University of Bath

PHD

# Role of the Chemokine Receptor CXCR3 in Human Mast Cell Degranulation and Signalling

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# Role of the Chemokine Receptor CXCR3 in Human Mast Cell Degranulation and Signalling.

A thesis submitted by

Ian Willox

For the degree of PhD
University of Bath
Department of Pharmacy and Pharmacology
September 2009

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## Abstract

The chemokine receptor CXCR3, which has three known variants (CXCR3-A, CXCR3-B and CXCR3-Alt), has been implicated in the recruitment of mast cells to tissues in many different chronic diseases with its agonists found in elevated levels in many pulmonary diseases. All three variants of CXCR3 were detected in cord blood-derived mast cells at the mRNA level. Using an antibody that is unable to distinguish individual CXCR3 isoforms, we detected a marked down-regulation of intracellular protein during maturation from progenitor cells, with no concomitant changes in the modest surface expression of CXCR3. The known CXCR3 agonists CXCL9, CXCL10 and CXCL11 as well as the reported CXCR3-B agonist CXCL4, were able to induce Akt and ERK1/2 phosphorylation, as well as partial degranulation. Responses to all agonists were inhibited by pre-treatment with selective CXCR3 antagonists and pertussis toxin. Use of novel isoform-selective inhibitors indicates that the p110γ isoform of PI3K is required for degranulation and signalling responses to CXCR3 agonists. Unexpectedly, dual (but not individual) isoform inhibition of the class I β and δ isoforms substantially inhibited signalling and degranulation responses, indicating a hitherto unrecognised synergy between these isoforms, which provide a conduit for CXCR3 signalling in mast cells.

**1. Introduction** 1
**1.1 The Immune System** 1
**1.1.1** Innate Responses 1
**1.1.2** Adaptive Immunity 2
**1.1.3** The Inflammatory Response 5
**1.1.4** The Resolution of Inflammation 6
**1.2 Mast Cells** **6**
**1.2.1** Historical Background 6
**1.2.2** What are Mast Cells? 6
**1.2.3** Mast Cell Subtypes 8
**1.2.4** Activation of Mast Cells 8
**1.2.5** IgE Independent Modes of Mast Cell Activation 10
**1.2.6** Mediator Release from Mast Cells 10
**1.2.7** Modulation of Immune Responses by Mast Cells 11
**1.3 How are Mast Cells Implicated in Disease?** **14**
**1.3.1** Type I Hypersensitivity Reaction 15
**1.3.2** Type II Hypersensitivity Reaction 16
**1.3.3** Type III Hypersensitivity Reaction 16
**1.3.4** Type IV Hypersensitivity Reaction 17
**1.3.5** Tumour development 17
**1.4 Mast cell models** **18**
**1.4.1** The Cytokine Milieu at the Precursor Stage 19
**1.4.2** The Importance of IL-4 Addition of Mast Cell Development 20
**1.5 Chemokines and Chemokine Receptors** **20**
**1.5.1** A Brief History of Chemokine/Chemokine Receptor System 20
**1.5.2** Nomenclature and Structural Characteristics of Chemokines 21
**1.5.3** Chemokine Receptor Structure 25
**1.5.4** Conserved Motifs in the Chemokine System 26
a) Functional motifs in agonists: 26
b) Receptor signalling motifs 27
- DRYLAIV 27
- NPXXY 27
**1.5.5** Silent Chemokine Receptors 28
**1.5.6** D6 Receptor 28

**1.5.7** DARC 29
**1.5.8** CCR11/CCX-CKR 30
**1.5.9** Glycosaminoglycans 30
**1.6 The Biological Function of Chemokines 31**
**1.6.1** Migration 31
**1.6.2** Extension of Pseudopodia 31
**1.6.3** Polarisation 32
**1.6.4** Actin Polymerisation 32
**1.6.5** Lymphocyte Modulation by Chemokines 33
**1.6.6** Lipid Chemoattractants 33
**1.6.7** Neuropeptides and Mast Cells 34
**1.6.8** The Chemokine Expression Profile of Mature Mast Cells 35
**1.6.9** The Role of Chemokines in Disease 35
**1.6.10** Dimerisation of Chemokine Receptors 40
**1.7 Conformation Changes of the Chemokine Receptor 41**
**1.7.1** G-protein Dissociation 41
**1.7.2** Phospholipase C/ Protein Kinase C 43
**1.8 Phosphoinositide 3-kinase 46**
**1.8.1** Overview 46
**1.8.2** Classes of PI3K 46
**1.8.3** PH Domains 49
**1.8.4** Protein Kinase B 52
**1.9 Assessing the role of PI3K 53**
**1.9.1** Pharmacological Intervention -The first generation 'dirty' PI3K inhibitors 53
**1.9.2** Second-Generation Inhibitors. 55
**1.9.3** Genetic Approaches 55
**1.9.4** RNA Interference 58
**1.10 Mitogen-Activated Protein Kinase 60**
**1.10.1** Extracellular Signal Regulated Kinase 60
**1.10.2** Rho in cell polarisation 61
**1.11 Mast Cell Signalling. 62**
1.12 **CXCR3** **66**
**1.12.1** CXCR3 and its Agonists on Mast Cells 69

**1.12.2** Therapeutic Potential of CXCR3 69
**1.12.3** CXCR7 71

**1.13 Regulation of GPCR signalling 72**
**1.13.1** Chemokine Receptor Internalisation and Intracellular Trafficking 73
**1.13.2** Pathways for Internalisation of Chemokine Receptors 73
**1.13.3** Regulation of Chemokine Receptor Trafficking by Rab GTPases 74
**1.13.4** Regulation and Functional Consequences of Internalisation 75
**1.14 Hypothesis and Aims 78**

**2. Methods and Materials 80**
**2.1 Methods 80**
**2.1.1** Cell Types and Culture Conditions 80
**2.1.2** Cord Blood Derived CD133+ Precursors 80
**2.1.3** THP-1 80
**2.1.4** HMC-1 81
**2.1.5** Evaluation of CD133+ precursor differentiation in to Immature mast cells. 81
**2.1.6** Evaluation of Mast Cell Maturity. 82
**2.1.7** Transmission electron microscopy (TEM) 82
**2.1.8** Freezing/thawing of cells 83
**2.1.9** Degranulation 83
**2.1.10** Determination of Protein Expression 84
**2.1.11** Immunoblotting 85
**2.1.12** Stimulation of cells and collection of whole cell extracts 85
**2.1.13** Electrophoretic Separation, Transfer and Immunoblotting of Cellular Proteins 86
**2.1.14** Membrane Stripping and Reprobing 87
**2.1.15** Flow Cytometry 87
**2.1.16** Determining Expression of Cell Surface Receptors 87
**2.1.17** Determination of Expression Levels of Intracellular Proteins 88
**2.1.18** RNA extraction with Trizol 86

**2.1.19** cDNA Synthesis from RNA 89
**2.1.20** Polymerase Chain Reaction (PCR) 89
**2.1.21** Agarose Gel Electrophoresis 90
**2.1.22** In Vitro cell migration assay 90
**2.1.23** Chemotaxis 90
**2.1.24** Chemotactic index 91
**2.1.25** Actin Polymerisation 91
**2.1.26** $Ca^{2+}$ via Flex Station 92
**2.2 Materials** **93**
**2.2.1** Antibodies and Secondary Detection Reagents 93
**2.2.2** Cell Culture 95
**2.2.3** Chemokines 96
**2.2.4** Chemicals 97
**2.2.5** PCR Sequences 98

**3. Characterisation of Mast Cell Models** **98**
**3.1 HMC-1 as a Model for Investigation of CXCR3 Signalling and Function on Mast Cells** **98**
**3.1.1** Introduction to Study 98
**3.1.2** CXCR3 Expression and Signalling on HMC-1 Cell Line 98
**3.1.3** CXCR3 Agonists Fail to Induce Migration of HMC-1 Cells 100
**3.2 Generation of Primary Mast Cell Model**
**3.2.1** Generation and Characterisation of Human Cord Blood Derived Mast Cells 102

**3.3 Characterisation of Primary Mast Cell Model** **107**
**3.3.1 Introduction to Study** **107**
**3.3.2** Antigen Induces Degranulation in Dose Dependent Manner 108
**3.3.3** Investigating the Mechanism of IgE Cross-Linking Induced Degranulation. 109
**3.3.4** The Role of Specific PI3K Isoforms in Degranulation by Cross-Linking of IgE. 111
**3.3.5** The Role of Rho Kinase in IgE/Antigen Induced Degranulation 115

**3.3.6** The Role of PLC in Mast Cell Induced Degranulation of Mast Cell by Antigen 117
**3.3.7** $IP_3$ in Mast Cell Induced Degranulation 119
**3.3.8** Calcium Influx is Key to Mast Cell Degranulation 120
**3.3.9** Summary 122
**3.4 Discussion 123**
**3.4.1** HMC-1 Cell Line makes a Poor Mast Cell Model for Investigating CXCR3 Function. 123
**3.4.2** Chemokine Receptors and Mast Cell Migration 124
**3.4.3** Development of Mast Cell Culture Systems 125
**3.4.4** Evaluation of Cord Blood Derived Mast Cell Model 125
**3.4.5** Role of PI3K Isoforms in Mast Cell Degranulation 126
**3.4.6** Role of Signalling PLC in Mast Cell Degranulation by Aggregation of FcεRI 127
**3.4.7** Reliance of Calcium Influx for Mast Cell Degranulation 129
**3.4.8** Rho kinase is crucial to mast cell degranulation by FcεRI aggregation 131
**Chapter 4 – Role of CXCR3 in Cord Blood Derived Mast Cells. 131**
**4.1 CXCR3 Function in Human Cord Blood Derived Mast Cells 131**
**4.1.1** Introduction to the Study 131
**4.1.2** Chemokine Expression Profile on Mature Human Cord Blood Derived Mast Cells 131
**4.1.3** Expression of CXCR3 Variants and CXCR7 on CD133+ during Differentiation and Maturation 133
**4.1.4** CXCR3 Agonists Induce Phosphorylation of the Protein Akt and ERK1/2 139
**4.1.5** CXCR3 Induced Partial Degranulation of Cord Blood Derived Mast Cells 140
**4.1.6** CXCR3 Induced Partial Degranulation of Human Mast Cells is PTX Sensitive 143
**4.1.7** CXCR3 Agonist Induced Degranulation is CXCR3 Dependent. 144

**4.1.8** CXCR3 Induced Biochemical Signalling are CXCR3 Dependent 146
**4.1.9** CCL2 Induced Partial Degranulation is CCR2 Dependent. 147
**4.1.10** Other Chemokines that are unable to Elicit Degranulation Responses can still Stimulate Biochemical Responses 148
**4.1.11** CXCR3 Agonists Augment Sub-Optimal Ag Induced Degranulation 149
**4.1.12** Up-regulation of Mast Cell Markers Stored within the Granules 152
**4.1.13** Reorganisation of the Cell Membrane is Critical for Degranulation 154
**4.1.14** Reorganisation of Actin by CXCR3 Agonists is CXCR3 Aependent 156
**4.1.15** Involvement of Rho kinase in Actin Reorganisation 158
**4.1.16** CXCR3 Agonists do not Induce Calcium Mobilisation in Mature Mast Cells 160
**4.1.17** The Role of PLC of CXCR3 Induced Mast Cell Degranulation 162
**4.2 The Role of PI3K in CXCR3 Induced Degranulation 166**
**4.2.1** Further Assessing the Role of p110δ and p110β in Mast Cell Degranulation 171
**4.2.2** Summary 174
**4.3 Discussion 175**
**4.3.1** Expression of CXCR3 Variants in Cord Blood Derived Mast Cells 175
**4.3.2** Up-Regulation of Chemokine Receptors after Mast Cell activation. 175
**4.3.3** Chemokine Agonists Induce Partial Degranulation of Human Mature Mast Cells 176
**4.3.4** The Role of CXCL4 in Mast Cells 178
**4.3.5** CXCR3 Agonists Induce a Short Signalling Profile 180
**4.3.6** CXCR3 Specificity of the CXCR3 Agonists 181
**4.3.7** CXCR3 Augmentation of Sub-Optimal Degranulatory 182

responses.

**4.3.8** Role of Activation Markers in Mast Cells 183

**4.3.9** Pharmacological Investigations into the PI3K Family 183

**4.3.10** A critical role for PI3Kγ in CXCR3 Agonists Induced Partial Degranulation 184

**4.3.11** What is the contribution of other Class 1 PI3K isoforms to CXCR3 Agonist-Induced Mast Cell Degranulation? 185

**4.3.12** A potential Synergy between p110δ and p110β 185

**4.3.13** Class1A and 1B PI3K Dependent Degranulation 186

**4.3.14** CXCR3 Reorganises the Actin Cytoskeleton. 187

**4.3.15** Clinical Importance of CXCR3 Agonists 188

**4.3.16** Cytosolic $Ca^{2+}$ not require for CXCR3 Function in Mast Cells 189

**4.3.17** Role of PLC in Degranulation 190

**4.4 Future Work** **192**

**4.4.1** The Role of CXCR3 in Precursor and Immature Mast Cell Stages 192

**4.4.2** Clarifying the Synergy of the PI3K Isoforms 192

**4.4.3** Other mast cells models 192

**5 Summary** **194**

**6 Appendix** **196**

**6.1 Buffers and Solutions** **196**

**6.1.1** Solutions and Buffers for SDS-PAGE and Western Blotting 196

**6.1.2** Recipes for SDS-PAGE Gels 197

**6.1.3** Immunoblotting Conditions for Specific Primary Antibodies 197

**6.1.4** Recipes for Buffers in the Flexstation $Ca^{2+}$ Readings 197

**7. References** **198**

**List of Figures**


| Figure Number | Description | Page number |
|---|---|---|
| 1.1 | Interaction of the innate and acquired immunity. | 4 |
| 1.2 | 2D Schematic of the FcεRI structure. | 9 |
| 1.3 | Variety of mast cell mediators. | 11 |
| 1.4 | The role of mast cells in the immune systems | 13 |
| 1.5 | Role of mast cells in initiating disease. | 15 |
| 1.6 | Characterisation of chemokine receptors by the role in immune responses. | 22 |
| 1.7 | Nomenclature of chemokine Receptors and their agonists. | 24 |
| 1.8 | Schematic depicting a typical GPCR. | 25 |
| 1.9 | Chemokine receptors and disease | 36 |
| 1.10 | Structural differences of PLC isoforms. | 43 |
| 1.11 | Overview of PLC activation. | 45 |
| 1.12 | The PI3K family | 48 |
| 1.13 | Pathways of phosphoinositide metabolism | 49 |
| 1.14 | Activation of Class 1 PI3K results in binding of PtdIns(3,4,5)$P_3$ to a wide variety of PH domains. | 50 |
| 1.15 | Signalling events following formation of phosphoinositide lipids by PI3K. | 51 |
| 1.16 | Structure of PI3K inhibitors. | 54 |
| 1.17 | Schematic representation of siRNA mediating complementary mRNA degradation. | 59 |
| 1.18 | Simplified GPCR activation of MAPK pathway. | 61 |
| 1.19 | Brief overview of signalling molecules involved in mast cell degranulation. | 65 |

| | | |
|---|---|---|
| 1.20 | 2D Schematic of the CXCR3 variants. | 68 |
| 2.1 | FlexStation 3 Microplate used to measure automated calcium responses | 92 |
| 3.1 | Expression and signalling of CXCR3 on HMC-1. | 99 |
| 3.2 | Migration of HMC-1 or THP-1 to different stimuli | 101 |
| 3.3 | Expression markers on CD133+ progenitors through to mature mast cells. | 103 |
| 3.4 | Characteristic tryptase staining of CD133+ precursors. | 104 |
| 3.5 | Characteristic May-Grunwald/Giemsa staining of CD133+ precursors. | 105 |
| 3.6 | TEM pictures of mast cells at different stages of differentiation and maturation. | 106 |
| 3.7 | IgE/Ag dose response | 108 |
| 3.8 | Cross-linking of IgE/Ag induced degranulation is PI3K dependent. | 110 |
| 3.9 | PI3K Isoform Specific IgE/Ag induced degranulation. | 114 |
| 3.10 | Involvement of Rho kinase in Antigen induced degranulation. | 116 |
| 3.11 | PLC Role of PLC in IgE/Ag induced degranulation. | 118 |
| 3.12 | Generation of IP3 is critical for FcεRI aggregation induced degranulation. | 119 |
| 3.13 | Varying $Ca^{2+}$ conditions in FcεRI aggregation induced degranulation. | 121 |
| 4.1 | Chemokine receptor expression profile on mature mast cells. | 132 |
| 4.2 | Expression of CXCR3 variants and CXCR7 at the mRNA in different stage of mast cell development. | 134 |
| 4.3 | Effect of differentiation/maturation on CXCR3 | 135 |

| | | |
|---|---|---|
| | expression in mast cells. | |
| 4.4 | Expression of CXCR3 variants and CXCR7 via western blots. | 137 |
| 4.5 | FACS expression of CXCR3B. | 138 |
| 4.6 | CXCR3 agonists induce phosphorylation of the protein Akt and ERK1/2. | 139 |
| 4.7 | Effect of CXCL9, CXCL10, CXCL11 and CXCL4 on mast cell degranulation. | 141 |
| 4.8 | Several chemokines induce partial degranulation of human mast cells. | 142 |
| 4.9 | Effect of PTX treatment on CXCR3 agonist induced partial degranulation. | 143 |
| 4.10 | Effect of CXCR3 antagonist on CXCR3 and CCR2 induced partial degranulation. | 145 |
| 4.11 | CXCR3 dependent signalling. | 146 |
| 4.12 | Effect of CCR2 antagonist on CXCR3 and CCR2 agonist induced partial degranulation. | 147 |
| 4.13 | CXCL12 elicits biochemical responses in mast cells | 148 |
| 4.14 | CXCR3 augmentation of sub-optimal IgE/Ag. | 149 |
| 4.15 | CXCR3 augmentation of sub-optimal IgE/Ag is CXCR3 dependent. | 151 |
| 4.16 | Expression of CD63 and CD203c after CXCR3 activation. | 153 |
| 4.17 | CXCR3 agonists reorganise actin filaments in mature cord blood derived mast cells. | 155 |
| 4.18 | CXCR3 agonists reorganise actin filaments in mature cord blood derived mast cells in a CXCR3 dependent manner. | 157 |
| 4.19 | Rho kinase inhibitor on CXCR3 induced partial degranulation. | 158 |
| 4.20 | Reorganisation of actin filaments in mature cord | 159 |

| | | |
|---|---|---|
| | blood derived mast cells in RHO kinase but not PI3K dependent. | |
| 4.21 | $Ca^{2+}$ with CXCR3 agonists mast cells. | 160 |
| 4.22 | $Ca^{2+}$ conditions degranulation. | 161 |
| 4.23 | Effect of PLC inhibitors on CXCR3 mediated degranulation. | 163 |
| 4.24 | Effect of $IP_3$ inhibition on CXCR3 induced degranulation. | 165 |
| 4.25 | Effect of PI3K inhibitor on CXCR3 agonist degranulation. | 166 |
| 4.26 | PI3K isoform specific inhibitor on CXCR3 degranulation. | 169 |
| 4.27 | Combination of PI3K isoform inhibitors on CXCR3 degranulation. | 171 |
| 4.28 | Effect of PI3K inhibitors on CXCL9, CXCL10, CXCL11, and CXCL4 induced phosphorylation of Akt. | 173 |
| 4.29 | Proposed model for PI3K signalling role in cord blood mast cell degranulation | 191 |

**List of Tables**


| Table Number | Description | Page Number |
|---|---|---|
| 1.1 | Examples of chemokine knockout murine model | 37 |
| 1.2 | Phenotypes of mice that have PI3K catalytic subunit genetically targeted. | 57 |
| 1.3 | Involvement of CXCR3 receptor in human disease. | 70 |
| 3.1 | $IC_{50}$ values (μM) for isoform-discriminating PI3K inhibitors. | 112 |
| 4.1 | $IC_{50}$ values for inhibition of degranulation were obtained by comparing concentration-dependent effects of individual inhibitors against optimal degranulation in response to 100 nM of specific agonist. | 170 |

## Abbreviations

| | |
|---|---|
| -RT | without reverse transcriptase |
| AA | Arachidonic Acid |
| Ab | antibody |
| Ag | antigen |
| APC | antigen presenting cell |
| BM | bone marrow |
| bp | basis pair |
| BSA | bovine serum albumine |
| $Ca^{2+}$ | calcium ion |
| CARD | Caspase-Recruiting Domain |
| CCPs | clathrin-coated pits |
| CCR | CC chemokine receptor |
| CCVs | clathrin-coated vesicles |
| CD | cluster of differentiation |
| CGRP | Calcitonin gene-related peptide |
| CNS | central nervous system |
| COPD | Chronic Obstructive Pulmonary Disease |
| COX | Cyclooxygenase |
| CX3CR | CX3C chemokine receptor |
| CXCR | CXC chemokine receptor |
| DAG | diacyl-glycerol |
| DARC | Duffy antigen/receptor for chemokines |
| DC | dendritic cells |
| $dH_2O$ | distilled water |
| DNA | deoxyribonucleic acid |
| EC | endothelial cell |
| ECM | extracellular matrix |
| *E. coli* | Escherichia coli |
| EDTA | ethylenediaminetetraacetic acid |
| EGFP | enhanced green fluorescent protein |
| ER | endoplasmic reticulum |

| | |
|---|---|
| ERK | Extracellular signal regulated kinase |
| EtOH | ethanol |
| FACS | fluorescence activates cell sorter |
| FITC | fluorescein isothiocyanate |
| FCS | foetal calf serum |
| GADS | GRB2-related adaptor protein |
| GAG | glycosaminoglycan |
| GAP | GTPase activating protein |
| GDP | guanine biphosphate |
| GEF | GDP exchange factor |
| GFP | green fluorescent protein |
| GPCR | G protein couples receptor |
| GRK | G protein coupled kinase |
| GRB2 | Growth-factor-receptor-bound protein 2 |
| GTP | guanine triphosphate |
| HRP | horseradish peroxidise |
| ICAM-1 | intracellular adhesion molecule |
| IFN-$\gamma$ | interferon gamma |
| IMDM | Iscove Modified Dulbecco Medium |
| Ig | immunoglobulin |
| IL | interleukin |
| $IP_3$ | inositol 1,4,5 triphosphate |
| IP-10 | IFN-$\gamma$-induced protein-10 |
| I-TAC | IFN-$\gamma$-inducible T cell $\alpha$ chemoattractant |
| ITAMs | Immunoreceptor tyrosine-based activation motifs |
| JAK | Janus-family tyrosine kinase |
| JNK | c-jun-$NH_2$-terminal kinase |
| kb | kilobase pair |
| kDa | kiloDalton |
| KO | knock out |
| L | litre |
| LN | lymph node |
| LPS | lipopolysaccharide |

| | |
|---|---|
| LAT | Linker for the Activation of T-cells), |
| LT | Leukotrienes |
| MAPK | mitogen activated protein kinase |
| MHC | major histocompatibility complex |
| Mig | monokine-induced by IFN-$\gamma$ |
| mRNA | messenger RNA |
| MS | multiple sclerosis |
| NGF | Nerve Growth Factor |
| NFAT | nuclear factor of activated T-cells |
| NK | natural killer |
| NTAL | Non-T-cell Activation Linker |
| NLR | NOD-Like Receptor |
| OD | optical density |
| ORF | open reading frame |
| PBMC | peripheral blood mononuclear cells |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PE | phycoerythrin |
| PFA | paraformaldehyde |
| pg | picogram |
| PH | pleckstrin homology |
| PI3K | phosphatidylinositol 3-kinase |
| $PIP_2$ | phosphatidylinositol 4,5-biphosphate |
| $PIP_3$ | phosphatidylinositol 3,4,5-biphosphate |
| PKA | protein kinase A |
| PKB | protein kinase B |
| PKC | protein kinase C |
| PF4 | Platelet Factor 4 |
| PLC | phospholipase C |
| PLD | phospholipase D |
| PTEN | phosphate and tensin homologue deleted on chromosome ten |
| PTX | pertussis toxin |
| RA | rheumatoid arthritis |

| | |
|---|---|
| RBC | red blood cell |
| rcf | gravitational force |
| RDC1 | receptor dog cDNA |
| RGS | regulator of G-protein signalling |
| RNA | ribonucleic acid |
| rpm | revolutions per minute |
| RPMI | Roswell Park Memorial Institute medium |
| RT | reverse transcriptase |
| RT-PCR | reverse-transcription PCR |
| SCF | Stem Cell Factor |
| SDF-1 | stromal cell-derived factor |
| SDS | sodium dodecyl sulphate |
| SEB | Staphylococcal Enterotoxin B |
| S1P | Sphingosine 1 phosphate |
| SHC | SH2-domain-containing leukocyte protein C |
| SLP76 | SH2-domain-containing leukocyte protein of 65kDa |
| SOS | Son of Sevenless homologue |
| SP | Substance P |
| STAT | signal transducer and activator of transcription |
| SYK | Spleen Tyrosine Kinase |
| TAE | Tris acetate EDTA |
| TBS | Tris buffered saline |
| TCR | T cell receptor |
| TEMED | tetramethylethylenediamine |
| TEM | Transmission electron microscopy |
| Th1 | T helper cell type 1 |
| Th2 | T helper cell type 2 |
| Tx | Thromboxanes |
| TLR | Toll-Like Receptors |
| TM | transmembrane |
| TNF-$\alpha$ | tumor necrosis factor-$\alpha$ |
| Tween-20 | polyoxyethylene-sorbitan monolaurate |
| VCAM-1 | vascular cellular adhesion molecule-1 |

| | |
|---|---|
| v/v | volume per unit volume |
| VIP | Vasoactive Intestinal Polypeptide |
| WT | wild type |
| w/v | weight per unit volume |

# 1. Introduction

## 1.1 The Immune System

The immune system is the body's defence mechanism which acts to protect the body from the potential threats of foreign pathogens. Two main branches of immunity are recognised: innate and adaptive immunity. A specific immune response — such as a generation of specific antibodies against a specific pathogen, for example — is known as an adaptive immune response, occurring during the lifetime of an individual as an adaption to infection with that pathogen. Adaptive immune responses are capable of generating lifelong protective immunity to re-infection of the same pathogen. This is in contrast to innate immunity, which is immediately available to combat a wide range of pathogens without requiring prior activation. Despite these systems having different immunological profiles, it is critical that they interact in such a way so as to initiate the body's full defence system.

The responses of both the innate and adaptive immunities depend on the activities of a group of white blood cells termed leukocytes. Immune responses are mediated by leukocytes, which derive from precursors in the bone marrow. A pluripotent hematopoietic stem cell gives rise to the lymphocytes responsible for adaptive immunity, and also myeloid lineages which participate in both the innate and adaptive immunity.

### 1.1.1 Innate Responses

Innate immune responses provide the first line of defence. This is proved by the simplest of barrier protection, which is provided by the skin and epithelial of the pulmonary and the gut; however, in the instance that these frontline defences are breached, the innate immunity then has a second line of defence which largely involves granulocytes. These leukocytes are a diverse collection of white blood cells whose prominent granules provide their characteristics. These cells are comprised of neutrophils and macrophages — the 'so-called' eaters of the innate immunity due to their ability to phagocytise invading pathogens and dendritic cell, and are the sentinels of the immune system.

The innate immune system discriminates between both self and non-self by utilising the receptors which bind features on micro-organisms with regular patterns known as Pathogen-Associated Molecular Patterns (PAMPs), which are recognised by Pattern-Recognition Receptors (PRRs). Amongst the membrane-bound PRRs, the best-known PRRs are the Toll-Like Receptors (TLRs) which sense a wide array of microbial ligands at the cell surface or within endosomes (Kawai and Akira, 2006). At present, 11 TLRs have been discovered in mammals (TLR1-TLR11). Interaction of TLRs with their specific PAMP induces NFκB signalling and MAP kinase pathways, and therefore the secretion of pro-inflammatory cytokines and co-stimulatory molecules. Cytoplasmic PRRs include the Caspase-Recruiting Domain (CARD) helicases — such as retinoic acid-inducible protein I and melanoma differentiation- associated protein 5, which are involved in antiviral responses (Kawai and Akira, 2006) — and the nucleotide binding oligomerisation domain NOD-Like Receptor (NLR) family, which recognises primary microbial molecules of bacterial origin (Inohara *et al*., 2005). There are reports of a number of PRRs which do not remain associated with the cell that produces them; these include complement receptors, collectins, pentraxin proteins and peptidoglycan recognition proteins, all of which are secreted proteins. One of the most well-known colectins is mannose-binding lectin, a major PRR binding to a wide range of bacteria, fungi and protozoa via sugar groups, phospholipids, nucleic acids or non-glycosylated proteins (Dommett *et al*., 2006).

These cells of the innate system are specifically inherited in the genome and, when fighting against further infection, will generate the same innate immunological response. This is in contrast to the adaptive immune response, which will adapt their response according to the specific invading particle.

### 1.1.2 Adaptive Immunity

Adaptive immunity is commonly split into two different classes: humoral and cell-mediated. Humoral responses are generated by B-cells which, upon activation, differentiate into plasma cells which secrete antibodies. Cell-mediated responses are generated by T-lymphocytes, which are further divided into two different subsets: the first, which, upon activation, differentiate into cytotoxic T-cells which

kill cells infected with viruses; the second, described as T-helper cells, activate other cells such as B-cells and those of the innate immunity.

Innate immunity acts as the activation trigger for the adaptive immune response by the production of pro-inflammatory cytokines. This action recruits further leukocytes to the point of inflammation. Dendritic cells play the leading role in the activation of the adaptive immunity. Immature dendritic cells recognise the conserved patterns on pathogens, activating maturation of the dendritic cell and expression of a range of co-stimulatory molecules (Akira *et al*., 2001; Vestweber, 2003). Subsequently, dendritic cells then migrate and present the processed antigen via the major histocompatibility complex to naïve T-lymphocytes, whilst a second co-stimulatory signal is received via the binding of B7 to CD28 (Banchereau and Steinman, 1998; Reis de Sousa *et al*., 2001). Figure 1.1 highlights the innate and acquired immune systems' interaction.

The complexity of the immune system is achieved by the dynamic and ever-evolving variety of cells which this system comprises. It is important to orchestrate this response in order to achieve a speedy reaction and to accordingly determine resolution of inflammation. One such cell which orchestrates the immune response is the mast cell. An orchestra comprises many different instruments; however, only one conductor directs the music they make. Following this analogy, mast cells are few but essential to promoting the immune and inflammatory responses (Kinet, 2007).

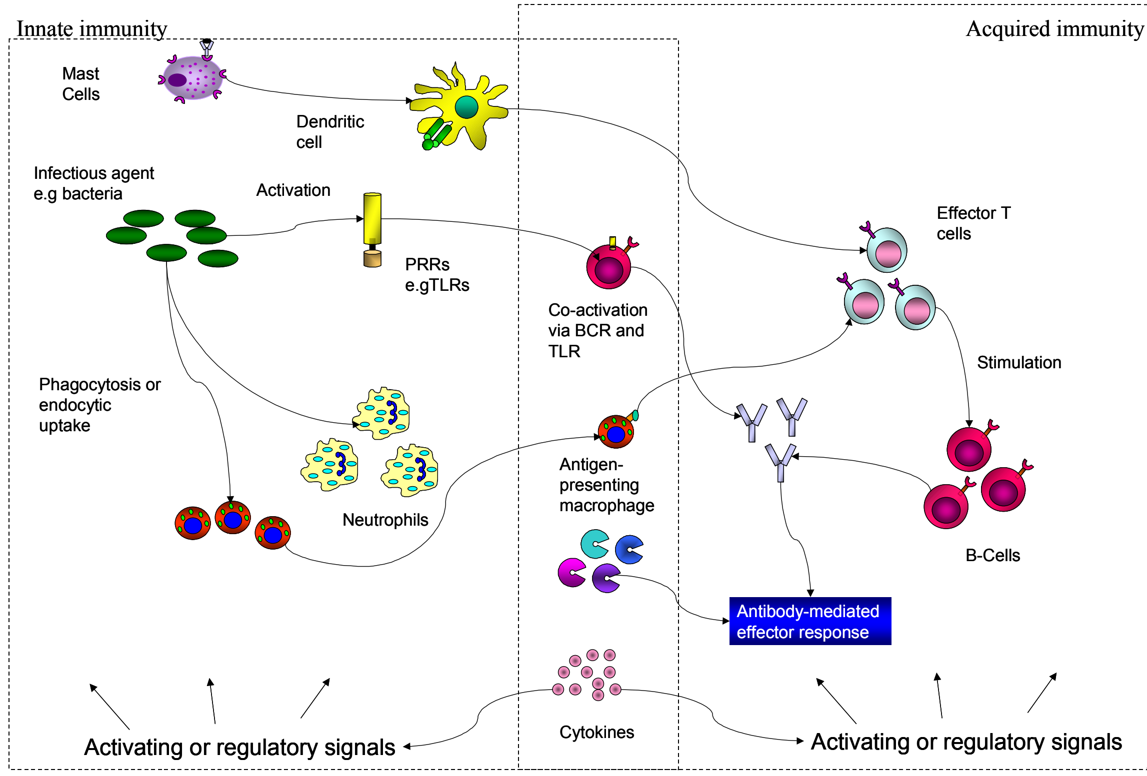



**Figure 1.1: Interaction of the innate and acquired immunity.** The innate and adaptive immune systems are interrelated in ways which have not yet been fully established. Antigens are phagocytosed or endocytosed in a non-specific manner by neutrophils, subsequently resulting in them being neutralised in the body. Alternatively antigens phagocytosed by macrophages can be presented to T-cells, generating a highly specific T-cell response. Responses such as these are partially dependent on PRRs — such as TLRs and NOD-like receptors — which recognise PAMPs presence on a variety of microorganisms. There are also a variety of soluble PRRs, such as complement proteins (C1Q), mannose-binding protein (MBP), and acute phase reactants, such as C-reactive protein (CRP), all of which play a role in innate responses by opsonising microorganisms and subsequently binding to apoptotic cellular debris in a non-specific manner. In addition, the co-stimulation of B-cells through TLRs (such as TLR9) can result in the production of specific antibodies to antigens. Moreover, cytokines such as interferons, Tumour Necrosis Factor (TNF), and interleukin 1 (IL1), might stimulate the activity of both the innate and adaptive immune responses. Antibody production can also reflex back on the innate immunity by activating mast cells, thereby resulting in the further recruitment and activation of the innate immunity. Adapted from Gregersen and Behrens, (2006).

### 1.1.3 The Inflammatory Response

The inflammatory response describes the complex biological response of the inflammatory tissues to harmful stimuli, such as pathogens or invading bacteria. It is the protective mechanism by which an organism attempts to remove potential harmful stimuli while, at the same time, inducing the healing process. Both the innate and acquired immunity are required in order for an immune response to be successful. Inflammatory responses have been classified into two types: acute and chronic.

Acute inflammation is a rapid response to an injurious agent which serves to deliver mediators of host defence-leukocytes and plasma proteins to the site of injury. The response comprises four components:

2. Alterations in vascular calibre which lead to an increase in blood flow.
3. Structural changes in the microvasculature which permit plasma proteins and leukocytes to leave the circulation.
4. Migration of phagocytes out of the venules and into interstitial spaces.
5. Recruitment of lymphocytes to the site of injury by cells of the innate immune system.

Once the inflammatory process has begun, it continues until the infection which originally caused the response has been eradicated. Phagocytes continue to consume and destroy bacteria whilst the acquired immune system binds and disposes of the harmful toxins; this is a fundamental process, as the inflammatory response should only last as long as the infection exists. Moreover, prolonged inflammatory responses result in chronic inflammation, subsequently leading to simultaneous destruction and repair of the infected tissues. Such inflammation which operates unchecked can consequently lead to a host of diseases, such as asthma, atherosclerosis and rheumatoid arthritis, to which mast cells are heavily linked. However, in the absence of the inflammatory response, infections would never heal, and the destruction of the infected tissues would seriously compromise the health of the individual.

### 1.1.4 The Resolution of Inflammation

The resolution of inflammation is initiated during the first few hours following the initiation of an inflammatory response. After entering tissues, granulocytes promote the switch of arachidonic acid-derived prostaglandins and leukotrienes to lipoxins, which in turn initiate the termination sequence. Neutrophil recruitment thus ceases, and programmed death by apoptosis is subsequently engaged. These events coincide with the biosynthesis, from omega-3 polyunsaturated fatty acids of resolvins and protectins, which critically shorten the period of neutrophil infiltration by initiating apoptosis (Serhan and Savill, 2005). Consequently, apoptotic neutrophils undergo phagocytosis by macrophages, thereby leading to neutrophil clearance and the release of anti-inflammatory and reparative cytokines, such as transforming growth factor-β1. The anti-inflammatory programme ends with the departure of macrophages through the lymphatics. Stromal cells — such as fibroblasts — also contribute to the resolution of inflammation by the withdrawal of survival signals and the normalisation of chemokine gradients, thereby allowing infiltrating leukocytes to undergo apoptosis or to otherwise leave the tissue through the draining lymphatics (Serhan et al., 2007).

## 1.2 Mast Cells

### 1.2.1 Historical Background

Mast cells were first described in the doctoral thesis of Paul Ehrlich in 1878, in which he refers to them as Mastzellen, most likely deriving from the German word ‘mastig’, meaning well-fed. They were initially given this name due to the misconception that these cells were over-fed due to their unique staining characteristics and large granules. The large amount of granules present in mast cells also accordingly led Ehrlich to the mistaken belief that they exist to nourish the surrounding tissue. Furthermore, the name ‘mast cell’ may have also arisen from the Greek word ‘masto’, literally meaning ‘to feed’.

