on the response of dexamethasone in ASMC of patients with severe asthma. Cells were pretreated with GW-856553 (10^{-8} or 10^{-6} M) and/or dexamethasone (10^{-9} - 10^{-6} M) for 2 hours and stimulated with TNF- α (10 ng/mL) for 24 hours. (A) CCL11 and (B) CXCL8 release was measured by ELISA. Points represent mean $\pm$ SEM of ASMC from 6 patients with severe asthma. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs vehicle.

5.2.7 Effect of JNK inhibition on chemokine release and suppressive effect of dexamethasone in ASMC of patients with severe asthma

To investigate a role for JNK activation in chemokine release from ASMC of patients with severe asthma, cells were pretreated with a selective JNK inhibitor, SP600125 (10^{-10} - 10^{-6} M) for 2 hours and stimulated with TNF- α (10 ng/mL) for 24 hours. CCL11 and CXCL8 release was measured by ELISA. CCL11 induced by TNF- α was not modulated by SP600125 (Fig. 5.11A), whereas TNF α -induced CXCL8 was slightly but significantly increased by the inhibitor at 10^{-6} M (Fig. 5.11B; $p < 0.01$).

To determine a role for JNK activation in corticosteroid insensitivity in ASMC of patients with severe asthma, cells were pretreated with SP600125 (10^{-10} or 10^{-8} M) and/or dexamethasone (10^{-9} - 10^{-6} M) for 2 hours and stimulated with TNF- α (10 ng/mL) for 24 hours. The suppressive effect of dexamethasone on induced CCL11 and CXCL8 was not affected by SP600125 (Fig. 5.12A and B).

5.2.8 Effect of ERK inhibition on chemokine release and suppressive effect of dexamethasone in ASMC of patients with severe asthma

To investigate a role for ERK activation in chemokine release, ASMC of patients with severe asthma were pretreated with a selective ERK inhibitor, U0126 (10^{-10} - 10^{-6} M) for 2 hours and stimulated with TNF- α (10 ng/mL) for 24 hours. CCL11 and CXCL8 release was measured by ELISA. U0126 partially inhibited TNF α -induced CCL11 in a concentration-dependent manner, with maximal suppression of 37% at 10^{-6} M (Fig.

5.13A). In contrast, TNF α -induced CXCL8 was not suppressed by U0126 at lower concentrations but was paradoxically potentiated by 76% at 10^{-6} M (Fig. 5.13B).

To determine a role for ERK activation in corticosteroid insensitivity in severe asthma, ASMC of patients with severe asthma were pretreated with U0126 (10^{-10} or 10^{-8} M) and/or dexamethasone (10^{-9} - 10^{-6} M) for 2 hours and stimulated with TNF- α (10 ng/mL) for 24 hours. The suppressive effect of dexamethasone on induced CCL11 (Fig. 5.14A) and CXCL8 (Fig. 5.14B) was not modulated by U0126.

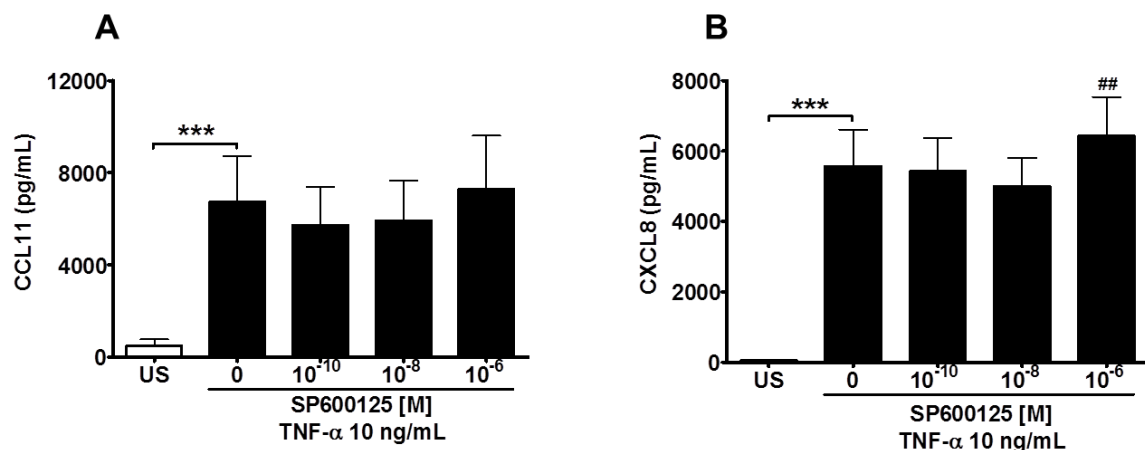


Figure 5.11. Effect of JNK inhibitor on chemokine release in ASMC of patients with severe asthma. Cells were pretreated with SP600125 (10^{-10} - 10^{-6} M) for 2 hours and stimulated with TNF- α (10 ng/mL) for 24 hours. (A) CCL11 and (B) CXCL8 release was measured by ELISA. Bars represent mean $\pm$ SEM of ASMC from 5-6 patients with severe asthma. ## $p < 0.01$ vs TNF- α alone. *** $p < 0.001$.

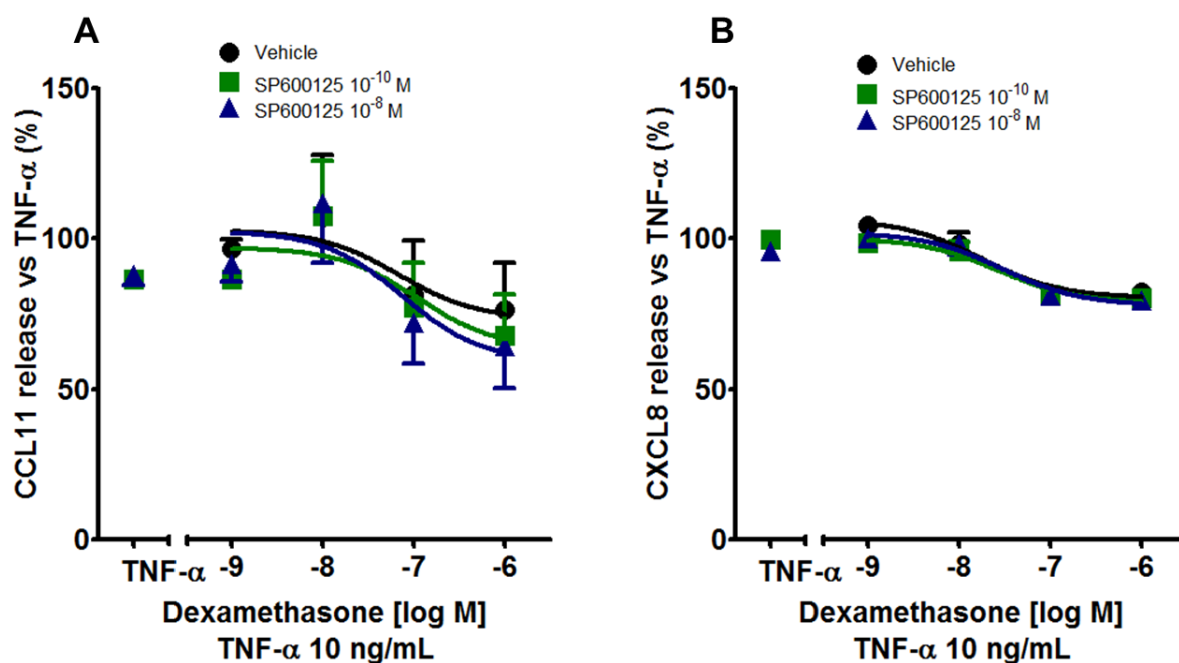


Figure 5.12. Effect of JNK inhibitor on the response of dexamethasone in ASMC of patients with severe asthma. Cells were pretreated with SP600125 (10^{-10} or 10^{-8} M) and/or dexamethasone (10^{-9} - 10^{-6} M) for 2 hours and stimulated with TNF- α (10 ng/mL) for 24 hours. (A) CCL11 and (B) CXCL8 release was measured by ELISA. Bars and points represent mean $\pm$ SEM of ASMC from 5-6 patients with severe asthma.

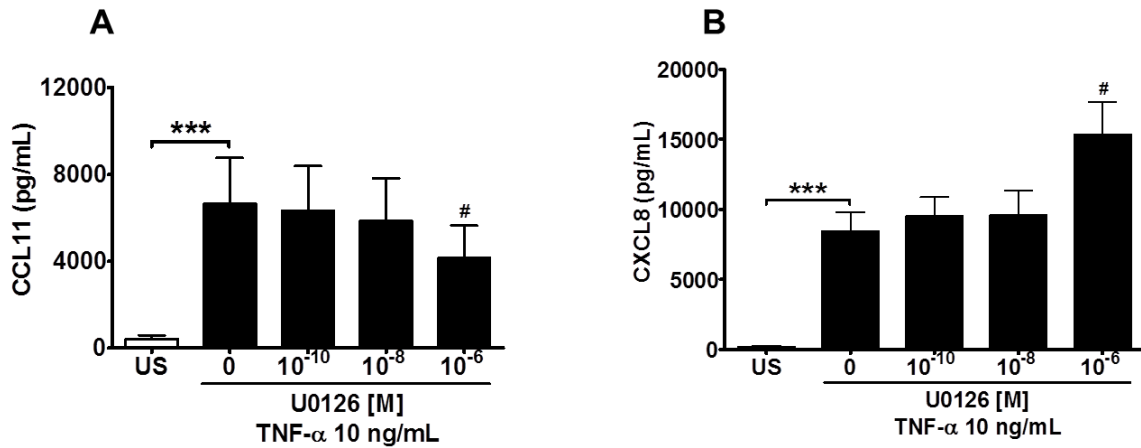


Figure 5.13. Effect of ERK inhibitor on chemokine release in ASMC of patients with severe asthma. Cells were pretreated with U0126 (10^{-10} - 10^{-6} M) for 2 hours and stimulated with TNF- α (10 ng/mL) for 24 hours. (A) CCL11 and (B) CXCL8 release was measured by ELISA. Bars represent mean $\pm$ SEM of ASMC from 6 patients with severe asthma # $p < 0.05$ vs TNF- α alone. *** $p < 0.001$.

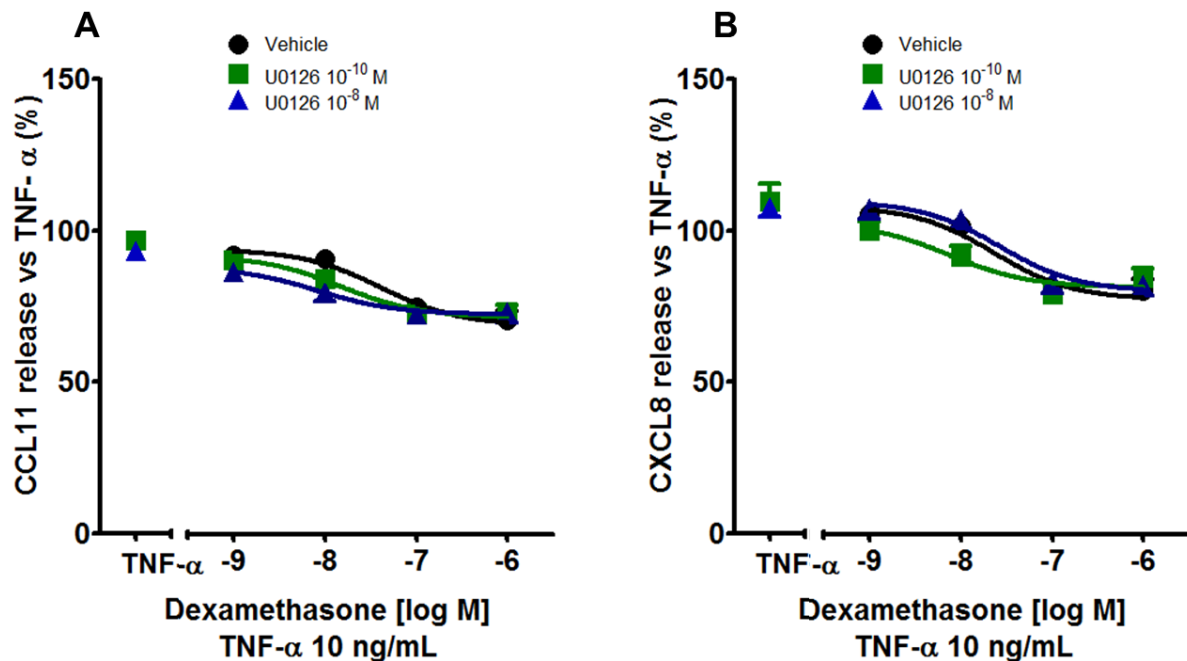


Figure 5.14. Effect of ERK inhibitor on chemokine release in ASMC of patient with severe asthma. Cells were pretreated with U0126 (10^{-10} or 10^{-8} M) and/or dexamethasone (10^{-9} - 10^{-6} M) for 2 hours and stimulated with TNF- α (10 ng/mL) for 24 hours. CCL11 (C) and CXCL8 (D) release was measured by ELISA. Points represent mean $\pm$ SEM of ASMC from 6 patients with severe asthma.