### 1.2.2 What are Mast Cells?

Since the first discovery of mast cells, it is now widely believed that mast cells are one of the key immune effecter cells in consideration of their primary function in host defence against parasites and other invading pathogens. Mast cells are

members of the innate immune system, and are often considered to be the first-line responders to immunological insults, simply because of their prevalence in areas highly exposed to the external environment. They originate from CD34+ hematopoietic pluripotent stem cells but do not mature until leaving the bone marrow, and circulate in peripheral blood as mast cell committed progenitors or 'immature' mast cells (Födinger *et al*., 1994; Kirshenbaum *et al*., 1991). This phenotype is, so far, poorly characterised. Progenitors are isolated from peripheral blood express c-Kit+, CD34+, CD117+ but lack expression of FcεRI and FcεRII. Studies have shown that CD34+ cells lacking c-Kit or CD13 will not develop into mast cells (Agis *et al*., 1993; Kirshenbaum *et al*., 1999).

It is from the peripheral blood that mast cells enter the tissues where the mast cell will develop into a fully mature cell. The tissue that a committed mast cell progenitor enters will 'tailor' the differentiation to distinct phenotypes — a phenomenon referred to as mast cell heterogeneity (Enerbäck, 1966). The key growth factor for mast cells is Stem Cell Factor (SCF), which will not only guide the differentiation of mast cells but also regulate all growth, survival, migration, adhesion and degranulation (Galli *et al*., 1994; Nilsson *et al*., 1999). Moreover, mature mast cells can normally be found widely distributed throughout the vascularised tissues, especially with numerous found beneath the epithelial surfaces of the skin, the respiratory system, the gastrointestinal and genitourinary tracts, and also in the central nervous system (Johnson and Krenger, 1992; Nilsson *et al*., 1999; Silver *et al*., 1996). These cells can be identified in most tissues as relatively large cells which express high levels of c-Kit (the receptor for SCF) and FcεRI (the high-affinity IgE receptor). Microscopically, these cells are easy to identify with toluidine blue or alcian blue dyes.

Despite being described as having an immobile nature (mature mast cells are generally found to be 'fixed' in the tissues and not circulating in the blood), mast cells are widely distributed throughout the body, therefore allowing them to encounter pathogens and environmental antigens invading the body. Mast cells are commonly found throughout the skin, mucosa of the genitourinary, respiratory and gastrointestinal tracts, as well as most vascularised tissues of mammals, including the lymphoid organs (Sayed *et al*., 2008).

### 1.2.3 Mast Cell Subtypes

Mature human mast cells can be divided into two distinct sub-categories depending on variations in the protease composition in the mast cell granules. These subsets are therefore defined as mast cells containing tryptase or those containing both tryptase and chymase (Irani *et al*., 1986). The Mast cell subset will depend largely on the type of tissue it resides in. Mast cells containing tryptase are predominately found in lung tissue and intestinal mucosa while mast cells containing both tryptase and chymase are found predominately in the skin and intestinal submucosa (Irani *et al*., 1986; Nilsson and Nilsson, 1995).

### 1.2.4 Activation of Mast Cells

Mast cells are very effective as both effector cells, which amplify inflammation, and as regulatory cells, which are able to suppress certain immune responses. Mature mast cells are primarily activated by IgE aggregation as a result of an antigen binding to a high affinity receptor FcεRI on the mast cell surface. The basic structure of an FcεRI receptor present on mast cell consists of four joined subunits — α, β and two γ subunits, as shown in Figure 1.2. Moreover, mature human mast cells constitutively express high affinity receptor FcεRI, with activation initiated when adjacent FcεRIs are cross-linked either by antigens interacting with receptor bound IgE or antibodies directed against either receptor bound IgE or the receptor itself. The cross-linking of these receptors induces a cascade of intracellular signalling events, which subsequently leads to the rapid release and de novo synthesis of mediators (Kuehn and Gilfillan, 2007; Nishida *et al*., 2005; Tkaczyk *et al*., 2002). Over recent years, it has also become clear that degranulation of mature mast cells can occur by IgG binding to FcγRI (Tkaczyk *et al*., 2002; Woolhiser *et al*., 2001).

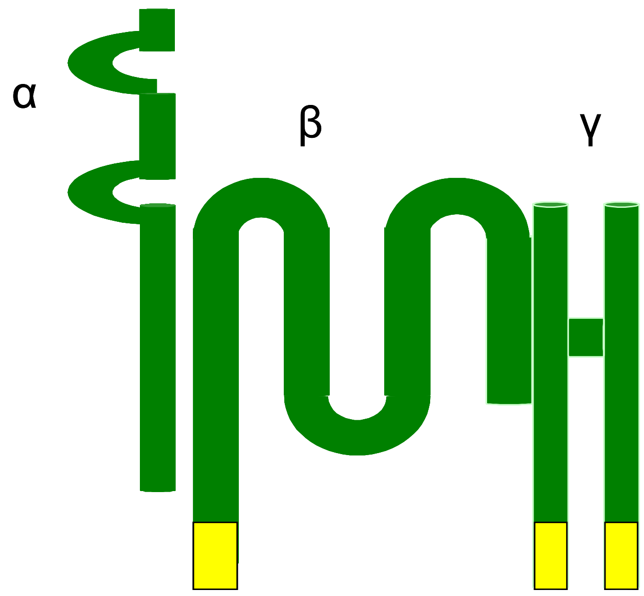



**Figure 1.2: 2D Schematic of the FcεRI structure**.The FcεRI is a heterotetrameric structure containing four polypeptide subunits: one α-chain, one β-chain, and two disulphide-linked γ chains. The α-chain provides the ligand-binding function of FcεRI**,** and has been recognised as a member of the Ig gene superfamily on the basis of its predicted amino acid sequence. The functions of the *β*- and γ-chains are unclear, although roles in cell surface trafficking and trans-membrane signalling have been suggested. Adapted from Pang *et al.*, (1993).

Similar to the two-signal model in the activation of T-cells, antigen signalling through the FcεRI is often modified by the co-inhibitory or co-stimulatory signals in mast cells. This has been noted in the administration of αCD28 to bone marrow-derived mast cells, which enhances TNF secretion (Tashiro *et al.*, 1997). TLR ligands have also been demonstrated in order to synergise with antigen to potentiate cytokine secretions by cross-linking of the FcεRI on mast cells. On the other hand, the Ig receptor FcγRIIB (Malbec and Daëron, 2007) and platelet endothelial cell adhesion molecule-1 (Wong *et al.*, 2002) utilise the ITIMs present on the FcεRIIgE so as to suppress mast cell function.

### 1.2.5 IgE Independent Modes of Mast Cell Activation

In addition to the activation by IgE/antigen, it is now known that mast cells can be stimulated by complementary components by both viral and bacterial pathogens, hormones, cytokines and chemokine. Mast cells express a wide range of complement receptors, including C3aR, C5aR CR2, CR4, and the recently described collectin/C1qR (Edelson *et al.*, 2006; Marshall, 2004). These receptors have been highlighted as playing an important role in cecal ligation and the puncture model of septic peritonitis in which complement-mediated activation of mast cells is essential for the clearance of invading bacteria. Furthermore, the range of TLRs present on mast cells have recently been shown to differentially activate mast cells through interactions between lipopolysaccharide and peptidoglycan (Prodeus *et al.*, 1997). Moreover, mast cells are directly activated through this mechanism by different bacteria, and act as a first-line defence against bacterial infections (Tkaczyk *et al.*, 2002). Other IgE independent mechanisms of mast cell activation by hormones include oestrogen inducing mast cell degranulation *in vitro* studies or the inhibition of histamine release (Theoharides *et al.*, 2006). Importantly, mast cells influence immune responses via the release of a wide range of mediators from within the granules store in the cytoplasm, whether their activation method is by IgE cross-linking of the FcεRI receptor or the IgE independent mechanisms, such as those highlighted above.

### 1.2.6 Mediator Release from Mast Cells

Mast cells are involved in a variety of biological processes, and their anatomical location close to epithelial surfaces in the skin, the respiratory system and gastrointestinal tract, makes mast cells a key frontline defence against invasion. In studies carried out by Malaviya *et al.* (1994) and Echtenacher *et al.* (1996), mast cells are described as having a key role in innate immunity. The primary way in which mast cells can modulate immune responses is their capacity to produce and secrete a large array of inflammatory mediators upon activation. The mediators which are released from mast cells stem from three different categories, as shown in Figure 1.3.

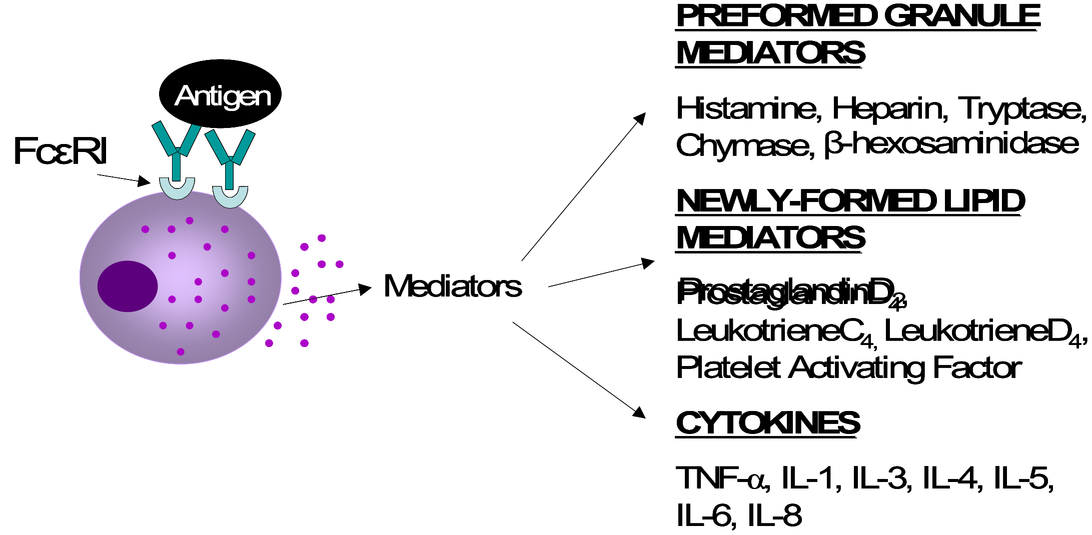



**Figure 1.3: Variety of mast cell mediators**. Mature mast cells release a wide range of mediators at different stages. The first mediators released are the preformed granule mediators, followed by the newly-formed mediators and, thirdly, the release of cytokines.

The three categories are:

1. Preformed secretary granule-associated mediators, such as histamine and heparin: these mediators are the first released by a mast cell following activation, and are of great importance during the early phases of acute allergic inflammatory reactions.
2. Newly-formed lipid mediators: these products result from the arachidonic acid metabolism following the activation of mature mast cells, and include the formation of leukotriene $C_4$ and prostaglandin $D_2$. These mediators are synthesised within minutes of activation, and their production may persist for 30 minutes or longer, thereby playing an important role in the acute inflammatory response (Secor *et al.*, 2000).
3. Cytokines, chemokines and growth factors: these are synthesised and secreted by the mast cells at a later stage, approximately 4-12 hours after initial activation. The formation of these mediators is critical when orchestrating the late phase response, which develops within a couple of hours of the induction of the inflammatory response. The sheer variety of

mediators can then be released from mast cells following activation, which allows mast cells to be versatile in the modulation of different immune responses.

### 1.2.7 Modulation of Immune Responses by Mast Cells

The key role of mast cells in innate immunity was first highlighted in studies on mast cell-deficient mice, therein demonstrating defective clearance and survival during bacterial infections. The possible role of mast cells in innate immunity was further suggested by evidence of complement fragment 3-induced degranulation of mature mast cells, and that the resulting release of TNF-a was a key factor in the recruitment of neutrophils and subsequent bacterial clearance (Prodeus *et al.*, 1997).

Our current understanding of how mast cells interact with micro-organisms by either direct or indirect interaction is that it most likely involves different receptors expressed on the surface of mast cells. These include CD48 recognising Fim H (a 29-kDa mannose binding lectin expressed by E.coil and other enterobacteria) (Malaviya *et al.*, 1994), toll-like receptors (Varadaradjalou *et al.*, 2003), and complement receptors (Prodeus *et al.*, 1997). The interaction between mast cells and micro-organisms induces the activation of mast cells and mediator release, inducing inflammatory responses or a direct killing of the pathogen, thereby resulting in bacterial clearance. The effects which mediators have on different cells of the innate immune system are highlighted in Figure 1.4.

This emerging evidence points to a pivotal role which mast cells play in innate immune responses. It has also become clear that mast cells are not limited to the innate immune system and are rather initiators of the acquired immune responses. As a part of the adaptive immune system, mast cells have the ability to present antigens to T-cells via either MHC class I or II molecules (Mekori and Metcalfe, 1999). A large array of co-stimulatory molecules have been identified on the surface of mast over the years, including ICAM-1, ICAM-3, CD43, CD80, CD86 and CD40L, all of which enable them to interact with a wide range of cells. The result of these interactions includes T-cell proliferation and cytokine release, immunomodulation towards Th2 phenotype, and the induction of IgE production by

B-cells (Gauchat *et al.*, 1993; Mekori and Metcalfe, 1999). Figure 1.4 provides an overview of the central role mast cell can play in modulating immune responses — both innate and acquired — interacting with a wide range of cell types.

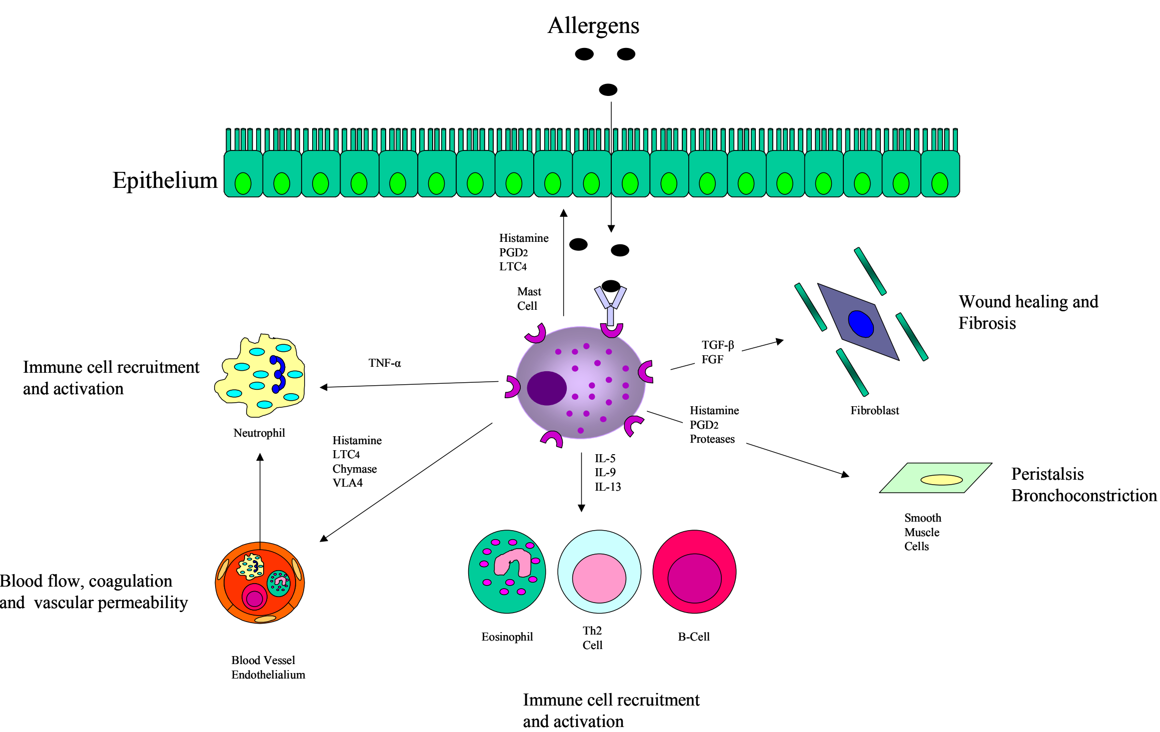



**Figure 1.4: The role of mast cells in immune system.** Mast cells act in such a way so as to orchestrate the immune system; this includes the regulation of epithelial, smooth muscle, endothelial function, activation of neutrophils, eosinophils lymphocytes and many tissue functions, such as wound-healing. Adapted from Bischoff, (2007).

Wound-healing or fibrosis is a very complex biological process which results from the interplay of different tissue structures and a large number of resident and infiltrating cells. It is the body's natural process of regenerating dermal and epidermal tissue. When an individual is wounded, a set of complex biochemical events takes place in a closely orchestrated cascade with the sole objective to repair the damage. These events overlap in time and may be artificially categorised into separate phases: inflammatory, proliferative, and remodelling. Ultimately, mast cells are considered to be important orchestrators in this whole

process by releasing mediators in three different ways and time points. The mast cell can orchestrate to limit damage, to re-vascular damaged tissue, to proliferate the connective cellular elements, and to remodel the matrix support (Metcalfe *et al.*, 1997; Noli and Miolo, 2001).

## 1.3 How are Mast Cells Implicated in Disease?

Mast cells can incur negative as well as positive immune responses, such that they both enhance and later suppress certain features of an immune response, primarily due to the fine balance between beneficial healing or impaired healing following the degranulation threshold being exceeded (Christy and Brown, 2007). At this point, mast cells turn into damaging effector cells, subsequently impairing the protective immune response.

Figure 1.5 demonstrates how mast cells can be at the centre of influence of many responses notable in diseases. Much of what is currently understood regarding the role of mast cells in disease comes from studies of hypersensitivity reactions; these are a group of pathogenic immune responses eliciting from antigens which are not inherently harmful; it is an overeager immune response that induces these pathological conditions. Hypersensitivity reactions have been classified into four types; type I-IV, determined by the major effecter mechanisms employed.

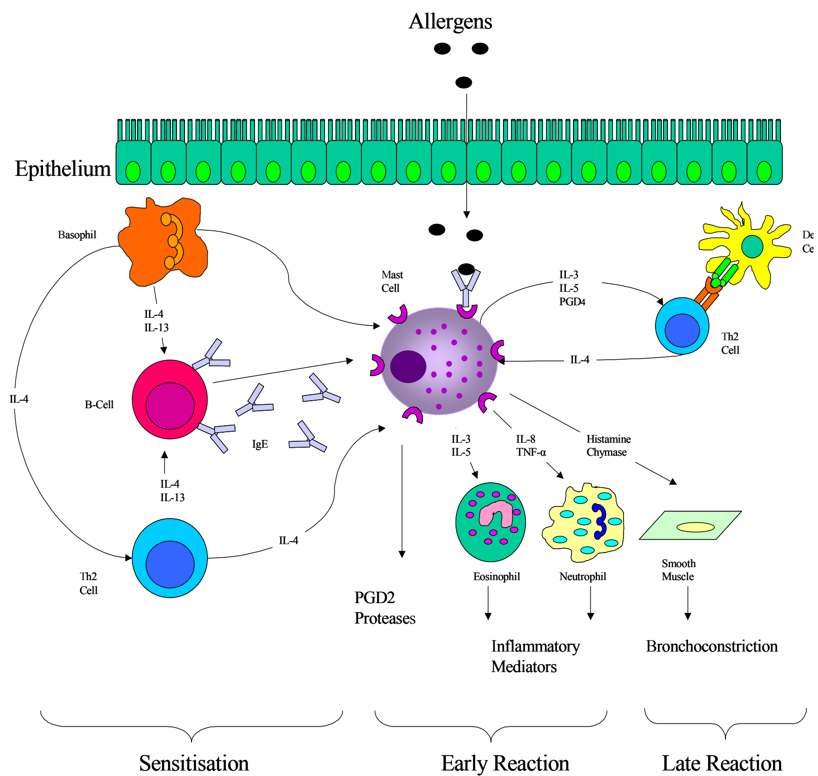



**Figure 1.5: Role of mast cells in initiating disease.** The activation of mast cells not only requires the synthesis of IgE by B-cells but also IL-4 released from T helper 2 cells in order to have full mediator release. The release of the newly formed mediators results in the early reaction normally seen in the skin and mucosal areas affecting blood vessels and sensory nerves resulting in pain. The cytokine release initiates the late phase reaction interacting with a wide range of cells. Adapted from Bischoff, (2007).

### 1.3.1 Type I Hypersensitivity Reaction

The term 'hypersensitivity' normally brings to mind allergic reactions (hay fever, atopic dermatitis and systemic anaphylaxis), all of which are classified into the Type I reactions. Mast cells are central to these responses. Type I reactions are noticeable within minutes of antigen activation of the mast cell due to the robust response induced by FcεRI cross-linking. During the initial sensitisation, certain individuals develop (for currently unknown reasons) IgE to these antigens, thereby upon subsequent re-exposure to the same antigen, robust mast cell responses. This includes early symptoms of vascular permeability and local swelling to the late phase responses by influx of inflammatory cells, tissue re-modelling, over

production of mucus in the airways, and local cutaneous swelling. Allergic inflammation is the most common of inflammatory diseases associated with mast cells, and is classified under the Type I hypersensitivity reactions. This is caused by the prolonged over-production of IgE in response to exposure to certain antigens; these can range from harmful antigens, such as venom and bacterial products, to harmless antigens, such as pollen, food and dust mites. Binding of IgE to these harmless antigens results in the cross-linking of FcεRI, therefore initiating a similar response to that of a harmful antigen, initiating the release of mediators, resulting in increased permeability of blood vessels, tissue oedema, leukocyte recruitment and inflammation. Typical examples of allergy reactions are hay fever, allergic asthma, atopic eczema and drug- and food-related allergies. These allergic responses are well-characterised in the context of Th2-type responses.

### 1.3.2 Type II Hypersensitivity Reaction

Type II hypersensitivity reactions are classical, and defined by interaction of IgG or IgM antibody with a cell surface antigen. This can be followed by the binding of complement-mediated cell lysis. Mast cells participate in Type II reactions, both directly though the binding of complement proteins, or otherwise via IgG antibody-inducing degranulation of mast cells. They can also influence Type II reactions by influencing T-cell responses, thereby helping B-cell isotype switching. Mast cells have been implicated in the Type II disease bullous pemphigoid, which is a chronic subepidermal blistering of the skin, classically characterised by the presence of IgG auto-antibodies to hemidesmosomal antigens BP20 and BP180. In the case of this disease, mast cells triggered by complement activation are a key source of the neutrophil chemoattractant CXCL8 (Nelson *et al.*, 2006).

### 1.3.3 Type III Hypersensitivity Reaction

Antigen-antibody complexes mediate the major immune destruction in Type III hypersensitivities by depositing in organs and tissues, binding complement, and attracting macrophages and neutrophils. Similar to Type II reactions, there are many reports to suggest a role for mast cells in Type III reactions. It has been shown that the ability of both antibodies and complement to induce the activation of mast cells resulting in the production of different chemoattractants, recruiting

pathology-effecter cells. Arthus reactions (a local antibody-mediated hypersensitivity reaction involving antibody-antigen complexes which fix complement, which is deposited in the walls of small vessels, causing acute inflammation with an infiltration of neutrophils), Rheumatoid Arthritis (RA) and systemic lupus erythematous, are such diseases classified as Type III reactions. The role of mast cells in RA is largely supported by the evidence that mast cell-deficient mice do not develop RA (Lee *et al.*, 2002). Developments in new treatments targeting RA are investigating ways in which to reduce the inflammation of the joints by reducing the number of mast cells present.

### 1.3.4 Type IV Hypersensitivity Reaction

Delayed-type hypersensitivities are also known as Type IV hypersensitivity reactions. These reactions often occur many days following initial exposure to antigen and are mediated by antigen-specific CD4+ T-cells. Multiple Sclerosis (MS), a CNS-demyelinating disease, is amongst the most widely studied of Type IV hypersensitivity reactions. The current evidence of the role of mast cells in MS is in both murine and human models, where mast cells accumulate at the site of inflammatory demyelination in the brain and spinal cord, and are often found de-granulated (Ibrahim *et al.*, 1996). High levels of the mast cell-specific protease tryptase are found in the cerebrospinal fluid of MS patients, indicating mast cell activation (Rozniecki *et al.*, 1995).

### 1.3.5 Tumour Development

Tumour development is a multi-step process, interacting genetically-altered malignant cells with the non-neoplastic cells in the surrounding area (Hanahan and Weinberg, 2000). A developing neoplasm often contains many different leukocytes; for example, macrophages, neutrophils, eosinophils and mast cells. The relationship between mast cells and tumours has long been established, since Westphal first reported the presence of A large number of mast cells in the periphery of tumours in 1891 (Prager and Bearden, 1962). Mast cells were originally thought to be a host response to neoplasia; however, evidence gathered over recent years has suggested that the actual role of mast cells may support tumour development (Dimitriadou and Koutsilieris; Ribatti *et al.*, 2001).

## 1.4 Mast Cell Models

Human mast cells utilised in studies can develop from heameopotiec progenitor cells found in cord blood, foetal liver, peripheral blood and bone marrow. Surface expression of the FcεRI receptor occurs constitutively, expressed at low levels in mature cells when differentiated from progenitor cells *in vitro* with recombinant human stem cell factor. This occurs in cells obtained from all of the abovementioned tissues aside from foetal liver. Addition of IL-4 induces the expression of FcεRI in foetal liver, and induces maximum regulation of FcεRI in mast cells derived from umbilical cord blood.

It has been shown that mast cells derived from umbilical cord blood precursors express CD13 and CD117. CD13 has been detected on cultured mast cells derived from foetal liver and cord blood derived precursors. CD13 is a 150kDa cell surface glycoprotein which is identical to the aminopeptidase N — a membrane bound glycoprotein thought to be involved in the regulatory metabolism of peptides by a wide range of cells; furthermore, it is also known to be expressed on stem cells during early stages of myeloid and lymphoid cell development. CD117 is also known as c-Kit, which is the receptor for the agonist SCF. Both the human and mouse c-Kit ligand induces differentiation of human mast cells in a long-term culture of the mononuclear cells of umbilical cord blood. Moreover, human mast cells are commonly characterised by the presence of two specific proteases: tryptase and chymase. The two subsets comprise mast cells, which just contain trypase, and those which contain both tryptase and chymase. Evidence suggests that it is possible to switch from single- to double-positive with prolonged culture with IL-4 (Toru *et al.*, 1998). It is thought that, in the majority of mast cells cultured under SCF conditions, the granule content will just develop tryptase.

The study of mast cells is challenging for a wide variety of reasons. Firstly, mast cells are found in the body in small numbers in both humans and experimental animals, and so it is difficult to obtain large numbers of such cells for experimental purposes. One solution for overcoming these problems is the development of mast cell culture systems. It was discovered that a large number of mast cells could be grown from the bone marrow of mice or rats by addition of IL-3 (Razin and Marx, 1984). This was the first reported mast cell model which enabled large-scale

experiments. There were, however, drawbacks to the system. Despite the expression of FcεRI, the cells generated only developed immature phenotypic characteristic. Furthermore, over time, there have been many other different methods of generating mast cells from many different sources, including foetal liver (Irani *et al.*, 1992b), bone marrow (Kirshenbaum and Metcalfe, 2006),cord blood (Durand *et al.*, 1994), as well as CD34+ peripheral blood (Kirshenbaum and Metcalfe, 2006). Additional to the development of these primary mast cells models, many mast cell line models exist; for example, HMC-1 (Butterfield *et al.*, 1990), LAD-2 (Kirshenbaum *et al.*, 2003) and the widely used rat mast cell line RBL-2H3 (Oliver *et al.*, 1988).

The utilisation of these primary mast cell models and mast cell lines has accordingly enabled the discovery of how mast cells respond to a wide range of different physiological and pathological mediators. These models have also subsequently enabled deeper understanding of the role of mast cells in inflammatory responses.

### 1.4.1 The Cytokine Milieu at the Precursor Stage

A previous study (Mitsui *et al.*, 1993) indicates that human mast cells differentiated from cord blood and developed by cytokine control are similar to human mast cells in the lung and gut mucosa according to similarities in sub-structural granule patterns of the cells. The majority of these cells contained only tryptase — unlike skin mast cells, which contain both tryptase and chymase (Mitsui *et al.*, 1993). Other studies, on the other hand, reveal that levels of chymase are almost doubled per individual mast cell in bone marrow derived compared to cord blood-derived mast cells (Shimizu *et al.*, 2002). This would indicate that the mast cell receptor profile is most likely shaped by the cytokines milieu present at the relevant progenitor stage.

Over the last decade and from a wide variety of sources, many studies have developed methods with the aim of culturing mast cells. Progenitor cells derived from cord blood are amongst the most widely studied. According to their neutral protease contents, two subtypes of human mast cells have so far been identified; those which are tryptase positive and those that are both chymase and tryptase

positive (Beil and Pammer, 2001; Craig and Schwartz, 1990; Forsythe and Ennis, 2000). There is the possibility that a third sub-set of mast cells exist which are chymase positive but tryptase negative (Li *et al.*, 1996). It is thought that mast cells derived from cord blood are believed to be predominately only tryptase positive, with low levels of mast cells containing both tryptase and chymase (Irani *et al.*, 1992a). Moreover, previous studies suggest that the majority of mast cells cultured under SCF will only develop tryptase (Mitsui *et al.*, 1993).

### 1.4.2 The Importance of IL-4 Addition of Mast Cell Development

The mast cells used in this study and the subsequent studies were generated from human cord blood derived CD133+ progenitors by culturing these cells in the presence of SCF and IL-6 in a serum-free medium for 8 weeks, with additional supplementing mature mast cells with serum and IL-4. The late addition of IL-4 is to maximise the expression of FcεRI and to enhance degranulatory responses when FcεRI becomes aggregate (Dahl *et al.*, 2002; Sayama *et al.*, 2002). Studies have revealed that addition of IL-4 in culture medium up regulates FcεRI (Kinet, 1999).

## 1.5 Chemokines and Chemokine Receptors

### 1.5.1 A Brief History of Chemokine/Chemokine Receptor System

The coordinated movement of the leukocytes is critical to both innate and adaptive immune systems, and is primarily mediated by the chemokine system. The history of chemokines first began in 1977, when the secreted platelet factor 4 (PF4/CXCL4) was purified without actual knowledge of its function or receptor (Walz *et al.*, 1977; Wu *et al.*, 1977). It was not until a decade later that the next chemokine (interleukin-8 (IL-8/CXCL8) was discovered, which showed chemotactic activity for neutrophils, establishing that chemokines are key elements in the control of leukocyte migration (Yoshimura *et al.*, 1987). It was not until 1992 at the 3rd International Symposium on Chemotactic Cytokines at Baden, Austria, that the term ‘chemokines’ was proposed and the name has remained. The next major finding in the chemokine field came with certain chemokines functioning as HIV-suppressive factors *in vitro* by blocking viral interaction with specific chemokine receptors, and that some chemokine receptors act as co-receptors for viral entry (Bleul *et al.*, 1996; Cocchi *et al.*, 1995; Feng *et al.*, 1996; Oberlin *et al.*,

1996). One year later, it followed with the discovery that CCR5 was a major co-receptor for the entry of HIV (Deng *et al.*, 1996). This research formed the initial foundation that would subsequently lead to the release of the first pharmaceutical chemokine target-based drug being approved for medical use — maraviroc, a CCR5 inhibitor used in the treatment of HIV — which has subsequently led the way for development of more chemokine-based treatments. The next reported chemokine target-based treatment is Schering-Plough's vicriviroc, which is showing potent antiretroviral activity in clinical trials, which is targeted against CCR5.

### 1.5.2 Nomenclature and Structural Characteristics of Chemokines

Following the first chemokine being discovered, their role became am interesting topic for investigation and debate as to their role and function in inflammatory responses (Thelen, 2001). Chemokines or chemotactic cytokines, as they are still sometimes referred to, are small secretary or membrane-bound proteins with molecular masses of 6-14kDa. There are around 50 chemokines which have been discovered to date, all of which have been traditionally divided into two major groups based upon their sequence homology and the position of the first two cysteine residues at the NH2 terminus, C-X-C chemokines (or $\alpha$ chemokines) and CC (or $\beta$ chemokines). The CXC chemokines contain a single non-conserved amino acid between the first two cysteines of this motif, whereas the CC chemokines have these residues juxtaposed. Two other minor groups of chemokines exist — C and CX3C, both having so far only one member per group. The former one lacks cysteines one and three of the typical chemokine structure (Kelner *et al.*, 1994), whereas the latter exhibits three amino acids between the first two cysteines and is also the only membrane-bound chemokine through a mucin-like stalk (Bazan *et al.*, 1997).

The expression profile of chemokines in a particular inflammatory site ultimately determines the type of infiltrating leukocyte; therefore, recruiting the correct cells to the right areas is done in order to reduce inflammation. As such, chemokines have been further characterised by their physiological features, considering the conditions and locations of chemokine production, as well as the cellular

distribution of chemokine receptors, subsequently leading to the separation of chemokines into so-called inflammatory (or alternatively called inducible) chemokines and homeostatic (or constitutive, housekeeping, or lymphoid) chemokines (Moser and Loetscher, 2001) (Figure 1.6).

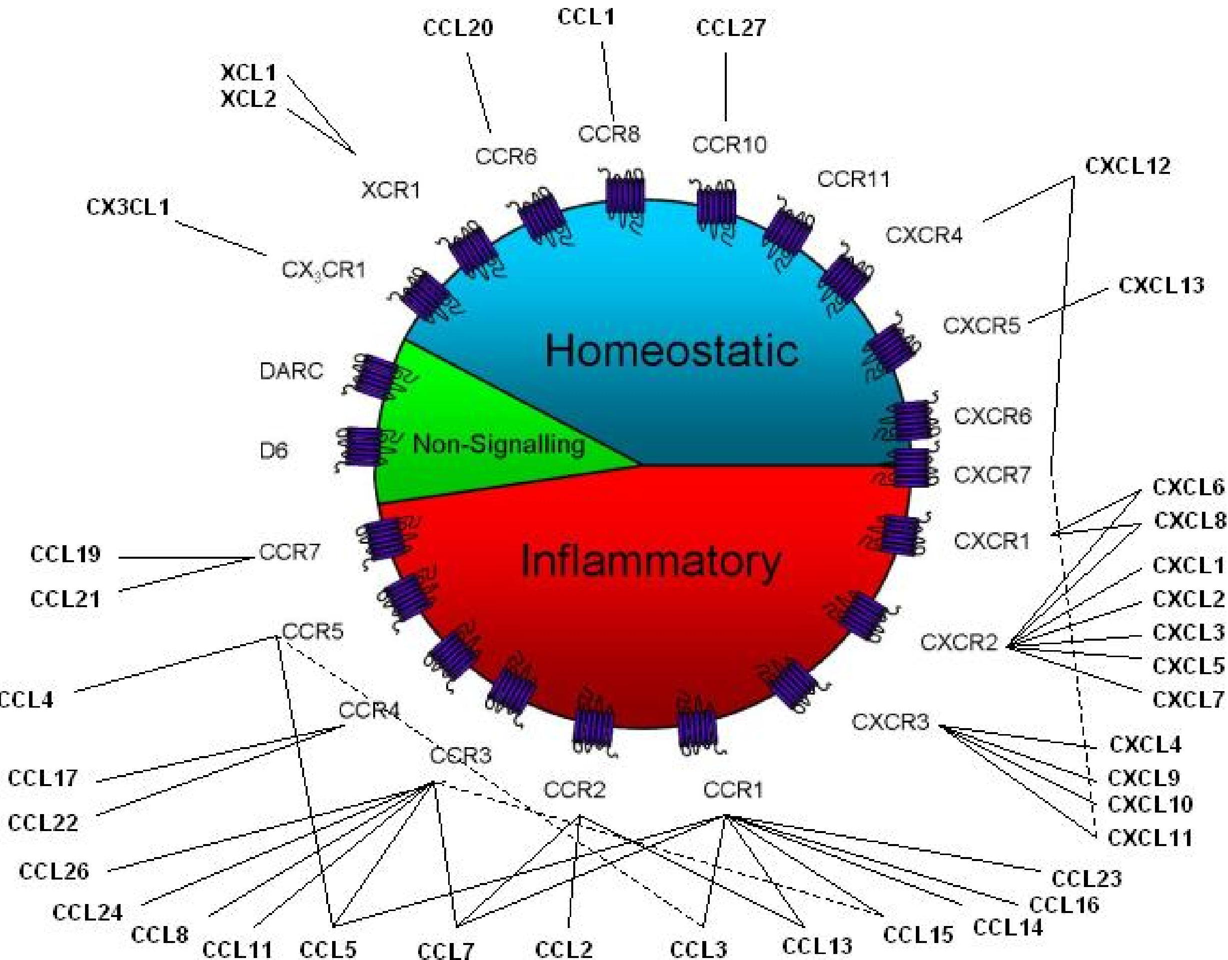



**Figure 1.6: Characterisation of chemokine receptors by the role in immune responses.** Chemokines are classified into three different charactertories: homeostatic, inflammatory and non-signalling. The classification depends on their characteristic features.

Inflammatory chemokines are expressed in inflamed tissues by resident and infiltrated cells on stimulation by pro-inflammatory cytokines or during contact with pathogenic agents. In contrast, homeostatic chemokines and their receptors are produced in discrete microenvironments within lymphoid or non-lymphoid tissues,

such as the skin and mucosa (Moser and Loetscher, 2001). These chemokines are involved in creating the directional map which the adaptive immune system follows during antigen surveillance.

Throughout history, many of these chemokines have had many different names assigned to them. It was the consensus of opinion within the field that their nomenclature be standardised based on their structure and how they were traditionally considered. The proposed chemokine nomenclature is based on the chemokine receptor nomenclature currently in use, utilising CC, CXC, XC or CX3C followed by an R (representing it is a receptor) followed by a number, therefore generating the current chemokine receptors known as CCR1-9, CXCR1-7, XCR1 and CX3CR1. The new nomenclature for chemokines follows a similar pattern, replacing the R with an L with the view of representing the fact that this is a ligand, not a receptor. Figure 1.7 highlights the old and new nomenclature of the human chemokine systems along with their respective receptor and genomic locations.