5.2.9 Effect of IFN- γ on MAPK activity induced by TNF- α in ASMC

Based on the observation that p38 phosphorylation was greater in ASMC of patients with severe asthma (5.3.2) and that p38 α/β inhibition improved the suppressive effect of dexamethasone on induced CCL11 and CXCL8 release in those of severe asthmatics (5.3.6), a role for heightened p38 activity in corticosteroid insensitivity in ASMC of severe asthmatics was implicated. Given that IFN- γ impaired suppressive of dexamethasone on the same chemokines (4.2.3.2 and 4.2.3.3), the effect of IFN- γ on induced MAPK was also investigated.

ASMC were stimulated with TNF- α and/or IFN- γ (10 ng/mL) for 15 minutes. Phosphorylated and total p38, JNK and ERK were measured by Western Blot. In ASMC of healthy subjects, phosphorylation of p38 was induced by TNF- α but not IFN- γ , and IFN- γ did not modulate TNF α -induced p38 phosphorylation (Fig. 5.15A). This effect was consistent also in cells of patients with non-severe and severe asthma (Fig. 5.15B and C).

Similarly, in ASMC of healthy subjects, TNF- α but not IFN- γ induced phosphorylation of JNK and ERK, and this phosphorylation was not influenced by IFN- γ . ASMC of non-severe and severe asthmatics responded similarly to IFN- γ (Fig. 5.16 and 5.17).

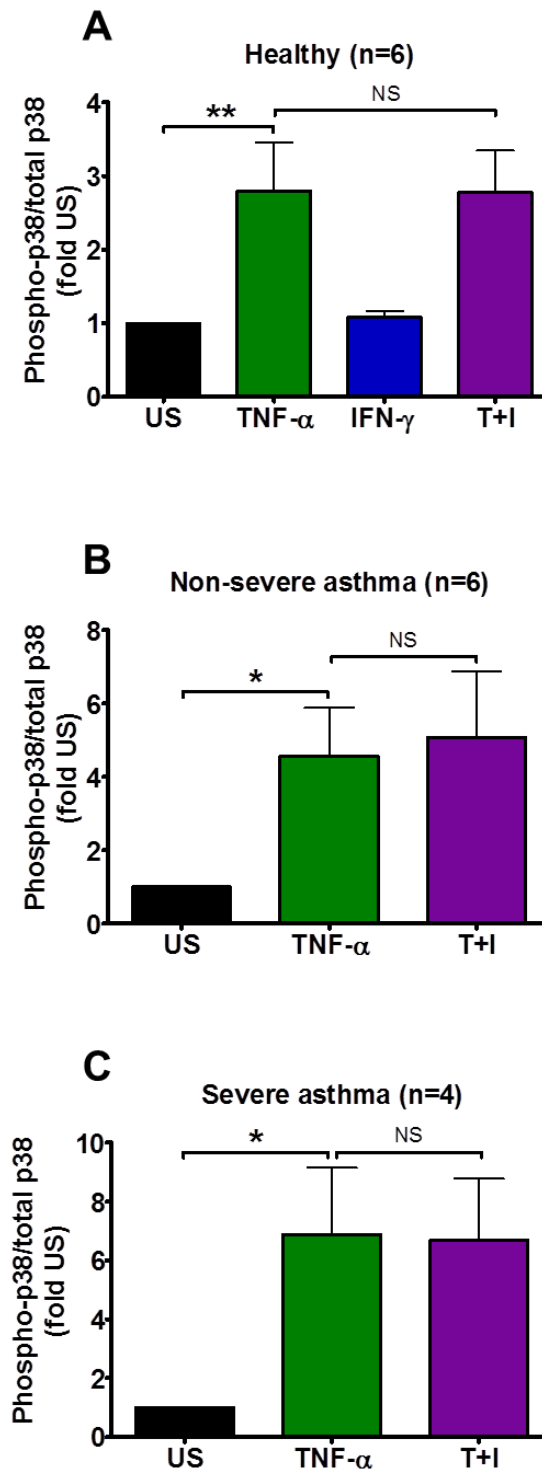


Figure 5.15. Effect of IFN- γ on TNF α -induced p38 MAPK phosphorylation. ASMC of (A) healthy subjects and patients with (B) non-severe and (C) severe asthma were stimulated with TNF- α and/or IFN- γ (10 ng/mL each) for 15 minutes. Phosphorylated and total p38 were measured by Western Blot. Bars represent mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$.

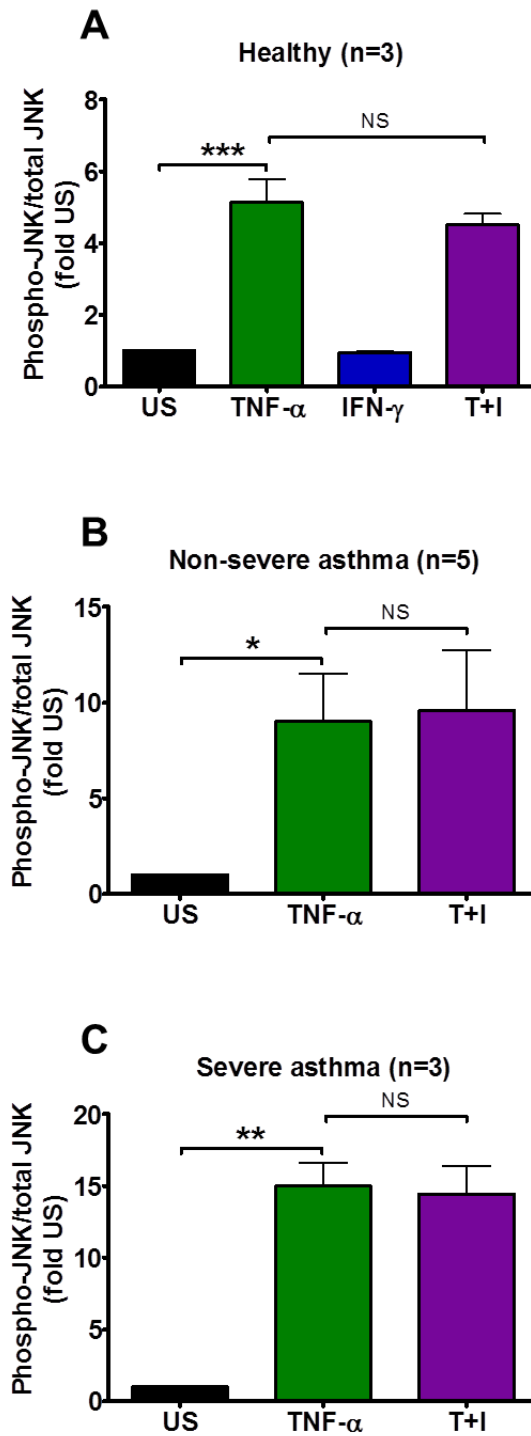


Figure 5.16. Effect of IFN- γ on TNF α -induced JNK phosphorylation. ASMC of (A) healthy subjects and patients with (B) non-severe and (C) severe asthma were stimulated with TNF- α and/or IFN- γ (10 ng/mL each) for 15 minutes. Phosphorylated and total JNK were measured by Western Blot. Bars represent mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

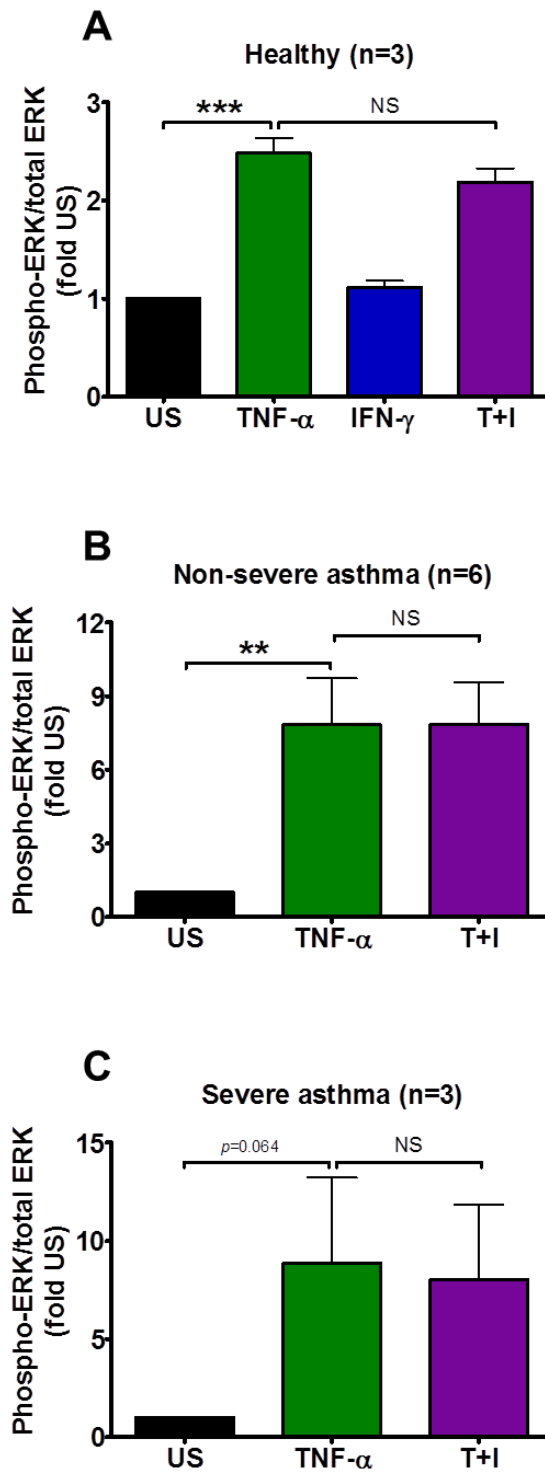


Figure 5.17. Effect of IFN- γ on TNF α -induced ERK phosphorylation. ASMC of (A) healthy subjects (A) and patients with (B) non-severe and (C) severe asthma were stimulated with TNF- α and/or IFN- γ (10 ng/mL each) for 15 minutes. Phosphorylated and total JNK were measured by Western Blot. Bars represent mean $\pm$ SEM. ** $p < 0.01$, *** $p < 0.001$.

5.3 Discussion

Summary of results:

TNF α -induced p38 MAPK activation was heightened in ASMC of patient with severe asthma, which inversely correlated to dexamethasone suppression of inflammatory chemokines. In contrast, activation of JNK and ERK was increased in ASMC of patients with asthma, while there was no difference in those between non-severe and severe asthma. Furthermore, inhibition of p38 α/β activity improved the suppressive effect of dexamethasone in severe asthma. IFN- γ , which impairs the suppressive effect of dexamethasone in ASMC, did not modulate MAPK activities.

Of the four isoforms of p38 MAPK, the α and β isoforms are highly effective at activating downstream kinases, such as MAPKAP 2/3 and MSK 1/2, and activate several transcription factors. In contrast, the γ and δ isoforms are less effective in activating downstream kinases (Adcock, Chung et al. 2006). Unlike the earlier compounds that caused undesirable liver toxicity, the second-generation of p38 MAPK inhibitors have considerably reduced effect on cytochrome P450 and result in less side effects (Adams, Boehm et al. 1998). GW-856553 (losmapimod), a selective p38 α/β inhibitor developed by GlaxoSmithKline (Aston, Bamborough et al. 2009), belongs to the nicotinamide class of p38 inhibitors. The therapeutic role of this drug has been extensively studied in clinical trials of several diseases including major depression, neuropathic pain, rheumatoid arthritis, myocardial infarction and airway diseases (Lomas, Lipson et al. 2012). In a phase II clinical trial, GW-856553 improves hyperinflation and reduces plasma fibrinogen in patients with COPD (Lomas, Lipson et al. 2012).

GW-856553 has a $pK_i=8.1$ and a $pK_i=7.6$ for $p38\alpha$ and $p38\beta$, respectively, and demonstrates 100-fold greater selectivity for both the α and β isoforms against a panel of 67 human kinases (Goldstein, Kuglstatter et al. 2010). The specificity of this molecule is further confirmed by the observations that GW-856553 suppresses $TNF\alpha$ -induced $p38$ phosphorylation to a baseline level, while induced JNK and ERK phosphorylation is not modulated by the inhibitor (5.2.6). This effect is consistent with that reported in PBMC (Bhavsar, Khorasani et al. 2010) and supported by studies demonstrating that $p38$ inhibitors prevent auto-phosphorylation (Galan, Garcia-Bermejo et al. 2000; Matsuguchi, Musikacharoen et al. 2000; Ge, Gram et al. 2002).

Heightened $p38$ activity in severe asthma (5.2.2) is now extended from alveolar macrophages (Bhavsar, Hew et al. 2008) and airway epithelial cells (Liu, Liang et al. 2008) to ASMC. A role for increased $p38$ activity in corticosteroid insensitivity in severe asthma is suggested by an inverse correlation between $p38$ phosphorylation and the suppressive effects of dexamethasone on inflammatory chemokines (5.2.5). This is further confirmed by the observations of improved dexamethasone suppression of CCL11 and CXCL8 release in ASMC of severe asthma (5.2.6) to a level which is achieved in those of non-severe asthma (4.2.2.1 and 4.2.2.2). These findings indicate that, in addition to $p38\gamma$ (Mercado, To et al. 2011), $p38 \alpha/\beta$ inhibition restores corticosteroid sensitivity in severe asthma, and this effect extends from PMBC (Bhavsar, Khorasani et al. 2010) to ASMC.

In contrast to increased JNK phosphorylation in other human cell types and tissue in corticosteroid resistant severe asthma (Sousa, Lane et al. 1999; Kobayashi, Mercado et al. 2011), despite increased JNK activation in ASMC of both non-severe and severe asthma, there is no difference between the two groups (5.2.3). Similarly, while ERK activity is greater in cells of asthma, which is consistent with the literature (Jude, Solway et al. 2010; Robins, Roussel et al. 2011), there is no difference between non-severe and severe asthma (5.2.4). These observations would suggest that there may only be a limited role for JNK or ERK activation in corticosteroid insensitivity in ASMC of patients with severe asthma, which is further confirmed by an absence of correlation between JNK/ERK phosphorylation and dexamethasone suppression of chemokines (5.2.5) and lack of any modulatory effect by specific inhibitors of JNK/ERK on the suppressive effect of dexamethasone in cells of severe asthma (5.2.8 and 5.2.9).

Whereas IFN γ impairs the suppressive effects of corticosteroid on several chemokines in ASMC (4.2.3.1-4.2.3.3), absence of its modulation on any of the MAPK activities (5.2.9) suggests that IFN- γ may contribute to corticosteroid resistance through signalling pathways which are not involved in severe asthma .

In summary, there is heightened p38 MAPK activity in ASMC of patients with severe asthma, which contributes to corticosteroid insensitivity. p38 inhibition restores corticosteroid sensitivity in severe asthma and thus suggests the potential as a therapeutic target.

Chapter 6.

Regulation of GR and NF- κ B (p65)

by corticosteroids in ASMC

Chapter 6. Regulation of GR and NF- κ B (p65) by corticosteroids in ASMC

6.1 Introduction

The anti-inflammatory effects of corticosteroids are mediated through GR, a ligand-activated transcription factor that modulates inflammatory and anti-inflammatory gene expression through a variety of molecular signalling pathways. Briefly, following activation by ligand binding of a glucocorticoid, GR dissociates from chaperone proteins and rapidly translocates into the nucleus. Within the nucleus, GR either binds to specific GRE on DNA within the promoter region of corticosteroid-responsive genes to enhance transcription of anti-inflammatory genes, or represses transcription of pro-inflammatory genes by interaction with inflammatory transcription factors, such as NF- κ B and AP-1. Both of the processes involve modification of histones and alteration in chromatin structure (Bloom 2008). Insensitivity to corticosteroid may develop at multiple points in anti-inflammatory signalling pathways, including GR abnormalities (e.g. reduced GR number and ligand binding affinity), decreased translocation of GR from the cytoplasm to the nucleus, altered GR interactions with other transcription factors, and abnormalities of histone-modifying enzymes (Fig. 6.1).

GR is a phosphoprotein, and changes in its phosphorylation pattern may affect all aspects of its function (Weigel and Moore 2007). The phosphorylation of these sites can be ligand dependent or independent. GR phosphorylation of specific amino acid residues has been associated with modulation of ligand binding, nuclear translocation, DNA binding, receptor dimerization, and interaction with general

transcription factors (Duma, Jewell et al. 2006). Of the six serine residues which are characterised as phosphorylation targets in human GR, phosphorylation of GR at S211 may correlate to ligand binding, nuclear translocation and transcriptional activity and thus is a hallmark for the transactivation potential of GR (Weigel and Moore 2007; Oakley and Cidlowski 2011).

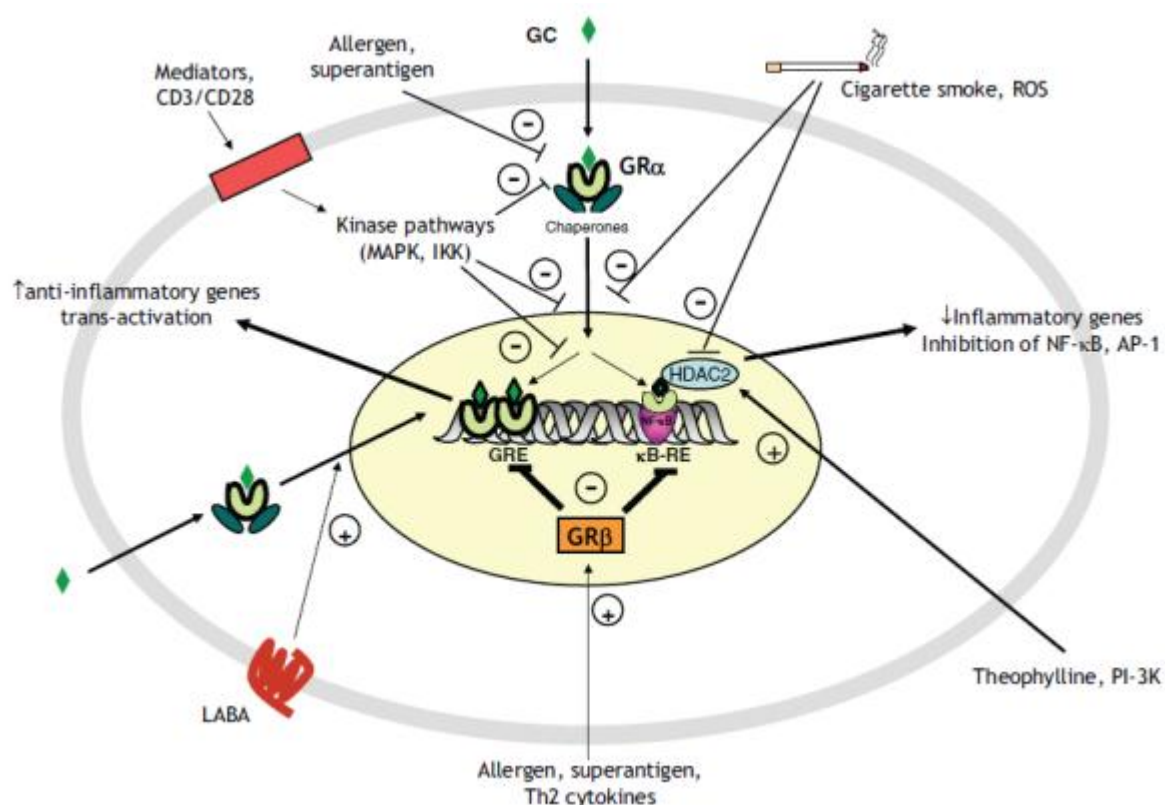


Figure 6.1. Mechanisms of glucocorticoid action by GR and sites of regulation in corticosteroid insensitivity. The activation of kinase pathways by inflammatory mediators or T-cell receptor coactivation (CD3/CD28) attenuates GR function by reducing ligand binding and nuclear translocation, or by mutually suppressing/interacting with NF-κB and AP-1. Allergens and superantigens not only affect GR ligand binding but also, along with Th2 cytokines, induce GRβ. Cigarette smoke and reactive oxygen species (ROS) stress prevent GR nuclear translocation or reduce the activity of HDAC2, reducing the ability of GR to switch off inflammatory genes (Adcock and Barnes 2008).

Glucocorticoids have a major effect on ASMC function due to high expression of GR (Adcock, Gilbey et al. 1996). However, the mechanisms by which corticosteroids modulate inflammatory genes in ASMC are not fully understood. It is also unclear whether ASMC display differential regulations of GR signalling pathways between the healthy and diseased subjects. Given the observations of impaired corticosteroid suppression of inflammatory chemokine expression (4.2.2.1, 4.2.2.2, 4.2.2.5 and 4.2.2.6), which correlates to heightened p38 activity (5.2.2, 5.2.5 and 5.2.6), in ASMC of patients with severe asthma, mechanisms of GR and NF- κ B regulation in response to corticosteroids are investigated in this chapter, in terms of expression, nuclear translocation and phosphorylation. The interaction between TNF α -induced p38 activity and nuclear translocation of GR are also explored.

6.2 Results

6.2.1 Investigation and comparison of GR regulation in ASMC

6.2.1.1 Comparison of GR abundance in ASMC of healthy subjects and patients with non-severe and severe asthma

The abundance of GR in ASMC at baseline was compared. After serum starvation for 24 hours, whole cell lysates were collected. Total GR and β -actin were measured by Western Blot and normalised to the standard protein (described in 2.2.7.1). GR expression in ASMC of patients with asthma was 49% of that expressed in cells of healthy subjects (Fig. 6.2 A and B; $p < 0.01$). However, there was no significant difference between patients with non-severe and severe asthma (Fig. 6.2 A and C).