**The Human Chemokine/ Chemokine Receptor family**

**CXC Chemokine/Receptor family**

| Systematic name | Human Chromosome | Human Ligand | Chemokine receptor(s) |
|---|---|---|---|
| CXCL1 | 4q12-q13 | GROα/MGSA-α | CXCR2 > CXCR1 |
| CXCL2 | 4q12-q13 | GROβ/MGSA-β | CXCR2 |
| CXCL3 | 4q12-q13 | GROγ/MGSA-γ | CXCR2 |
| CXCL4 | 4q12-q13 | PF4 | Unknown |
| CXCL5 | 4q12-q13 | ENA-78 | CXCR2 |
| CXCL6 | 4q12-q13 | GCP-2 | CXCR1, CXCR2 |
| CXCL7 | 4q12-q13 | NAP-2 | CXCR2 |
| CXCL8 | 4q12-q13 | 1L-8 | CXCR1, CXCR2 |
| CXCL9 | 4q21.21 | Mig | CXCR3 |
| CXCL10 | 4q21.21 | IP-10 | CXCR3 |
| CXCL11 | 4q21.21 | I-TAC | CXCR3 |
| CXCL12 | 10q11.1 | SDF-1α/β | CXCR4 |
| CXCL13 | 4q21 | BLC/BCA-1 | CXCR5 |
| CXCL14 | Unknown | BRAK/bolekine | Unknown |
| CXCL15 | Unknown | Unknown (Lungkine in mouse) | Unknown |

**C Chemokine/Receptor family**

| Systematic name | Human Chromosome | Human Ligand | Chemokine receptor(s) |
|---|---|---|---|
| XCL1 | 1q23 | Lymphotactin | XCR1 |
| XCL2 | 1q23 | SCM-1b | XCR1 |

**CX3C Chemokine/Receptor family**

| Systematic name | Human Chromosome | Human Ligand | Chemokine receptor(s) |
|---|---|---|---|
| CX3CL1 | 16q13 | Fractaline | CX3CR1 |

**CC Chemokine/Receptor family**

| Systematic name | Human Chromosome | Human Ligand | Chemokine receptor(s) |
|---|---|---|---|
| CCL1 | 17q11.2 | I-309 | CCR8 |
| CCL2 | 17q11.2 | MCP-1/MCAF | CCR2 |
| CCL3 | 17q11.2 | MIP-1α /LD78α | CCR1, CCR5 |
| CCL4 | 17q11.2 | MIP-1β | CCR5 |
| CCL5 | 17q11.2 | RANTES | CCR1, CCR3, CCR5 |
| (CCL6) | | Unknown (mouse only) | Unknown |
| CCL7 | 17q11.2 | MCP-3 | CCR1, CCR2, CCR3 |
| CCL8 | 17q11.2 | MCP-2 | CCR3 |
| (CCL9/CCL10) | | Unknown (mouse only) | Unknown |
| CCL11 | 17q11.2 | Eotaxin | CCR3 |
| (CCL12) | | Unknown (mouse only) | Unknown |
| CCL13 | 17q11.2 | MCP-4 | CCR2, CCR3 |
| CCL14 | 17q11.2 | HCC-1 | CCR1 |
| CCL15 | 17q11.2 | HCC-2/Lkn-1/MIP-1δ | CCR1, CCR3 |
| CCL16 | 17q11.2 | HCC-4/LEC | CCR1 |
| CCL17 | 16q13 | TARC | CCR4 |
| CCL18 | 17q11.2 | DC-CK1/PARC/AMAC-1 | Unknown |
| CCL19 | 9p13 | MIP-3β /ELC/exodus-3 | CCR7 |
| CCL20 | 2q33-q37 | MIP-3α /LARC/exodus-1 | CCR6 |
| CCL21 | 9p13 | 6Ckine/SLC/exodus-2 | CCR7 |
| CCL22 | 16q13 | MDC/STCP-1 | CCR4 |
| CCL23 | 17q11.2 | MPIF-1 | CCR1 |
| CCL24 | 7q11.23 | MPIF-2/Eotaxin-2 | CCR3 |
| CCL25 | 19p13.2 | TECK | CCR9 |
| CCL26 | 7q11.23 | Eotaxin-3 | CCR3 |
| CCL27 | 9p13 | CTACK/ILC | CCR10 |

**Figure 1.7: Nomenclature of Chemokine Receptors and their agonists.** Chemokines can be classed according to the number of amino acids separating the first two cysteine residues at the NH2 terminal. The receptors for CXC subclasses are shown in green; the C-C subclasses are in light blue; and the minor subclasses are in blue. The pairing of chemokines to their receptors is principally based on receptor-binding assays, and has identified receptors which have just one agonist, whereas other receptors have multiple. Each of the agonists throughout history has been recorded with a common name and systemic name. Adapted from Murphy *et al.*, (2000).

The chemokine family can be loosely grouped as a group of three gene clusters representing duplicated genes with similar function and a smaller number of isolated genes representing highly conserved chemokines, with no functional and little or no receptor sharing, allowing broad grouping of the chemokines according to function. The CXC 4q12-13 cluster chemokines act mainly on neutrophils, while the CC 17q11.2 cluster chemokines are predominantly monocyte and effector T-cell chemoattractants. The CXC chemokines in the 4q21.21 mini-cluster act specifically as T-cell chemoattractants, as highlighted in Figure 1.7.

### 1.5.3 Chemokine Receptor Structure

Chemokines mediate their effects by binding to G-protein coupled, 7-transmembrane serpentine receptors called chemokine receptors. The classical structure of a chemokine receptor is highlighted in Figure 1.8. Studies published in 1991 describe the first chemokine receptors (CXCR1 and CXCR2) which, like all signalling chemokine receptors identified since, belong to the family of G protein-coupled receptors (Holmes *et al.*, 1991; Murphy and Tiffany, 1991).

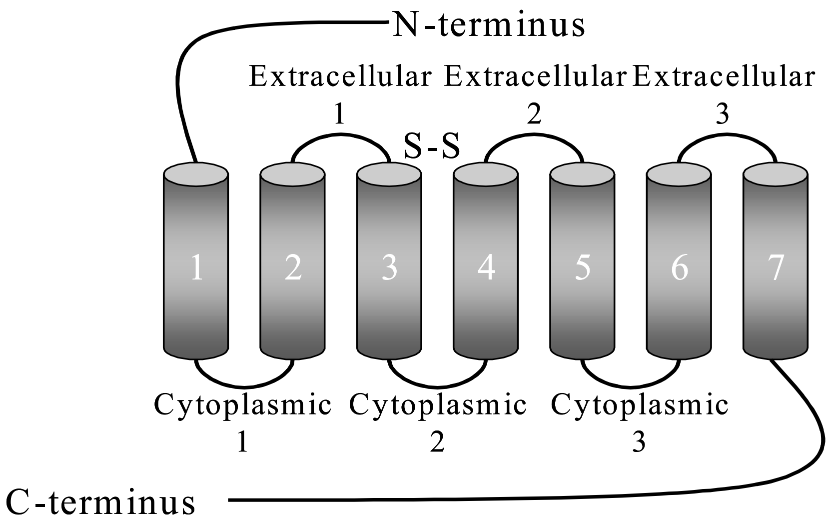



**Figure 1.8: Schematic depicting a typical GPCR.** A representational diagram of an unfolded chemokine receptor, highlighting the 7-transmembrane domains, the intra and extracellular loops with the second intracellular loop being critical for signalling of GPCRs. The N-terminus hangs on the outside of the cell with the C-terminus stored intracellularly.

The agonist-receptor network is complex: some chemokine receptors have just a single ligand, e.g. CXCR4 only mediates its effect via CXCL12, whereas other receptors can have multiple ligands; for example, CXCR3 mediating its effect via 4 different agonists, CXCL9, CXCL10, CXCL11 and CXCL4. It is common for single chemokines to act as ligand for multiple different receptors, and that each receptor can bind an array of chemokines.

The complexity of the chemokine/chemokine receptor system may provide a very complicated but nevertheless effective way of regulating the cellular responses and functions of different cell types, thereby creating a robust system of over-lapping ligands and receptors which protect the host. For example, CXCR3 agonists CXCL9, CXCL10 and CXCL11 are involved in responses driven by Th1 cells, and are accordingly reported to be antagonists for CCR2, which is thought to play a role in Th-2 driven response (Loetscher and Clark-Lewis, 2001). Furthermore, CCL11 as an agonist for CCR3 has been shown to be a partial agonist for CCR2 and CCR5 (Martinelli *et al.*, 2001). Cross-reactivity seems to be a fundamental property of the chemokine/chemokine receptor system acting as a self-regulating system to protect the host.

Chemokine receptors are expressed on the majority of the cells involved in the immune system, with each type of cell having a unique and distinct array of chemokine receptors depending on the function of the cell. Chemokine receptors share 25-80% identity at an amino acid level, indicating a common ancestor with several similar features and motifs shared amongst different chemokine receptors.

### 1.5.4 Conserved Motifs in the Chemokine System

With similar homology throughout the chemokine receptor system, certain key motifs have been highly conserved through the evolution of the chemokine/chemokine receptor linage. Such important conversed motifs include the DRYLAIVHA motif, the NPXXY motif and the Gly at junction of 7$^{th}$ TM and cyto domain.

#### a) Functional motifs in agonists:

The CXC chemokines are further sub-classified according to the presence or absence of ELR motif in the N-terminal, Glu-Leu-Arg (ELR)$^{+}$ and Glu-Leu-Arg (ELR)$^{-}$. the ELR$^{+}$ group of chemokines includes CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8 and CXCL15, all of which have been shown to have angiogenic activity and attract mainly neutrophils and polymorphonuclear (PMN) leukocytes to the sites of inflammation. ELR$^{-}$ chemokines — to which CXCL4, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, CXCL14 and CXCL16 belong —

have mainly angiostatic properties and attract lymphocytes and monocytes with poor chemotactic ability for neutrophils (Laing and Secombes, 2004).

### b) Receptor signalling motifs

**DRYLAIV:**

The sequence DRYLAIVHA is found at a length of 340 to 370 amino acids — an acidic flexible N-terminal segment or a variation n the second extracellular loop. This flexible N-terminus is important during activation. The DRYLAIV motif is conserved in all signalling chemokine receptors, and is a variant of the acidic residue arginine aromatic residue motif found at this position. In all signalling chemokine receptors, it is considered necessary for G protein coupling and calcium signalling. Exceptions to this rule include the non-signalling promiscuous chemokine receptor-like molecules, Duffy Antigen Receptor for Chemokines (DARC) and D6, which will be discussed later on in this paper.

The interaction between R of the DRY motif with its adjacent D/E residue at position 3.49 and an additional D/E at position 6.30 near the cytoplasmic end of TM6 is known as the ionic lock (Ballesteros *et al.*, 2001). Charge-neutralising mutation of D/E6.30 in TM6 results in increased constitutive activity (Ballesteros *et al.*, 2001; Montanelli *et al.*, 2004). Another characteristic feature of chemokine receptors is the highly conserved NPXXY motif found within TM7, in which N residue acts as an on/off switch by adopting two active and inactive conformational stages (Govaerts *et al.*, 2001; Urizar *et al.*, 2005).

**NPXXY**

The NPXXY motif (with the X representing amino acid) in the 7$^{th}$ trans-membrane domain is highly conserved within the chemokine receptor family. This motif is thought to contribute to the internalisation and signal transduction of chemokine receptors (He *et al.*, 2001). Positions of Asn, Pro, and Tyr residues are almost invariable within the motif. The inner two residues are generally hydrophobic in nature, whilst the residues that flank Tyr and Asn may vary (Probst *et al.*). Asn7.49 of the highly conserved NPxxY motif in TM 7 acts as an on/off switch by adopting alternative conformations in the inactive and active receptor states (Govaerts *et al.*, 2001; Urizar *et al.*, 2005). Upon receptor activation, N7.49 is proposed to adopt

the trans-conformation to interact with D2.50 of the (N/S)LxxxD motif in TM2 (Remko *et al.*, 2007). The NPXXY motif has been associated with receptor trafficking (Barak *et al.*, 1995).

### 1.5.5 Silent Chemokine Receptors

The inflammatory process depends on the precise control of each part. It has been long thought that chemokine receptors play a role in the recruitment of inflammatory cells to the site of infection or injury; however, not all chemokine receptors which have been so-far discovered to date have this function; these are so-called atypical chemokine receptors and have apparent non-signalling or 'silent' properties, and raise the question as to the function of these abundant receptors.

The chemokine system includes at least three 'silent' receptors — DARC, D6 and CCX CKR — each of which has distinct specificity and tissue distribution (Locati *et al.*, 2005). Mechanistically, DARC and D6 represent a subclass of chemokine internalising receptors, 'interceptors,' taking chemokines into nucleated cells in the apparent absence of signalling (Nibbs *et al.*, 2003). These receptors have moderate-to-high homology when compared to classical receptors, but ultimately lack in G protein-coupling motifs and are incapable of eliciting chemotactic or activating responses to a ligand (Liu *et al.*, 2006). These receptors are different from their chemotactic counterparts, as they show a mutation in the second intracellular loop. Typically, chemokine receptors display the motif 'DRYLAIV' in their second intracellular loop, with this motif accordingly acting as a key mediator in the coupling to downstream signalling.

### 1.5.6 D6 Receptor

The D6 receptor is a typical chemokine receptor but has a mutation in its DRYLAIV motif: it has been mutated to DKYLEIV and also has replaced an aspartic acid residue to an asparagine in the second trans-membrane domain — both key components of G-protein dependent signalling. The D6 receptor shares similarities in terms of structure with other chemokines, binding at least 13 CC chemokines (Nibbs *et al.*, 1997), all of which are known to be inflammatory. Curiously, D6 has been demonstrated as being unable to bind constitutive chemokines (Fra *et al.*, 2003). Chemokines which bind to D6 are also rapidly

internalised, followed by dissociation from the receptor, and remain trapped within the cell for degradation. Meanwhile, D6 recycles back to the plasma membrane for further ligand sequestration (Hansell *et al.*, 2006).

*In vivo* studies reveal that deletion of D6 can result in the increased susceptibility to skin cancer and inflammatory similar to psoriasis (Nibbs *et al.*, 1997). Anti-inflammatory tumour suppressor properties of D6 are also supported by *in vivo* with null mice studies (Bonecchi *et al.*, 2004; Jamieson *et al.*, 2005). D6 is expressed at low levels by circulating leukocytes (Borroni *et al.*, 2006); however, high levels of D6 were found on endothelial cells of lymphatic afferent vessels in the skin, gut and lungs (Nibbs *et al.*, 2001) and in the placenta (Bonini *et al.*, 1997). D6 cannot couple with signalling pathways used by chemokines, but instead poses unusual intracellular trafficking properties to mediate repeated rounds of chemokine internalisation (Fra *et al.*, 2003; Weber *et al.*, 2004; Bonecchi *et al.*, 2004). Moreover, D6 undergoes rapid constitutive ligand-independent trafficking both to and from plasma membrane, utilising β-arrestin and clathrin dependent route of internalisation; this feature is unique amongst mammalian chemokine receptors (Weber *et al.*, 2004).

### 1.5.7 DARC

Similar to the D6 receptor, DARC has its DRYLAIV motif mutated. DARC has the motif DRYLGH resulting in the receptor being unable to signal. DARC may act as either a reservoir or transporter to both CC and CXC chemokines, maintaining the presence of chemokines with the circulation, releasing them when required. This mechanism can also aid the desensitisation of leukocytes. DARC also uses chemokine sequestration in order to control the CC and CXC chemokines which it binds (Nibbs *et al.*, 2003; Rot, 2005; Du *et al.*, 2002); this limits chemokine availability and regulates blood chemokine levels (Fukuma *et al.*, 2003; Jilma-Stohlawetz *et al.*, 2001). Moreover, it has been suggested that DARC promotes chemokine transcytosis across blood vessel endothelial cells. Furthermore, whilst chemokines internalised by D6 are degraded, the fate of chemokines internalised by DARC may be cell context-dependent, and it is further possible that they can maintain their biological activities. Therefore, it has been stated that DARC

expressed on erythrocytes and vascular endothelial cells may act as either biological sink or a transporter for both CC and CXC chemokines, respectively. This suggests that, in contrast to chemokine decoy/scavenger D6, DARC plays a more complex role in chemokine homeostasis (Pruenster and Rot, 2006).

### 1.5.8 CCR11/CCX-CKR

Recent work carried out by Comerford *et al* suggests that, along with D6 and DARC, CCX-CKR also shows biochemical properties of a chemokine-sequestrating atypical chemokine receptor (Comerford *et al.*, 2006). In contrast to previously described 'atypical' receptors, CCX-CKR binds homeostatic chemokines, namely CCL19, CCL21 and CCL25, and also exhibits weak interactions with human CXCL13 (Gosling *et al.*, 2000). Similar to DARC and D6, CCX-CKR does not couple with typical signalling pathways, and displays alterations within DRYLAIVHA motif. CCX-CKR has been shown to internalise and degrade CCL19 *in vitro* high efficiency and in β-arrestin independent manner; however, in contrast to most chemokine receptors, this process was abolished through caveolin-1 manipulations.

### 1.5.9 Glycosaminoglycans

Chemokines do not only bind to GPCRs: they are also known to be capable of binding to proteoglycans. This process is facilitated by highly acidic glycosaminoglycan (GAG) chains (Handel *et al.*, 2005). The majority of chemokines need high (in the micro-molar range) concentrations to bind to GAGs, although it has been demonstrated that CXCL4 can bind GAGs in a nano-molar range. It is thought that the interaction of chemokines with the endothelial cell-expressed proteoglycans is to immobilise high concentrations of locally generated chemokines on the luminal surface of the microvascular endothelium. It has been proposed that chemokines are present on the surface of the endothelial, triggering the signalling in leukocytes resulting in the up-regulation of selecting molecules resulting in the adhesion and migration of leukocytes through the endothelial barrier and into the tissue.

## 1.6 The Biological Function of Chemokines

The immune system of the body is a dynamic system. In order to effectively fight infections and diseases, cells must first migrate around the body to the area under attack. and not wait until the fight comes to them. Cell movement is not only essential for encountering pathogens, but also for initiating the complex interactions between different immune cells. Chemokine receptors and their agonists create the navigation system of the body and induce the migration of immune cells.

### 1.6.1 Migration

Migration is a multi-step process involving the co-ordinated interaction of adhesion molecules and chemokines and their receptors between leukocytes and endothelial cells, and the regulated migration from the bone marrow to the blood, and further from the blood to the tissue. The cytoskeletal re-organisation and polarisation of leukocytes requires integrin activation by chemokines, thereby allowing firm adhesion and migration.

There are several defined mechanisms of migration. The term ‘chemotaxis’ is the directed migration of a cell towards a chemotactic gradient; similarly, haptotaxis also describes the directional movement of a cell towards a chemotactic gradient but on an immobilised substrate. Chemokinesis involves the random movement of cell which is independent of any chemotactic gradient. Chemofugetaxis is the active movement of a cell away from a chemokine source. This phenomenon was first described following the observation that high (but not low) concentrations of CXC12 has the ability to induce chemofugetaxis in a sub-population of T-cells; furthermore, it is also thought to contribute to thymic emigration (Poznansky *et al.*, 2000). Directional migration requires three distinct features of a cell, including the extension of pseudopodia, gradient sensing and polarisation, and directional movement.

### 1.6.2 Extension of Pseudopodia

In chemokinesis, pseudopodia are extending and contracting from the cytoplasm over the cell surface. Following cell activation, these pseudopodia are focused towards the leading edge of a cell due to the accumulation of such signalling

molecules; for example, PtdIns $(3,4,5)_3$. This movement and rearrangement of signalling molecules is considered critical in the initiation of directional migration.

### 1.6.3 Polarisation

The process of polarisation in directional migration in leukocytes requires the cell to be polarised, with the molecular processes at the front (leading edge) of a moving cell being distinct from those at the back (uropod) (del Pozo *et al.*, 1995). This polarised shape of the cell is the result of a redistribution of F-Actin from a fairly uniformed distribution throughout the cell, to being concentrated within the leading edge (Coates *et al.*, 1992; Howard and Oresajo, 1985; Parent and Devreotes, 1999). Furthermore, it is known that leukocytes do possess some intrinsic cell polarity where there is differential sub-cellular localisation of the F-actin filaments and assembled myosin, thereby allowing rapid responses to chemoattractant gradients.

### 1.6.4 Actin Polymerisation

During the process of migration, the polymerisation of actin filaments is critical to establishing and maintaining cell polarity. The functional unit of actin is known as globular action (G-actin), and is the polymerisation of these intrinsically polarised units by the action of ATP. Once bound together, these generate filamentous action (F-actin), which is generated by the polymerisation at one end of the filament (known as the barbed end), whereas depolymerisation occurs at the opposing or pointed end. The formation of F-actin at the leading edge drives the plasma membrane forward, subsequently resulting in the protrusion of the cell. The growth of F-actin is only halted by the binding of a capping protein on the barbed end of the filament, thereby maintaining or stabilising the filament or promoting depolymerisation. The phosphorylation and dephosphorylation rates are relatively equal, thus ensuring the maintenance of the current filament length. This process is known as tread-milling (Revenu *et al.*, 2004; Vicente-Manzanares *et al.*, 2005).

Despite the fact that inducing the migration is the primary function of chemokine, there is nevertheless increasing evidence for the role of chemokines in non-

migration responses which are also modulated by chemokines, including lymphocyte differentiation and effecter functions (Luther and Cyster, 2001).

### 1.6.5 Lymphocyte Modulation by Chemokines

During T-cell activation, CCR5 and CXCR4 are recruited to the immunological synapse and, in this context, as opposed to delivering signals competing with those induced by TCR and adhesion molecules, they function as T-cell co-stimulators (Molon *et al.*, 2005). Indeed, heterodimerisation of CCR5 with other chemokine receptors, such as CXCR4 or CCR2, elicit the coupling of Gq to the receptor, and this change in G protein results in preferential chemokine-induced cell adhesion rather than chemotaxis (Mellado *et al.*, 2001a; Mellado *et al.*, 2001b). It has also been demonstrated that T-cell chemokine receptors coupled with Gq and/or G11 protein are recruited to the immunological synapse by a Gi-independent mechanism (Molon *et al.*, 2005). CXCR4 has been also shown to be physically associated with the T-cell Receptor to induce signalling events in T-cells (Kumar *et al.*, 2006).

Further evidence shows that some chemokines can act as modulators of angiogenesis, thereby promoting inflammatory responses, tumour growth or interference with angiogenesis, thereby inhibiting tumour growth (Homey *et al.*, 2002). Furthermore, several chemokines have been shown to be potent activators of innate immune cell, such as basophils and eosinophils, mediating different release responses (Bischoff *et al.*, 1993; Jinquan *et al.*, 2000; Kampen *et al.*, 2000).

Many different molecules can be found in the directed migration of motile cells. Several different molecules exist, with the most notable being the chemokine family, as described in detail in this study.

### 1.6.6 Lipid Chemoattractants

Mast cells are a key source of other mediators besides chemokines which are able to induce migration. These potent mediators of inflammation are derivatives of Arachidonic Acid (AA) a 20-carbon unsaturated fatty acid produced from membrane phospholipids.

The principal pathways of arachidonic acid metabolism are:

- the 5-lipoxygenase pathway, which produces a collection of leukotrienes (LT).
- the cyclooxygenase (COX) pathway, which produces prostaglandin $H_2$ ($PGH_2$). $PGH_2$ serves as the substrate for two enzymatic pathways; one leading to the production of several prostaglandins (PG); the other leading to the production of thromboxanes (Tx).

The release of leukotriene $B_4$ — which has been demonstrated as a potent mediator of neutrophil chemotaxis and stimulator of leukocyte adhesion to endothelial cells — is of particular interest. Leukotriene $B_4$ mediates its effect via GPCRs B-LT1 and B-LT2. The receptor B-LT1 has been shown to be restricted to leukocytes (Kamohara *et al.*, 2000; Tager *et al.*, 2003). The release of this mediator from mast cells has been implicated in a wide range of inflammatory diseases; for example, increased levels in murine models of Chronic Obstructive Pulmonary Disease (COPD), asthma and RA (Crooks *et al.*, 2000; Montuschi and Barnes, 2002). This evidence has since been further backed-up in studies by the administration of $LTB_4$ antagonist in models of RA, reducing levels of inflammation (Griffiths *et al.*, 1995).

### 1.6.7 Neuropeptides and Mast Cells

Human skin mast cells respond to neuropeptide stimulation with a rapid release of histamine and minimal generation of $PGD_2$ and $LTC_4$ (Bischoff, 2004). As such, they are uniquely positioned in such a way so as to respond to neuropeptides produced by nearby neurons. Mast cells express receptors for neuropeptides, such as Substance P (SP), Nerve Growth Factor (NGF), calcitonin gene-related peptide (CGRP) and Vasoactive Intestinal Polypeptide (VIP) (Kulka et al., 2008). These neuropeptides are believed to activate mast cells either by direct G protein binding or by ligating specific surface receptors (Ferry, 2002). The neuropeptide activation of human mast cells not only activates degranulation and release of preformed granule contained mediators, but can also induce the production of

cytokines and chemokines, including GM-CSF, IL-3, MCP-1, IP-10, RANTES and IL-8. These mediators can recruit and activate T-lymphocytes, eosinophils and other inflammatory cells.

### 1.6.8 The Chemokine Expression Profile of Mature Mast Cells

Many factors can influence the range of chemokine receptors which are expressed on the surface of a specific cell. The health and age of the host and anatomical location of specific cell types are contributing factors. In the case of *in vitro* studies, the activation and cytokine milieu which are exposed to the cell are also considered to be contributing factors that can ultimately determine the chemokine profile, as well as the mast cell sub-type that will develop. At least nine chemokine receptors (CXCR1, CXCR2, CXCR3, CXCR4, CX3CR1, CCR1, CCR3, CCR4 and CCR5) have been described as being expressed by human mast cells of different origins (Juremalm and Nilsson, 2005). Moreover, seven chemokines (CXCL1, CXCL5, CXCL8, CXCL14, $CX_3CL1$, CCL5 and CCL11) have been shown to act on some of these receptors and to induce mast cell migration (Juremalm and Nilsson, 2005).

### 1.6.9 The Role of Chemokines in Disease

Due to the sheer number and variety of biological functions which involve the chemokine system, mis-regulation of this system can result in great harm to the host. Chemokines have been implicated in a wide range of inflammatory diseases, including autoimmune, cardiovascular, cancer, transplantation, neuro-inflammation, HIV and allergic inflammatory diseases (Gerard and Rollins, 2001). Figure 1.9 provides an overview of certain chemokine receptors involved in the case of different diseases.

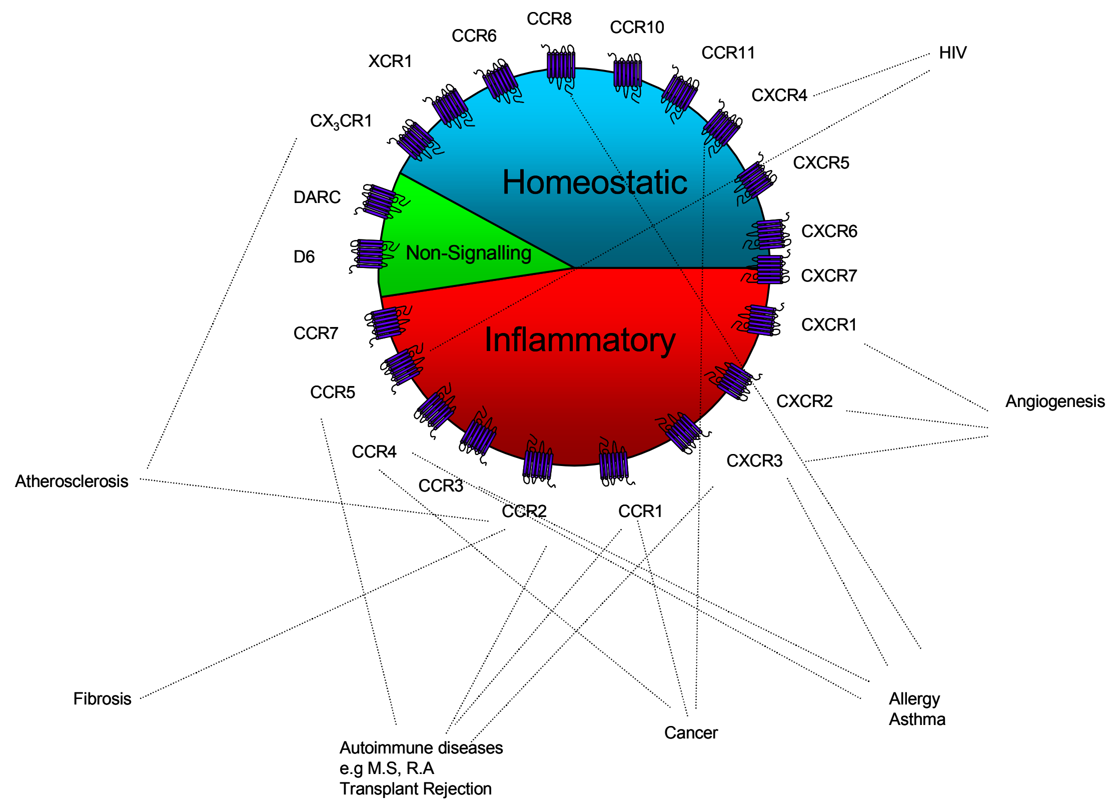



**Figure 1.9: Chemokine receptors and disease.** Chemokine receptors can be classified according to their function. Homeostatic chemokine receptors are responsible for orchestrating trafficking and homing of leukocytes during their immune surveillance role. The inflammatory chemokine receptors are important components of the immune system. Non-signalling chemokine receptors act as a chemokine sink to remove excess chemokines from the system. Adapted from Johnson *et al.*, (2004).

One strategy for investigating the role of chemokines in disease is the implementation of the knockout murine models. Gene-targeting approaches have proved particularly useful when dissecting the role of specific chemokines and their receptors *in vivo*. The ability to remove or alter with precision a single gene by gene-targeting or knockout is now a routine technique which is commonly adopted when creating animal models that can be used to study the pathophysiology of various diseases. The aim of a gene knockout is to ablate the function of the targeted gene in order to discern its role *in vivo*. Table 1.1 provides an overview of certain chemokine knockout models which have been developed.

| Targeted Subunit | Immunological Phenotype | References |
|---|---|---|
| CCR1 | Imbalance in Th1/Th2 cytokines. Prolonged allograft survival in a transplantation model, resistance to EAE development and decreased pancreatitis-associated lung injury. Increased glomerular injury in nephrotoxic nephritis and increased susceptibility to A. fumigatus and T. gondii infection compared to wild-type mice. | Blease *et al.*, 2000b; Gao *et al.*, 2000; Gerard *et al.*, 1997; Khan *et al.*, 2001; Rottman *et al.*, 2000; Topham *et al.*, 1999. |
| CCR2 | Defects in macrophage recruitment and in the production of Th1 cytokines, such as interferon-γ (IFNγ). Unable to clear infection by Listeria monocytogenes and more susceptible to the injurious effects of intrapulmonary challenge with Aspergillus fumigatus spores. Resistant to experimental autoimmune encephalitis induced by the MOG peptide. | Blease *et al.*, 2000a; Boring *et al.*, 1997; Fife *et al.*, 2000; Izikson *et al.*, 2000; Kurihara *et al.*, 1997. |
| CCR3 | Eosinophil recruitment to the lung is severely curtailed, with eosinophils remaining in the sub-endothelial space; paradoxically, there is an increase in airway hyper-responsiveness. Eosinophils were absent in the CCR3 knockout mice, mast cell numbers and IL-4 expression were normal in allergic skin inflammation model. | Ma *et al.*, 2002. |
| CCR4 | No effect on Th2 differentiation. Resistant to LPS-induced endotoxin shock in both low- and high-dose models. | Chvatchko *et al.*, 2000. |
| CCR5 | Impaired macrophage function and reduced efficiency in the clearance of Listeria infection as well as enhanced T cell-dependent | Zhou *et al.*, 1998; Zhou *et al.*, 1998; Tran *et al.*, 2000; |

| | | |
|---|---|---|
| | immune response (DTH reaction). No protection against the development of EAE or against the development of multiple sclerosis, although they do show delayed disease onset. | Barcellos *et al.*, 2000. |
| CCR6 | 2–15-fold increase in specific T cell subsets within the mucosa (CD4+ and CD8+ ah-TCR T cells) Develop more severe and persistent inflammation than wild type mice in 2.4-DNFB-induced contact hypersensitivity. Conversely in a DTH model, developed no inflammation. Reduced airways resistance, fewer eosinophils around the airways, reduced IL-5 in the lung and reduced serum IgE compared to wild type mice. | Cook *et al.*, 2000, Varona *et al.*, 2001, Lukacs *et al.*, 2001. |
| CCR7 | Severely delayed kinetics regarding antibody responses and lack of contact sensitivity and DTH reactions. Due to impaired lymphocyte migration, there are profound morphological alterations in all secondary lymphoid organs, and upon activation, DCs fail to migrate into the draining lymph nodes. | Forster *et al.*, 1999. |
| CCR8 | Defective Th2 immune responses in models of Schistosoma mansonii-soluble egg antigen (SEA)-induced granuloma formation as well as in ovalbumin and cockroach antigen-induced airways inflammation, resulting in 50–80% reduction in eosinophil recruitment. Th2 cells developed normally, they showed aberrant Th2 cytokine production. | Chensue *et al.*, 2001. |
| CCR9 | Appear phenotypically normal. | Wurbel *et al.*, 2001; |
| CXCR2 | Decreased neutrophil recruitment but also an increase in the number of B cells, and lymphadenopathy and splenomegaly. | Cacalano *et al.*, 1994; Del Rio *et al.*, 2001; Frendeus *et* |

| | | |
|---|---|---|
| | Increased susceptibility to Toxoplasma gondii infection, urinary tract infection and is necessary for normal wound healing. Reduces the progression of atherosclerosis, implying a role for this receptor in macrophage recruitment to atherosclerotic plaques. | *al.*, 2000; Devalaraja *et al.*, 2000; Boisvert *et al.*, 2000. |
| CXCR3 | Resistance to development of acute allograft rejection, and CXCR3 deficient allograft recipients treated with a brief, sub-therapeutic course of cyclosporin A maintained their allografts permanently and without evidence of chronic rejection. | Hancock *et al*, 2000; Widney *et al.*, 2005. |
| CXCR4 | CXCR4 embryonic lethal embryo lethal. | Ma *et al.*, 2002; Tachibana *et al.*, 1998; |
| CXCR5 | Altered B cell migration and abnormal germinal centre formation in the spleen, and lack of Peyer's patches. | Forster *et al.*, 1996. |
| CX3CR1 | Increased graft survival time. Selective reduction in natural killer cells. | Haskell *et al.*, 2001; Jung *et al.*, 2000. |

**Table 1.1: Examples of chemokine knockout murine model.** The table above provides certain examples of several different murine models available for each chemokine receptor, and the insights which these models have provided into the role of their specific receptor.

Chemokines influence allergic diseases, not only by the recruitment of leukocytes but also the activation of different leukocytes, inducing the release of a wide range of inflammatory mediators inducing Th2 promotion and IgE synthesis. It was originally believed that the CC-chemokines were the primary chemokines involved in allergic response, with CCL5 and CCL5 being the best known as chemo-attractants for inflammatory cells, such as eosinophils, basophils and mast cells. Other CC-chemokines, such as CCL3, CCL7 and CCL13, have also been associated with leukocyte recruitment in the case of allergic diseases. The majority

of these chemokines are known to bind CCR3 — which is expressed on Th2-cells, basophils, mast cells — and is a major receptor on eosinophils (Kitaura *et al.*, 1996). Therefore, this has led to the belief that antagonists against CCR3 may be a good target as an alternative approach in the treatment of allergic disorders (Pease and Williams, 2001).

### 1.6.10 Dimerisation of Chemokine Receptors

The simplest way to view GPCR signalling is according to the model of a monomeric GPCR binding an agonist on the N-terminus side of the receptor. This induces conformational change of the receptor, altering the 7 trans-membrane domains in such a way so as to allow interaction of the G-proteins (Armbruster and Roth, 2005). Signalling is not only restricted to monomeric receptors; these receptors will form homodimers and heterodimers with other receptors (Breitwieser, 2004). The precise structural characteristics of dimerisation are not fully understood, although two models have been proposed: 1) contact dimerization; and 2) domain-swapping. For contact dimerisation, each receptor is an individual entity, contacting each other by non-covalent interactions between trans-membrane helices. Domain-swapping shares helices which form covalent links between two polypepetide chains; however, there is no clear evidence to support either of these theories with regards to chemokine dimersation, but provides useful theoretical knowledge to envisage dimerisation. Hernanz-Falcón *et al.* (2004) have suggested that the trans-membrane domains 1 and 4 are involved in the dimerisation of CCR5. Furthermore, dimerisation has also been observed between CXCR1 and CXCR2, thereby forming both homo- and heterodimers. Vila-Coro *et al* also report CXCL12 stimulated dimerisation of CXCR4 receptor (Vila-Coro *et al.*, 1999b). Although heterodimerisation can occur with certain chemokine receptors, this cannot be a generalisation for all chemokine receptors, but it is most likely they can only couple to a few others, indicating the need for each case to be individually examined.

The functional relevance of dimerisation may be important in many aspects of leukocyte biology. Chemokines are bound to the surface of the epithelium and may induce the characteristic rolling of the cell. Dimerisation of receptors may not

only decrease the required threshold for activation, but may also provide increased sensitivity and specificity within the system (Rodríguez-Frade *et al.*, 2001).

## 1.7 Conformation Changes of the Chemokine Receptor

There is a two-step model of receptor activation for chemokines. The amino-terminus of the receptor is thought to tether the chemokine with high affinity, following which the chemokine N-terminus activates a ligand-binding pocket with the TM helices. The resulting conformational changes result in the recruitment of heterotrimeric G proteins and subsequent downstream signalling.

### 1.7.1 G-protein Dissociation

Chemokine receptors — like other members of the GPCR family — transduce signals via heterotrimeric G-proteins. The name ‘G-protein’ is short for guanine nucleotide-binding proteins, which act as ‘molecular switches’, mediating their effects by switching between an inactive Guanosine Diphosphate (GDP) and active Guanosine Triphosphate (GTP) bound state. The active state results in the activation of many different downstream cell processes.