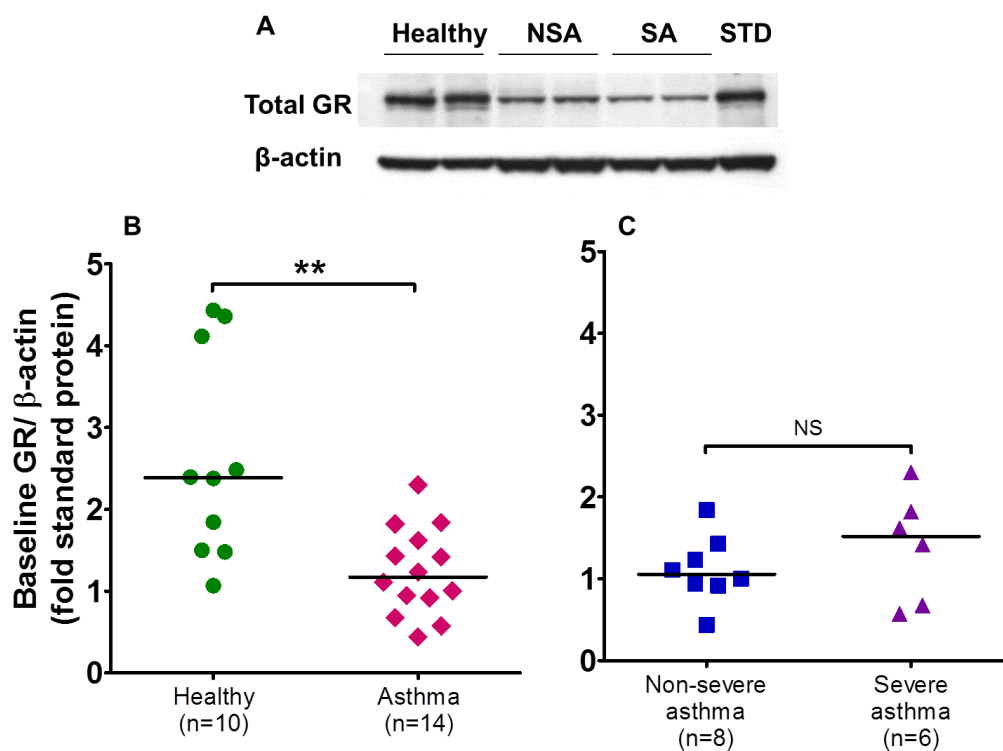


Figure 6.2. Comparison of baseline GR expression in ASMC of healthy subjects and patients with non-severe (NSA) and severe asthma (SA). GR and β -actin in the whole cell lysates were measured by Western Blot. (A) A representative blot with duplicates for each group. (B) Comparison of ASMC of healthy subjects and patients with asthma. (C) Comparison of ASMC of non-severe and severe asthmatics. Horizontal lines represent median. ** $p < 0.01$.

6.2.1.2 Effect of dexamethasone on GR expression in ASMC

To determine the effect of dexamethasone on total GR expression in ASMC, cells of healthy subjects were stimulated with dexamethasone (10^{-6} M) over a time course (5 minutes to 24 hours). Total GR and β -actin expression in the whole cell lysates was measured by Western Blot. Dexamethasone did not influence GR expression up to 4 hours post-stimulation. However, after 24 hours, the baseline abundance of total GR was suppressed by 57.5% (Fig. 6.3; $p < 0.01$).

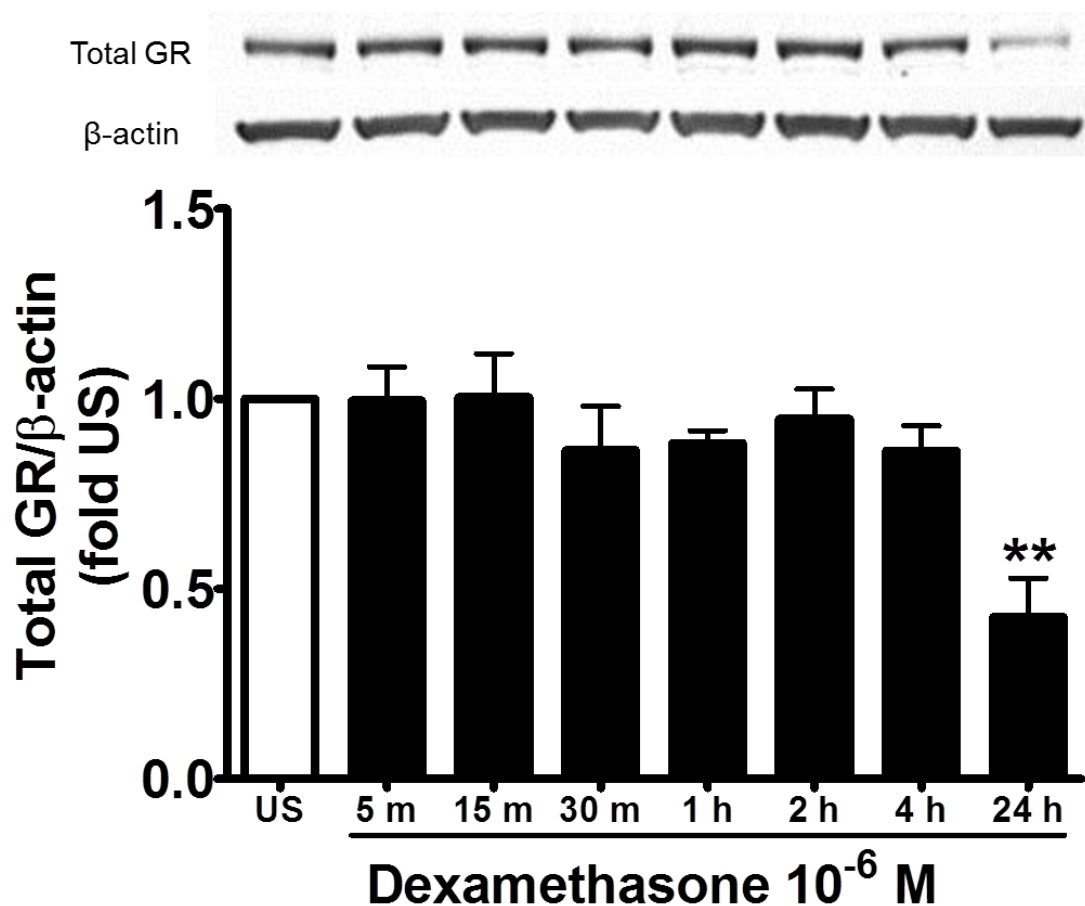


Figure 6.3. Effect of dexamethasone on GR expression in ASMC. Cells were stimulated with dexamethasone (10^{-6} M) for time indicated. Total GR and β -actin in the whole cell lysates were measured by Western Blot. Bars represent mean $\pm$ SEM of ASMC from 3 healthy subjects. ** $p < 0.01$ vs unstimulated.

6.2.1.3 Effect of dexamethasone on nuclear translocation of GR in ASMC

To investigate the effect of dexamethasone on nuclear translocation of GR in ASMC, cells of healthy subjects were stimulated with dexamethasone (10^{-7} M) for 30 minutes to 4 hours. GR, TBP and α -tubulin in both nuclear and cytoplasmic extracts were measured by Western Blot. Nuclear GR abundance was increased by dexamethasone at 30 minutes and was sustained for 4 hours, while cytoplasmic GR displayed a reciprocal effect (Fig. 6.4). These results confirm that nuclear translocation of GR is induced by dexamethasone in ASMC.

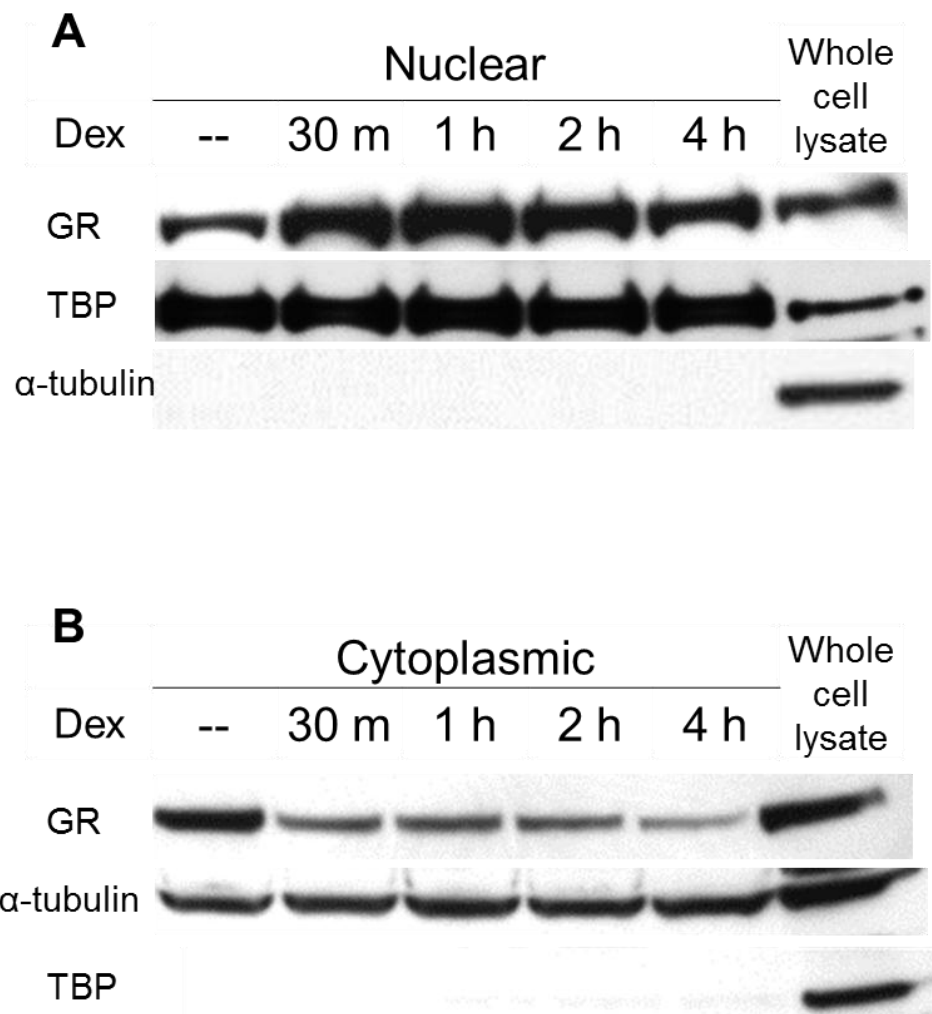


Figure 6.4. Effect of dexamethasone on nuclear translocation of GR in ASMC. Cells were stimulated with dexamethasone (10^{-7} M) for time indicated. GR, TBP and α -tubulin in (A) nuclear and (B) cytoplasmic extracts were assessed by Western Blot. A representative blot of ASMC from 2 healthy subjects is shown.

6.2.1.4 Comparison of baseline nuclear abundance of GR in ASMC of healthy subjects and patients with non-severe and severe asthma

Baseline nuclear GR abundance in ASMC at baseline was compared between the three groups. After serum starvation for 24 hours, nuclear extracts were collected. GR and TBP were measured by Western Blot and normalised to the standard protein (described in 2.2.7.1). The quantity of nuclear GR was similar among ASMC of healthy subjects and patients with non-severe and severe asthma (Fig. 6.5).

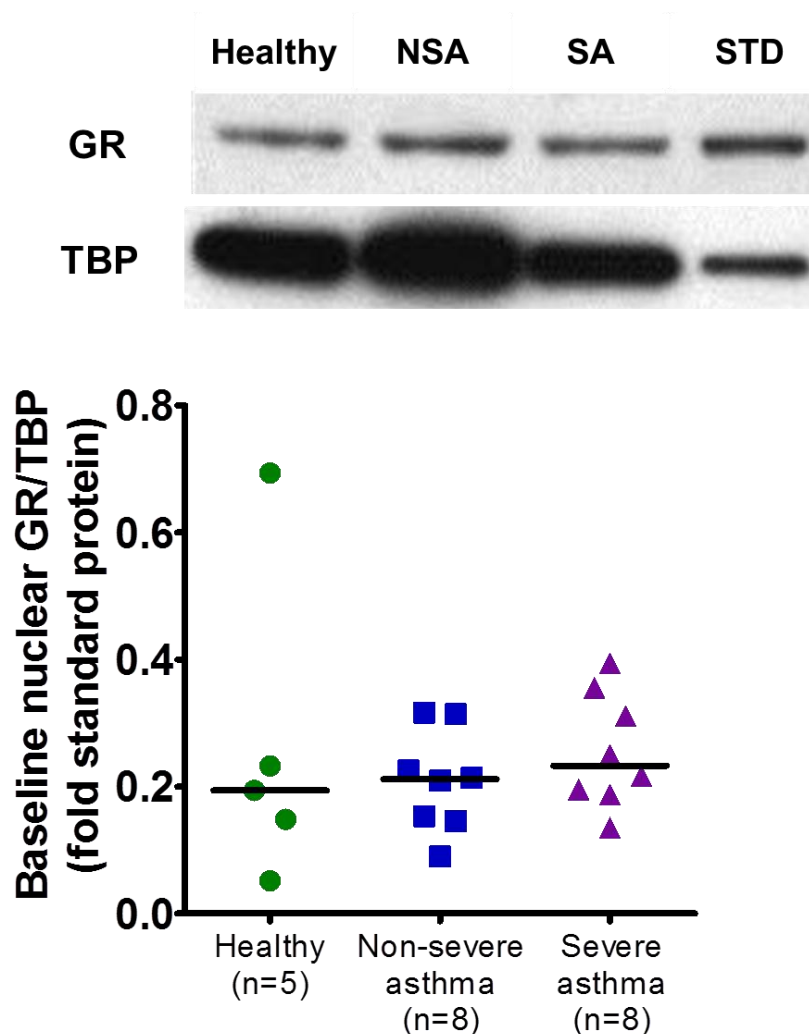


Figure 6.5. Comparison of baseline abundance of nuclear GR in ASMC of healthy subjects and patient with non-severe (NSA) and severe asthma (SA). GR and TBP in the nuclear extracts were measured by Western Blot. A representative blot is shown. Horizontal lines represent median. STD: standard protein.