The interaction of an agonist with its respective chemokine receptor and the alteration of the 7 trans-membrane receptors all occur at the cell’s surface. The agonist and receptor complex must undergo a specific process in order to turn this into a cellular function. Moreover, activation of the receptor occurs after the coupling of an agonist. After activation, the second messenger signalling cascade is provided by a heterotrimeric guanine nucleotide binding protein (G-protein) just under the surface of the membrane (Neer, 1995). The G-protein accordingly acts as the molecular switch to initiate different functions within a cell. The G-protein comprises $\alpha$, $\beta$ and $\gamma$ subunits. Activation of a chemokine receptor initiates the exchange of GDP for GTP on the $\alpha$ subunit. The result of this exchange removes the hydrophobic pocket to which the $\beta\gamma$ subunit binds, subsequently reducing the affinity between G$\alpha$-GTP and $\beta\gamma$-subunit; the result of this is the dissociation of the two complexes (Lambright *et al.*, 1994).

After the disassociation event, the β-γ subunit is free to activate or inhibit downstream signalling molecules. Termination of this response is rapid due the GTP being hydrolysed back to GDP through innate GTPase activity, thereby allowing the re-association of the α and βγ-complex (Hamm, 1998; Sprang, 1997).

The specific downstream effectors depend on the G-protein which is activated. G-proteins are characterised by their G-α subunits. Initial experiments employing Pertussis toxin(PTX) blockade suggests that $G\alpha_{i/O}$ proteins are primarily responsible for downstream signalling, as physiological responses — such as chemotaxis — were readily inhibited by pre-incubation of cells with the toxin (Thelen *et al.*, 1988); however, it is now known that there are three other classes: $G_s$, $G_q$ and $G_{12/13}$. PTX catalyses the ADP-ribosylation of the α subunits of the G-proteins $G_i$, and $G_o$ resulting in the inability of the G-protein to interact with the cell membrane. All of these subunits and the βγ-subunit couple to and activate many different signalling pathways, including PLCβ2 and β3 (Katz *et al.*, 1992), PI3K (Stephens *et al.*, 1994; Tang and Downes, 1997), MAPK cascade (Inglese *et al.*, 1995), adenylyl cyclase (Tang and Gilman, 1991), β-adrenergic receptor kinase, and $K^+$ and $Ca^{2+}$ channels (Cabrera-Vera *et al.*, 2003).

### 1.7.2 Phospholipase C/ Protein Kinase C

Phospholipase C/Protein Kinase C cascade has been demonstrated to activate downstream of many different chemokine receptors. PLC is a crucial modulator of phosphoinositides at the plasma membrane. There are 6 different isoforms of PLC, β, γ, δ, ε, ζ and η. The structural differences of PLC isoforms are highlighted in Figure 1.10.

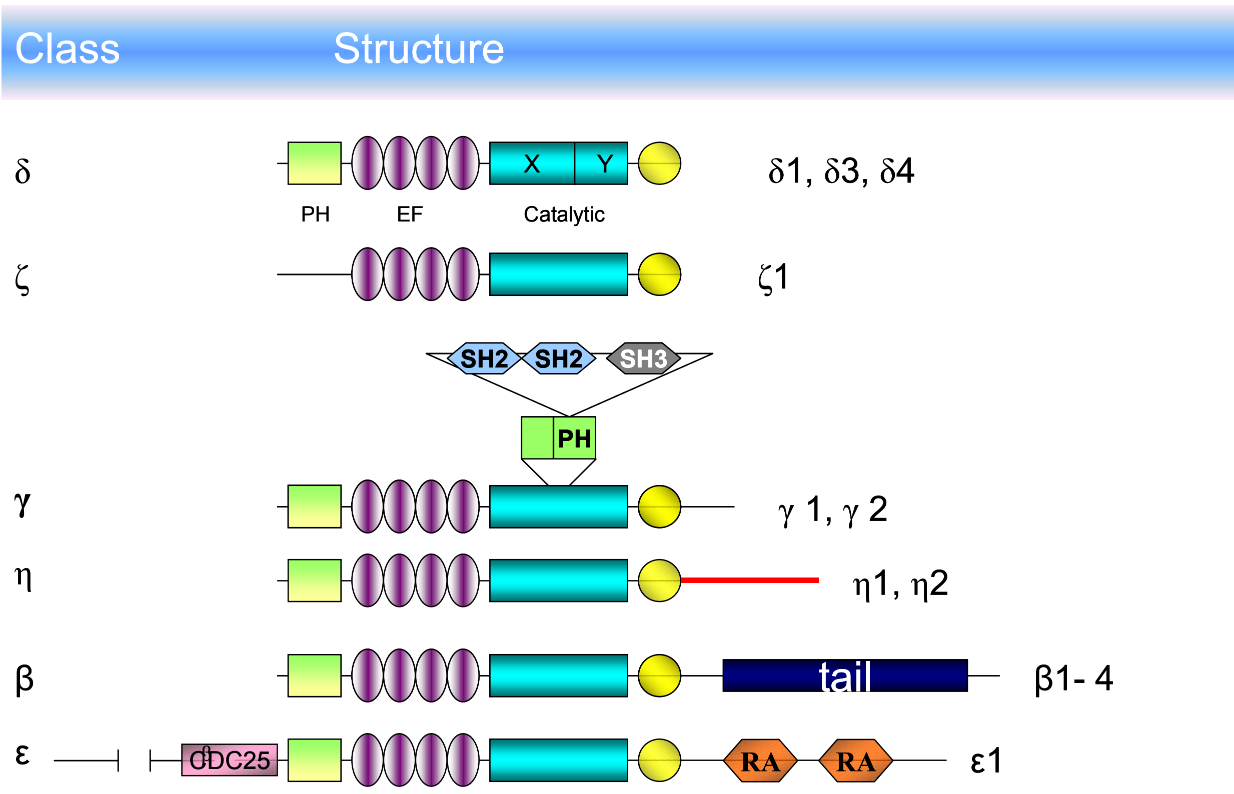



**Figure 1.10: Structural differences of PLC isoforms.** The four domains of PLCδ, the PH domain, EF-hands (EF), catalytic domain (containing highly conserved X and Y regions) and the C2 domain (C2) are also present in most isoforms of other PLC families. The unique region of PLCγ, inserted through a flexible loop of the catalytic domain, includes the second PH domain: two SH2 (Src homology 2) regions and one SH3 (Src homology 3) region. PLCε contains the domain with a guanyl-nucleotide exchange factor activity (CDC25) and two predicted RA (Ras association) domains which are implicated in the binding of small GTPases from the Ras family. In PLCβ, the unique region (tail) is present at the C-terminus. The sequences unique to PLCη (tail) are, as in PLCβ, located at the C-terminus. Adapted from Katan, (2005).

Most chemokines share the ability to bind to chemokine receptors which trigger these downstream cascades, rapidly activating phosphoinositide-specific phospholipase C-ß$_2$ (PLC-ß$_2$) and PLC-ß$_3$ isoenzymes, which subsequently leads to inositol-1,4,5-triphosphate($IP_3$) and diacylglycerol (DAG) formation and to a transient rise in the concentration of intracellular free calcium ($Ca^{2+}$) by utilising PtdIns(4,5)$P_2$ as a substrate (Hawkins *et al.*, 1984; Cicchetti *et al.*, 2002). In the neutrophils of mice that lack the genes encoding PLC-ß$_2$ and PLC-ß$_3$, the chemokine-induced calcium elevation is fully suppressed, which further supports the conclusion that PLC-ß$_2$ and PLC-ß$_3$ are the sole PLC isoforms activated by chemokines in immune cells (Li *et al.*, 2000).

$IP_3$, by interaction with its receptor, induces the mobilisation of cytosolic calcium. This results in the entry of $Ca^{2+}$ ions through Calcium Release-Activated Channels (CRAC channels) (Luik and Lewis, 2007; Luik *et al.*, 2006). The result of these channels opening is directly related to reduce the levels of calcium from the intracellular stores such as the endoplasmic reticulum. There are two key components which are considered critical to mediating this process: firstly, Stromal Interaction Molecule 1 (STIM1), which is located in the endoplasmic reticulum and acts as a calcium senor; and ORAI1, which is a functional component of the $Ca^{2+}$ release-activated $Ca^{2+}$ (CRAC) channels (Roos *et al.*, 2005). Furthermore, it is believed that the depletion of calcium from the intracellular stores results in the re-localisation of STIM1 from a diffuse distribution to a focal point near the plasma membrane. The redistribution of STIM1 to the plasma membrane allows the interaction with the CRAC channels, thereby allowing the replenish of the intracellular stores (Figure 1.11) (Luik *et al.*, 2006).

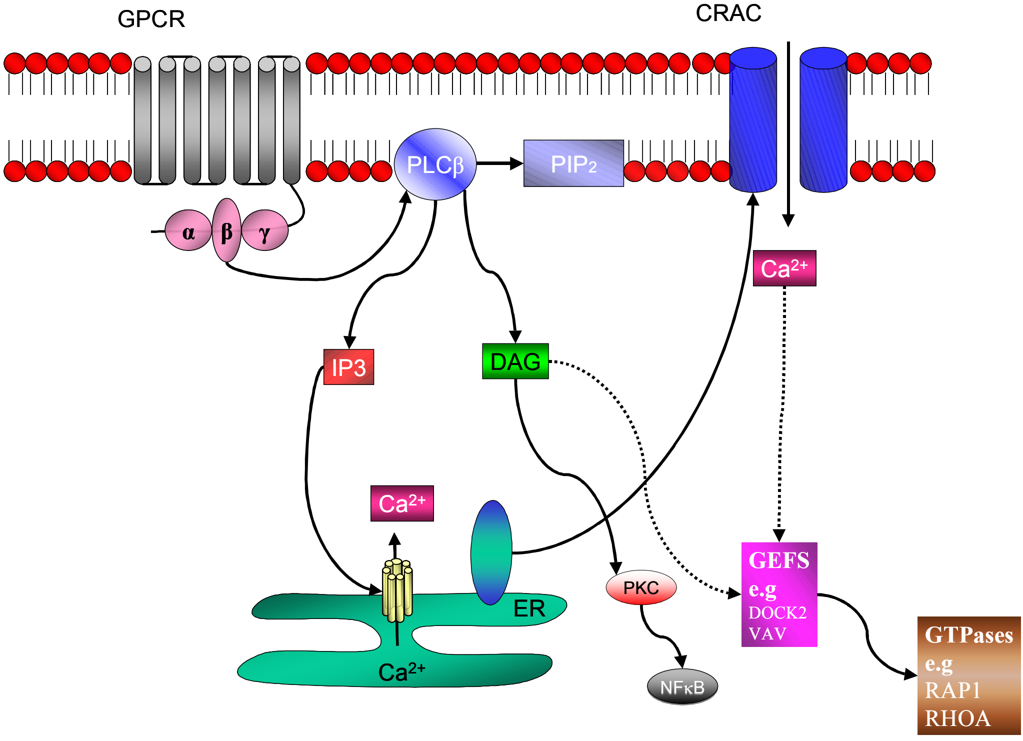



**Figure 1.11: Overview of PLC activation.** The G-protein subunit of the GPCR activates phospholipase C (PLC), which cleaves phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2) to produce inositol-1,4,5-trisphosphate (InsP3) and diacylglycerol (DAG). IP3 causes the release of calcium from stores within the endoplasmic reticulum, which is detected by STIM1. This causes the opening of CRAC channels in the plasma membrane. Adapted from Ley *et al.*, (2007).

## 1.8 Phosphoinositide 3-Kinase

### 1.8.1 Overview

The early research on phosphorlayted forms of phosphoinositide lipids focuses on the conversion of $PIP_2$ by PLC. In the mid-1980s, researchers discovered that the lipid $PIP_2$ can serve as a substrate for the phosphoinositide 3-kinase or PI3K family, which is characterised by their ability to catalyse the phosphorylation of D3 of the inositol ring on membrane-bound phosphoinositide lipids to produce PI-3,4,5-$P_3$ ($PIP_3$). The first known receptor to associate with and activate PI3K was platelet-derived growth factor (Auger *et al.*, 1989; Kaplan *et al.*, 1987). $PIP_3$ acts as a secondary messenger, which actively recruits intracellular signalling molecules, such as Ser/Thr protein kinase B/Akt, Tec family tyrosine kinases and the Grp1/Arf exchange factor to the plasma membrane.

### 1.8.2 Classes of PI3K

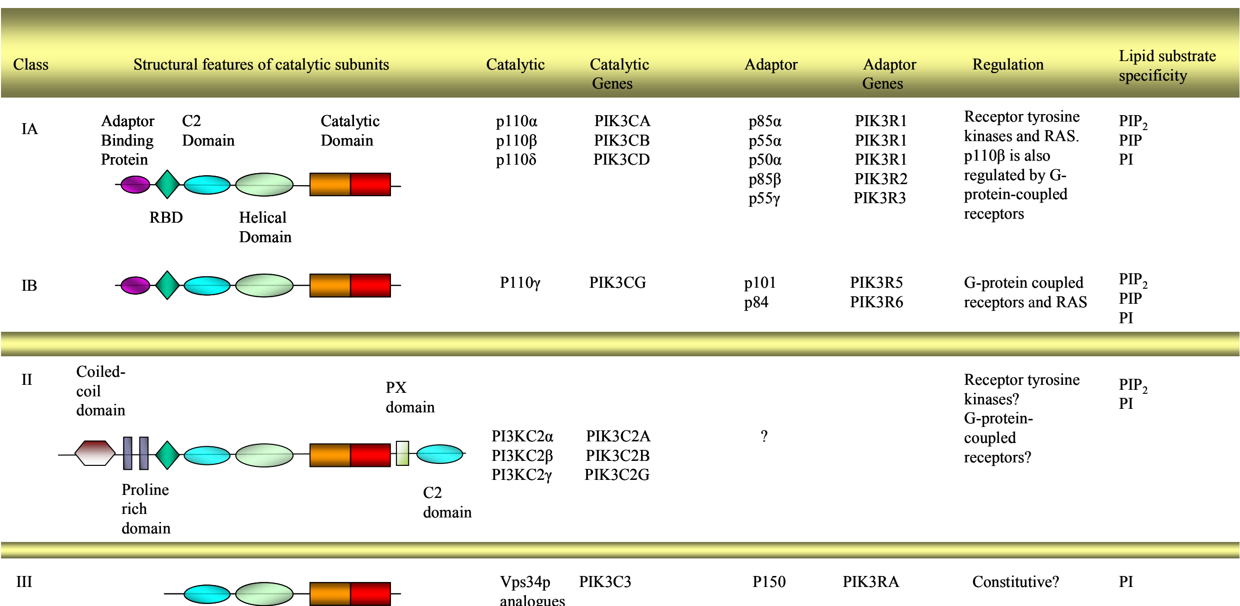
Mammals have 8 isoforms of PI3K and are subdivided into three main classes according to their varying *in vivo* substrates and structural characteristics. The subset of PI3K enzymes which are acutely activated by membrane bound receptors are known as Class 1 PI3Ks. Of these, the Class IA PI3Ks signal the downstream of tyrosine kinases and consist of a catalytic subunit, p110α (Hiles *et al.*, 1992), p110β (Hu *et al.*, 1993), and p110δ (Vanhaesebroeck *et al.*, 1997), and are complexed to one of five regulatory subunits (collectively known as p85s) containing a Src homology 2 (SH2) domain (Stoyanov *et al.*, 1995). The p85s are encoded by three genes: the first three regulatory subunits are all splice variants of the same gene (*Pik3r1*); the other two are expressed by other genes (Pik3r2 and Pik3r3, p85β and p55γ, respectively) (Carpenter *et al.*, 1990; Fruman *et al.*, 1996; Inukai *et al.*, 1996; Pons *et al.*, 1995; Skolnik *et al.*, 1991). Of the regulatory subunits, p85α is the most highly expressed. The three catalytic subunits are expressed by separate genes (Pik3ca, Pik3cb and Pik3cd for p110α, p110β and p110δ, respectively).

The Class 1B family has only one member, p110γ (Krugmann *et al.*, 1999; Stoyanov *et al.*, 1995), which can interact with one of two regulatory subunits (p84/87 or p101 encoded by the PIK3R6 and PIK3R5 gene respectively) and is activated by Gβγ subunits downstream of G-protein coupled receptors. The p110γ,

like Class 1A isoform, forms heterodimers but varies from Class 1A isoforms as it lacks the N-terminus p85. *In vitro* Class 1A and 1A isoforms have been shown to catalyse PtdIns, PtdIns(4)P and PtdIns (4,5)$P_2$ into their respective products; however, *in vivo*, their preferred substrate is PtdIns (4,5), which is converted into PtdIns (3,4,5)$P_3$ (Fruman and Cantley, 2002; Vanhaesebroeck *et al.*, 1999). Most of the ligands which activate Class IB PI3K are involved in coordinating the body's response to injury and infection.

Class II PI3Ks are encoded according to three separate genes: PI3K Class II α, β, γ. The Class II PI3K α and β are ubiquitously expressed, whereas the Class II γ has been shown to be almost solely in the liver. *In vitro* assays demonstrate that the Class II isoforms can bind phospholipids in a calcium-independent manner via a C-terminal domain; compared to the Class I PI3Ks, Class II PI3Ks demonstrates lipid specificity utilising Ptd to PtdIns(3)P and PtdIns(4)P to PtdIns(3,4)$P_2$ (MacDougall *et al.*, 1995). It is therefore likely that the Class II PI3Ks will induce the accumulation of lipids with a profile distinct from that stimulated by the Class I PI3Ks (Vanhaesebroeck and Waterfield, 1999).

The Class III PI3K is the least well-known and least studied of the isoforms in the PI3K family. Class III PI3Ks are only thought to comprise one member — the human homologue of the yeast vesicular protein-sorting protein, Vps34 (vascular protein sorting 34) — which, both *in vitro* and *in vivo*, only catalyses PtdIns and is thought to play a role in intracellular trafficking events (Yan and Backer, 2007). There structural differences of the different PI3K isoforms are highlighted in Figure 1.12.



| Class | Structural features of catalytic subunits | Catalytic | Catalytic Genes | Adaptor | Adaptor Genes | Regulation | Lipid substrate specificity |
|---|---|---|---|---|---|---|---|
| IA | Adaptor Binding Protein; C2 Domain; Catalytic Domain; RBD; Helical Domain | p110α<br>p110β<br>p110δ | PIK3CA<br>PIK3CB<br>PIK3CD | p85α<br>p55α<br>p50α<br>p85β<br>p55γ | PIK3R1<br>PIK3R1<br>PIK3R1<br>PIK3R2<br>PIK3R3 | Receptor tyrosine kinases and RAS. p110β is also regulated by G-protein-coupled receptors | $PIP_2$<br>PIP<br>PI |
| IB | | P110γ | PIK3CG | p101<br>p84 | PIK3R5<br>PIK3R6 | G-protein coupled receptors and RAS | $PIP_2$<br>PIP<br>PI |
| II | Coiled-coil domain; PX domain; Proline rich domain; C2 domain | PI3KC2α<br>PI3KC2β<br>PI3KC2γ | PIK3C2A<br>PIK3C2B<br>PIK3C2G | ? | | Receptor tyrosine kinases?<br>G-protein-coupled receptors? | $PIP_2$<br>PI |
| III | | Vps34p analogues | PIK3C3 | P150 | PIK3RA | Constitutive? | PI |

**Figure 1.12: The PI3K family**. Class 1 PI3K are heterodimeric enzymes consisting of a catalytic and adaptor subunit. The adaptor subunit comprises a N-terminal p85-binding domain, which binds a p85 regulator subunit, a RBD (RAS binding domain) mediating activation by GTPase RAS, a C2 domain and a helical domain. Class 1B PI3Ks is similar to the Class 1A, but lacks the p85 binding domain, instead having p101 as its adaptor subunit. P101 subunits enable the interaction between p110y and the BY subunits of the G proteins, which are activated by G-protein coupled receptors. Class II PI3K comprises three members and are similar to Class IB but contain additional PX and C2 domains towards the C-terminal. Class III comprise a single member with a Vps34 catalytic subunit. Adapted from Bader *et al.*,(2005).

Mammalian cells express PtdIns(3)P constitutively; however, once these cells are stimulated, PtdIns(3,4)$P_2$ and PtdIns(3,4,5)$P_3$ are expressed in abundance. This difference in expression profile would suggest that PtdIns(3,4)$P_2$ and PtdIns(3,4,5)$P_3$ play a role as intracellular mediators (Figure 1.13).

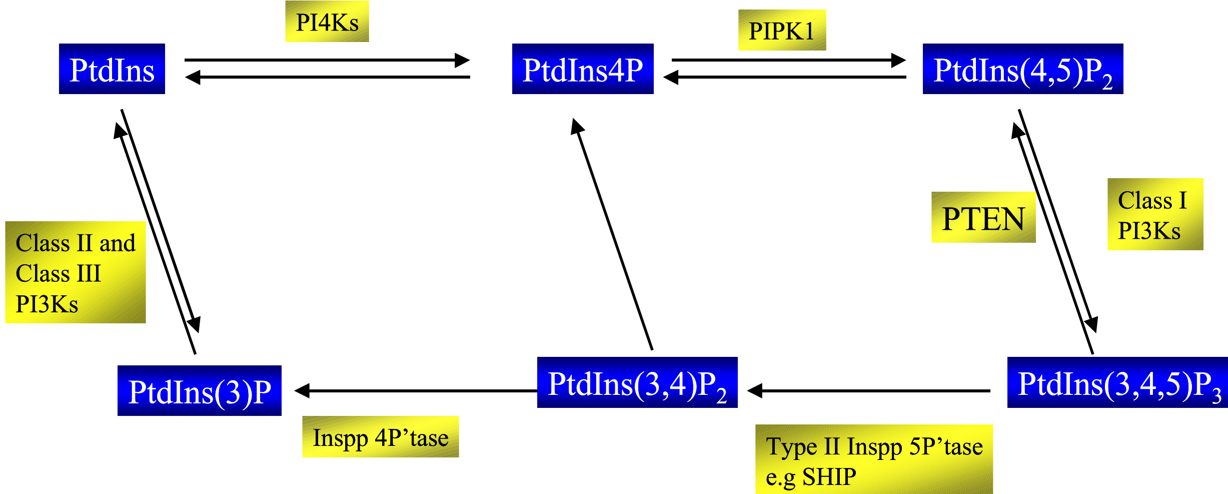



**Figure 1.13: Pathways of phosphoinositide metabolism.** The major routes of phosphoinositide metabolism thought to operate in mammalian cells are depicted; where known, the main enzyme activities thought to catalyse these conversions are also noted. The activation of Class I PI3Ks by cell-surface receptors is shown, thus leading to the rapid synthesis of PtdIns(3,4,5)$P_3$ and, via the action of 5 phosphatases, the synthesis of PtdIns(3,4)$P_2$. Adapted from (Hawkins *et al.*, 2006).

### 1.8.3 PH Domains

The activation of PI3K results in the accumulation of PtdIns(3,4)$P_2$ and PtdIns(3,4,5)$P_3$ at the plasma membrane, which then recruits proteins via the lipid binding domains (known as Pleckstrin Homology (PH) domains). A protein domain of approximately 120 amino acids occurs in a wide range of proteins involved in intracellular signalling or as a constituent of the cytoskeleton. Work from a number of different laboratories has built the concept that: (i) some PH domains bind phosphoinositides; (ii) a subset of these PH domains bind PtdIns(3,4,5)$P_3$ and/or PtdIns(3,4)$P_2$ with high affinity and specificity; and (iii) several proteins which contain these PtdIns(3,4,5)$P_3$/PtdIns(3,4)$P_2$-specific PH domains play key roles in mediating Class I PI3K-dependent regulations of important cellular responses (Hawkins *et al* 2006). However, it is still unclear precisely how PtdIns(3,4,5)$P_3$/PtdIns(3,4)$P_2$ engagement with the PH domain of an effector protein regulates its activity. This interaction is of sufficient affinity that the target

protein undergoes a net change from a predominantly cytosolic to a predominantly plasma membrane location.

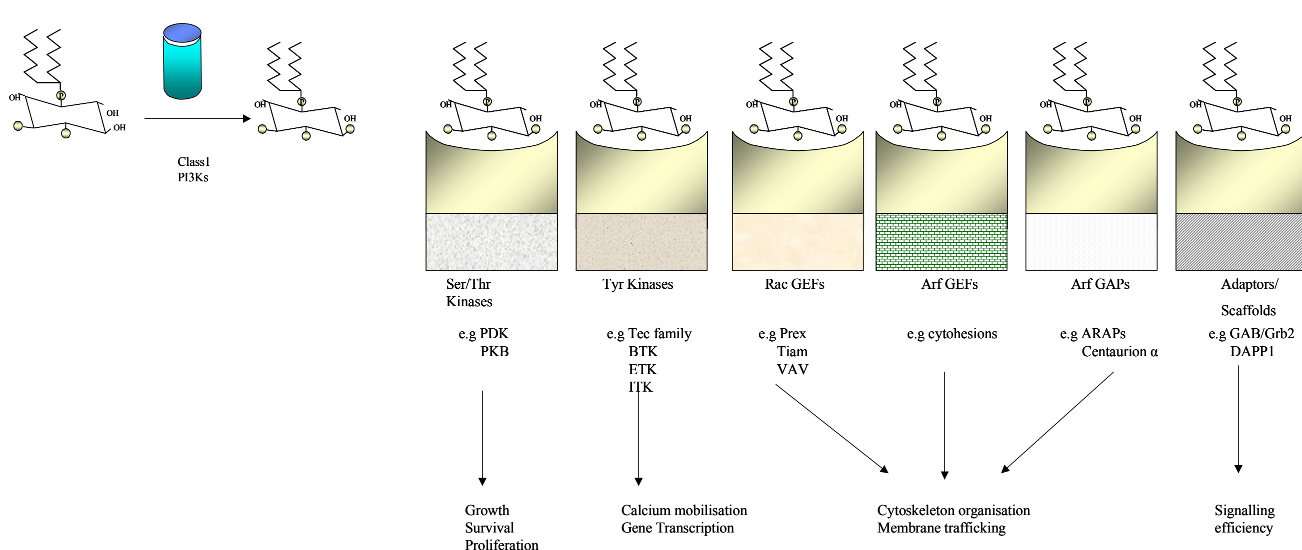



**Figure 1.14: Activation of Class 1 PI3K results in binding of PtdIns(3,4,5)$P_3$ to a wide variety of PH domains.** Phosphorylation of PIP2 to PIP3 culminates in increased levels, thus resulting in the sensing by PH domain with appropriate affinity and specification, leading to the activation of a wide range of PH domains. These PH-domain-containing effectors belong to several different protein families, some of which are shown together with specific examples and an indication of the downstream responses which they are thought to regulate. Adapted from Hawkins *et al.*, (2006).

It is now also thought that PtdIns(3,4,5)$P_3$/PtdIns(3,4)$P_2$ binding to PH domains can often derepress an intramolecular inhibition mediated by the PH domain, subsequently inducing significant activation of the effector. Examples of this are apparent in the activation of GEFs (guanine nucleotideexchange factors) for small GTPases. PtdIns(3,4,5)$P_3$ binding is thought to relieve a PH-domain-mediated inhibition of the Dbl catalytic domain and to also bring the GEF into proximity with its lipid-tethered GTPase (Welch *et al.*, 2003). Furthermore, PDK (Phosphoinositide-Dependent Kinase)-mediates phosphorylation and activation of PKB (also known as Akt) (Komander *et al.*, 2004; McManus *et al.*, 2004; Mora *et al.*, 2005). Both PDK and PKB possess PH domains which bind PtdIns(3,4,5)$P_3$/PtdIns(3,4)$P_2$, thus allowing an increase in their relative effective concentration on Class I PI3K activation. PDK appears to be ‘constitutively active’,

and its activity is ultimately considered independent of phosphoinositide/PH domain binding. Moreover, it also has a major role to play in PI3K-independent phosphorylation of the activation loop in several ‘AGC family’ kinases. In contrast, however, the ability of PDK to phosphorylate PKB in its activation loop is highly dependent on Class I PI3K activity. PtdIns(3,4,5)$P_3$/PtdIns(3,4)$P_2$ binding to the PH domain of PKB is required to make Thr308 in the activation loop available for phosphorylation by PDK. Figure 1.14 highlights the PtdIns(3,4,5)$P_3$/PtdIns(3,4)$P_2$ binding to PH with the wide downstream effectors activated. The PI3K family has been implicated in a wide range of biological processes, such as cell survival, proliferation, cell migration and degranulation with the signalling pathways highlighted in Figure 1.15.

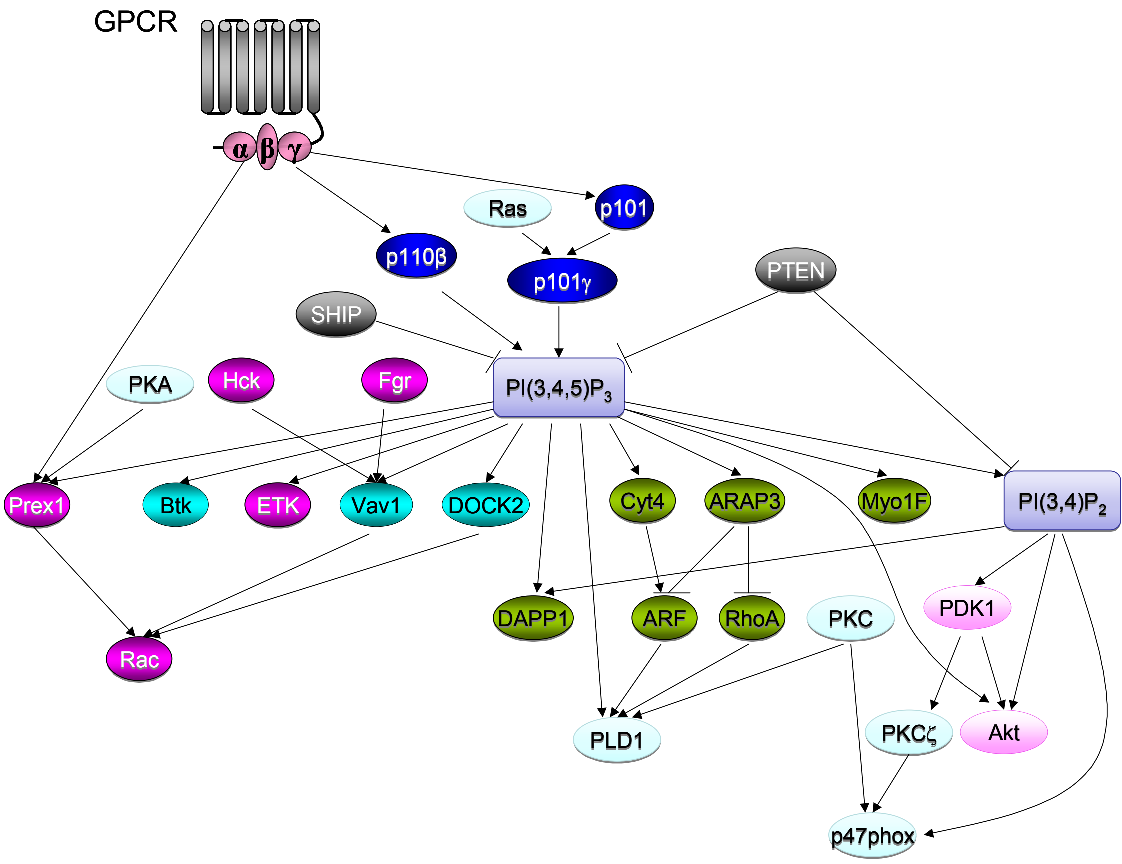



**Figure 1.15: Signalling events following formation of phosphoinositide lipids by PI3K.** Agonist stimulation of chemokine receptor activated class IB PI3K via action of Gβγ subunits and the Ras. This leads to accumulation of PI3K products PtdIns(3,4,5)$P_3$ and indirectly PtdIns(3,4)$P_2$ in the membrane and interaction with variety of effector proteins by binding to the PH domain.

### 1.8.4 Protein Kinase B

One of the most widely studied molecules phosphorylated by the activation of PI3K is the serine/threonine kinase, Protein Kinase B (PKB, also known as Akt) (Burgering and Coffer, 1995). There are three known members in the Akt family: Aktα, Aktβ and Aktγ, all of which share close homology to the protein kinases A, G and C, collectively form the AGC kinase family (Manning and Cantley, 2007). The precise activation of the kinases is not yet fully understood; however, phosphorylation of Akt is thought to occur at two key sites — the Ser473 in the hydrophobic region of the C-terminal regulatory down and Thr308 within the kinase activation loop (Toker and Newton, 2000). Alteration of Akt by activation of PI3K results in accumulation of PtdIns(3,4,5)P3 at the plasma membrane. After relocation to the plasma membrane, this lipid recruits Akt via its PH domain. Upon reaching the plasma membrane, it is thought that PDK-1 induces the phosphorylation of the Thr308, whereas the rictor-mTOR complex directs phosphorylates Ser473 in the hydrophobic region of Akt (Sarbassov *et al.*, 2005). In order for Akt to be fully active, both of these residues have to become phosphorylated. Moreover, the regulation of Akt activation has been shown to be controlled by the dephosphorlaytion of PtdIns(3,4,5)$P_3$ by the cellular phosphatase SHIP, resulting in decreased levels of PtdIns (3,4,5)$P_3$ at the cellular membrane leaving the remaining Akt in its inactive form in the cytoplasm (Stambolic *et al.*, 1998). Moreover, activation of Akt is known to induce the phosphorylation of a number of proteins further downstream of itself, particularly those involved in the regulation of glucose metabolism and cell survival (Wymann *et al.*, 2000). Further downstream signalling pathways include mTOR.

A role for Akt activation within the degranulation of mast cells has appeared over the last decade, and is currently being investigated (Ali *et al.*, 2008; Wymann *et al.*, 2003). Akt can be directly dephosphorylated, thus inactivating Akt; this is induced by the PH domain leucine-rich repeat protein phosphatase (PHLPP), which specifically dephosphorylates the hydrophobic motif of Akt (Ser473 in Akt1) (Gao *et al.*, 2005). PHLPP levels are markedly reduced in the instance of several colon cancers and glioblastoma cell lines, which have elevated Akt phosphorylation, induced apoptosis and restricted tumour growth. Moreover, the

reintroduction of PHLPP into a glioblastoma cell line causes a dramatic suppression of tumour growth (Gao *et al.*, 2005).

## 1.9 Assessing the Role of PI3K

Many different approaches have been studied through PI3K in order to assess the role of a particular gene. Each of the methods has its advantages and disadvantages, all of which will be discussed in the following section. The use of antibodies or inhibitors has been used to block the function of proteins by affecting the proteins from binding. Other studies have utilised the use of knock-in/knock-out animal models. Further studies, in the middle of these two extremes in order to try and utilise RNA interference against specific isoforms of interest — transcribed but degraded before generation of the protein.

### 1.9.1 Pharmacological Intervention: first generation 'dirty' PI3K inhibitors

The PI3K family comprises a group of enzymes possessing several structural elements and binding domains. Studies aiming to reveal the role of the PI3K family have utilised pharmacological inhibitors. Moreover, the majority of studies have used the broad-spectrum isoform PI3K inhibitors Wortmannin and LY294002.

Wortmannin, a metabolite of Penicillium funiculosum, is one such molecule. Wortmannin's anti-inflammatory actions were first recognised in 1974 by Wiesinger *et al.* (1974) and, approximately a decade later, it was described as a potent inhibitor of the respiratory burst in neutrophils and monocytes (Baggiolini *et al.*, 1987). It was not until 1993 that PI3K was recognised as one of the many molecular targets of Wortmannin (Arcaro and Wymann, 1993). The action of Wortmannin involves interacting with the ATP-binding pocket by forming a covalent interaction with the catalytic lysine residue, and it is likely that the reactive nature of the Wortmannin structure makes it prone to forming unexpected and possibly hazardous combinations with other biological molecules (Wymann *et al.*, 1996).

LY294002 was first described as a PI3K inhibitor by the Lilly Research Laboratory, in 1994. LY294002 is ATP-competitive, has low molecular weight and planar with

relatively low potency against the PI3Ks. Moreover, it has been found to inhibit — or at least interact with — a plethora of other intracellular targets at concentrations little removed from those required for PI3K inhibition (Gharbi *et al.*, 2007). Despite the limitations of these first generation PI3K inhibitors — such as poor selectivity, toxic effects and the inability to distinguish between specific PI3K isoforms, for instance — they have nevertheless been proven as pivotal tools in research when striving to fully understand the role of PI3K (Rommel *et al.*, 2007; Ward and Finan, 2003). The pre-treatment of murine T and B lymphocytes, freshly isolated T lymphocytes with LY294002 or Wortmannin demonstrates a role for PI3K in the peak chemotactic response (Reif *et al.*, 2004; Sotsios *et al.*, 1999). Moreover, in recent years, a new general broad-spectrum inhibitor, ZSTK474, is reported to lack the off-target limitations of the widely used inhibitors LY294002 and Wortmannin (Kong and Yamori, 2007). Similar to LY294002, ZSTK474 is also an ATP-competing inhibitor of PI3K. The chemical structure of each of the three PI3K general inhibitors is highlighted in Figure 1.16.

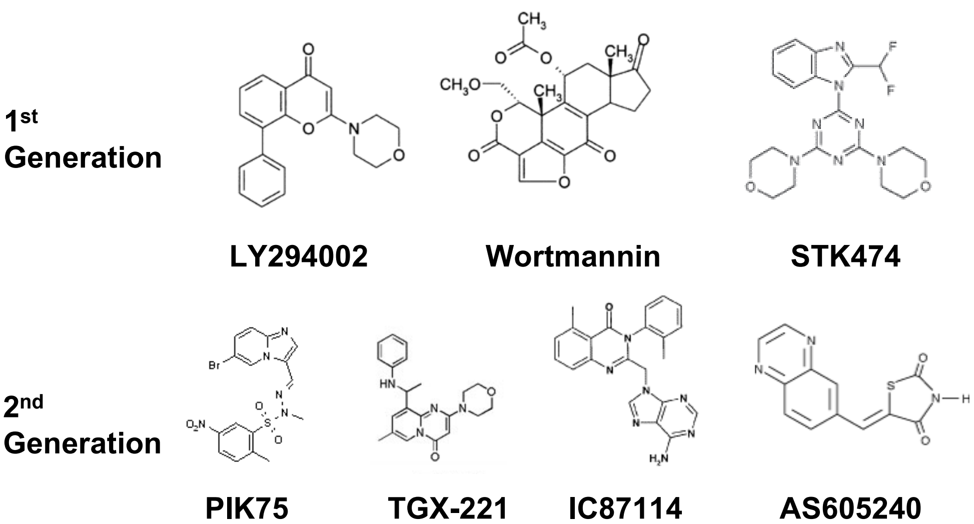



**Figure 1.16: Chemical structure of PI3K inhibitors.**

The use of these inhibitors is limited due to their broad-spectrum nature, which has consequently led to confusion as to which specific PI3K isoforms are involved in different cellular functions. This has subsequently led to questions posed concerning exactly which class of PI3K can be involved, and therefore underplays the contribution of these family members within different models. In more recent

years, however, there has been much excitement concerning the development of PI3K isoform specific inhibitors — the so-called second-generation inhibitors.