6.2.1.5 Comparison of nuclear translocation of GR induced by dexamethasone in ASMC of healthy subjects and patients with non-severe and severe asthma

To compare nuclear translocation induced by dexamethasone in ASMC, cells were stimulated with dexamethasone (10^{-7} M) for 30 minutes to 4 hours. GR and TBP in the nuclear extracts were measured by Western Blot. In ASMC of healthy subjects and patients with non-severe asthma, dexamethasone induced approximately a 3-fold increase in GR abundance at 30 minutes, which was maintained to one hour post-stimulation, followed by a gradual decrease. Of importance, in ASMC of patients with severe asthma, nuclear GR was induced by less than 2-fold; this was significantly decreased compared to those of healthy subjects and patients with non-severe asthma at all time points (Fig. 6.6).

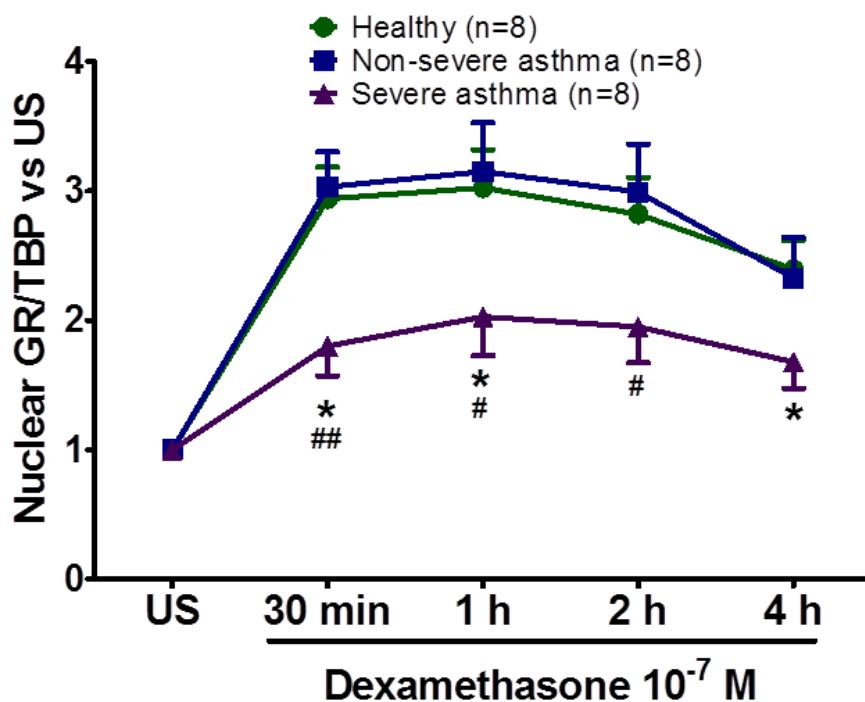


Figure 6.6. Comparison of dexamethasone-induced GR nuclear translocation in ASMC of healthy subjects and patients with non-severe and severe asthma. ASMC were stimulated with dexamethasone (10^{-7} M) for time indicated. GR and TBP in the nuclear extracts were measured by Western Blot. Points represent mean $\pm$ SEM. * $p < 0.05$ vs healthy subjects. # $p < 0.05$, ## $p < 0.01$ vs patients with non-severe asthma.

6.2.1.6 Effect of dexamethasone on phosphorylation of GR at S211 in ASMC

To investigate the effect of dexamethasone on phosphorylation of GR at the serine residue 211 (S211), ASMC of healthy subjects were stimulated with dexamethasone (10^{-6} M) for 5 minutes to 4 hours. Phosphorylated and total GR in the whole cell lysates were measured by Western Blot. GR phosphorylation was significantly induced by dexamethasone at 30 minutes ($p<0.01$) post-stimulation, which was further increased (5.2-fold) at one hour and maintained for 4 hours (Fig. 6.7; $p<0.001$).

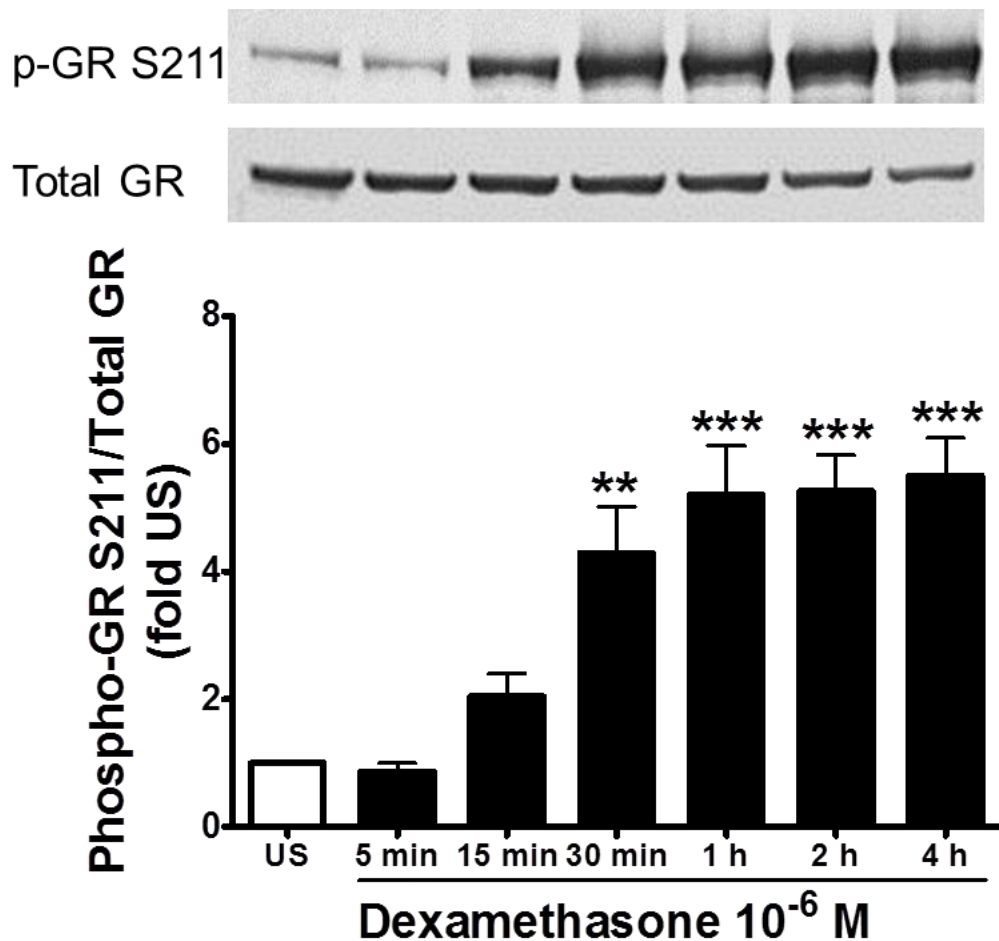


Figure 6.7. Effect of dexamethasone on GR phosphorylation at S211 in ASMC. Cells were stimulated with dexamethasone (10^{-6} M) for time indicated. Phosphorylated and total GR in the whole cell lysates were measured by Western Blot. Bars represent mean $\pm$ SEM of ASMC from 3 healthy subjects. ** $p<0.01$, *** $p<0.001$ vs unstimulated (US).

6.2.1.7 Comparison of phosphorylation of GR at S211 induced by dexamethasone in ASMC of healthy subjects and patients with non-severe and severe asthma

To compare phosphorylation of GR at S211 induced by dexamethasone in ASMC, cells were stimulated with dexamethasone (10^{-6} M) for 2 hours. Total and phosphorylated GR in the whole cell lysates were measured by Western Blot. In ASMC of healthy subjects, dexamethasone induced a 6-fold increase in GR phosphorylation at S211, and this induction was similar between the cells of the healthy and patients with asthma (Fig. 6.8).

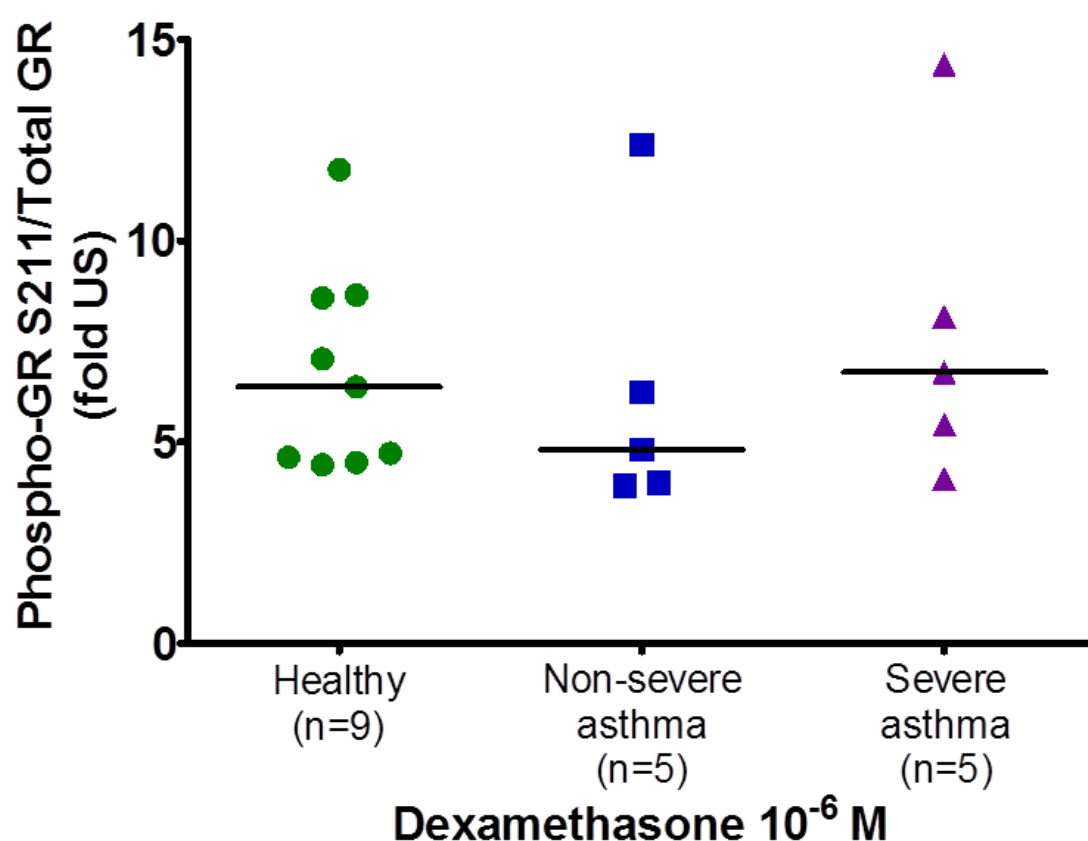


Figure 6.8. Comparison of dexamethasone-induced GR phosphorylation at S211 in ASMC of healthy subjects and patients with non-severe and severe asthma. Cells were stimulated with dexamethasone (10^{-6} M) for 2 hours. Phosphorylated and total GR in whole cell lysates were measured by Western Blot. Horizontal lines represent median.