### 1.9.2 Second-Generation Inhibitors

Given the high degree of similarity that exists between the amino acids forming the ATP-binding pockets of the four class I PI3Ks, it was expected that isoform selective inhibitors with at least 50-fold difference in potency would be difficult to obtain; this, however, was disproved with the discovery of the quinazolinone purine series, exemplified by IC87114 and described by the ICOS Corporation (Sadhu *et al.*, 2003). Although only these compounds have been used within the last decade, they have already significantly contributed to the current body of knowledge regarding the different isoform specific functions. PIK75 has been noted as a class 1α inhibitor, and has accordingly demonstrated a role in insulin-signalling (Knight *et al.*, 2006). IC87114 and AS605240 are selective inhibitors for the δ and γ isoforms respectively, and have accordingly highlighted a role for these isoforms in the case of neutrophil trafficking (Camps *et al.*, 2005; Puri *et al.*, 2004). Moreover, each of these inhibitors appears to mediator their effect by competitively binding at the ATP binding site. The advancement of PI3K isoform-specific inhibitors has subsequently allowed, and is continuing to be critical in, unravelling the roles of each of the specific isoforms.

### 1.9.3 Genetic Approaches

The contribution of knock-out and knock-in mice models has greatly increased our knowledge regarding many different systems and their particular isoforms, each with many different signalling pathways. The PI3K family is just one of these. In order to produce knockouts using recombinant DNA technology, the normal DNA sequence of the gene being studied is altered so as to prevent the synthesis of a normal gene product. Cloned cells in which this DNA alteration is successful are then injected into mouse embryos in order to produce chimeric mice; the chimeric mice are then bred with the aim of yielding a strain in which all the cells of the mouse contain the disrupted gene.

The advantages of knockout mice are:

- The ability to study gene function in a living animal;
- The ability to test drugs and therapies; and
- Ease of breeding and maintaining.

Disadvantages:

- 15% of gene knockouts are lethal;
- There is only the ability to study embryonic development, as genes may have different functions in adults;
- The knockout may produce no observable change(s); and
- The knockout may illustrate no correlation with human gene activity.

The kinase dead or knock-in models, at which point mutation within the gene of interest, is generated resulting in the transcription of the isoform and production of protein but yet it provides no functional signals. The knock-in method was developed in which a mutated DNA sequence was exchanged for the endogenous sequence without any other disruption of the gene. Some knock-in strategies rely on the use of gene vectors with flanking sequences — termed *loxP* — which, upon exposure to an enzyme called Cre recombinase, undergoes reciprocal recombination, subsequently leading to the deletion of the intervening DNA. With this method, it is possible to replace a gene sequence with a sequence of the investigator’s choice and to accordingly delete unnecessary sequences. The advantages of knock-in mice are:

- Specific integration site;
- The transgene genetic environment is totally controlled; and
- Only one transgenic line is necessary.

The major drawback of this method is that the molecular biology work is considered to be significantly more complex and can take longer to perfect. However, it is worth noting that compensatory mechanisms in protein expression by other PI3K isoforms exist. In an attempt to overcome this, these knock-in

murine models have needed to maintain protein stoichiometry. A list of some of the PI3K murine models are summarised in Table 1.2.

| Targeted Subunit | Genetic Approach | Viability | Immunological Phenotype | References |
|---|---|---|---|---|
| P110α | KO | Embryonic lethal | Not applicable | Bi *et al.*, 1999. |
| P110β | KO | Embryonic lethal | Not applicable | Bi *et al.*, 2002. |
| P110δ | KO | Viable | ↓ neutrophil tethering and trafficking across inflamed venules. ↓ B cell number and proliferation | Puri *et al.*, 2004; Jou *et al.*, 2002; Clayton *et al.*, 2002. |
| P110δ | KI | Viable | ↓ neutrophil migration and $PIP_3$ generation. ↓ B and T cell number and proliferation | Okkenhaug *et al.*, 2002. |
| P110γ | KO | Viable | ↓ neutrophil and macrophage migration in vivo and in vitro. ↓ T proliferation | Hirsch *et al.*, 2000; Li *et al.*, 2000; Rodriguez-Borlado *et al.*, 2003. |
| P110γ | KI | Viable | ↓ Decrease in vivo and in vitro chemotaxis | Patrucco *et al.*, 2004. |
| Class II α | Not yet generated | Not applicable | Not applicable | |
| Class II β | KO | Viable | Wound healing unaffected | Harada *et al.*, 2005. |
| Class III | Not yet generated | Not applicable | | |

**Table 1.2: Phenotypes of mice that have PI3K catalytic subunit genetically targeted.** Adapted from Vanhaesebroeck *et al.*, (2005).

A review reports that the use of knock-out murine models allows a potential functional redundancy between the PI3K isoforms (Vanhaesebroeck and Waterfield, 1999). The variation in species used to generate these murine models also has drawbacks; this is where the 'middle ground' in studying PI3K becomes an invaluable tool, as it allows specific targeting on gene function in humans.

### 1.9.4 RNA Interference

Murine models are not perfect and, like any technique implemented, have disadvantages. One problem which has been associated with knockout models is functional redundancy between particular isoforms. If one particular gene is knock-out, other isoforms within this family subsequently compensate for the absence of this gene and may artificially drive functionality through other members. Moreover, RNA interference could potentially overcome this problem as it has only a of knockdown 80-90% of normal levels, thereby allowing background function of the selected isoforms; therefore, any potential compensatory mechanisms by other isoforms with the same family may not become active.

RNA interference is a naturally occurring event in which double-stranded RNA molecules induce the degradation of the corresponding mRNA. This, in turn, inhibits the translation of mRNA. RNA interference is the silencing of gene by target messenger RNA, and this technique has been used in research for many years, initially being utilised in plants, followed in the 1990s in invertebrates. The switch to mammalian systems has proved a much bigger challenge: the biggest problem was the struggle to introduce long, double-stranded RNA in mammalian cells. As a result of this, the cells' initiation interferon response led to wide protein inhibition (Bass, 2001); however, this issue has been overcome during the last decade as a result of the utilisation of small double-stranded RNA molecules, normally 21-23 base pairs long. These small double-stranded RNA molecules (siRNA) have been utilised in order to decrease target mRNA levels correlating to a decrease in protein expression of up to 90% (Gresch *et al.*, 2004). The mechanism of siRNA action is highlighted in Figure 1.17.

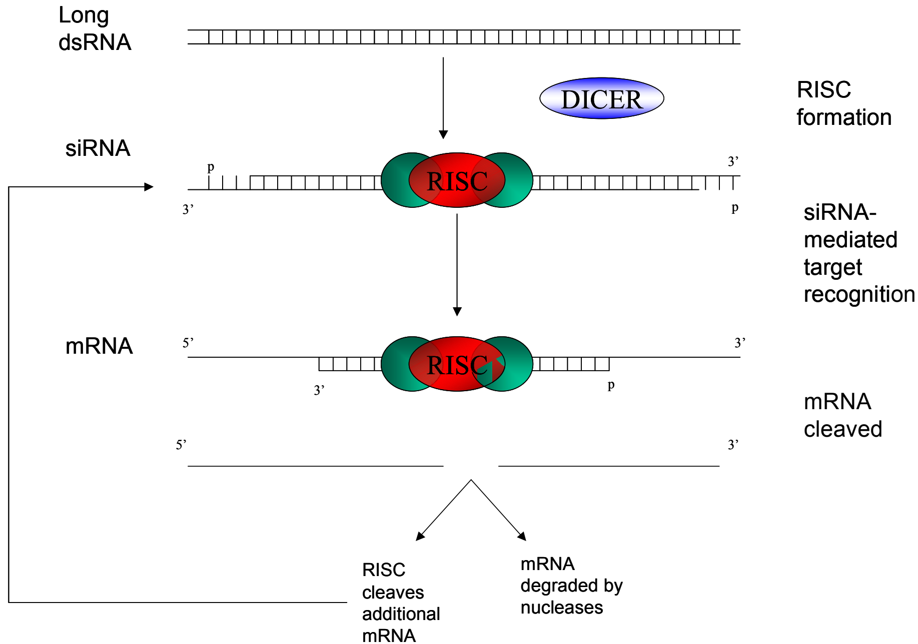



**Figure 1.17: Schematic representation of siRNA mediating complementary mRNA degradation.** Long dsRNA are introduced into the cell and are cleaved by enzymes, known as Dicers, into siRNA of 21-23 nucleotides in length. These siRNAs are then incorporated into the RISC complex, where the two strands are unwound, thereby allowing one strand to bind to complementary mRNA within the cells. The mRNA is then cleaved and degraded by nucleases, with the RISC free to bind further siRNAs. Abbreviations of dsRNA — double-stranded RNA, siRNA — short interfering RNA, RISC — RNA-induced silencing complex. Adapted from Sioud and Sørensen, (2004).

The principle that RNA interference is based upon is that only specific target gene will be down-regulated. The majority of studies focus on one target gene and several additional genes; however, recent data suggests that care must be taken in order to avoid off-target effects. Furthermore, it is possible to knock-down genes with homology similar to the target gene, and they are therefore knocked down as so-called off-target (Jackson and Linsley, 2004).

There are several methods available in order to facilitate the entry of siRNA into mammalian cells *in vitro*; lipid based carriers and viruses, for instance, have been used with varying success. Moreover, the direct application of siRNAs onto mucosal membranes has been demonstrated to be an efficient up-take of siRNA,

thereby helping to explain why the lung has shown the most successful application of siRNA technology *in vivo* to date.

The application of siRNA possesses many challenges, especially in the case of humans; lipid-based carriers and viruses, for example, have been used with varying success. It has been demonstrated in Phase I results of the first two therapeutic siRNA trials demonstrated that siRNAs are well-tolerated and have suitable pharmacokinetic properties. Moreover, Kumar *et al.* (2008) demonstrate that siRNA treatment can dramatically suppress HIV infection in T-cells, which therefore highlights the importance of siRNA as a potential powerful therapeutic target over the next decade.

## 1.10 Mitogen-Activated Protein Kinase

The Mitogen-Activated Protein Kinase (MAPK) pathway is made up of many different subunits. The most widely studied of these is the ERK (extracellular signal regulated kinase), JNK (c-jun-$NH_2$-terminal kinase), and the p38 kinases. These different subunits each plays a key role in signal transduction from the plasma membrane to the nucleus. Moreover, MAPKs primarily reside in the cytoplasm; however, upon activation, these molecules have the capacity to phosphorylate nuclear substrates, such as protein kinases, cell-cycle regulators and transcription factors. Furthermore, activation of MAPKs induces a cascade of phosphorylation events via proteins kinases. Numerous protein kinases are involved in different stages of this process, each regulated through G-proteins, scaffolds, substrates, adaptors and regulator proteins. This, in turn, induces a wide range of cellular functions, including differentiation, cell proliferation, inflammatory responses and apoptosis (Weston *et al.*, 2002).

### 1.10.1 Extracellular Signal Regulated Kinase

ERK is the most well-known member of the MAPK family. Activation of this protein is carried out after stimulation via receptor tyrosine kinases, GPCRs and integrins. The subsequent activation of ERK is catalysed upstream by the kinases MEK1 or MEK2, subsequently resulting in the homodimerisation of ERK1/2 and translocation to the nucleus where they can further stimulate other kinases and transcription factors (Kolch, 2005). CXCR3 agonists demonstrate the ability to

activate the MAPK pathway, utilising the ERK1/2 response as a read-out for MAPK activation (Figure 1.18) (Smit *et al.*, 2003).

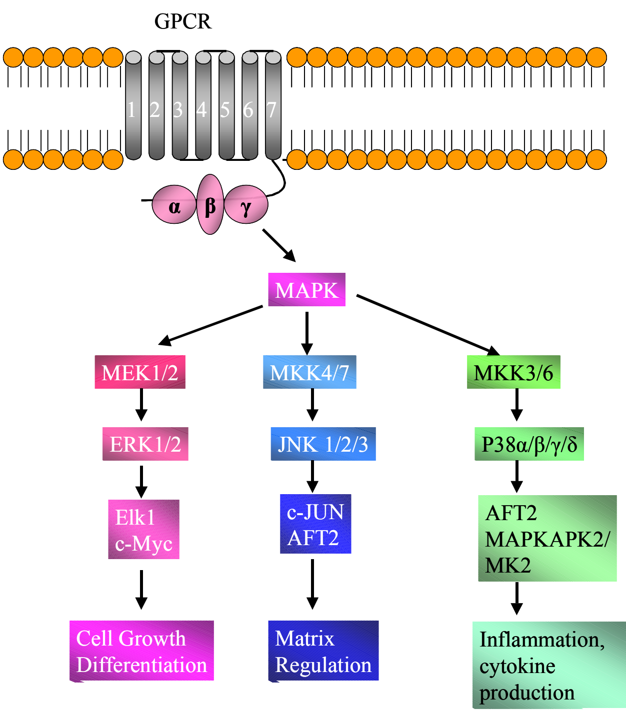



**Figure 1.18: Simplified GPCR activation of MAPK pathway.** The activation of GPCR accordingly activates the MAPK family, which subsequently results in the activation of the three main MAPK families — ERK, JNK and p38. The primary but overlapping responses include cell growth and differentiation (ERK), matrix regulation (JNK), and inflammatory cytokine production (p38). Adapted from Sweeney and Firestein, (2007).

### 1.10.2 Rho in Cell Polarisation

Cell polarity is essential for the process of cell migration and degranulation, and is regulated by conserved protein complexes, including the Par complex, Rho GTPases and their regulators. Increasing evidence indicates that differential sub-cellular localisation and activity of the Par complex — consisting of Par3, Par6, and atypical protein kinase C (aPKC) — are critical for polarisation (Kraynov *et al.*, 2000).

It is also known that the spatial coordination of Rac and Rho activity is essential for degranulation. The molecular mechanisms regulating these GTPases during degranulation are unknown. Members of the Rho family of GTPases are emerging

as key regulators of cell migration; specifically, Rac activity is increased at the leading edge of a migrating cell (Wang *et al.*, 2002). Depending on the physiological context, Rac and Cdc42 can function as mediators and/or regulators of the Par complex. Moreover, PI3K has been shown to predominantly activate Rac or Cdc42 (Raftopoulou and Hall, 2004; Srinivasan *et al.*, 2003; Weiner, 2002).

This activity drives the actin polymerisation which underlies lamellipodia formation and subsequent forward protrusions (Xu *et al.*, 2003). Rac activity leads to lamellipodia formation and forward protrusion. Moreover, rho activity is hypothesised to be required later in order to stabilise the cell during the contractile events of migration.

A large body of evidence has indicated that Rac proteins, and/or components of F-actin networks which are dependent on Rac activity, may form part of a positive-feedback loop, capable of stimulating PtdIns(3,4,5)$P_3$ production preferentially at the leading edge (Park *et al.*, 2004; Sasaki *et al.*, 2004). It has been further demonstrated that RhoA and its downstream effector ROCK regulates the assembly and activity of the Par complex during cell migration.

### 1.11 Mast Cell Signalling

Antigen-dependent mast cell activation is regulated by a complex series of intracellular signalling processes which are activated following antigen/IgE cross-linking to FcεRI. The proximal-signalling events required to release the mediators seem to be common for all categories of mediator, whereas the distal signalling events diverge to regulate different mechanisms, by which the mediators are then released (Gilfillan and Tkaczyk, 2006).

One of the main families of kinases which are involved in mast cell signalling is the SRC family kinases which, during the initial stages, is LYN and mainly resides in lipid rafts (Kovarova *et al.*, 2006). The exact involvement of LYN is unclear; however, it has been reported that antigen-mediated allergic reactions are absent in $LYN^{-/-}$ mice (Hibbs *et al.*, 1995). One possibility for the involvement of LYN in activating both positive and negative pathways for FcεRI mediated degranulation,

with the negative pathways being mediated by the PI3K regulator SHIP (Hernandez-Hansen *et al.*, 2004).

The activation of LYN has been shown to phosphorylate the tyrosine residues in the FcεRI β and γ-chains. Following phosphorylation, the immunoreceptor tyrosine-based activation motifs (ITAMs) of the FcεRI β and γ-chains provide docking sites for the SH2 domains of LYN and ZAP70-related tyrosine kinase SYK (spleen tyrosine kinase) (Chen *et al.*, 1996; Furumoto *et al.*, 2004). The activation of SYK and/or LYN induces the phosphorylation of the trans-membrane adaptor molecule LAT (Linker for the Activation of T-cells), and is critical for the guidance and direction of downstream signalling (Saitoh *et al.*, 2000).

Phosphorylation of LAT results in the recruitment of several molecules such as cytosolic adaptor molecules; for example, GRB2 (growth-factor-receptor-bound protein 2), GADS (GRB2-related adaptor protein), SHC (SH2-domain-containing leukocyte protein C), and SLP76 (SH2-domain-containing leukocyte protein of 65kDa); guanine-nucelotide-exchange factors and adaptor molecules, such as SOS (Son of Sevenless homologue) and VAV; and signalling enzymes, such as phospholipase $C\gamma_1$ ($PLC\gamma_1$). The results of all the molecules being recruited subsequently enables the formation of macromolecules through either direct or indirect binding to LAT, thereby allowing diversification of the downstream signalling required for the release of all the various mediators stored in the cytoplasm of mast cells.

The crucial residues of LAT involved in regulating signalling and degranulation are Y132, Y171, Y191 and Y226; these 4 residues interact though direct or indirect interaction with the signalling enzyme PLCγ, the result of which induces the mobilisation of intracellular calcium and activation of PKC. There are two forms of PLC expressed within mast cells: PLCγ1 and PLCγ2; both of these require re-localisation to the plasma membrane prior to their activation. The precise role of PLCγ has been difficult to assess in the case of mast cells, as knock-out of PLC in mice is lethal (Ji *et al.*, 1997). However, a study suggests that, in bone marrow-derived mast cells in PLC-deficient mice, reduced degranulation response when challenged with antigen has been observed (Wang *et al.*, 2000). LAT is now

known to be not the only adaptor molecule required for mast cell activation. Studies in LAT-deficient bone marrow-derived mast cells still demonstrate residual calcium mobilisation and degranulation, (Saitoh *et al.*, 2000).

Another trans-membrane adaptor molecule, Non-T-cell Activation Linker (NTAL) — sometimes referred as LAT2 due to the structural similarity to LAT — has been identified as a key component for mast cell activation by providing key docking sites. NTAL has been shown to rapidly phosphorylate tyrosine residues in an LYN- and SYK-dependent manner following FcεRI activation (Brdicka *et al.*, 2002; Tkaczyk *et al.*, 2004). However, it is believed that NTAL has a role to play in the negative regulation of mast cell induced degranulation due to the increased capacity to signal and de-granulate in NTAL knock-out mice (Volná *et al.*, 2004). Moreover, it is also believed that NTAL and LAT compete for the same pool of associating signalling molecules in lipid rafts (Tkaczyk *et al.*, 2004). NTAL functions in a similar way to LAT by recruiting GRB2. Despite the similarity between LAT and NTAL, they do not bind to exactly the same molecules. LAT has a site (Y132) which, when phosphorylated, allows binding of PLCγ, whereas NTAL, on the other hand, has a region between 104 and 114, containing the sequence YIDP allowing the binding of SRC-family kinases such as LYN and FYN (Linnekin, 1999).

Studies reveal that the absence of the FceRI β chain only partially limits mast cell activation, whereas knock-out of the γ chain completely abolishes mast cell activation. This has subsequently led to the conclusion that the γ chain is required for degranulation, whereas the β chain is involved in the amplification of the signals (Ra *et al.*, 1989; Repetto *et al.*, 1996; Takai *et al.*, 1994). This amplification pathway is thought to involve another SRC-family kinase FYN, which doesn't activate LAT but another kinase family — the PI3Ks (Gu *et al.*, 2001; Parravicini *et al.*, 2002). An overview of mast cell signalling is highlighted in Figure 1.19.

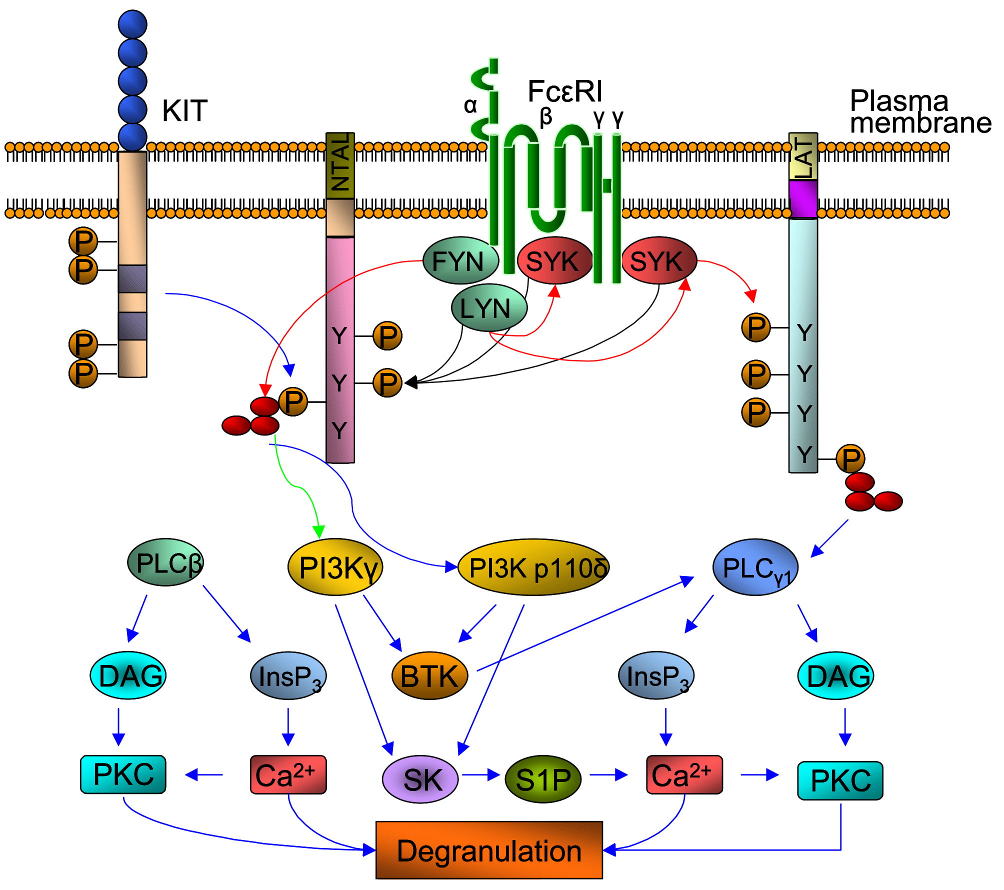



**Figure 1.19: Brief overview of signalling molecules involved in mast cell degranulation.** Activation of FcεRI and c-Kit result in the activation of both the principle signalling pathways (red arrows) and the complementary pathways (black arrows). The role that NTAL might have to play in these pathways is yet to be confirmed experimentally. The blue and green arrows indicate the activation of downstream signalling pathways, which subsequently leads to degranulation (adapted from Gilfillan and Tkaczyk, 2006).

PI3K provides a docking site for PH domains of several signalling molecules which are considered important for mast cell activation containing these PH domains, including PLCγ 1 and 2, VAV and Burtons tyrosine kinase (BTK), (Iwaki *et al.*, 2005; Salojin *et al.*, 2000). There have been many different approaches utilised over the years with the aim of deciphering the role of PI3K in mast cell activation and subsequent degranulation. Initial studies began with the broad-spectrum PI3K inhibitors, Wortmannin and LY294002, which were observed to inhibit both antigen-mediated calcium mobilisation and degranulation (Barker *et al.*, 1995; Barker *et al.*, 1998). Moreover, it is widely recognised that these inhibitors have a wide range of off-target effects hitting other kinases. Genetic approaches have

generated mice lacking the p85α or p85β subunits individually, mice lacking catalytic p110γ, p110δ subunits, or mice with a knock-in point mutation of p110δD910A, thereby revealing the involvement of p110δ but not p110γ as the key PI3K isoform involved in mast cell activation. Mast cells derived from the bone marrow of p85α and p85β knock-out mice show normal antigen mediated calcium flux and degranulation.

PI3K plays a multiple role in mast cells — not only in terms of the release of mediators but also in chemotaxis, adhesion and homeostasis. As with all PI3K-induced responses, specific isoforms are responsible for mediating different reactions. Examples of this are in p110δ-deficient bone marrow-derived mast cells, where chemotaxis and adhesion are severely diminished compared to wild type mast cells (Jolly *et al.*, 2004). *In vivo* studies also demonstrate the loss of peritoneal and gastrointestinal mast cells in p85α-deficient mice (Kim *et al.*, 2004).

## 1.12 CXCR3

Historically, CD183 was the third CXC chemokine receptor discovered and was subsequently given the name CXCR3 as it is now more commonly known. CXCR3 is a PTX-sensitive, seven-transmembrane domain-spanning G protein-coupled receptor which binds the pro-inflammatory non-ELR motif CXC chemokines: monokine, induced by human IFN-γ (Mig)/CXCL9, IFN-inducible 10-kDa protein (IP-10)/CXCL10 and IFN-inducible T-cell α chemo-attractant (I-TAC)/CXCL11. CXCL9, CXCL10 and CXCL11 (Loetscher *et al.*, 2000; Murphy *et al.*, 2000; Zlotnik *et al.*, 1999);(Khan *et al.*, 2000). CXCR3 was cloned and identified as a receptor for CXCL9 and CXCL10 by Loetscher *et al.* (1996) and was subsequently identified as a receptor for CXCL11 (Cole *et al.*, 1998). CXCL9, CXCL10 and CXCL11 are selective, potent agonists of CXCR3 (Kd 0.1–5 nM) (Cole *et al.*, 1998; Loetscher *et al.*, 1996). Additional chemokines have been reported to bind to CXCR3 (e.g., CXCL13 (Jenh *et al.*, 2001) and CCL11 (Xanthou *et al.*, 2003). Notably, the reported affinities are generally weak and the biological significance of the interactions is questionable. Similarly, the agonists for CXCR3 have been reported to be antagonists of CCR3 (Loetscher *et al.*, 2001) and CCR5 (Petkovic *et al.*, 2004), but high concentrations of the CXCR3 ligands are required to achieve inhibition of CCR3 or CCR5 biological functions. CXCL11 has a higher binding

affinity for CXCR3 than either CXCL9 or CXCL10 and is a more potent activator of CXCR3 (Clark-Lewis *et al.*, 2003; Meyer *et al.*, 2001; Sauty *et al.*, 2001). Similarly, CXCL11 is a more potent antagonist to CCR3 than CXCL9 or CXCL10 (Loetscher *et al.*, 2001).

Recent studies show that different CXCR3 ligands exhibit unique temporal and spatial expression patterns, suggesting that they have non-redundant functions *in vivo*. Moreover, the CXCR3 ligands share low sequence homology (around 40% amino acid identity) and exhibit differences in their potencies and efficacies at CXCR3, with CXCL11 being the dominant ligand in several assays (Cole *et al.*, 1998; Meiser *et al.*, 2008)**.** CXCR3 is expressed in a wide variety of cells, including activated T lymphocytes, NK cells, malignant B lymphocytes, endothelial cells, and thymocytes (Loetscher *et al.*, 1996; Loetscher *et al.*, 1998; Qin *et al.*, 1998; Romagnani *et al.*, 2001; Trentin *et al.*, 1999; Van Der Meer *et al.*, 2001).

The binding of chemokines to CXCR3 induces cellular responses which are involved in leukocyte traffic, most notably integrin activation, cytoskeletal changes and chemotactic migration (Loetscher *et al.*, 2000; Murphy *et al.*, 2000; Zlotnik *et al.*, 1999). Signal transduction has not been further analysed but may include the same enzymes (including phospholipases, protein kinase B and C, PI3K, MAP kinases, G protein-coupled receptor kinases, and small GTPases) identified in the signalling cascade as induced by other chemokine receptors (Loetscher *et al.*, 2000; Murphy *et al.*, 2000; Zlotnik *et al.*, 1999).

In recent years, however, two main variants of CXCR3 receptor have been identified, namely CXCR3-B (Lasagni *et al.*, 2003) and CXCR3-alt (Ehlert *et al.*, 2004). Both variants are generated via alternative splicing of mRNA, encoding the original CXCR3 receptor (henceforth referred to as CXCR3-A). In the case of CXCR3-B, alternative splicing results in the translation of CXCR3-B mRNA generating a 416–amino acid receptor containing a longer $NH_2$-terminal extracellular domain different from the CXCR3-A sequence in the first 52 amino acid residues; the remaining receptor at a protein level, on the other hand, was identical (Lasagni *et al.*, 2003). Moreover, it has also been reported that CXCR3-B could be bound to a G protein that is not $G\alpha_i$ as CXCR3-B is not pertussis toxin-sensitive. Pertussis toxin insensitive hetrotrimetric G proteins include members of

the Gα12 and Gq families and the Gi family member Gz, although the latter is predominately expressed in neurons and platelets (Kouroumalis *et al.*, 2005). CXCR3-B is expressed at a lower concentration than CXCR3-A, but has been shown to be functional against all classical CXCR3 ligands, CXCL9, CXCL10 and CXCL11. This form of receptor has been shown to bind Platelet Factor 4 (PF4/CXCL4) in addition to the three classical CXCR3 agonists (Lasagni *et al.*, 2003). Figure 1.20 highlights the structural differences between the CXCR3 isoforms.

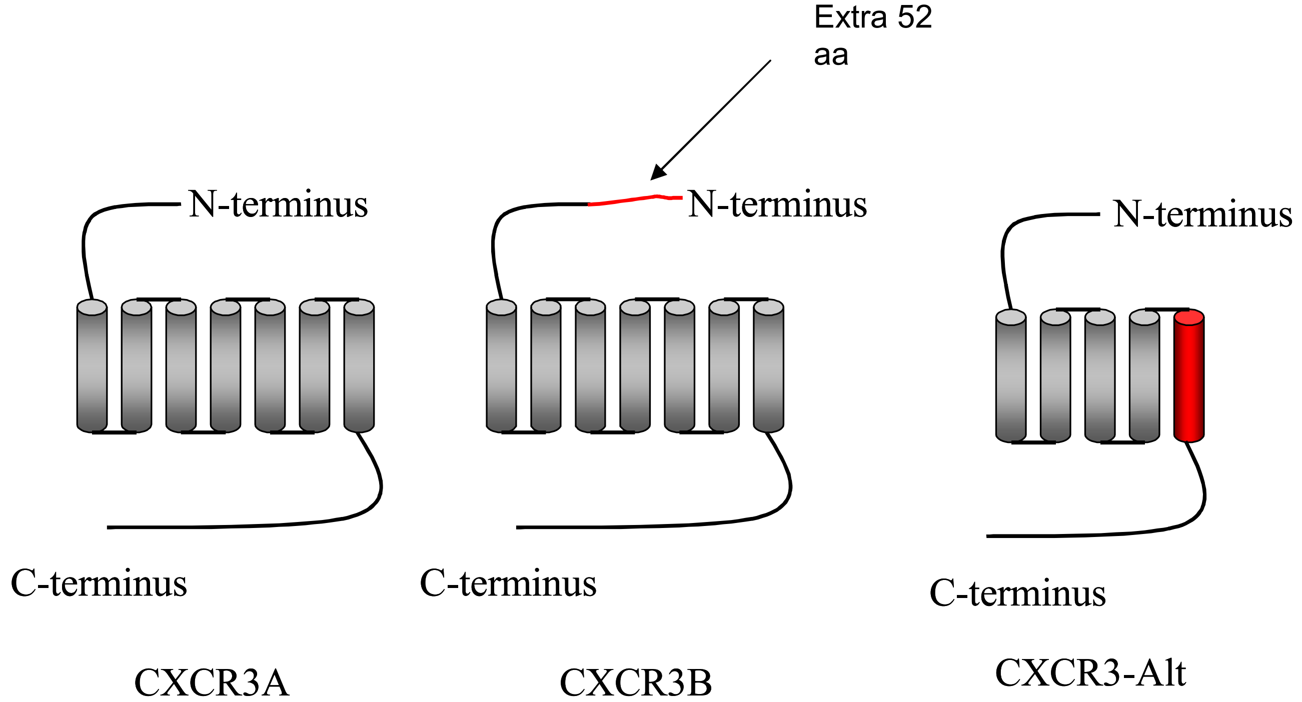



**Figure 1.20: 2D Schematic of the CXCR3 variants.** The three variants of CXCR3 have structure differences. CXCR3B has a slightly longer NH2 terminus, by 52 aa when compared to CXCR3A. CXCR3 is the truncated form of CXCR3, and has a predicted four, possibly five, transmembrane spanning domains.

In contrast, CXCR3-alt is a truncated version of CXCR3 (lacking 101 amino acids), which consequently exhibits a dramatically altered C terminus and has a predicted 4-5 transmembrane domain structure (Ehlert *et al.*, 2004). Despite this drastically modified structure, CXCR3-alt has nevertheless been shown to bind and respond to CXCL11, yet is unresponsive to CXCL9 and CXCL10. In addition, CXCL11 — but not other CXCR3 agonists — also binds to CXCR7; a receptor which has been

associated with increased adhesiveness, invasiveness and reduced apoptosis of human umbilical vein endothelial cells and tumour cells (Burns *et al.*, 2006).

### 1.12.1 CXCR3 and its Agonists on Mast Cells

Previous studies carried out on mast cells reveal that CXCR3 is the most abundantly expressed, functional chemokine receptor on human lung mast cells. In contrast, CXCR3 expression by bone marrow mast cells is low (Brightling *et al.*, 2005). It is thought from this study that the CXCR3 activation may facilitate the migration of mast cells within tissue, and may therefore be important in the micro-localisation of mast cells within specific tissue structures. However, although CXCR3 was the chemokine receptor most highly expressed, human lung mast cells is also expressed in the chemokine receptors CCR3, CXCR1, and CXCR4 in more than 10% of cells (Brightling *et al.*, 2005).

It has been widely reported that CXCR3 agonists play a key role in the recruitment of CXCR3 positive mast cells to the tissues in many diseases (Ruschpler *et al.*, 2003; Wardlaw *et al.*, 1986; Yousem, 1997). Such diseases include Rheumatoid Arthritis, (Ruschpler *et al.*, 2003), post-lung transplant bronchiolitis obliterans syndrome (Belperio *et al.*, 2003), and sarcoidosis (Miotto *et al.*, 2001). ASM bundles are contracted in asthma and is predominately mediated by activation of mast cell CXCR3 by ASM-derived CXCL10, but not CXCL9 or CXCL11, therefore suggesting a different role for the various CXCR3 agonists in diseases.

### 1.12.2 Therapeutic Potential of CXCR3

Studies using CXCR3-KO (CXCR3$^{-/-}$) show a delayed acute or chronic rejection of cardiac or pancreatic allograft in murine models of transplant rejection. In some cases — especially in combination with immunosuppressive therapy — CXCR3$^{-/-}$ mice were able to maintain the allograft. Moreover, in the case of similar models, treatment with an antibody directed against either CXCR3 or CXCL10 significantly prolongs allograft survival. In addition, anti-CXCL10 antibodies inhibited chronic experimental colitis, and a Phase II clinical trial has been launched with the aim of further studying the effects of one of the antibodies (MDX1100) in the treatment of

ulcerative colitis. The same antibody will also be tested in a Phase II trial for rheumatoid arthritis.

| Disease | References |
|---|---|
| Multiple sclerosis | Sorensen *et al.*, 1999. |
| Rheumatoid arthritis | Qin *et al.*, 1998. |
| Atherosclerosis | Mach *et al.*, 1999. |
| Chronic obstructive pulmonary disease | Saetta *et al.*, 2002. |
| Inflammatory bowel disease | Yuan *et al.*, 2001. |
| Psoriasis | Rottman *et al.*, 2001. |
| Hepatitis C | Shields *et al.*, 1999. |
| Sarcoidosis | Agostini *et al.*, 1998. |
| SARS | Glass *et al.*, 2004; Danesh *et al.*, 2008. |
| Transplant rejection | Hancock *et al.*, 2000; Hancock *et al.*, 2001; Inston *et al.*, 2007. |
| Metastasis of melanoma and colon cancer cells to the lymph nodes | Kawada *et al.*, 2004; Kawada *et al.*, 2007. |
| Metastasis of breast cancer cells to the lungs | Walser *et al.*, 2006. |
| HIV | Hatse *et al.*, 2007. |

**Table 1.3: Involvement of CXCR3 in human disease.** Adapted from Wijtmans *et al.*, (2008).

Use of protein-based CXCR3 antagonists also confirms that the blocking of this receptor helps to reduce skin inflammation (Proudfoot and Kosco-Vilbois, 2003) and neuro-inflammation in mice model (Vergote *et al.*, 2006).