6.2.2 Investigation of mechanisms of suppression of NF- κ B activity by corticosteroid

6.2.2.1 Comparison of the suppressive effect of dexamethasone on induced p65 protein and mRNA expression in ASMC of healthy subjects and patients with non-severe and severe asthma

To compare the suppressive effect of dexamethasone on induced p65 protein expression, ASMC were pretreated with dexamethasone (10^{-7} M) for 2 hours and then stimulated with TNF- α (10 ng/mL) for 24 hours. p65 and TBP in the whole cell lysates were measured by Western Blot. In ASMC of healthy subjects, p65 protein expression induced by TNF- α was suppressed by dexamethasone by 21.3% ($p<0.05$). This suppressive effect was similar in cells of patients with non-severe asthma ($p<0.001$) and was maintained in those of severe asthma ($p<0.01$) (Fig. 6.9A).

To compare the suppressive effect of dexamethasone on induced p65 mRNA expression, ASMC were pretreated with dexamethasone (10^{-10} - 10^{-6} M) for 2 hours and then stimulated with TNF- α (10 ng/mL) for 24 hours. p65 mRNA and 18S rRNA were measured by RT-qPCR. In ASMC of healthy subjects, TNF α -induced p65 mRNA was inhibited in a concentration-dependent manner, with maximal suppression of approximately 50% by dexamethasone at 10^{-7} and 10^{-6} M ($p<0.01$, respectively). This suppressive effect was similar in cells of patients with non-severe asthma and was maintained in those of severe asthma (Fig. 6.9B).

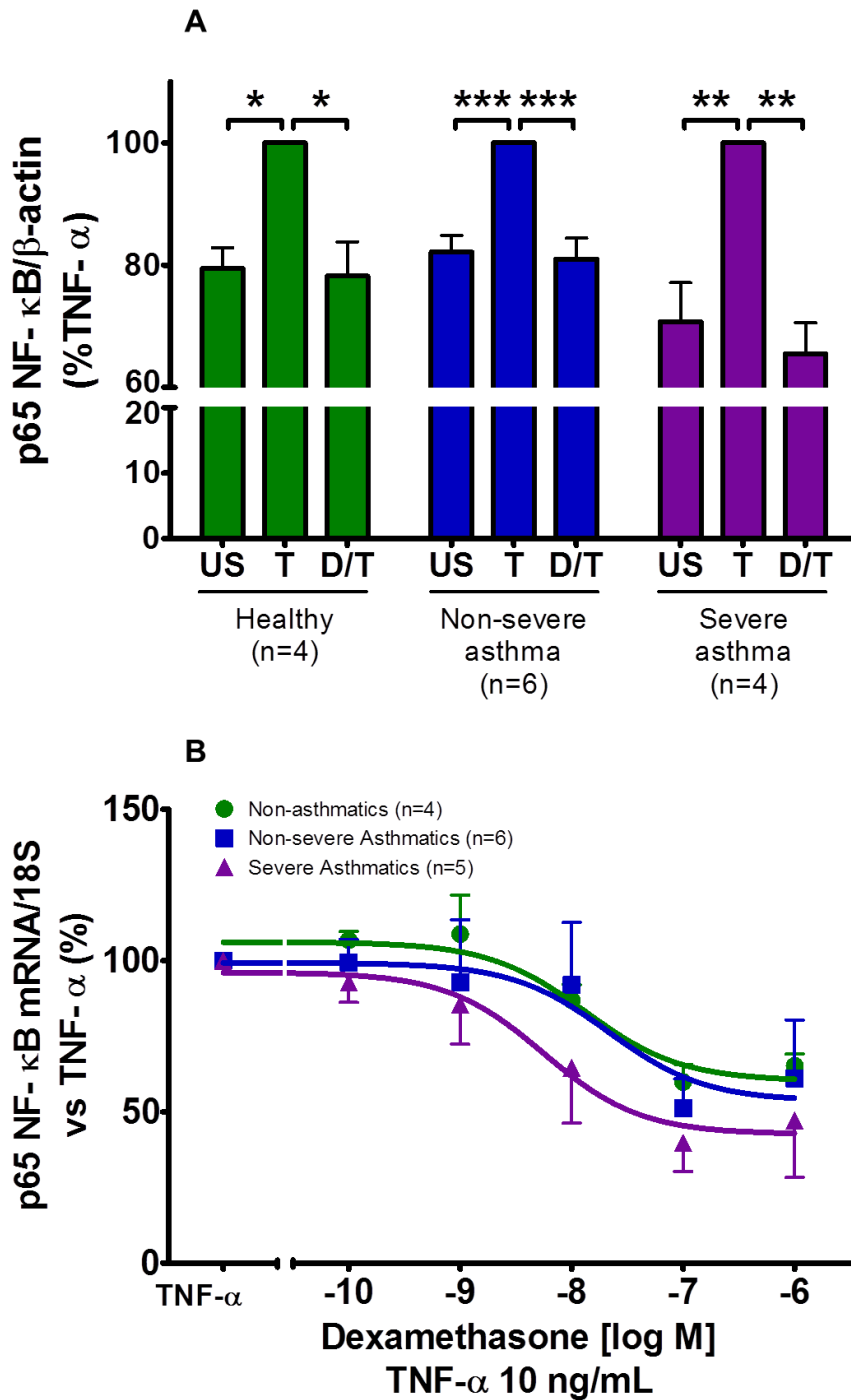


Figure 6.9. Comparison of the suppressive effect of dexamethasone on induced p65 expression in ASMC of healthy subjects and patients with non-severe and severe asthma. Cells were pretreated with dexamethasone (10^{-7} M for protein and 10^{-10} - 10^{-6} M for mRNA) for 2 hours and TNF- α (10 ng/mL) for 24 hours. p65 protein/TBP and p65 mRNA/18S were measured by Western Blot and RT-qPCR, respectively. Bars and points represent mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

6.2.2.2 Effect of dexamethasone on TNF α -induced nuclear translocation of p65 in ASMC of healthy subjects and patients with non-severe and severe asthma

To investigate the effect of dexamethasone on nuclear translocation of p65 induced by TNF- α in ASMC, cells were stimulated with dexamethasone (10^{-7} M) or TNF- α (10 ng/mL) alone or in combination for 30 minutes. p65 and TBP in nuclear extracts were measured by Western Blots. TNF- α increased nuclear abundance of p65, which was not affected by dexamethasone. This effect was similar in ASMC of healthy subjects and patients with non-severe and severe asthma (Fig. 6.10 A-C).

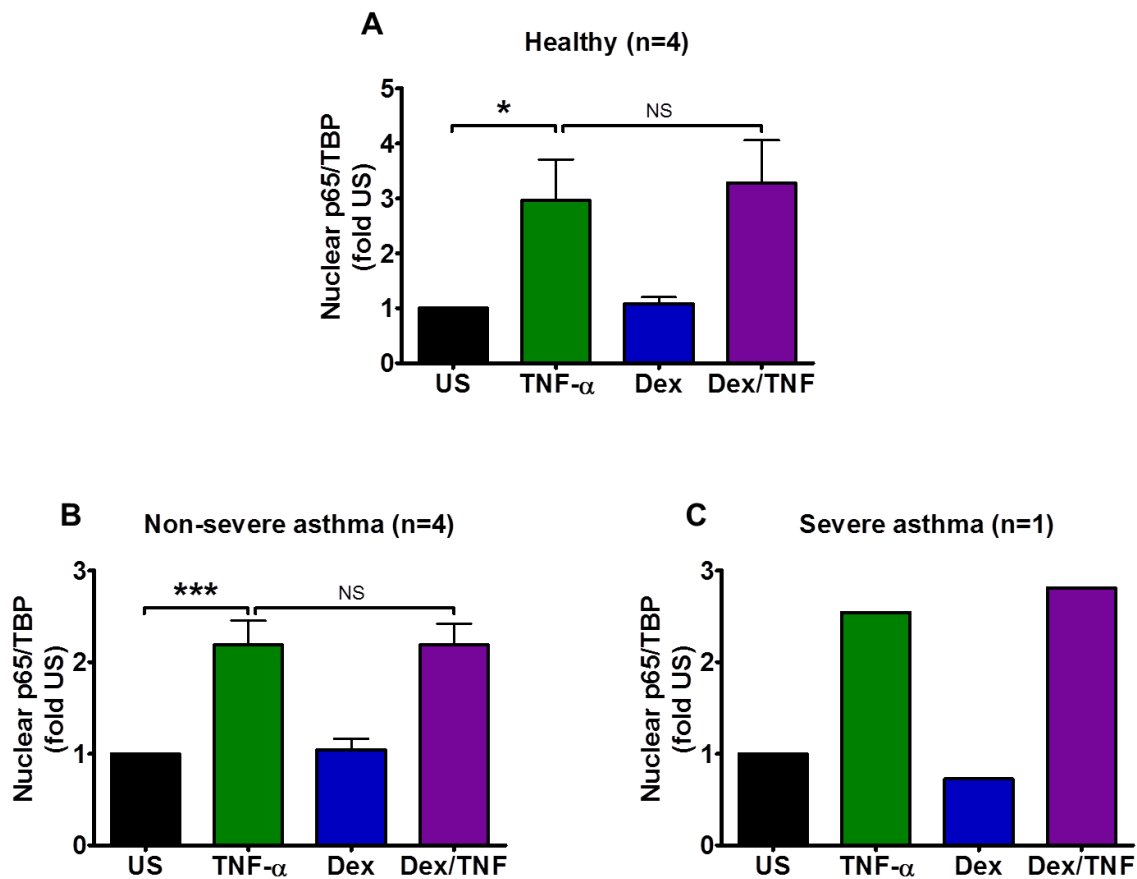


Figure 6.10. Effect of dexamethasone on TNF α -induced nuclear translocation of p65 NF- κ B in ASMC. Cells were stimulated with TNF- α (10 ng/mL) and/or dexamethasone (10^{-7} M) for 30 minutes. Nuclear p65 and TBP from ASMC of (A) healthy subjects and patients with (B) non-severe and (C) severe asthma were measured by Western Blot. Bars represent mean $\pm$ SEM. * $p < 0.05$, *** $p < 0.001$.

6.2.2.3 Effect of dexamethasone on p65 recruitment to the CCL11 promoter in ASMC of healthy subjects and patients with asthma

Having shown that dexamethasone suppresses TNF α -induced CCL11 release (4.3.2.1) and mRNA expression (4.3.2.4) in ASMC, an investigation into the effect of dexamethasone on induced p65 recruitment to the gene promoter of CCL11 was performed. Cells were pretreated with dexamethasone (10^{-6} M) for 2 hours and then stimulated with TNF- α (10 ng/mL) for one hour. p65 recruitment to the CCL11 promoter was measured by ChIP assay. In ASMC of healthy subjects, TNF α -induced a 15-fold increase in the recruitment of p65, which tended to be partly suppressed by IFN- γ (Fig. 6.11A; $p=0.061$). This effect was similar in ASMC of a patient with non-severe asthma, in which IFN- γ suppressed TNF α -induced p65 recruitment by 38% (Fig. 6.11B).