A hallmark of CXCR3 is its prominent expression in *in vitro* cultured effector/memory T-cells, and in T-cells present in many types of inflamed tissues. In addition, CXCL9, CXCL10 and CXCL11 are commonly produced by local cells in inflammatory lesion, therefore suggesting that CXCR3 and its chemokines participate in the recruitment of inflammatory cells. Therefore, CXCR3 is a target

for the development of small molecular weight antagonists which may be used in the treatment of diverse inflammatory diseases. Essentially, a better understanding of all the CXCR3 variants is needed in order to fully develop any therapeutic potential of CXCR3. CXCR3 and its agonists have been implicated in several immune-mediated inflammatory diseases, and, similarly, a few reports describe the vast differences in potencies of the three CXCR3 ligands (Heise *et al.*, 2005). Most of the therapeutic studies based around CXCR3 concentrate on the therapeutic potential of CXCR3 within tumours. Moreover, studies have looked at CXCR3 ligands as potent inhibitors of tumour growth in mice (Arenberg *et al.*, 2001) to the attraction of CD8+ T-lymphocytes by CXCR3 ligands as potent anti tumour activities (Hensbergen *et al.*, 2005). A number of studies also consider the development of CXCR3-antagonists, as a high prevalence of CXCR3 ligand expression is usually observed, especially in inflamed joints of RA patients (Wedderburn *et al.*, 2000) in MS lesions (Balashov *et al.*, 1999) during pancreatitis in Type 1 diabetes (Frigerio *et al.*, 2002) and during allograft rejection in animal models and transplantation patients (Agostini *et al.*, 2001). Table 1.3 highlights some of the diseases which have been linked with CXCR3 involvement.

### 1.12.3 CXCR7

CXCR7 is a 7-transmembrane receptor member of the G-protein coupled receptor family. It was previously thought that this protein was a receptor for VIP and was considered to be an orphan receptor (Law and Rosenzweig, 1994). CXCR7 is encoded in humans in the second chromosome — the same chromosome where the genes to CXCR1, CXCR2 and CXCR4 are encoded. The binding of the chemokines CXCL12/SDF-1 and CXCL11 has been demonstrated by various scholars, including Balabanian *et al.* (2005) and Burns *et al.* (2006). Recent findings in zebra fish suggest that CXCR7 primarily functions by sequestering the chemokine CXCL12. Membrane-associated CXCR7 has also been found to be expressed on many tumour cell lines, on activated endothelial cell, and on foetal liver cells. Unlike many other chemokines receptors, CXCR7 has not yet been shown to induce cell migration or to otherwise induce the mobilisation of intracellular calcium (Dambly-Chaudière *et al.*, 2007). Furthermore, it has been recently demonstrated that CXCR7 also regulates the expression of the pro-angiogenic factors interleukin-8 or vascular endothelial growth factor, which are

likely to participate in the regulation of tumour angiogenesis and the activation AKT pathways (Wang *et al.*, 2008).

### 1.13 Regulation of GPCR Signalling

The expression of GPCRs on the cell surface is a finely controlled process. The first step is the generation of a receptor in the endoplasmic reticulum, where the GPCR is assembly-ready for transportation to the cellular membrane. The newly assembled glycosylated receptor is then considered ready to interact with its specific agonist. Signalling via GPCRs is very sensitive following activation. Moreover, the sensitivity or negative regulation has been termed desensitisation.

Desensitisation occurs very quickly following activation by its agonist. This activation event reduces the responsiveness of the receptor to further stimulation. The reduction in responsiveness is due to the G protein-coupled receptor kinases phosphorylating the receptor induced conformational changes, which results in the binding of inhibitory β-arrestin protein that induces the dissociation of the G-protein (Pao and Benovic, 2002; Vroon *et al.*, 2006). The disassociation of the G-proteins can also occur via second messenger-dependent kinases; for example, protein kinase A and C. In contrast to the G-protein receptor kinases, this disassociation occurs in the presence or absence of agonist, and has been named heterologous desensitisation. This type of desensitisation is responsible for cellular hypo-responsiveness (Kristiansen, 2004). Moreover, GPCR signalling is further controlled by the internalisation of the receptor; this control process of GPCR signalling is longer than desensitisation and can take up to several minutes (Luttrell and Lefkowitz, 2002).

Once a GPCR has been internalised, there are two resulting outcomes: recycling of the receptor back to the surface, or degradation of the receptor. Recycling of the receptor back to the surface requires the receptor to undergo dephosphorylating and the removal of the β-arrestin, thereby allowing the resensitation of the receptor to its appropriate agonist. Use of modified chemokines demonstrates longer recycling times, thereby indicating different rates depending on the agonist stimulating the receptor (Pastore *et al.*, 2003). Degradation of the receptor is via a lysomal degradation pathway.

### 1.13.1 Chemokine Receptor Internalisation and Intracellular Trafficking

The expression of the chemokine receptor on the surface is a balance between the rate of internalisation and the rate of replacement (recycling or synthesis of new protein) (Mueller *et al.*, 2002). Chemokine receptors undergo a basal level of internalisation and the degradation or recycling in the absence of the agonist. Agonist-binding can enhance the internalisation and trafficking of these receptors, and can accordingly increase the rate of receptor sensitisation versus desensitisation, and recycling versus degradation (Neel *et al.*, 2005). Two major routes of receptor trafficking exist: clathrin-mediated endocytosis, and lipid raft/caveolae dependent internalisation. Moreover, it is possible that certain receptors can internalise via both pathways whilst others may utilise one preferred pathway. The pathway of internalisation selected by a particular receptor will partially depend on the specific cell type and the different expression of specific adaptor proteins, membrane lipid composition in close location to the receptor or other unknown factors (Neel *et al.*, 2005).

### 1.13.2 Pathways for Internalisation of Chemokine Receptors

The internalisation of the receptor and its agonist by clathrin-coated pits is the best characterised endocytic process (Mukherjee *et al.*, 1997). Activation of a receptor by its agonist induces a phosphorylation of Ser and Thr residues in intracellular loops and C-terminus of the receptor due to action of GRKs (G protein-coupled receptor kinases, which are activated by the kinase activity of second messengers, such as PKC) which subsequently leads to the uncoupling of the G protein from the receptor and, in some cases, the receptor desensitisation. Receptor phosphorylation and/or presence of di-leucine motif in the C-terminus of chemokine receptor are crucial for the binding of adaptor proteins which link the receptor to a lattice of clathrin that ultimately facilitates its internalisation (Neel *et al.*, 2005). Adaptin-1 and β-arrestin have been found to play an important role in chemokine receptor internalisation. β-arrestin binding to receptor occurs through phosphorylated residues in the C-terminus, but can also occur through the intracellular loops. Furthermore, it has also been reported that CXCR4 β-arrestin binds to both carboxyl-terminus and the third intracellular loop (Cheng *et al.*,

2000). AP-2 binds to some chemokine receptors, including CXCR2 and CXCR4, via highly conserved Leu-Leu, Ile-Leu and Leu-Ile motif within the C-terminus (Heilker *et al.*, 1996; Fan *et al.*, 2001). Association of the adaptor proteins is required for the recruitment of clathrin and through action of dynamin, formation of clathrin-coated vesicles (Mousavi *et al.*, 2004). Following receptor internalisation, clathrin-coated vesicles are uncoated and receptor-ligand complexes are directed to the early endosomal compartment. The receptor can then be directed to the perinuclear recycling compartment and can be recycled back to the plasma membrane or can otherwise enter the late endosomal compartment and be directed to the lysosomal compartment for degradation.

An alternative pathway for the internalisation of a chemokine receptor may occur via lipid rafts or through cholesterol enriched structures, called caveolae (Parton and Simons, 2007; Palade, 1953). Caveolae are stabilised by caveolin-1 (CAV-1) and CAV-2 cholesterol-binding proteins and form characteristic flask-shape structures with no obvious coat (Stan, 2005). Once internalised, some receptors can enter the compartment, known as caveosome, and fuse with early endosomes also used in clathrin-dependent pathways (Sharma *et al.*, 2003). Despite some reports that chemokine receptors — such as CCR5 and CXCR4 (Manes *et al.*, 1999; Manes *et al.*, 2000; Nguyen and Taub, 2002) — have been found to some degree in lipid rafts; coveolae/lipid rafts-dependent internalisation does not appear to be a common feature for the chemokine receptors.

### 1.13.3 Regulation of Chemokine Receptor Trafficking by Rab GTPases

Rabs are small GTPases which are involved in the regulation of a variety of cellular trafficking events. The exchange of GDP for GTP, GTP hydrolysis w and GDP displacement are regulated by Guanine nucleotide Exchange Factors (GEFs), GTPase-Activating Proteins (GAPs) and GDP Dissociation Inhibitors (GDIs), respectively. Rab5 mediates early endocytic responses which are required for the fusion of early endosomes (Rybin *et al.*, 1996). Moreover, Rab5 interacts with Class I of PI3K and induces the production of $PI_3P$. Rab5 and $PI_3P$ recruit EEA-1 (Early Endosomal Antigen) and other proteins involved in fusion with early endosomes. Furthermore, the internalisation of CXCR2, CXCR4 and CCR5

requires activity of Rab5 and is significantly blocked by expression of Rab5 dominant negative mutant (Fan *et al.*, 2003; Venkatesan *et al.*, 2003).

There are two types of endocytic recycling — rapid and slow pathway — to which Rab4 and Rab11a contribute respectively (Ullrich *et al.*, 1996; Sheff *et al.*, 1999; Sonnichsen *et al.*, 2000). The slow recycling pathway involving localised on perinuclear compartment Rab11a seems to be important in the case intracellular trafficking of chemokine receptors. This is shown for the case of CXCR2 which, upon agonist-induced internalisation, localises to Rab11a-containing compartment (Fan *et al.*, 2003). Rapid recycling pathway through Rab4 positive endosomes bypasses the perinuclear endosomes and occurs in PI3K-dependent manner (Hunyady *et al.*, 2002). It is not known which mechanisms regulate these different recycling pathways. Rab7 is involved in the directing of late endosomes to the lysosomal compartment. Prolonged exposure of chemokine receptor to ligand may result in their degradation in lysosomes. Moreover, Rab7 is thought to be important in the process of lysosomal sorting. Involvement of Rab7 is shown for the CXCR2 receptor where the blocking of Rab7 activity results in the decreased localisation of this receptor to the lysosomal compartment, and its accumulation in early and recycling endosomes (Fan *et al.*, 2003).

### 1.13.4 Regulation and Functional Consequences of Internalisation

Receptor internalisation following ligand-binding is most likely the reason for down-regulation of most chemokine receptors (Neel *et al.*, 2005). The rate of this process can be dependent on multiple factors involving C-terminus of receptor, the type of ligand, cell type or phosphorylation status. Phosphorylation of Ser and Thr residues and presence of di-leucine motif in the carboxyl-terminus is shown to be required for the internalisation of some chemokine receptors. This has been reported for CXCR4 receptor, for example (Neel *et al.*, 2005). In contrast, the internalisation of CXCR3 by any of its ligands is not affected by the mutational change in the LLLRL motif located in the C-terminus. However, mutational change of C-terminal Ser and Thr residues inhibited CXCL9- and CXCL10-nduced internalisation with no effect of CXCL11, whereby action was dependent on the third intracellular loop of CXCR3 in 300-19 cells (Colvin *et al.*, 2004). The rate of internalisation also depends on the type of ligand. Most chemokine receptors bind

with high affinity more then one chemokine, and these chemokines may differentially induce the internalisation of the receptor. This situation is observed for CXCR2, which binds two ligands — CXCL6 and CXCL8 — with high affinity; however, the latter is more efficacious in inducing internalisation of CXCR2 (Feniger-Barish *et al.*, 2000). Similar findings have been reported for the CXCR3 receptor, which becomes internalised by CXCL9, CXCL10 and CXCL11, with CXCL11 being the principal chemokine responsible for CXCR3 internalisation (Sauty *et al.*, 2001). Cell context may also affect the rate of internalisation and the pathway through which a receptor becomes internalised; this may be due to the availability of different endocytic compartments, such as caveolae, and the expression of various proteins, such as β-arrestin and other adapt proteins. For example, CXCL11 reduces surface expression of endogenous CXCR3 in T-cells down to 20% whereas, in transfected L1.2 cells, only 50% reduction of basal expression of surface CXCR3 is detected (Meiser *et al.*, 2008). Moreover, the internalisation of some receptors — such as CCR5 — has been reported to occur via both clathrin and caveolae-dependent pathway. However, in some cell types, such as Chinese Hamster Ovary (CHO), CCR5 internalises mainly clathrin-dependent endocytosis (Signoret *et al.*, 2005); in other cell types, on the other hand, namely Human Embryonic Kidney cells (HEK293), the dominant pathway for CCR5 internalisation is via caveoli (Venkatesan *et al.*, 2003).

The factors which mediate the fate of internalised receptors are largely unknown. They possibly include the duration and concentration of ligand stimulation, and different sorting motifs present within intracellular domains of the receptor. As is the case of the CCR5 receptor, short stimulation leads to the direction of recycling endosomes, whereas longer stimulation cause its localisation to late endosomal compartment (Signoret *et al.*, 2000). Similarly, at the early time of stimulation with CXCL8, CXCR2 enters recycling endosomes while the extended stimulation time leads to its direction to the lysosomes (Fan *et al.*, 2003). Moreover, intracellular trafficking is an important aspect of the chemokine receptor function, and has been studied for multiple receptors. Some findings suggest the role of internalisation of chemokine receptors in trans-endothelial migration (Sauty *et al.*, 2001). However,

the requirements for internalisation in chemotaxis and signalling remain controversial and still need to be addressed.

## 1.14 Hypothesis and Aims

The aims of the first part of this thesis were as follows:

- To establish the expression of CXCR3 on the human mast cell line, HMC-1, and the primary mast cell model derived from cord blood mast cells, and to determine their suitability as a mast cell model.

Hence, the following hypotheses were proposed:

- All variants of CXCR3 will be expressed on HMC-1 and will induce migration of the HMC-1 cell line.
- The primary mast cell model will mirror more closely mast cells in the human body than the cell line, HMC-1.

The following objectives were established in order to test these hypotheses:

- Examine the expression of CXCR3 variants on the HMC-1 cell line by investigating expression at the mRNA and protein level.
- Investigate the signalling profile of CXCR3 agonists in the HMC-1 cell line.
- Analyse functional responses of CXCR3 on the HMC-1 cell line by examining chemotactic responses.
- Examine cell surface markers on primary mast cell models so as to ensure correct differentiation from precursors to mature mast cells.
- Establish the mast cell phenotype generated in the primary model and check if the function mirrors the current understanding.
- Analyse certain signalling pathways involved in FcεRI via western blotting, and utilise available inhibitors to establish their importance in mast cell degranulation.

The second aim of this thesis was to establish the role of CXCR3 on the primary mast cell model derived from cord blood. As such, the following hypotheses were proposed:

- CXCR3 variants will all be expressed on the primary mast cell model, and will augment sub-optimal FcεRI induced degranulation.

- CXCR3 activation will induce PI3K and PLC activation, which will be critical to the release of mediators from mast cells.

The following tasks were subsequently established to test these hypotheses:

- Examine CXCR3 at mRNA and protein levels.
- Induce the degranulation of primary mast cell models utilising sub-optimal antigen concentration, then stimulate with CXCR3 agonists.
- Examine any augmentation of degranulation by CXCR3 in order to determine if this response is CXCR3-specific, utilising small molecular CXCR3 antagonists.
- Investigate the pathways activated by CXCR3, and accordingly examine their involvement in degranulation of these pathways, utilising specific isoform inhibitors.

# 2. Methods and Materials

## 2.1 Methods

### 2.1.1 Cell Types and Culture Conditions

The focus of this work was to characterise the signalling and function of CXCR3 and its variants in human mast cells. The models that were used were mast cells differentiated and matured for CD133+ precursor cells derived from human cord blood or the human leukemic mast cell line, HMC-1. Furthermore, the THP-1 cell line was utilized to investigate the presence of variants of CXCR3 in other leukocytes. All primary cells and cell lines were routinely maintained in their respective media supplemented with antibiotics and 10% foetal bovine serum as described in detail below. Cells were cultured ever 3-4 days as required and maintained at 37$^{o}$C and 5% $CO_2$. Prior to experimental procedures, cell viability was determined using trypan blue to stain any dying cells.

### 2.1.2 Cord Blood Derived CD133+ Precursors

CD133+ progenitor cells (Stem Cell Technologies) were grown in presence of Stem Cell Factor (SCF) (100 ng/ml); IL-6 (100 ng/ml) and IL-3 (30 ng/ml). Cytokines were applied in Stem pro serum-free medium containing 50U/ml penicillin, 50µg/ml streptomycin and 1% l-glutamine. Cultures were maintained at 37 °C and 5% $CO_2$ in 6-well plates at cell density $0.5 \times 10^6$/ml. Cells were fed twice per week during the first 4 weeks of culturing, and thereafter once per week by removing half of the volume of the cytokine-supplemented medium and adding fresh medium containing 2× cytokine cocktail. After 8 weeks, cultures were supplemented with 5% FCS and IL-4 (10ng/ml) (Mirkina et al., 2007).

### 2.1.3 THP-1

THP-1 cells(Sigma-Aldrich, Poole, UK) were maintained in 175cm$^2$ tissue culture flask in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS), 50U/ml penicillin and 50µg/ml streptomycin. Cells were grown to a maximum of 1.5 x $10^6$ cells and diluted with fresg complete medium to 5x$10^5$ cells every 3-4days. THP-1 cells were maintained in a humidified incubator at 37$^{o}$C and 5% $CO_2$.

### 2.1.4 HMC-1

HMC-1 cells (Mayo Foundation for Medical Education and Research, Rochester, USA, MMV-88-049) were maintained in 175cm$^2$ tissue culture flask in Iscove's modified Dulbecco's medium(IMDM) without phenol red medium supplemented with 10% foetal bovine serum (FBS), 50U/ml penicillin, and 50μg/ml streptomycin. Cells were grown to a maximum of 1.5 x 10$^6$ cells and diluted with fresh complete medium to 5x10$^5$ cells every 3-4days. HMC-1 cells were maintained in a humidified incubator at 37$^o$C and 5% $CO_2$.

### 2.1.5 Evaluation of CD133+ Precursor Differentiation in to Immature Mast Cells.

In order to verify that the differentiation procedure was successful, cells off all ages were routinely stained for the presence of the mast cell specific marker tryptase.

1 x 10$^5$ of primary mast cells were washed twice in RPMI and centrifuged onto polylysine-coated glass slides in a Cytospin3 cytocentrifuge at 250g. The resulting smears were then air-dried and fixed by submersion in an acetone: methanol solution (1:1) for 1 minute. The smears were then carefully washed in TBS buffer (pH 7.6) and a circle drawn around the outside of the smear using a SuperPap pen to create a hydrophobic ring around the smear in order to generate a concentrated pool of antibody over the smear. 100μl of anti-human mast cell tryptase mAb or isotype matched control was added to the appropriate smear and incubated at 4$^o$C overnight.

To visualise the presence of tryptase within human mast cells, prepared smears were stained using the alkaline phosphatase/anti-alkaline phosphatase (APAAP) method according to the manufacturer's (DAKO) instructions, briefly outline as follows. After incubation overnight, excess anti-human tryptase antibody was washed away using TBS buffer (pH 7.6) and then a drop of Dako REAL™ Link, Secondary Antibody (LINK) (Bottle A) was added to each smear and left at room temperature for 30 minutes. After incubation, excess secondary antibody was washed off and a drop of Dako REAL™ APAAP Immunocomplex (APAAP) (Bottle

B) was added to each smear for 30 minutes at room temperature. During this time the Chromogen Red-containing Substrate Working Solution (CHROM) was prepared by mixing thoroughly 750µL AP Substrate Buffer (Bottle F) with 30 µL Chromogen Red 1 (Bottle C), 30µL Chromogen Red 2 (Bottle D) and 30 µL Chromogen Red 3 (Bottle E) in that exact order and with thorough mixing after the addition of each chromogen. Use CHROM within 20 minutes. Any volume of CHROM that was not used was discarded. Smears were rinsed again in TBS (pH7.6) to remove the excess APAAP and a drop of CHROM was added to each smear for 20 minutes at room temperature. Slides were then washed again in TBS (pH 7.6) and air dried then analysed microscopically.

### 2.1.6 Evaluation of Mast Cell Maturity.

1 x $10^5$ of primary mast cells were washed twice in RPMI and centrifuged onto polylysine-coated glass slides in a cytospin3 cytocentrifuge at 250g. The resulting smears were then air-dried and fixed by submersion in an acetone: methanol solution (1:1) for 1 minute. Smears were then air-dried before submersion in May-Grunwald's eosine-methylene blue solution modified for 1 minute. Smears were rinsed with $dH_2O$ and air-dried. Smears were then submersed in Giemsa's azur eosin methylene blue solution for 1 minute. After incubation period, excess stain was washed off using $dH_2O$ and again left to air dry.

Slides were then analysed microscopically.

### 2.1.7 Transmission Electron Microscopy (TEM)

TEM is a microscopy technique whereby a beam of electrons is transmitted through an ultra thin specimen, interacting with the specimen as it passes through it. An image is formed from the electrons transmitted through the specimen, magnified and focused by an objective lens and appears on an imaging screen, a fluorescent screen in most TEMs, plus a monitor, or on a layer of photographic film, or to be detected by a sensor such as a CCD camera.

A biocompatible surfactant-n-dodecylammonium α-glutamate was diluted to a 2.5% final concentration in complete culture medium. 1x$10^6$/ml were re-suspended in this medium to fix the cells at room temperature over a two hours period then

stored overnight at $4^oC$. Samples were washed three times, each for a period of 5 minutes in 0.1 Sodium Cacodylate buffer. Samples were post fixed in 1% Osmium Tetroxide + 1% Potassium Ferrocyanide for 1 hour before washed three times with $dH_2O$. After post fixation, each sample was encapsulated in 3% agarose and cut into cubes. Each cube was subsequently washed in $dH_2O$ twice each for a period of five minutes. 1% aqueous Uranyl Acetate was applied to each cube before stored at room temperature in the dark for one hour. The cubes were then dehydrated using increasing concentrations of acetone – 50, 70, 90 and 95% at $4^oC$, cubes were treated with each dilution twice over a period of ten minutes per concentration before final dehydration in 100% dry acetone at room temperature. This final dehydration with 100% acetone was applied to each cube 4 times over a period of twenty minutes. After dehydration samples were infiltrated in Spurrs Epoxy Resin: 100% dry acetone 1:1 for a period of 1-2 hours then 100% Spurrs Epoxy resin for 2 hours then fresh resin overnight. Each sample was then embedded in moulds and cured in a $70^oC$ oven for 8 hours.

### 2.1.8 Freezing/Thawing of Cells

For storage, $1x10^6$ cells/ml in exponential growth were re-suspended in freeze medium containing 10% dimethyl sulpohoxide (DMSO), and 90% foetal calf serum. The cell suspension was transferred to cryotube (1ml/tube), cooled overnight at $1^oC$/minute in isopropanol to $-80^oC$ and transferred to liquid nitrogen tanks for long-term storage. For resuscitation of cells from liquid nitrogen, cells were rapidly defrosted for one-minute in a $37^oC$ water bath, washed once in RPMI-1640 and re-suspended in correct medium and volume and cultured as previously stated.

### 2.1.9 Degranulation

Mature mast cells were passively sensitised overnight in culture media containing chimeric human Fc anti-4-hydroxy-3-nitrophenylacetyl (NP)-specific IgE (1 µg/ml). The mature mast cells were then washed twice in RMPI-1640 without phenol red, 0.1% BSA and re-suspended at $1x10^6$/ml in RPMI-1640 without phenol red, 0.1% BSA. 90µl of cell suspension was added to each well. Degranulation was induced by addition of 10x NP-BSA or specific chemokine. When the effects of inhibitors

were examined, these, or controls, were added 30 min prior to the addition of NP-BSA or specific chemokine. Degranulation was performed at 37$^{o}$C, 5% $CO_2$ for 150 minutes, after which β-hexosaminidase release was measured as a readout of degranulation. To measure total β-hexosaminidase release, control well were treated with 1% TRITON-X solution.

To measure β-hexosaminidase release, 90 µl of cell supernatants were transferred to 96-well flat bottom plates and mixed with 90 µl of chromogenic β-hexosaminidase substate *p*-nitrophenyl-*N*-acetyl-β-d-glucosaminide (1.3 mg/ml in 0.1 M sodium citrate buffer, pH 4.5). Incubation of supernatant and substrate took place at 37$^{o}$C, 5% $CO_2$ for 90 minutes. After incubation for 1.5 h at 37 °C, the reaction was stopped by the addition of 90 µl of 2M NaOH, and the release of the product *p*-nitrophenol was determined by monitoring the O.D. at 405 nm in an ELISA reader.

### 2.1.10 Determination of Protein Expression

Immunoblotting and flow cytometry are techniques which can be utilised to facilitate the detection of a protein of interest. As the techniques are also used in the analysis of signalling pathways and functional studies, these topics will be discussed in much greater detail in the section below with specifics to this study, however, a brief overview of these techniques will be presented here. Immunoblotting uses whole cell lysates which are then analysed for the expression of a particular protein using electrophoresis to separate the proteins according to their size. The transfer of these proteins onto a nitrocellulose membrane facilitates their detection by incubating this membrane in the presence of antibodies against the protein of interest which can be detected by the use of HRP-secondary antibody. Flow cytometry also uses whole cells which has the advantage as the antibodies can be added directly to the sample without the need for protein separation.

### 2.1.11 Immunoblotting

Immunoblotting or western blotting is a technique that facilitates the detection of a protein of interest or the phosphorylated form of that protein. Within this study, cells were stimulated with numerous chemokines triggering downstream signalling events characterised by the phosphorylation of proteins within that pathway. By lysing the cells, the mixture of proteins contained within the cell can be extracted. The whole cell lystates are then dispensed into wells within a polyacrylamide gel. Upon applying an electrical current across the gel, the proteins separate according to their molecular weight, with smaller proteins migrating the furthest. These separated proteins are then transferred onto a nitrocellulose membrane which can be 'probed' using an antibody against a protein of interest. Following incubation and binding of the primary antibody to the protein of interest, a secondary antibody is applied which is coupled to a horseradish peroxidise tag. Upon delivering a chemiluminescent agent there is a reaction which produces luminescence that is in relation to the amount of protein detected. This luminescence is detected using a sensitive photographic film, allowing visualisation of the protein of interest.

Immunoblotting was utilised in this study to allow the visualisation of the phosphorylation of Akt, ERK1/2 and S6.This phosphorylation was used as a readout of chemokine stimulation (CXCL4, CXCL9, CXCL10, CXCL11, CXCL12, CXCL16 and CCL2) in mature mast cells and THP-1 cell line. This method was also used to visualise the variants of CXCR3 and CXCR7. Furthermore, to determine the involvement of different proteins involved in mast cell signalling needed for a functional response, we utilised this method to indentify key components of this signalling pathway through inhibition of specific molecules.

### 2.1.12 Stimulation of Cells and Collection of Whole Cell Extracts

1 x $10^6$ of primary mast cells or THP-1 cells per point were washed twice in RPMI 1640 and incubated at 37$^o$C in serum free RMPI 1640 for 60 minutes. Cells were or were not stimulated with specific chemokine diluted in RPMI 0.1% BSA. Stimulations were terminated by aspiration of the supernatant followed by the addition of ice cold lysis buffer (50mM Tris-HCL ph 7.5, 150mM sodium choride, 1% Nonidet P40, 10% Glycerol, 5mM EDTA, 1mM sodium vanadate, 1mM sodium

molybdate, 10mM sodium fluoride, 40µg/ml PMSF, 0.7µg/ml Pepstatin A, 10µg/ml aprotinin, 10µg/ml leupeptin, 10µg/ml soyabean trypsin inhibitor). Lysates were rotated at 4$^{o}$C for fifteen minutes, followed by centrifugation at 17,500g. The protein rich supernatant were diluted in 5x loading buffer (10% SDS, 50% glycerol, 200mM Tris HCL pH 6.8, Bromophenol blue) heated to 95$^{o}$C for 5 mins and stored at -20$^{o}$C.

### 2.1.13 Electrophoretic Separation, Transfer and Immunoblotting of Cellular Proteins

Solubilised proteins were electrophoresed on a one dimensional 10% sodium dodecyl sulphate-polyacrlamide gel (SDS-PAGE). This was carried out using the Bio-Rad Mini Protean II system. Samples were loaded into a stacking gel and run at 80V in running buffer containing 25mM Tris base, 192 mM Glycine and 0.1%(w/v) SDS. Upon reaching the resolving gel, samples were electrophoresed at 180V. The proteins were transferred by electoblotting for 60 minutes at 40mV onto nitrocellulose membrane 0.45µM soaked in semi-dry transfer buffer (70% H2O, 20% methanol and 10% blot buffer (39mM Glycine, 48M Tris base and 0.0375% SDS)). Membranes were incubated for 60 minutes at room temperature in block buffer (TBS-tween containing 5% milk), with slight agitation and rinsed three times for five minutes in TBS-tween. The membrane was incubated in the specified 1$^{o}$ antibody dilution 1:1000 in TBS-tween supplemented with 0.01% sodium azide and 5% milk overnight with slight agitation. The membrane was washed three times for five mintues in TBS-Tween and incubated in the 2$^{o}$ antibody couple to horse radish peroxidise (HRP) diluted 1:10,000 in block buffer for 60minutes at room temperature and washed in TBS-Tween three times for five minutes. Visualisation of the protein was performed by incubating the membrane in 5ml of Enhanced Chemiliminescent reagent (ECL), for one minute and exposing to Fuji medical x-ray film.

### 2.1.14 Membrane Stripping and Reprobing

In order to verify whether the samples were equally loaded the membranes were stripped of antibody and reprobed with a pan antibody that detects absolute levels of protein. Firstly the membrane was rehydrated in TBS for 10 minutes and then placed in 100mls of stripping buffer to remove bound antibody, (100mM 2-Mecaptoethanol, 2% SDS, 62.5mM Tris-HCL pH 6.7) and incubated at 60$^{o}$C for 30 minutes. After extensive washing with TBS-Tween, the membrane was incubated for 60 minutes at room temperature in block buffer, washed three times in TBS-Tween. Blots were re-probed with a different primary antibody and the immunoblotting procedure is carried out as described above.

### 2.1.15 Flow Cytometry

Flow cytometric analysis is a highly selective technique used to count, examine and sort single cells with use of a laser. Within this study this technique was utilised to determine the expression of cell surface receptors and also used to investigate proteins levels at an intracellular level.

The sample being analysed is directly into a stream of fluid that passes through a number of detectors, which can analyse the properties of the particles that pass through it, such as size and granularity. With the use of fluorescently labelled antibodies surface receptors or intracellular proteins can be detected and the expression levels compared to other samples. For example, incubation with a specific anti-human CXCR3 can be compared to its specific isotype control to determine the proteins levels of CXCR3 on the cell.

### 2.1.16 Determining Expression of Cell Surface Receptors

Mast cell models and THP-1 cell line were washed twice in PBS 5% BSA and re-suspended in 100μl. 5μl of PE-conjugated anti-human antibodies against the receptor of interest or the respective immunoglobulin isotype control was added and incubated on ice and darkness for 30mins. Cells were washed twice in PBS 5% BSA and re-suspended in 300μl for analysis.

### 2.1.17 Determination of Expression Levels of Intracellular Proteins

1 x $10^6$ mast cell models or THP-1 cell line were incubated at 37°C in serum free RPMI for 30 minutes. Cells were centrifuged at 17,500g, and fixed by aspiration of the supernatant and addition of 2% formaldehyde in PBS for 10 minutes at room temperature followed by 1 min on ice. Cells were washed in PBS and permeabilised by the addition of 90% methanol at –20oC, which slowly vortexing followed by incubation at 4°C for 30minutes. Cells were washed, re-suspended in 100μl of PBS 0.1% BSA and blocked for 10 minutes at room temperature. 5μl of specific PE-conjugated antibody or appropriate PE-conjugated isotype control added (1 in 20 dilution) and incubated at on ice and in the dark for 30minutes. Cells were washed twice and re-suspended in 400μl PBS, for flow cytometric analysis using a Becton Dickinson FACS Canto flow cytometer and analysed using FACSDiva software.

### 2.1.18 RNA Extraction with Trizol

10x$10^6$ were homogenised with 1ml of Trizol tm in a sterile RNAase free microfuge tube by pipetting vigorously up and down. RNA was then extracted according to the Trizol manufacturer's instructions. In brief, samples were incubated at room temperature for 5 mins, spun at 17,500g for 10 minutes at 4°C and the top layer collected and placed in a fresh tube. 0.2 chloroform was added to samples which where then shaken vigorously for 15 seconds and incubated at room temperature for 2 minutes. Samples were then centrifuged in a chilled microfuge for 15 minutes at 17,500g before the upper layer carefully removed and placed in fresh tube. 0.5ml of propan-2-ol was added to each sample which were then incubated at room temperature for 10 minutes to allow nucleic acid precipitation. Samples were then centrifuged at 9000g, for 10 minutes at 4°C. RNA pellets were washed in 75% EtOH, air dried and re-suspended in the RNA storage solutions. The optical absorbance at 260nM and 280nM was measured using a spectrophotometer and used to determine the RNA concentration. Purity was estimated from the OD260/OD280.

RNA samples were DNAase treated using the DNA-freeTM kit according to the manufacturer's instructions (Invitrogen). In brief, 5μg of each RNA sample was

diluted in 1xDNAaseI buffer containing 1μl of DNAaseI with the total volume made up to 50μl with diethylpyroCarbonate (DEPC) treated water. Sample were mixed and incubated at 37oC for 30 minutes. 5μl of DNAase inactivation reagent was then added and samples mixed thoroughly during incubation at room temperature for 2 minutes. Samples were centrifuged at 6000g for 1.5 minutes and the supernatant containing DNAase treated RNA, placed in a fresh tube.

### 2.1.19 cDNA Synthesis from RNA

cDNA was reversed transcribed from DNAase-treated RNA using the superscript TM first strand synthesis system for RT-PCR according to manufacturer's instructions (Qiagen). Briefly, 5μg of each RNA sample was mixed with 10μg/ml Oligo(dT)$_{12\text{-}18}$, 200nM dNTP mix and the volume made up to 10μl with DEPC treated water. Samples were incubated at 65$^{o}$C for 5 minutes then placed on ice for ~1 minute. To each sample, 9μl of a mixture containing the following components (final concentrations) was added: 1xRT-buffer, 5mM $MgCl_2$, 10nM DTT, and 1μl of RNaseOUT TM recombinant Rnase inhibitor. Samples were incubated at 42$^{o}$C for 2 minutes and then 1μl (50 units) of superscript TM II reverse transcriptase was added to each sample (except –RT controls). Reactions were incubated at 42$^{o}$C for 50 minutes before being terminated by incubation at 70$^{o}$C for 15 minutes. Samples were collected by centrifugation and 1μl of RNAaseH added to each one. These were incubated at 37$^{o}$C for 20 minutes and then stored at –20$^{o}$C until used for PCR amplification.

### 2.1.20 Polymerase Chain Reaction (PCR)

PCR for all applications was performed using a NewEngland Biolabs master mix. . Reaction mix for PCR was as follows: 2x NewEngland Biolabs master mix (12.5 μL), 10μM 5' primer (2 μL), 10μM 3' primer (2 μL), approximately 1 μg cDNA and distilled water (up to 25 μL volume). All reactions were mixed well by pipetting before being placed in a thermocycler.

Appropriate thermocycler programs were used according to the application: Amplification of cDNA: Initial denaturation: 94°C 5 mins, (denaturation: 94°C 1

min, annealing: 60°C 30 sec, extension: 72°C 1 min) x 35, final extension: 72°C 10 min, final hold: 4°C; PCR products were then analysed by agarose gel electrophoresis as described below.

### 2.1.21 Agarose Gel Electrophoresis

1.2% of agarose was prepared in 100 ml of 1xTAE buffer and melted by heating in a microwave for 2 minutes, followed by gentle mixing until completely dissolved. 2 μL of ethidium bromide (10 mg/mL) was added to 100 mL of cooled agarose solution, mixed and slowly poured in an appropriate tank. Required combs were placed into the gel and any air bubbles were removed. Gel was left to set for 30-60 minutes. 1x TAE buffer (running buffer) was added to an appropriate gel running tank. The gel was then placed in the tank and the comb removed. PCR products were mixed with 6 x gel loading buffer and loaded on to the gel alongside with DNA ladder. Electrophoresis was preformed at 90V in the TAE running buffer until bromophenol blue (from the loading buffer) has run 3/4 the length of the gel. After that point gel (preferably within its holder) was carefully transferred to visualise and photograph PCR products on the UV transluminator with photo camera build.

### 2.1.22 In Vitro Cell Migration Assay

The ChemoTx System is a disposable 96-well format migration assay. The system is set up with the appropriate agonists in the lower wells, over which a 5μm pore size filter is placed, through which the cell migrate. This filter is designed to mirror the 96 well plate structure beneath. Surrounding each well on the filter is a hydrophobic material that focuses the cell suspension directly over the ligand below, eliminating the need for the upper chambers. Following the incubation time, the migrated cells were collect and counted.

### 2.1.23 Chemotaxis

Chemotaxis assays were performed in 96-well chemotaxis chambers with polycarbonate membranes (5μm pore size). The lower chambers were filled with 29μl of chemokine diluted in RPMI-1640, 0.1% BSA and carefully overlaid with the polycarbonate membrane. Cells were washed twice in RPMI-1640 and re-

suspended in RPMI-1640, 0.1% BSA at a concentration of $3.2x10^6$ cells/ml. 25µl of the cell suspension was loaded on top of the filter.

Migration was performed at $37^oC$, 5% $CO_2$ for 180 minutes, after which non-migrated cell on the top of the filter were rinsed off with PBS. Following centrifugation (350g, 10 minutes) the filter was removed and the migrated cells were re-suspended in 300µl PBS, 0.1% BSA. Analysis of migration was performed using FACS Canto (BD Biosciences, UK). Samples were performed in triplicate with each being analysed for 60 seconds. Data is expressed as chemotactic index.

### 2.1.24 Chemotactic Index

The chemotatic index was calculated as follows: Number of cells migrated/basal levels of migration.