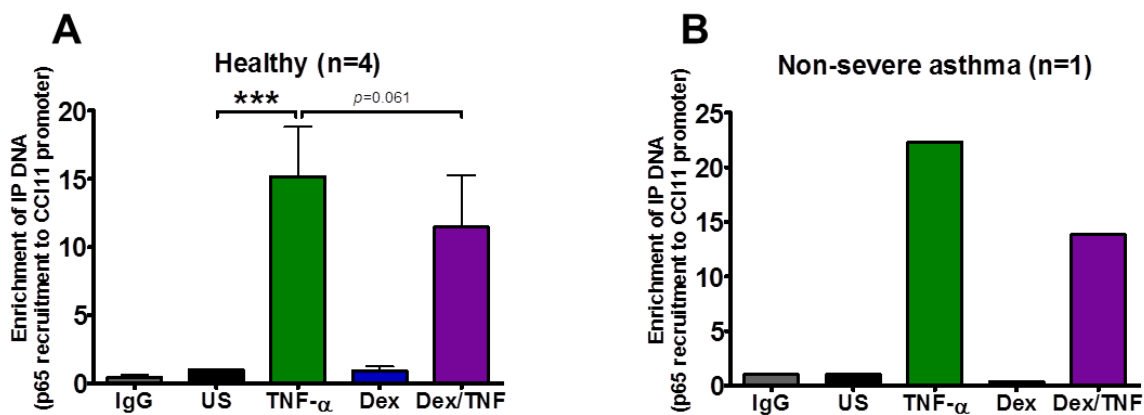


Figure 6.11. Effect of IFN- γ on TNF α -induced p65 recruitment to the CCL11 promoter in ASMC of healthy subjects and patients with asthma. Cells were stimulated with TNF- α and/or IFN- γ (10 ng/mL each) for one hour. p65 recruitment to the CCL11 promoter in ASMC of (A) healthy subjects and (B) a patient with non-severe asthma was measured by ChIP assay. Bars represent mean $\pm$ SEM. *** $p<0.001$.

6.2.3 Investigation of mechanisms by which TNF α -induced p38 activation modulates corticosteroid response in ASMC

6.2.3.1 Effect of TNF- α on dexamethasone-induced nuclear translocation of GR in ASMC of healthy subjects and patients with non-severe and severe asthma

Given the observation that i) p38 phosphorylation induced by TNF- α is associated with the suppressive effect of dexamethasone in ASMC (5.3.5) and ii) induced GR nuclear translocation is impaired in ASMC of patients with severe asthma (6.3.1.5), the effect of TNF- α on nuclear translocation of GR was investigated. Cells were stimulated with dexamethasone (10^{-7} M) or TNF- α (10 ng/mL) alone or in combination for 30 minutes. GR and TBP in the nuclear extracts were measured by Western Blot. In ASMC of healthy subjects, dexamethasone, but not TNF- α , increased nuclear abundance of GR by 3-fold. However, this induction was not modulated by TNF- α (Fig. 6.12A). This effect was similar in ASMC of patients with non-severe (Fig. 6.12B) and severe asthma (Fig. 6.12C), where induced nuclear translocation of GR was not altered by TNF- α .

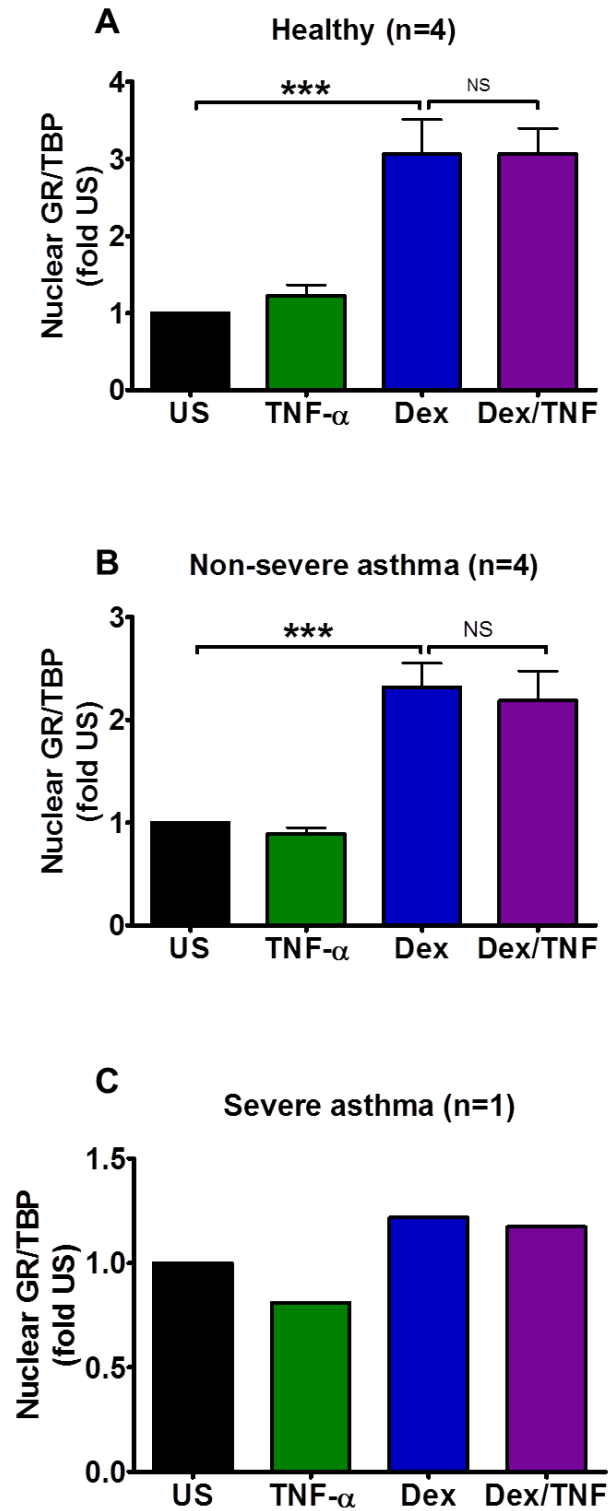


Figure 6.12. Effect of TNF- α on induced nuclear translocation of GR in ASMC. Cells were stimulated with dexamethasone (10^{-7} M) and/or TNF- α (10 ng/mL) for 30 minutes. GR and TBP in the nuclear extracts in (A) the healthy and patients with (B) non-severe and (C) severe asthma were measured by Western Blot. Bars represent mean $\pm$ SEM. *** $p < 0.001$.

6.2.3.2 Effect of p38 inhibition on dexamethasone-induced nuclear translocation of GR in ASMC

Given the observations that i) corticosteroid sensitivity in ASMC of patients with severe asthma is restored by p38 α inhibition (5.3.6) and ii) nuclear translocation of GR is impaired in cells of patients with severe asthma (6.3.1.5), the effect of p38 inhibition on induced GR nuclear translocation was examined. ASMC of patients with severe asthma were stimulated with dexamethasone (10^{-7} M), in the presence or absence of the p38 α inhibitor GW856553, for 30 minutes to 2 hours. GR and TBP in the nuclear extracts were measured by Western Blot. Dexamethasone increased nuclear abundance of GR at 30 minutes, which was maintained to 2 hours, whereas GW-856553 did not modulate this effect (Fig. 6.13).

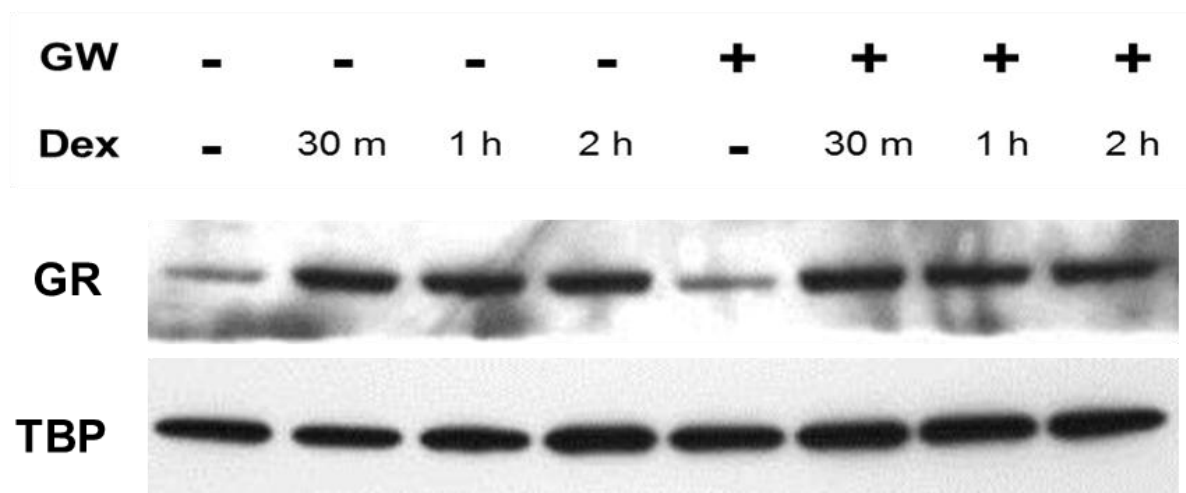


Figure 6.13. The effect of p38 α inhibitor on nuclear translocation of GR in ASMC. Cells were stimulated with dexamethasone (10^{-7} M) with or without GW-856553 for time indicated. GR and TBP in the nuclear abstracts were assessed by Western Blot. A representative blot of ASMC from 2 patients with severe asthma is shown.

6.3 Discussion

Summary of results:

GR abundance was reduced in ASMC of patients with asthma. GR expression was not modulated by dexamethasone until 24 hours post-stimulation. Nuclear translocation of GR was impaired in ASMC of patients with asthma compared to those of healthy and non-severe asthmatic subjects. Phosphorylation of GR at S211 was not different between groups. In addition, p65 expression was suppressed by dexamethasone. Whereas dexamethasone did not modulate p65 nuclear translocation, it attenuated recruitment of p65 to the CCL11 promoter. Finally, TNF α -induced p38 activation did not modulate nuclear translocation of GR.

6.3.1 Differential expression and regulation of GR in ASMC

The mechanism underlying decreased expression of GR in ASMC of patients with asthma (6.2.1.1) is uncertain, yet this might account for failure of corticosteroid suppression of serum-induced ASMC proliferation in those of asthma (Roth, Johnson et al. 2004). In contrast, the statistical equivalence of GR expression in ASMC between patients with non-severe and severe asthma is supported by reports of no difference in either GR α or GR β mRNA expression in peripheral lymphocytes of severe or moderate asthmatic subjects (Jakiela, Bochenek et al. 2010) and similar protein and mRNA expression of both GR α and GR β in PBMC of steroid-dependent or well-controlled asthma (Gagliardo, Chanez et al. 2000).

The abundance of GR is reduced by dexamethasone at 24 hours post-stimulation (6.2.1.2), suggestive of a down-regulatory effect of glucocorticoids on GR expression.

GR mRNA is also exposed to negative regulation by glucocorticoids (Shimojo, Hiroi et al. 1995). This might be explained by the presence of negative GRE, AP-1, NF- κ B, and CREB regulatory motifs in the promoter of GR, all of which are negatively regulated by glucocorticoids (Duma, Jewell et al. 2006). Alternatively, glucocorticoids may regulate the expression of the GR gene post-transcriptionally, perhaps via destabilization of the GR mRNA (Vedeckis, Ali et al. 1989). Moreover, ligand-activated GR protein is degraded upon prolonged exposure to glucocorticoids by the proteasome-ubiquitin degradation pathway (Alarid 2006).

The observation of reduced dexamethasone-induced nuclear abundance of GR in severe asthma (6.2.1.5) extends impaired GR nuclear translocation from PBMC (Matthews, Ito et al. 2004; Mercado, To et al. 2011) to ASMC of patients with severe asthma. The defective GR nuclear translocation in corticosteroid insensitive severe asthma may be partly attributed to hyper-phosphorylation of GR (Mercado, To et al. 2011), and this can contribute to impaired effect of corticosteroids via either decreased GR-GRE binding (Adcock, Lane et al. 1995) or failure to suppress HAT activity induced by inflammatory stimuli (Matthews, Ito et al. 2004). Nuclear translocation of GR could also be affected by i) GR nitrosylation, which leads to reduced dissociation of GR from hsp90 (Galigniana, Piwien-Pilipuk et al. 1999); ii) GR phosphorylation by MAPK p38, JNK and ERK (Barnes 2010) and iii) involvement of JAK3/STAT5 pathway (Goleva, Kisich et al. 2002). The roles of these signalling pathways for corticosteroid insensitivity in severe asthma have yet to be determined.