### 2.1.25 Actin Polymerisation

1 x $10^6$ of primary mast cells or THP-1 cells per point were washed twice in RPMI 1640 and incubated at $37^oC$ in serum free RMPI 1640 for 60 minutes. When the effects of inhibitors were examined, these, or controls, were added 30 min prior to stimulation with chemokine. Cells were or were not stimulated with specific chemokine diluted in RPMI 0.1% BSA at a specific time period. After stimulation cells were fixed in 4% paraformaldehyde (w/v) for 10 minutes at room temperature before centrifuged at 10,000 rpm for 10 seconds before resuspended in 0.2% Triton X-100 (v/v) at room temperature for 5 min to permeabilise the samples. After washing three times with PBS, the samples were blocked in 1% FBS in PBS for 30 min at 37°C and then incubated with TRITC-conjugated phallodin (0.1 µg/ml for 1 h at room temperature. Cells were washed twice in PBS 5% FBS and re-suspended in 300µl for analysis. Flow cytometric analysis using a Becton Dickinson FACS Canto flow cytometer and analysed using FACSDiva software.

### 2.1.26 $Ca^{2+}$ via Flex Station

Mast cells were loaded with Fluo-4 AM and $[Ca^{2+}]i$ was measured as described previously in conjunction with a fluorometric imaging plate reader (FLIPR; Molecular Devices Ltd., Wokingham, UK) (Cronshaw et al., 2006). Mature mast cells or THP-1 cells were washed twice and resuspended at 10 x $10^6$ cells/ml in 5 ml loading buffer (RPMI without phenol Red indicator supplemented with 10% FCS, 2.5mM probenecid and 20 mM HEPES). This cell suspension was loaded with 5 mM Fluo-4 AM in the dark for 1 hour at 37°C. Cells were washed twice in assay buffer (Hanks' balanced saline solution/20 mM HEPES without phenol Red indicator) and resuspended at 7 x $10^6$/ml. Cell suspension (50 µl; 350,000 cells) was added to each well of a black-walled, clear-bottomed plate (Corning Costar UK Ltd., High Wycombe). After loading, the plate was centrifuged at 1200 revolutions per minute for 5 min at room temperature and then incubated at 37°C for 1 hour. The cell plate was then loaded into the FLIPR instrument. The cells were then excited at 488 nm using the FLIPR laser, and the fluorescence emission wavelength was at 525nm. Fluorescence readings were taken at 1-s intervals for times indicate. Agonist (50 ml) was added (dispense speed, 30 ml/s) after 1 min using the FLIPR. Raw fluorescence data were exported for each well and tabulated versus time within Microsoft Excel (Microsoft Corp).

**Figure 2.1 – FlexStation 3 Microplate used to measure automated calcium responses.** Picture taken from Bucher Biotech.

## 2.2 Materials

### 2.2.1 Antibodies and Secondary Detection Reagents

| Antibody | Species | Conjugate | Supplier | Country |
|---|---|---|---|---|
| Anti-phospho-S6 ribosomal protein | Rabbit | N/A | Cell Signalling Technologies | Hitchin, UK |
| S6 Ribosomal Protein (5G10) | Rabbit | N/A | Cell Signalling Technologies | Hitchin, UK |
| Anti-phospho-Akt | Rabbit | N/A | Cell Signalling Technologies | Hitchin, UK |
| Pan Akt | Goat | N/A | Santa Cruz | Wembley, UK |
| Anti-ERK1/2(p42/p44) | Rabbit | N/A | Cell Signalling Technologies | Hitchin, UK |
| Anti-Human Mast Cell Tryptase | Mouse | N/A | Dako | Cambridge, UK |
| mouse IgG1 | Mouse | N/A | Dako | Cambridge, UK |
| Chimeric human Fc anti-4-hydroxy-3-nitrophenylacetyl (NP)-specific IgE | | N/A | AbD Serotec | Oxford, UK |
| Anti-human CCR1 | Mouse | PE | R and D | Abingdon, UK |
| Anti-human CCR2 | Mouse | PE | R and D | Abingdon, UK |
| Anti-human CCR3 | Mouse | PE | R and D | Abingdon, UK |
| Anti-human CCR5 | Mouse | PE | R and D | Abingdon, UK |
| Anti-human CCR7 | Mouse | FITC | R and D | Abingdon, UK |
| Anti-human CXCR3 | Mouse | PE | R and D | Abingdon, UK |
| Anti-human CXCR3 | Mouse | N/A | R and D | Abingdon, UK |
| Anti-human CXCR4 | Mouse | FITC | R and D | Abingdon, UK |
| Anti-human CXCR6 | Mouse | PE | R and D | Abingdon, UK |
| Anti-human CXCR7 | Mouse | PE | R and D | Abingdon, UK |
| Anti-human CXCR7 | Mouse | N/A | R and D | Abingdon, UK |
| Mouse IgG1 isotype | Mouse | PE | R and D | Abingdon, UK |

| | | | | |
|---|---|---|---|---|
| control | | | | |
| Mouse IgG2a isotype control | Mouse | PE | R and D | Abingdon, UK |
| Mouse IgG2a isotype control | Mouse | FITC | R and D | Abingdon, UK |
| Anti-human CD133 | Mouse | PE | Miltenyi Biotec | Surrey, UK |
| Anti-human CD13 | Mouse | PE | Beckon Dickenson | Oxford, UK |
| Anti-human CD54 | Mouse | PE | Beckon Dickenson | Oxford, UK |
| Anti-human CD117 | Mouse | PE | Beckon Dickenson | Oxford, UK |
| Anti-human CD63 | Mouse | PE | Beckon Dickenson | Oxford, UK |
| Anti-human CD203c | Mouse | PE | Miltenyi Biotec | Watford, UK |

### 2.2.2 Cell Culture

| Item/Reagent | Supplier | Country |
|---|---|---|
| 10ml plastic pipettes | Greiner Bio-one | Stonehouse, UK |
| 25ml plastic pipettes | Greiner Bio-one | Stonehouse, UK |
| 50ml tubes | Greiner Bio-one | Stonehouse, UK |
| Pasteur Pipette | Fisher Scientific | Loughborough, UK |
| 200mM L-Glutamine | Invitrogen | Paisley, UK |
| 6 well tissue culture plates | Nunc(Fisher Scientific) | Loughborough, UK |
| 96 microwell plates | Nunc(Fisher Scientific) | Loughborough, UK |
| Cryotubes | Nunc(Fisher Scientific) | Loughborough, UK |
| RPMI-1640 | Gibco | Paisley, UK |
| Foetal Bovine Serum | Gibco | Paisley, UK |
| IMDM | Gibco | Paisley, UK |
| Penicillan/Streptomycin | Gibco | Paisley, UK |
| Sterile Phosphate buffered saline(PBS) | Gibco | Paisley, UK |
| Stem-Pro | Invitrogen | Paisley, UK |
| IL-3 | Novartis Institutes for Biomedical Research (NIBR) Vienna | Vienna, Austria |
| IL-6 | Novartis Institutes for Biomedical Research (NIBR) Vienna | Vienna, Austria |
| SCF | Novartis Institutes for Biomedical Research (NIBR) Vienna | Vienna, Austria |
| IL-4 | Novartis Institutes for Biomedical Research (NIBR) Vienna | Vienna, Austria |
| Cord Blood CD133+ Cells | Stem Cell Technologies | Grenoble, France |
| THP-1 | Sigma-Aldrich | Poole, UK |
| HMC-1 | Mayo Foundation for | Rochester, USA |

| | | |
|---|---|---|
| | Medical Education and Research. | |
| 175cm2 tissue culture flasks | Nunc(Fisher Scientific) | Loughborough, UK |
| 75cm2 tissue culture flasks | Nunc(Fisher Scientific) | Loughborough, UK |
| RPMI-1640 w/o Phenol Red | Gibco | Paisley, UK |
| Polysine Slides | VWR International | West Sussex, UK |
| SuperPap Pen | Zymed Laboratories (Invitrogen) | Paisley, UK |
| May-Grunwald's eosine-methylene blue solution modified | Merck (VWR International) | West Sussex, UK |
| Giemsa's azur eosin methylene blue solution | Merck(VWR International) | West Sussex, UK |
| 96-well chemotaxis chambers | Neuro Probe (Receptor Technologies) | Leamington Spa, UK |

### 2.2.3 Chemokines

| Chemokine | Supplier | Country |
|---|---|---|
| Human recombinant CXCL4 | Peprotech | London, UK |
| Human recombinant CXCL9 | Peprotech | London, UK |
| Human recombinant CXCL10 | Peprotech | London, UK |
| Human recombinant CXCL11 | Peprotech | London, UK |
| Human recombinant CXCL12 | Peprotech | London, UK |
| Human recombinant CXCL16 | Peprotech | London, UK |
| Human recombinant CCL2 | Peprotech | London, UK |
| Human recombinant CCL5 | Peprotech | London, UK |
| Human recombinant CCL11 | Peprotech | London, UK |
| Human recombinant CCL22 | Peprotech | London, UK |

### 2.2.4 Chemicals

| Chemical | Supplier | Country |
|---|---|---|
| Bromophenol Blue | BDH-International | Leicestershire, UK |
| Bovine Serum Albumin | Sigma-Aldrich | Poole, UK |
| Dimethyl sulfoxide | Sigma-Aldrich | Poole, UK |
| EGTA | Sigma-Aldrich | Poole, UK |
| Ethanol | Fisher Scientific | Loughborough, UK |
| Methanol | Fisher Scientific | Loughborough, UK |
| Glycerol | Sigma-Aldrich | Poole, UK |
| Tris-HCL | Sigma-Aldrich | Poole, UK |
| Trizol Reagent | Invitrogen | Paisley, UK |
| Tween-20 | Sigma-Aldrich | Poole, UK |
| Triton-X | Sigma-Aldrich | Poole, UK |
| Phallodin | Sigma-Aldrich | Poole, UK |
| Sodium Azide | Sigma-Aldrich | Poole, UK |
| Paraformaldehyde | Sigma-Aldrich | Poole, UK |
| 4-Nitrophenyl n-acetly-B-D-Glucosaminide | Sigma-Aldrich | Poole, UK |
| NP-BSA | Biosearch Technologies | Novato, CA, USA |
| Fluo-4-AM | Invitrogen | Paisley, UK |
| Sodium Hydroxide | Sigma-Aldrich | Poole, UK |
| Sodium dodecyl sulphate | Sigma-Aldrich | Poole, UK |
| Precision protein all blue standards | Biorad | Hemel Hempsted, UK |
| ECL Advance western blotting detection reagent | GE Healthcare | Slough, UK |
| X-Ray Film | Fuji | Bedfordshire, UK |
| Pertussis toxin | Calbiochem | Leicestershire, UK |
| ZSTK-474 | Axxora Platform | Nottingham, UK |
| T487 | UCB | Cambridge, UK |
| NBI-74330 | UCB | Cambridge, UK |

| | | |
|---|---|---|
| N-oxide metabolite of NBI-74330 | UCB | Cambridge, UK |
| AS605240 | Echelon Bioscience (Tebu-bio) | Le Perray en Yvelines Cedex, France |
| IC87114 | Symansis (Caltagmedsystems) | Buckingham, UK |
| PIK75 | Axon Medchem | Groningen, The Netherlands |
| TGX-221 | Cayman Chemical | Michigan, USA |
| TGX-121 | Novartis Institutes for Biomedical Research (NIBR) Vienna | Vienna, Austria |
| U73122 | Calbiochem | Leicestershire, UK |
| U73343 | Calbiochem | Leicestershire, UK |
| Y27632 | Sigma-Aldrich | Poole, UK |
| 2-APB | Cayman Chemical | Michigan, USA |

### 2.2.5 PCR Sequences

| **Oligo** | **Sequence 5' to 3'** |
|---|---|
| CXCR3A/Alt | CCAAGTGCTAAATGACGCCG |
| CXCR3B | GACAGTTATAGGAGGAGCTGCTC |
| CXCR7 | GCCGTCATTTGATTGCCCGC |
| B-Actin | ATGGATGATGATATCGCCGCG |
| CXCR3A/Alt | GCAAGAGCAGCATCCACATC |
| CXCR3B | CAGTGTCAGCACCAGCAGC |
| CXCR7 | CGAAGAGGTTGATGGAGAAG |
| B-Actin | CTAGAAGCATTTGCGGTGGAC |

# 3 Characterisation of Mast Cell Models

## 3.1 HMC-1 as a Model for Investigation of CXCR3 Signalling and Function on Mast Cells

### 3.1.1 Introduction to Study

HMC-1 is a unique human mast cell line which can serve as a resource for mast cell proteins including granule proteins and mast cell genetic material (DNA and RNA). The HMC-1 line was derived from a patient with mast cell leukaemia and it is believed it is comprised of immature mast cells. These cells may respond in different ways compared to mature mast cells. HMC-1 cells lack a functional IgE receptor and have two point mutations in the intracellular juxtamemberane domain (Val560Gly) and in the catalytic domain (Asp816Val) of the c-Kit receptor (Furitsu *et al.*, 1993).

### 3.1.2 CXCR3 Expression and Signalling on HMC-1 Cell Line

To investigate if the HMC-1 cell line was an appropriate model to study responses induced by CXCR3 agonists, mRNA and cell surface expression of CXCR3 and its variants were examined. Figure 3.1a reveals that only 2 variants of CXCR3 are present within the HMC-1 cells. CXCR3A and CXCR3B mRNA were detectable but there was no expression of CXCR3-alt (Figure 3.1a). At least one of these variants is translated to a protein level. Use of a non-isoform discriminating antibody revealed expression of CXCR3 at a surface level (Figure 3.1b)

Biochemical signals robustly activated by chemokine agonists include phosphorylation and activation of Akt (a downstream readout for PI3K activation) and the ribosomal protein S6. HMC-1 cells are a poor model for investigating phosphorylation events due to very high basal levels of phosphorylation (Figure 3.1c). Using CXCL9 as a representative agonist of CXCR3 basal levels of phosphorylation, it was not possible to determine elevation of phosphorylation of these proteins above basal levels by CXCL9 (Figure 3.1c).

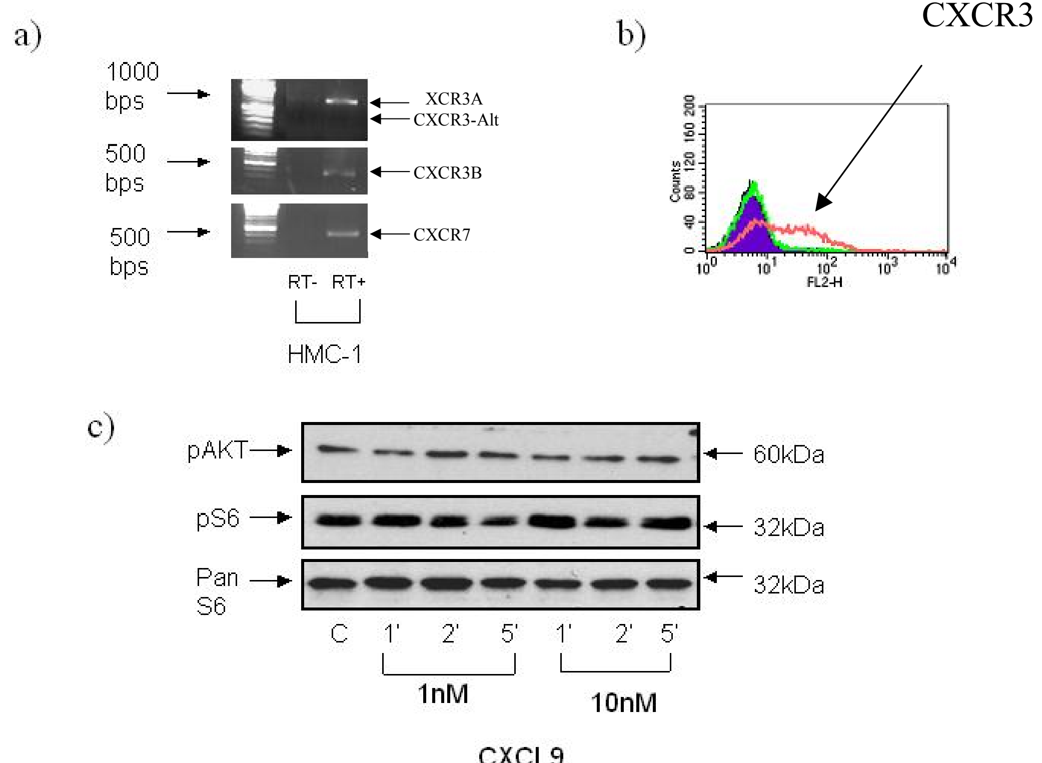



**Figure 3.1: Expression and signalling of CXCR3 on HMC-1**. a) mRNA expression of CXCR3 variants in HMC-1 cells. mRNA (5µg) from 5 x $10^6$ HMC-1 was extracted and reverse transcribed in the presence (+) or absence (–) of reverse transcriptase to ensure that samples were not contaminated with genomic DNA. mRNA levels of CXCR3 variants and CXCR7 were examined using specific primers as described in materials and methods. Arrows point to bands of mRNA expression for each CXCR3 variant or CXCR7 respectively. b) Cell surface expression of CXCR3 (orange) on HMC-1 cells. Briefly 1x$10^6$ cells were washed in PBS and incubated with the respective PE conjugated antibody at 4$^o$C, cells were washed in PBS and analysed on a FACS Calibre using Cell Quest software. Unstained (purple) and non-specific isotype stained (green) populations were used as negative controls. c) CXCL9 signalling in HMC-1 cells. Aliquots of HMC-1 (1×$10^6$ cells/ml) were left untreated or stimulated at 37 °C with CXCL9 for 1-5 min and lysed by the addition of 1× sample buffer. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with a phospho-specific Akt or S6 Ab and proteins were visualised with ECL. The blots were stripped and reprobed with anti-S6 Ab to verify equal loading and efficiency of protein transfer (lower panel). The data are representative of three other experiments.

### 3.1.3 CXCR3 Agonists Fail to Induce Migration of HMC-1 Cells

To further evaluate if HMC-1 could act as an appropriate mast cell model I examined their function by investigating if CXCR3 agonists could induce migration of HMC-1 cells. Figure 3.2 demonstrates that all four CXCR3 agonists failed to induce migration of HMC-1 cells. It was possible to induce migration of HMC-1 cells to the mast cell growth factor SCF. The migration observed was induced at 10nM, peaked at 100nM and started to decrease at 300nM (Figure 3.2a). The THP-1 cell line was utilised through out this thesis to act as a comparison of CXCR3 responses during evaluation of the mast cell model. In THP-1 cells, all CXCR3 agonists were capable of inducing chemotactic responses (Figure 3.2b). It was notable that CCL2 was a more potent chemotactic agent than the CXCR3 agonists in inducing migration of THP-1 cells. Figure 3.2b reveals that the peak response was 7.8 on the chemotactic index, clearly demonstrating a stronger response compared to the greatest response induced by CXCR3 agonists.

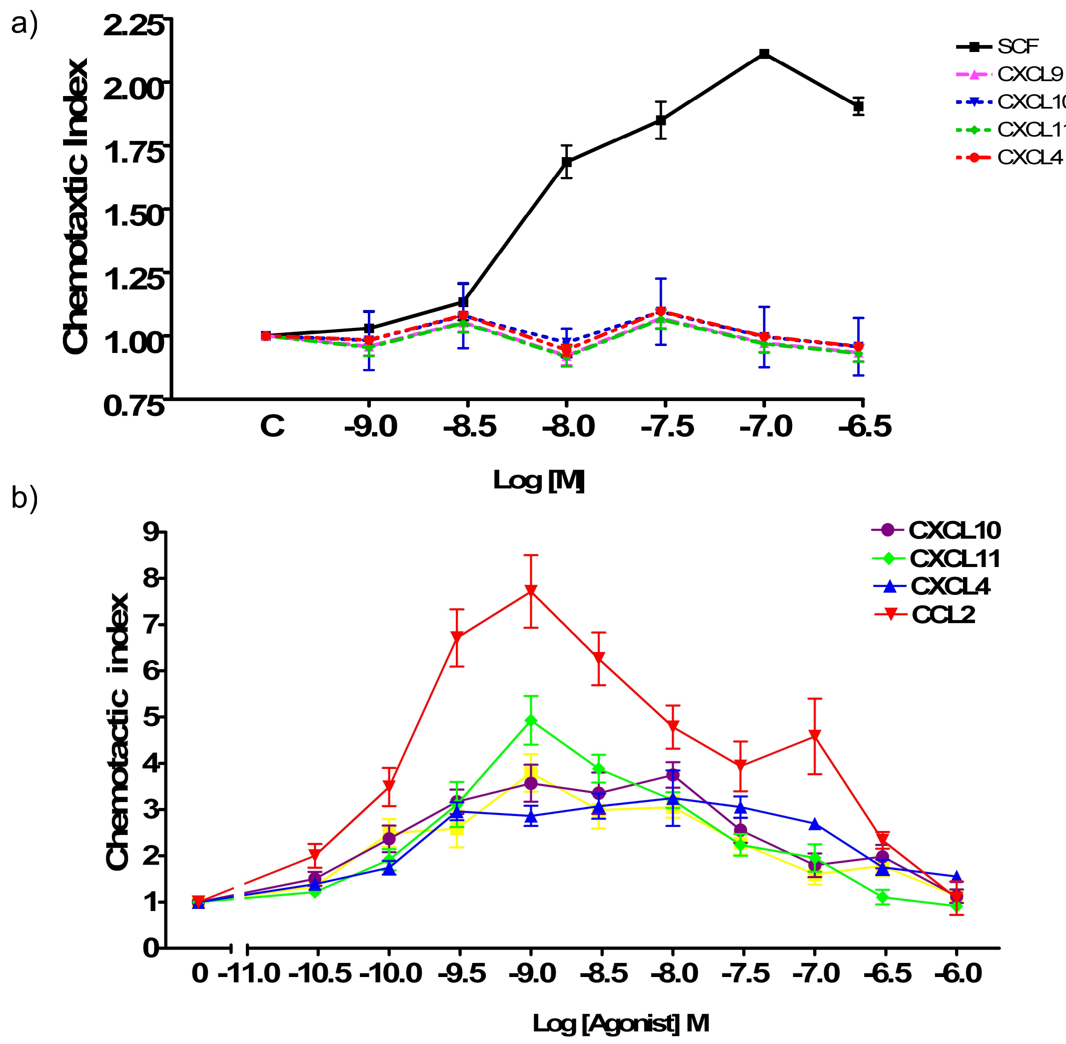



**Figure 3.2: Migration of HMC-1 or THP-1 to different stimuli**. a) HMC-1 cells or b) THP-1 cells ($8x10^4$/25µl) were placed on the upper membrane of a 96-well chemotaxis plate above lower chambers containing varying concentrations of CXCR3 agonists or SCF. Chemotaxis across a 5µm pore size membrane was determined after 3 hour incubation at $37^oC$ in 5% $CO_2$ as described in material and methods. Data shown is derived from a single experiment performed in triplicate; data is representative of three experiments.

## 3.2 Generation of Primary Mast Cell Model

### 3.2.1 Generation and Characterisation of Human Cord Blood Derived Mast Cells

CD133+ progenitor cells were differentiated into human mature mast cells by growing CD133+ progenitor cells in serum-free Stempro medium in the presence of SCF and IL-6 for 8 weeks. In addition IL-3 was added for the first 2 weeks to induce proliferation and IL-4 and serum were added to this cocktail after the initial 8-week period had passed. This method of initial serum-free conditions, followed by a subsequent serum supplementation has previously been reported to support the formation of functional human mast cells (Dahl et al., 2002). Figure 3.3 demonstrates the expression of surface markers that indicate the differentiation of CD133+ cells into mature mast cells. Precursors (0 – 2 wks) stained positive for the myeloid antigen CD13, the mast cell growth receptor CD117, CD133 (the progenitor marker used to isolate the cells) and IL-3R a growth marker for myeloid cells. Precursor cells however did not stain positive for the FcεRIa receptor commonly associated with mature mast cells. During differentiation and maturation a loss of CD133 and IL-3R expression is observed and accompanied by an upregulation of the FcεRIa. Mature mast cells (>9 wks) are observed to have total loss of CD133 and IL-3R expression, whereas the FcεRIa is highly expressed, which is a key characteristic of mature mast cells.

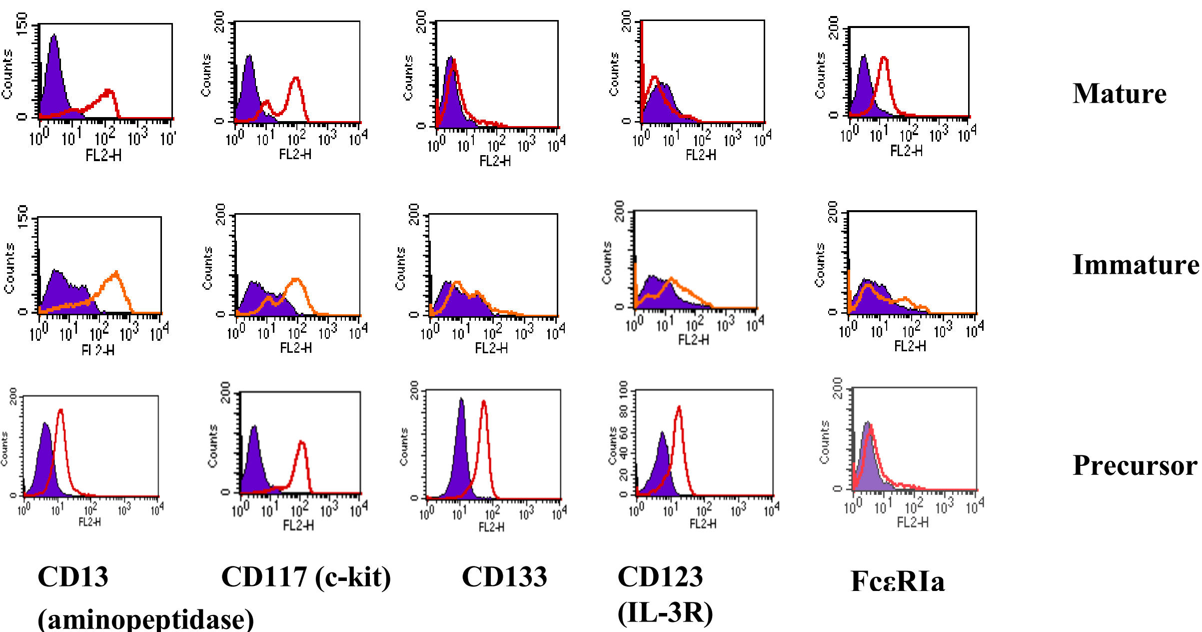



**Figure 3.3: Expression markers on CD133+ progenitors through to mature mast cells.** Representative surface expression profiles of CD13, CD117, CD133 CD123 and FcεRIa on precursors (0-2 weeks), immature (~2-9 weeks) and mature (9 weeks +) cells. The respective immunoglobulin isotype controls are shown in blue. Briefly $1x10^6$ cells were washed in PBS and incubated with the respective PE conjugated antibody at $4^oC$, cells were washed in PBS and analysed on a FACS Calibre using Cell Quest software.

Further to the expression of these cells surface markers the mature cells were observed to display typical phenotypic features of mature mast cells such as metachromasia and the abundant expression of granular mast cell tryptase (Figure 3.4). Tryptase was detected in both immature and mature cells. However the mild staining observed in the precursor stage was thought to be due to non-specific binding of the substrate system. Staining was visually more intense in the mature cells compared to immature cells, with 90 % of the mature population staining positive for human mast cell tryptase (Figure 3.4).

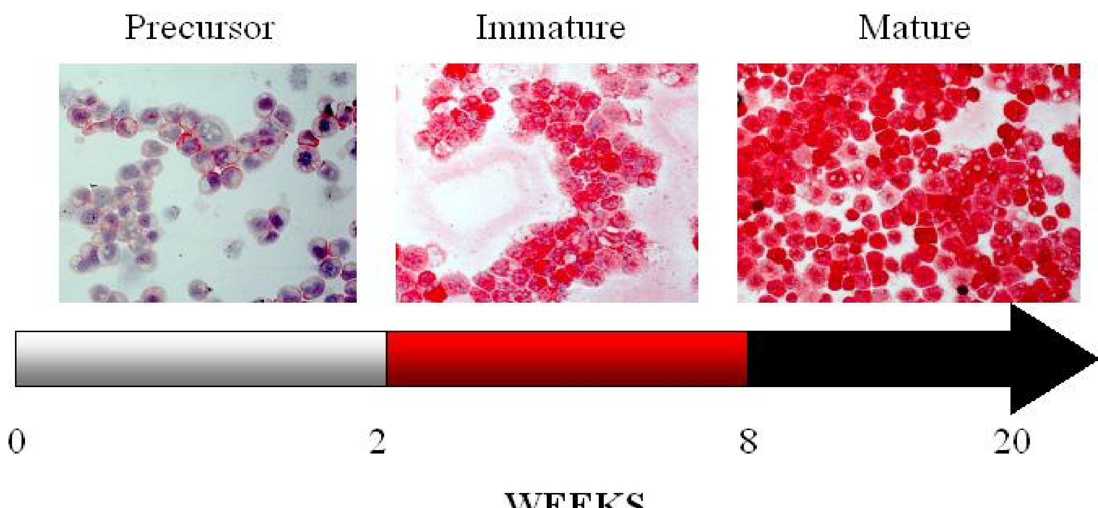



**Figure 3.4: Characteristic tryptase staining of CD133+ precursors.** Representative images demonstrating characteristic tryptase staining throughout development of CD133+ progenitor cells into mature human mast cells. Briefly 1 x $10^5$ of primary mast cells were washed twice in RPMI and centrifuged onto poly-L-lysine-coated glass slides in a cytospin3 cytocentrifuge at 250g. The resulting smears were air-dried and fixed by submersion in an acetone-methanol solution for 1 min. The smears were then carefully washed in TBS buffer (pH 7.6) and 100µl of anti-human mast cell tryptase mAb was added to the appropriate smear and incubated at $4^oC$ overnight. To visualise the presence of tryptase within human mast cells, prepared smears were stained using the alkaline phosphatase/anti-alkaline phosphatase (APAAP) method according to the manufacturer's instructions. Magnification is at x20

A further defining characteristic of a mature mast cell is the ability to release a vast range of pre-formed and newly formed mediators. Using a light microscopy and a May-Grunwald/Giemsa stain (Figure 3.5). I was able to demonstrate that CD133+ cells have a large nucleus but little sign of any preformed granules. Immature cells have faint granule staining which is in contrast to the heave staining of the mature mast cells stain, displaying the presence of a wide range of mediators stored within the cytoplasm of these mast cells.

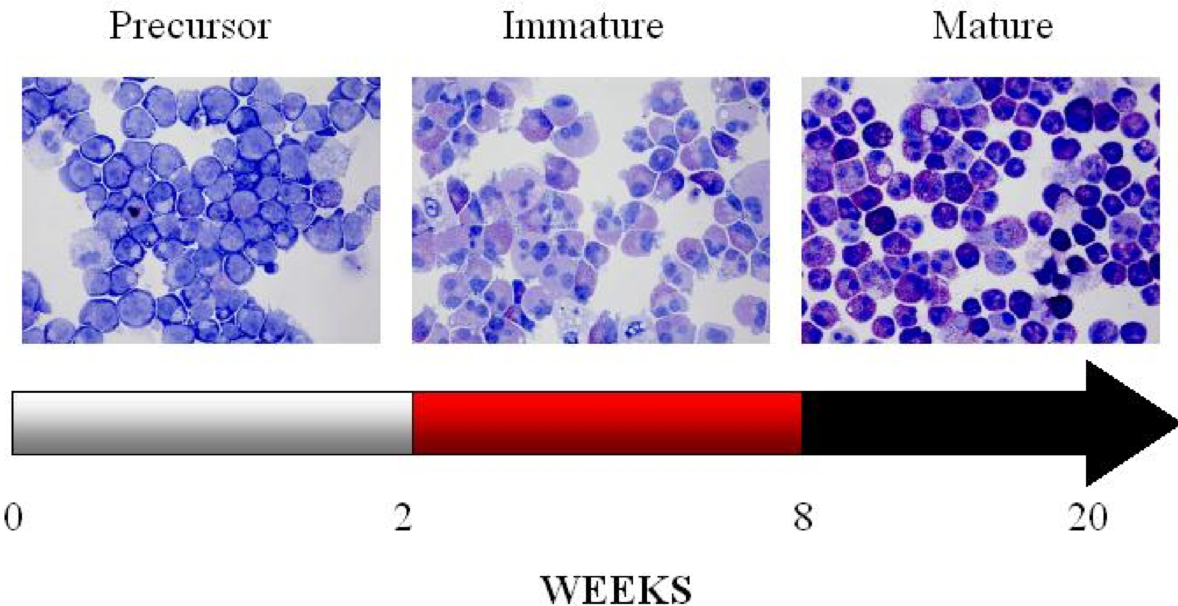



**Figure 3.5: Characteristic May-Grunwald/Giemsa staining of CD133+ precursors.** Representative images demonstrating, characteristic May Grunwald/Giemsa staining throughout the development of CD133+ progenitor cells into mature human mast cells. Briefly 1 x $10^5$ of primary mast cells were washed twice and centrifuged onto poly-L-lysine-coated glass slides at 250g. The resulting smears were then air-dried and fixed by submersion in an acetone: methanol solution (1:1) for 1 minute. Smears were then air-dried before submersion in May-Grunwald's eosine-methylene blue solution modified for 1 minute. Smears were rinsed with $dH_2O$ and air-dried. Smears were then submersed in Giemsa's azur eosin methylene blue solution for 1 minute. After the incubation period, the excess stain was washed off using $dH_2O$ and again left to air dry. Magnification is 40x.

Finally, in the characterisation of mast cells, ultra-thin slices were taken at different stages of differentiation. These slices were analysed by TEM and revealed that precursors have a large nucleus and little else present in their cytoplasm (Figure 3.6). The immature mast cells were observed to have clear compartments where the granules of the mast cells are stored. Whereas the mature mast cells have developed larger compartments, which are filled with dark stained matter indicative of the presence of granules.

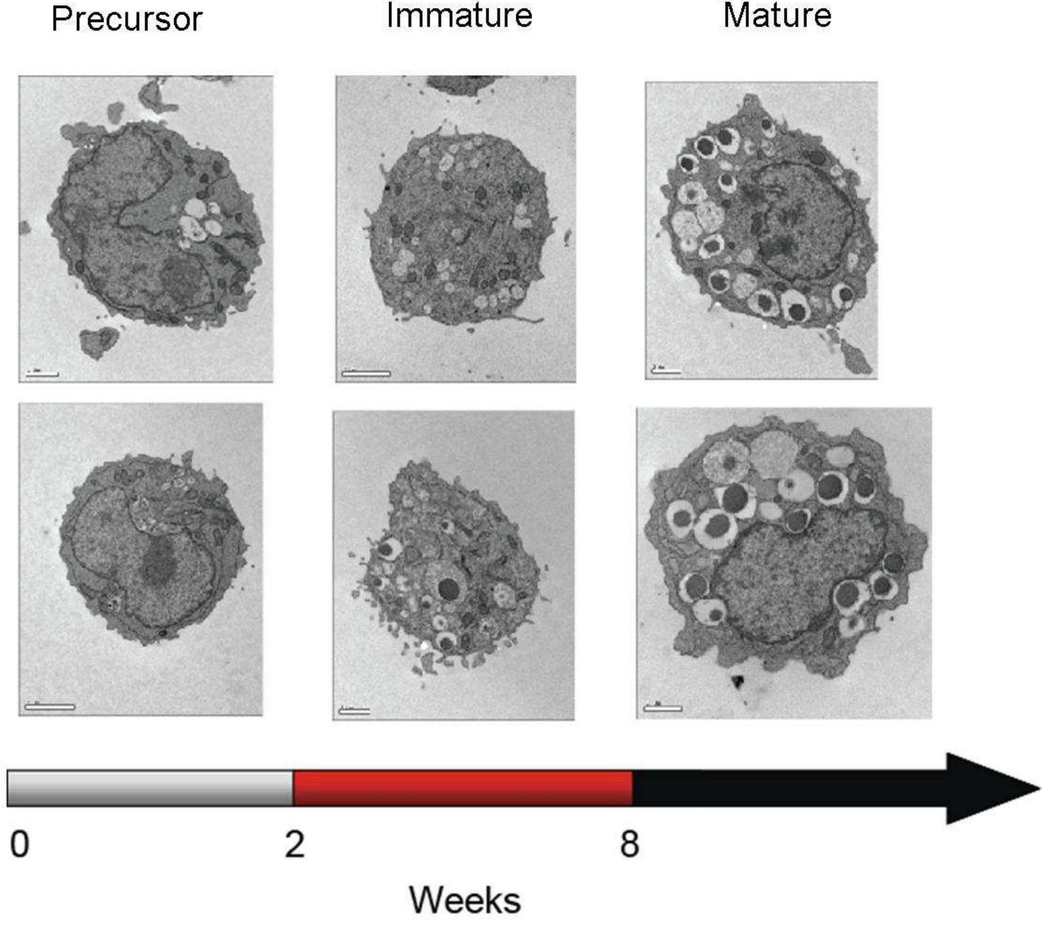



**Figure 3.6: TEM pictures of mast cells at different stages of differentiation and maturation.** Examples of mast cells from different stages of development were prepared for imaging under TEM as described in *Materials and Methods.* Each stage has two different mast cells pictured under TEM. Measurement bar is 1μm.

## 3.3 Characterisation of Primary Mast Cell Model

### 3.3.1 Introduction to Study

Prior to investigating CXCR3 expression and function of this primary mast cell model it was important to validate this human cord-blood derived primary mast cell model. The cross-linking of the IgE-loaded high-affinity IgE receptor by multivalent antigens has been demonstrated to result in mast cell activation and subsequent release of multiple pro-inflammatory mediators. Other membrane activation events can either prime mast cells for subsequent degranulation or can act in synergy with FcεRI signal transduction.

An allergen is able to bind to antigen-binding sites, situated on the variable regions of the IgE molecules on the mast cell surface allowing the clustering of the intracellular domains of the cell-bound Fc receptors, which are associated with the cross-linked IgE molecules. This induces a complex sequence of reactions inside the mast cell that lead to its activation. The dose-response curve for FcεRI-mediated degranulation is bell-shaped regardless of whether the IgE or the Ag concentration is varied. Moreover, certain early signalling events continue to increase whereas degranulation drops under sub-optimal conditions (Gimborn *et al.*, 2005).