Whereas phosphorylated GR at S211 is proposed as a hallmark for the transcriptional potential of GR (Beck, Vanden Berghe et al. 2009), absence of differential degrees of phosphorylation between groups (6.2.1.7) suggests GR phosphorylation at this serine residue may not contribute to corticosteroid insensitivity in severe asthma.

6.3.2 Regulation of NF- κ B (p65) by dexamethasone in ASMC

TNF α -induced expression of the p65 and p50 components of NF- κ B is suppressed by dexamethasone in ASMC of healthy subjects at both protein and mRNA levels (Kang, Tirumurugaan et al. 2006). This is further confirmed in those of asthma (6.2.2.1). Interestingly, whereas corticosteroid insensitivity is displayed in ASMC of patients with severe asthma in terms of suppressing some of the NF κ B-mediated genes such as CCL11 and CXCL8 (4.2.2.1, 4.2.2.2, 4.2.2.5 and 4.2.2.6), the suppressive effect of dexamethasone on p65 expression is not dissimilar between any of the groups (6.2.2.1). This suggests that reduced response to corticosteroids in severe asthma is not displayed in terms of suppression of overall NF- κ B expression.

Inability of dexamethasone to inhibit induced p65 nuclear translocation in ASMC (6.2.2.2) has been previously reported (Nie, Knox et al. 2005) and is consistent in A549, a human lung epithelial cell line (Bhavsar, Sukkar et al. 2008). Interestingly, in human lung myofibroblasts, TNF α -induced nuclear translocation of NF- κ B is inhibited by fluticasone (Baouz, Giron-Michel et al. 2005). This suggests the existence of cell-specific regulatory effects of corticosteroids on NF- κ B nuclear translocation.

Consistent with the absence of a modulatory effect of dexamethasone on p65 nuclear translocation, the literature reports that dexamethasone does not prevent TNF α -induced NF- κ B DNA binding or NF κ B-mediated reporter activity in ASMC (Amrani, Lazaar et al. 1999). Intriguingly, TNF α -induced p65 recruitment to the CCL11 promoter is attenuated by dexamethasone (6.2.2.3). This regulatory effect is consistent with reported studies and is associated with reduced acetylation of H4, probably resulting in conformation change of chromatin and prevention of p65 recruitment (Nie, Knox et al. 2005), and is now extended from ASMC of healthy subjects to patients with non-severe asthma. In contrast, induced p65 recruitment to the promoter of inflammatory genes, such as CXCL1, is not suppressed by dexamethasone (Issa, Xie et al. 2006), suggesting that despite widespread recognised corticosteroid suppression of NF- κ B activity (Barnes 2006), the regulatory signalling pathways are sophisticated and gene-specific. It would be worthwhile to examine whether the effect of dexamethasone on p65 recruitment to the CCL11 promoter in ASMC of patients with severe asthma is different, based on the observation of impaired corticosteroid suppression of induced CCL11 expression in those of severe asthma (4.2.2.1 and 4.2.2.5).

6.3.3 Role of p38 MAPK activity for GR nuclear translocation in ASMC

Dexamethasone-induced nuclear translocation of GR is not modulated by TNF- α in ASMC (6.2.3.1), which is supported by evidence from confocal microscopic studies (Issa, Xie et al. 2006). In addition, p38 inhibition does not modulate nuclear translocation of GR in response to dexamethasone in ASMC of patients with severe asthma (6.2.3.2), suggesting impaired GR nuclear translocation is not an effect of

heightened p38 activity in ASMC of severe asthma. This is in contrast to the findings in T-cells, where p38 inhibition reverses corticosteroid insensitivity and reduced GR nuclear translocation induced by IL-2 and IL-4 (Goleva, Li et al. 2009). This suggests while IL-2 and IL-4 display greater expression in the airways of severe asthma (Leung, Martin et al. 1995) and contribute to corticosteroid insensitivity *in vitro*, the cytokines may not underlie the mechanism of corticosteroid in severe asthma.

In summary, there is impaired nuclear translocation of GR in ASMC of patients with severe asthma. This may underlie the mechanism of corticosteroid insensitivity in severe asthma but it is not mediated by p38 MAPK activity. Whereas dexamethasone does not modulate nuclear translocation, DNA binding, or even the reporter activity of NF- κ B, it suppresses transcription of specific inflammatory gene(s) through attenuation of p65 recruitment to the gene promoter. Comparison of the regulatory effects between non-severe and severe asthma will provide further mechanistic insights into the phenomenon.

Chapter 7.

General discussion

Chapter 7. General discussion

Asthma is a chronic disease characterised by airway inflammation, hyper-responsiveness and remodelling. Airway smooth muscle participates in all of these processes, and thus ASMC provide a suitable model for the investigation of the pathophysiology of the disease. TNF- α and IFN- γ are important pro-inflammatory cytokines involved in the immunopathogenesis of asthma, including exacerbation and severe disease (Barnes 2008), but the interaction between the two has yet to be fully understood in the context of ASMC. While IFN- γ attenuates TNF α -induced p65 acetylation (Keslacy, Tliba et al. 2007), TNF- α /IFN- γ mutually up-regulate the receptors of their counterpart (Krakauer and Oppenheim 1993; Kost, Mutch et al. 1999). The proposed mechanisms based on these observations are contradictory and may be too simplified to account for the differential regulation of TNF α -induced chemokine expression by IFN- γ in ASMC. Investigations into modulatory effects at individual genes, using techniques such as the ChIP assay, are essential for clearer insights into the gene-specific regulatory mechanisms. IFN- γ suppresses TNF α -induced CCL11 (3.2.1.1 and 3.2.1.9) and CXCL8 expression (3.2.1.2 and 3.2.1.10) by attenuating p65 recruitment to the gene promoters (3.2.1.12 and 3.2.1.13), while the cytokine potentiates TNF α -induced CXCL10 expression (3.2.1.3) by synergistic recruitment of CBP (Clarke, Clifford et al. 2010). The mechanisms by which TNF- α and IFN- γ synergistically induce CX3CL1 expression (3.2.1.4 and 3.2.1.8) has yet be determined.

Patients with non-severe and severe asthma display different clinical phenotypes (Wenzel and Busse 2007) and distinct inflammatory profiles (Macedo, Hew et al.

2009). Both baseline and TNF α -induced CCL11 expression is greater in ASMC of non-severe asthma (3.2.2.1), indicating inherently different phenotypes between healthy subjects and patients with non-severe and severe asthma. In contrast, only IFN γ -induced CXCL10 release is increased in cells of severe asthma (3.2.2.3). Hence, the differential expression of pro-asthmatic chemokines in ASMC of healthy and diseased subjects is displayed in response to specific cytokine stimulation. Increased p65 expression in cells of severe asthma (3.2.3.1) does not account for individual underlying signalling pathways. Although p65 recruitment to each gene promoter is not different (3.2.3.5), the possibility of differential NF- κ B activities between groups may not be excluded, as the activity of the transcription factor can also be regulated by post-translational modification without interfering with DNA-binding affinity (discussed in 3.3.2).

Patients with severe asthma are barely or even not controlled by high-dose steroid treatment (Bousquet, Mantzouranis et al. 2010). The suppressive effects of dexamethasone on induced CCL11 and CXCL8 expression are impaired in ASMC of patients with severe asthma (4.2.2.1, 4.2.2.2, 4.2.2.5 and 4.2.2.6). This is the first study which reveals that ASMC of severe asthma display corticosteroid insensitivity and extends the findings from PBMC (Hew, Bhavsar et al. 2006) and alveolar macrophages (Bhavsar, Hew et al. 2008; Bhavsar, Levy et al. 2010) to an airway structural cell at passage 4-5, reinforcing inherent differential phenotypes between non-severe and severe asthmatic subjects. Furthermore, heightened p38 activity is displayed in ASMC of severe asthma (5.2.2), and inhibition of p38, rather than JNK or ERK, improves corticosteroid suppression of inflammatory chemokines in those of

severe asthma to an extent achieved in non-severe asthma (5.2.6-5.2.8). This indicates the therapeutic potential of p38 inhibitors in the treatment of corticosteroid-insensitive severe asthma.

Despite the observation that TNF- α /IFN- γ (Howarth, Babu et al. 2005; Shannon, Ernst et al. 2008) and IL-2/IL-4 (Leung, Martin et al. 1995) display greater expression in the airways of severe asthma, and that these cytokine combinations cause corticosteroid resistance in association with induction of p38 activity *in vitro*, their involvement in inducing corticosteroid insensitivity in severe asthma is still arguable: i) not all of the TNF α -induced chemokines whose response to steroids are impaired by IFN- γ (4.2.3.1-4.2.3.3) are insensitive to corticosteroid suppression in severe asthma (4.2.2.3) and ii) while IL2 and IL4-mediated reduction in GR nuclear translocation and decreased anti-inflammatory effects is reversed by a p38 inhibitor (Goleva, Li et al. 2009), the former effect is not displayed in cells of severe asthma (6.2.3.2). These discrepancies highlight the value of studies based on specimens obtained from diseased subjects instead of those from the healthy or immortal cell lines. Furthermore, whereas nuclear translocation of GR is impaired in ASMC of severe asthma (6.2.1.5), the impact on glucocorticoid-regulated genes may vary, depending on the cofactors recruited and/or gene-specific GR-DNA binding affinity. This may explain the observations that not all glucocorticoid-mediated chemokines respond inefficiently to corticosteroids in severe asthma (4.2.2.3, 4.2.2.4 and 4.2.2.7).

One of the limitations of this research is that the cultured human ASMC at passage 4-5 may not reflect the situation *in vivo*. However, these *in-vitro* data are concordant

with the clinical observation that these patients with severe asthma are under suboptimal control despite corticosteroid therapy. The causes of chronic airflow obstruction observed in severe asthma include enhanced airway smooth muscle contraction, airway wall inflammation and oedema as well as intraluminal mucus. The lack of effect of corticosteroids in suppressing pro-asthmatic chemokine expression from ASMC of patients with severe asthma may contribute further to the severity of asthma. Reversibility of corticosteroid insensitivity in airway smooth muscle could lead to better control of severe asthma.

The results and discussion of the studies presented herein indicate a number of directions for future research, either in the form of new work or to clarify existing controversies:

1) Mechanisms underlying differential expression of CCL11 and CXCL10 in ASMC of healthy subjects and patients with non-severe and severe asthma

Investigate and compare i) IFN γ -induced STAT1 and RNA polymerase II recruitment to the CXCL10 promoter, ii) post-translational modification of p65, such as phosphorylation and acetylation, and iii) mRNA half-lives in ASMC between groups.

2) Mechanisms underlying corticosteroid insensitivity in terms of suppressing CCL11 and CXCL8 in severe asthma

Investigate and compare the effect of dexamethasone on i) recruitment of p65, CBP to the gene promoters and ii) acetylation of histone H4 in ASMC between groups

3) Mechanisms by which heightened p38 MAPK activity contributes to corticosteroid insensitivity in severe asthma

Investigate and compare i) the effects of dexamethasone on p38 MAPK activation and mRNA half-lives of induced CCL11 and CXCL8, ii) a role for p38 MAPK in mRNA half-lives of the chemokines and iii) a role for MKP-1 in regulation of p38 activity and corticosteroid response in ASMC between groups.

4) Mechanisms by which TNF- α and IFN- γ synergistically induce CX3CL1 expression

Investigate i) STAT1, IRF-1, CBP and RNA polymerase II recruitment to the CX3CL1 promoter and ii) acetylation of the histone protein H4 in the presence of TNF- α or IFN- γ alone or in combination.

5) Mechanism by which dexamethasone potentiates TNF α and IFN γ -induced CX3CL1 expression in ASMC

Investigate i) recruitment of p65, STAT1, IRF-1, CBP and RNA polymerase II to the CX3CL1 promoter in the presence of TNF- α /IFN- γ or dexamethasone alone or in combination and ii) compare the regulatory effects between ASMC and airway epithelial cells.

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