### 3.3.2 Antigen Induces Degranulation in Dose Dependent Manner

Treatment of mature mast cells with NP-BSA (0.1 – 1000 ng $ml^{-1}$, 2 hrs, 37°C?) resulted in a dose-dependent increase in beta-hexaminidase release (Figure 3.7) This was observed to reach maximal release using 100 ng $ml^{-1}$ NP-BSA with concentrations greater than this resulting in lesser beta-hexaminidase release. The curve generated from this dose response treatment started to exhibit bell-shaped characteristics similar to results previously discovered in other studies.

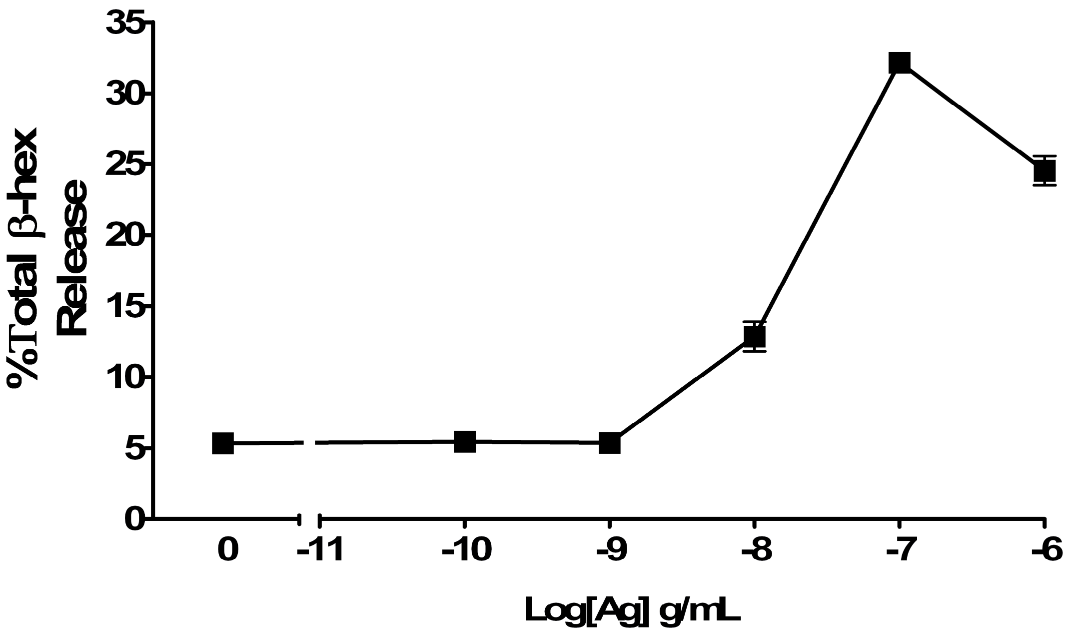



**Figure 3.7: IgE/Ag dose response.** Mature mast cells ($1x10^6$/ml) were treated overnight with chimeric human Fc anti-4-hydroxy-3-nitrophenylacetyl (NP)-specific IgE (1 µg/ml). Cells were stimulated with NP-BSA for 2 hours. Samples were centrifuged for 10mins and supernatants transferred to new wells before addition of β-hexosaminidase substrate for 1.5 hours to measure β-hexosaminidase release. Data are expressed as % the total β-hexosaminidase release. Results are expressed as mean ± SEM of 4 experiments performed in triplicate.

### 3.3.3 Investigating the Mechanism of IgE Cross-Linking Induced Degranulation.

PI3K has recently been described as a key-signalling molecule in the degranulation mechanism in mast cells (Wymann et al., 2003). Therefore it was important in this study to evaluate the role of the PI3K family in mast cell degranulation induced by cross-linking of FcεRI.

FcεRI initiate their signalling processes via activation of tyrosine kinases, either intrinsically or by recruitment of cytosolic kinases (Gilfillan and Tkaczyk, 2006; Roskoski, 2005). They have been demonstrated to utilise the class 1A PI3Ks to mediate subsequent downstream signalling events. GPCRs, such as those for adenosine, prostaglandin (PG)E2, sphingosine 1 phosphate (S1P) and complement component C3a, mediate their responses via class 1B PI3K. (Wymann et al., 2003). Utilising the general isoform non-selective inhibitor of the PI3K family, ZSTK474, revealed that at least one isoform of PI3K is involved in the degranulation of mast cells by cross linking of IgE by treatment with 100 ng/ml of antigen (Figure 3.8). Pre-treatment with concentrations < 1 μM demonstrated little inhibition of degranulation whilst concentrations $\geq$ 1 μM, demonstrated clear inhibition of degranulation induced by treatment with antigen. 100% inhibition was only achieved at 10 μM with an $IC_{50}$ of 1.15 μM. This is above the $IC_{50}$ of the compound to be specific for PI3K indicating the involvement of other signalling molecules in the mechanism of degranulation. ZSTK474 $IC_{50}$ for PI3K is between 10 and 50 nM.

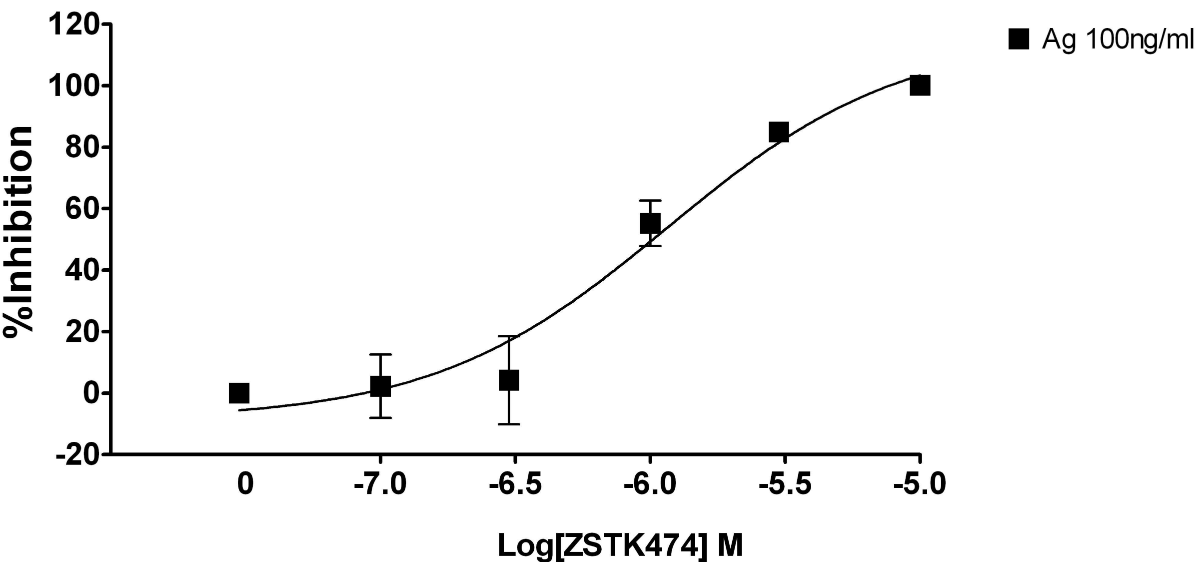



**Figure 3.8 – Cross-linking of IgE/Ag induced degranulation is PI3K dependent.** Mature mast cells ($1x10^6$/ml) were treated overnight with chimeric human Fc anti-4-hydroxy-3-nitrophenylacetyl (NP)-specific IgE (1µg/ml). Cells were treated with ZSTK474 at concentrations indicated for 30mins before stimulation with 100 ng/ml NP-BSA for 2 hours. Samples were centrifuged for 10mins and supernatants transferred to new wells before addition of β-hexosaminidase substrate for 1.5 hours to measure β-hexosaminidase release. Data are expressed as % inhibition compared to the control IgE/Ag response. Results are expressed as mean ± SEM of 4 experiments performed in triplicate.

### 3.3.4 The Role of Specific PI3K isoforms in Degranulation by Cross-Linking of IgE.

PI3K can be considered a central regulator of critical downstream signalling processes for receptor-mediated mast cell responses. Evidence from murine studies appears to indicate that *in vitro* studies both p110δ and p110γ are required for FcεR1-driven mast cell degranulation, whereas *in vivo*, p110δ (but not p110γ) is indispensable for allergic responsiveness (Ali et al., 2004; Ali et al., 2008; Laffargue et al., 2002). The role of PI3K in human mast cells has yet to be fully elicited. This study aims to highlight potential key PI3K isoforms in FcεRI induced degranulation in the human mast cell model.

Isoform specific PI3K inhibitors were used to investigate the role of individual PI3K catalytic isoforms in IgE/Ag-induced degranulation. A recent comprehensive analysis of inhibitor selectivity revealed that the most selective compounds include the quinazolinone purine inhibitors of PI3K such as IC87114 which targets PI3Kδ , the chromones that preferentially target PI3Kβ/δ (TGX-121), the pyridinylfuranopyrrimidine that preferentially targets PI3Kα (PIK75) and the thiazolidinediones that preferentially target PI3Kγ (AS605240) (Knight et al., 2006; Smith et al., 2007). At least one compound from each of these chemotypes exhibits 10-100-fold selectivity between their primary target(s) versus other class I PI3Ks (Table 3.1).

| | **Class I PI3K isoforms $IC_{50}$(μM)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **α** | | **β** | | **γ** | | **δ** | |
| | $IC_{50}$ | F.S | $IC_{50}$ | F.S | $IC_{50}$ | F.S | $IC_{50}$ | F.S |
| PIK75 | 0.0078 | 1 | 0.343 | 44 | n/a | n/a | 0.907 | 116 |
| TGX-221 | 5 | 714 | 0.007 | 1 | 3.5 | 500 | 0.1 | 14 |
| IC87114 | > 200 | >1539 | 16 | 123 | 61 | 469 | 0.13 | 1 |
| TGX-121 | >5 | >100 | 0.05 | 1 | >50 | >1000 | 0.05 | 1 |
| AS605240 | 0.06 | 7.5 | 0.27 | 34 | 0.008 | 1 | 0.3 | 38 |
| ZSTK474 | n/a | n/a | 0.012 | 2 | 0.053 | 9 | 0.006 | 1 |

**Table 3.1: $IC_{50}$ values (μM) for isoform-discriminating PI3K inhibitors.** All values are based on in vitro assays of inhibitor activity against purified protein activity. F.S represents the fold selectively of each inhibitor. (Knight et al., 2006; Smith et al., 2007).

PIK75 which displays around 116-fold selectivity against PI3Kα versus the δ isoform respectively had no inhibitory effect on mast cell degranulation at concentrations known to be specific for p110α (Figure 3.9a). It is notable however that inhibition of degranulation by PIK75 starts to occur at concentrations predicated to impinge on other PI3K isoforms. In rank order of potency target, p110δ is the next isoform inhibited, indicating a potential role for p110δ in IgE/Ag induced degranulation.

Use of TGX-221, which has approximately 10-fold selectivity for p110β versus p110δ (Knight et al., 2006), had no effect on degranulation responses to IgE/Ag, even at concentrations predicted to impinge on p110δ activity (Figure 3.9b). It is notable, however, that inhibition of degranulation by TGX-221 starts to occur at concentrations predicated to impinge on other PI3K isoforms. In rank of order-of-potency target, p110δ is the next isoform inhibited, again highlighted as a potential role for p110δ. Interestingly, the p110δ-discriminating inhibitor IC87114 inhibits IgE/Ag-induced degranulation with an $IC_{50}$ of 10-20 μM (Figure 3.9d) markedly higher than its reported $IC_{50}$ for p110δ. obtained against purified enzyme (Table 3.1). This is

surprising as there is usually good concordance between $IC_{50}$'s obtained for IC87114 against p110δ using purified enzyme (Crabbe et al., 2007; Knight et al., 2006; Smith et al., 2007), versus $IC_{50}$'s derived from cell based assays. Given the reported $IC_{50}$ for IC87114 versus p110β is 16 μM, it was important to assess the role of p110β in IgE/Ag agonist-stimulated mast cell degranulation. This was further investigated using 2 inhibitors known to target p110β. Firstly, TGX-121 displays equipotency against p110β and p110δ with approximately 100-fold selectivity against these isoforms versus p110α and p110γ. TGX-121 inhibited IgE/Ag degranulation, (Figure 3.8c). The $IC_{50}$ for TGX-121 is comparable to that predicted for inhibition of p110β and p110δ (Table 3.1).

AS605240 displays around 10-fold selectivity against PI3Kγ versus α isoforms but approximately 35-fold selectivity vs. δ/β. AS605240 inhibited IgE/Ag agonist induced β-hexosaminidase release with an $IC_{50}$ comparable to reported its $IC_{50}$ for p110γ (Figure 3.9e). Possible concerns about off-target effects of AS606240 on p110α at concentrations >60nM seem unfounded, as the p110α-targeting inhibitor PIK75 had no effect on degranulation until ranges which are known to impinge on other PI3K isoforms (Figure 3.9e).

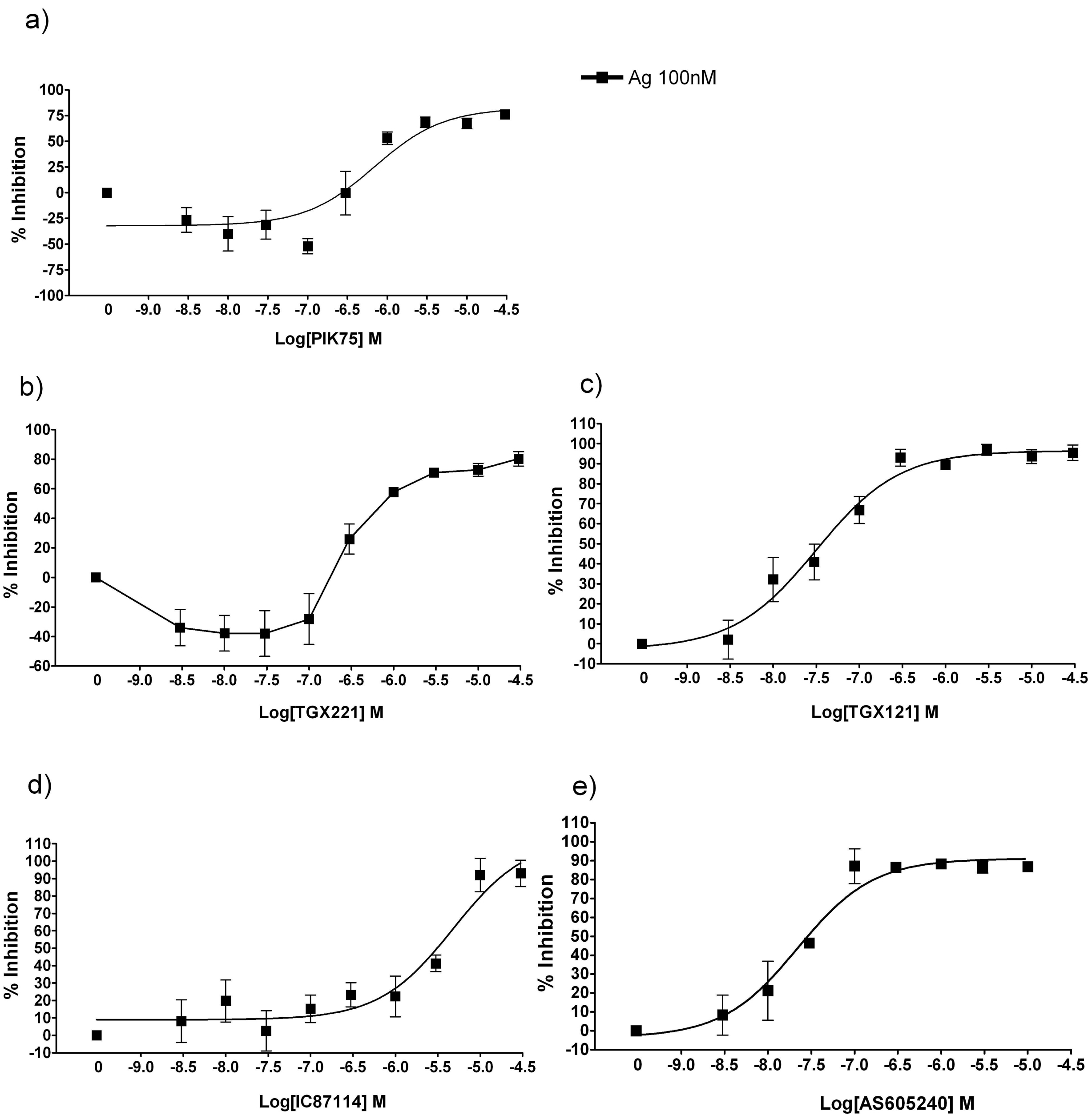



**Figure 3.9: PI3K Isoform Specific IgE/Ag induced degranulation.** Mature mast cells ($1x10^6$/ml) were treated overnight with chimeric human Fc anti-4-hydroxy-3-nitrophenylacetyl (NP)-specific IgE (1μg/ml). Cells were treated with specific PI3K inhibitor at concentration indicated for 30mins before stimulation with 100 ng/ml NP-BSA for 2 hours. Samples were centrifuged for 10mins and supernatants transferred to new wells before addition of β-hexosaminidase substrate for 1.5 hours to measure β-hexosaminidase release. Data are expressed as % inhibition compared to the control IgE/Ag response. Results are expressed as mean ± SEM of 4 experiments performed in triplicate.

### 3.3.5 The Role of Rho Kinase in IgE/Antigen Induced Degranulation

Signalling by the FcεRI receptor manages the cytoskeletal re-arrangements which are associated with degranulation of mature mast cells. There are two important features of the microtubles in the degranulation of mast cells. Firstly, tubulin polymerisation-inhibiting agents have been shown to inhibit the crosslinking of FcεRI (Martin-Verdeaux et al., 2003; Nielsen and Johansen, 1986; Tasaka et al., 1991). Secondly, the mobilisation of granules by FcεRI induced activation has been demonstrated in the mast cell line RBL to be dependent on the microtubles (Smith et al., 2003), highlighting a crucial role of cytoskeletal re-arrangements in mast cell degranulation. One such pathway which has been implicated as key to the cytoskeletal rearragnement is the Rho kinase pathway (Nishida et al., 2005). Rho-kinase is an effector molecule of RhoA, a monomeric GTP-binding protein causing $Ca^{2+}$ sensitization via inactivation of myosin phosphatase. The major physiological functions of Rho-kinase include contraction, migration and proliferation in cells. These actions are thought to be related to the pathophysiological features of asthma, i.e., airflow limitation, airway hyper-responsiveness, β-adrenergic desensitization, eosinophil recruitment and airway remodeling. (Kume, 2008). This study aimed to investigate the role of Rho kinase in degranulation induced by cross-linking of the FcεRI by IgE/Antigen complexes.

Figure 3.10 indicates that Rho kinases play a key role in the degranulation of human mature mast cells induced by IgE/Antigen complexes. Pre-treatment of mast cells with the inhibitor Rho kinase Y27632 was observed to inhibited degranulation by 90 %. This suggests that Rho kinase plays a major role in mast cell degranulation; however other signalling pathways could be involved.

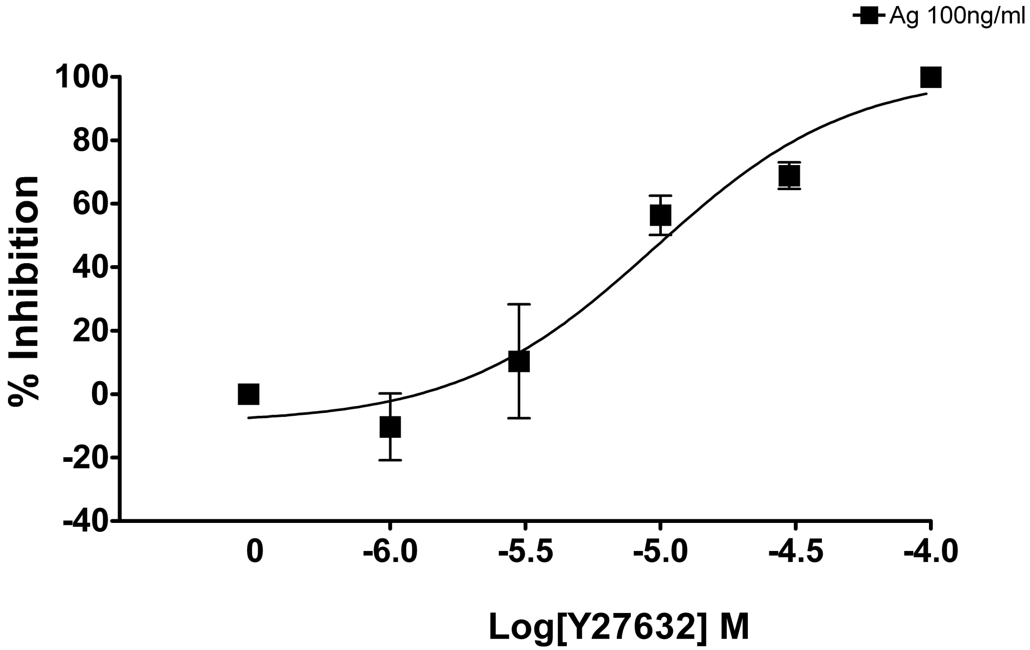



**Figure 3.10: Involvement of Rho kinase in Antigen induced degranulation.** Mature mast cells ($1x10^6$/ml) were treated overnight with chimeric human Fc anti-4-hydroxy-3-nitrophenylacetyl (NP)-specific IgE (1µg/ml). Cells were treated with Y27632 at concentration indicated for 30mins before stimulation with 100ng/ml NP-BSA for 2 hours. Samples were centrifuged for 10mins and supernatants transferred to new wells before addition of β-hexosaminidase substrate for 1.5 hours to measure β-hexosaminidase release. Data are expressed as % inhibition compared to the control IgE/Ag response. Results are expressed as mean ± SEM of 4 experiments performed in triplicate.

### 3.3.6 The Role of PLC in Mast Cell Induced Degranulation of Mast Cell by Antigen

Mast cell degranulation following FcεRI-aggregation is generally believed to be dependent on PI3K mediated PLCγ activation. Mast cells are one of the few cell types that express both PLCγ1 and PLCγ2 isoforms (Barker et al., 1998). In primary cultures of human mast cells, FcεRI aggregation induced a rapid translocation and phosphorylation of $PLC\gamma_1$, and subsequent $IP_3$ production that preceded PI3K related signals (Tkaczyk et al., 2006). Therefore with different subsets of mast cells portraying different characteristics it was of interest to investigate if PLC is as crucial a signalling molecule in cross-linking of FcεRI to induce degranulation in human cord blood derived mast cells.

Figure 3.11 demonstrates that PLC plays a key role in degranulation of cord blood derived mast cells by aggregation of FcεRI. Pre-treatment of cells with the aminosteroid U73122 which is reported to act as a specific inhibitor of PLC was observed to inhibit mast cell degranulation whereas use of the inactive analogue U73343 in the same concentration range failed to inhibit degranulation.

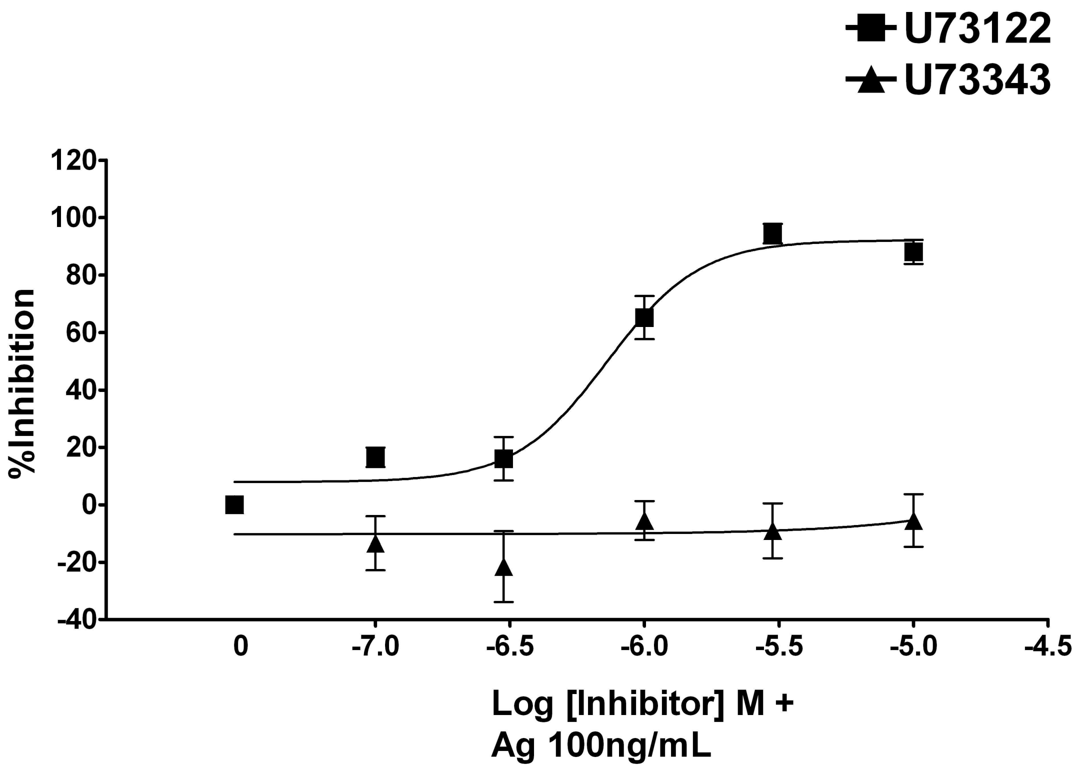



**Figure 3.11: Role of PLC in IgE/Ag induced degranulation.** Mature mast cells ($1x10^6$/ml) were treated overnight with chimeric human Fc anti-4-hydroxy-3-nitrophenylacetyl (NP)-specific IgE (1μg/ml). Cells were treated with specific PLC inhibitor at concentration indicated for 30mins before stimulation with 100ng/ml NP-BSA for 2 hours. Samples were centrifuged for 10mins and supernatants transferred to new wells before addition of β-hexosaminidase substrate for 1.5 hours to measure β-hexosaminidase release. Data are expressed as % inhibition compared to the control IgE/Ag response. Results are expressed as mean ± SEM of 4 experiments performed in triplicate.

### 3.3.7 $IP_3$ in Mast Cell Induced Degranulation

The activation of mast cells by aggregation of the FcεRI increases levels of $IP_3$ due to the activation of phospholipases. This in turn increases the calcium flux which triggers the movement of intracellular secretary molecules towards the plasma membrane and subsequent fusion of the granular membrane with the plasma membrane. This is a key process in the degranulation process.

Figure 3.12 reveals that $IP_3$ is critical to the degranulation of mast cells by FcεRI. Pre-treatment of mature mast cells with the $IP_3$ inhibitor 2-AB strongly inhibits mast cell degranulation induced by aggregation of FcεRI.

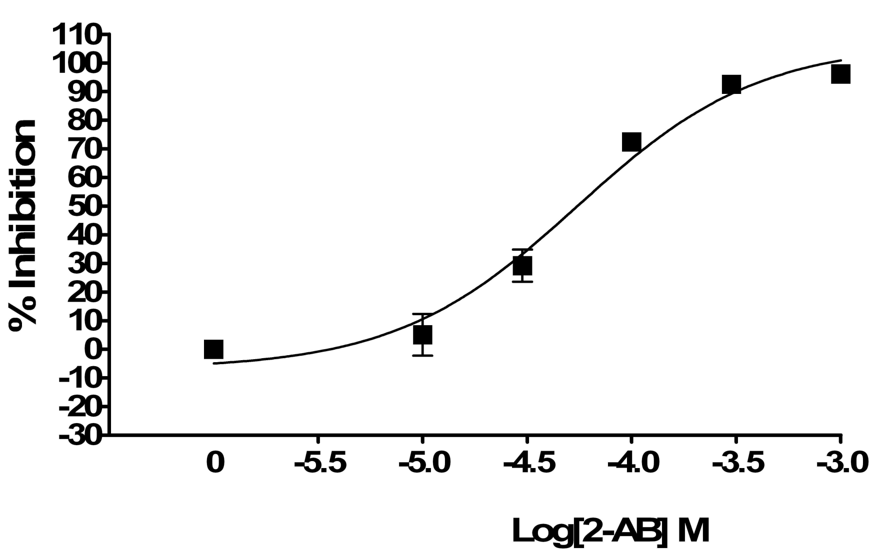



**Figure 3.12: Generation of $IP_3$ is critical for FcεRI aggregation induced degranulation.** Mature mast cells (1x$10^6$/ml) were treated overnight with chimeric human Fc anti-4-hydroxy-3-nitrophenylacetyl (NP)-specific IgE (1µg/ml). Cells were treated with specific IP3 inhibitor, 2-AB at concentration indicated for 30mins before stimulation with 100ng/ml NP-BSA for 2 hours. Samples were centrifuged for 10mins and supernatants transferred to new wells before addition of β-hexosaminidase substrate for 1.5 hours to measure β-hexosaminidase release. Data are expressed as % inhibition compared to the control IgE/Ag response. Results are expressed as mean ± SEM of 4 experiments performed in triplicate.

### 3.3.8 Calcium Influx is Key to Mast Cell Degranulation

Evidence exists for the importance of calcium dependent and independent pathways both being critical for mast cell degranulation by FcεRI. It was therefore essential to investigate if the role of calcium in the extracellular medium and its importance in FcεRI aggregation induced degranulation. Figure 3.13 reveals that the absence of calcium in the extracellular medium strongly restricts the degranulatory response induced by aggregation of FcεRI. Aggregation of FcεRI in medium without $Ca^{2+}$ only induced minimal release of β-hexaminidase above basal levels. In completely calcium free conditions, calcium free media was pre-treated with 1mM EGTA to sequester any remaining calcium ions, only trace amount of β-hexaminidase release were detectable above basal levels. These findings clearly indicate the importance of calcium in the extracellular media to induce degranulation.

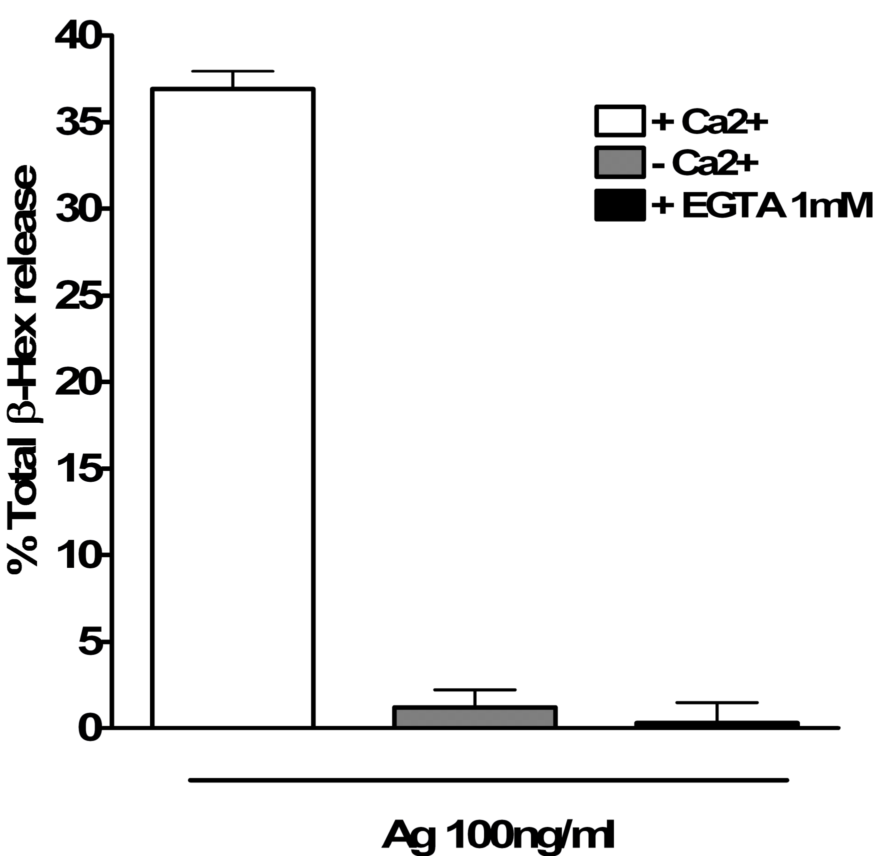



**Figure 3.13: Varying $Ca^{2+}$ conditions in FcεRI aggregation induced degranulation.** Mature mast cells ($1x10^6$/ml) were treated overnight with chimeric human Fc anti-4-hydroxy-3-nitrophenylacetyl (NP)-specific IgE (1μg/ml). $1x10^6$ human mature cord blood-derived mast cells in appropriate buffer, (RPMI,RPMI without $Ca^{2+}$ or RPMI without $Ca^{2+}$and 1mM EGTA) and before stimulation with 100ng/ml NP-BSA for 2 hours. Samples were centrifuged for 10mins and supernatants transferred to new wells before addition of β-hexosaminidase substrate for 1.5 hours to measure β-hexosaminidase release. Data is expressed as % of total β-hexosaminidase content. Results are expressed as mean ± SEM of 4 experiments performed in triplicate.

### 3.3.9 Summary

- FcεRI aggregation by antigen induces degranulation of mature human mast cells derived from cord blood in a dose dependent manner.
- FcεRI aggregation induced degranulation of mast cells is dependent on the γ, β and δ isoforms of PI3K.
- PLC is a key signalling molecule needed to be activated in order to induce mast cell degranulation via FcεRI aggregation.
- This activation results in the formation of $IP_3$ which is highlighted as another key signalling molecule required in mast cell degranulation
- Calcium mobilisation is crucial to mast cell activation.

## 3.4 Discussion

The aim of this study was to successfully evaluate a mast cell model to investigate CXCR3 expression and its function. Two mast cell models were evaluated: HMC-1 and primary human mast cell derived from human CD133 precursor cell isolated from cord blood. Investigations began by characterising the HMC-1 cell line as an appropriate mast cell model.

### 3.4.1 HMC-1 Cell Line makes a Poor Mast Cell Model for Investigating CXCR3 Function.

The HMC-1 cell line was derived from a patient with mast cell leukaemia which is comprised mostly of immature mast cells. Far from being ideal as a model for investigating mast cell function the cells may respond very differently to mature mast cells. HMC-1 cells lack a functional IgE receptor and have mutations in the coding sequence of the c-Kit proto oncogene (Furitsu et al., 1993). The benefits of utilising a cell line are in the numbers that can be generated and maintenance of a cell line is far less than the cost of the primary mast cell line utilised in this study. Under normal circumstances, SCF binds to c-Kit, inducing homodimerization of the receptor that leads to intrinsic kinase activity and results in autophosphorylation of tyrosine residues (Blechman et al., 1993). c-Kit then becomes the docking site for various Src homology domain 2 (SH2) domain signalling molecules. The mutations in the coding sequence produce high spontaneous tyrosine phosphorylation of c-Kit resulting in phosphorylation of down stream signalling proteins (Ma et al., 1999). Although CXCR3 was expressed at an mRNA and surface level, the mutation in the c-Kit results in the high basal activation of many down stream signalling proteins exposed the HMC-1 model as an inappropriate mast cell model to study CXCR3 signalling events.

Despite the mutated c-Kit, in other studies and in this study, SCF through the binding of c-Kit is able to induce a chemotactic response in HMC-1 cells. SCF falls into the growth factor category with receptors endowed with tyrosine kinase activity that also stimulate cell migration. Stimulation of c-Kit with SCF induces dimerisation and

activation of its intrinsic tyrosine kinase activity, leading to auto-phosphorylation and to the phosphorylation of a number of substrates (Blume-Jensen et al., 1991; Herbst et al., 1991; Lev et al., 1991). In this pathway is protein kinase C, a serine/threonine kinase that is involved in the control of cell proliferation, differentiation, and motility (Nishizuka, 1988). It was discovered, however, that CXCR3 agonists failed to induce the migration of HMC-1 cell line. This inability to induce the migration of HMC-1 has also been noted in response to other chemokine agonists (Nilsson et al., 1994).

### 3.4.2 Chemokine Receptors and Mast Cell Migration

Chemokine receptors whose expression is inducible rather than constitutive, such as CCR1, CCR2 (receptor for monocyte chemoattractant protein 1 [MCP-1], also designated CCL2), CCR5, and CXCR3 agonists are known to play a prevalent role in the control of inflammation (Amoura et al., 2003). Airway inflammation is characterised by selective recruitment of mononuclear and granulocytic cells. This recruitment is mediated by the action of chemotactic cytokines, such as chemokines. A number of chemokines and their receptors have been identified and proposed as potential therapeutic targets to help resolve allergic airway inflammation. Chemokines that have been associated with allergic inflammatory diseases such as asthma and allergic rhinitis are CXCR3 and CCR2 agonists.

It is widely recognised that members of the CC subfamily of the chemokine family exert strong chemoattractant activities on monocytes, T cells and natural killer cells (Rollins, 1996). This study clearly demonstrated the functional responses of CCL2 in the monocyte cell line, THP-1 with functional response seen in both $Ca^{2+}$ mobilisation and chemotatic assay.

In addition to promoting the transmigration of circulating monocytes into tissues, CCL2 has been shown to exert various effect on monocytes, including superoxide anion induction, chemotaxis, and calcium flux (Rollins, 1996). CCL2 plays a critical role in the regulation of human monocyte function and has largely been associated with modulating monocyte migration in response to inflammation (Rollins et al., 1991).

CCL2 also induced characteristic monocyte responses, such as $Ca^{2+}$ mobilisation and respiratory burst (Rollins, 1996)., thus making this specific chemokine an appropriate positive control in this study.

### 3.4.3 Development of Mast Cell Culture Systems

Since the HMC-1 cell line model proved to be an inappropriate mast cell model to study CXCR3 signalling and function, the focus of this study moved to evaluate a primary mast cell model. The utilisation of these primary mast cell models has allowed the discovery of how mast cells responded to a wide range of different physiological and pathological mediators. These models have also allowed the understanding the role of mast cells in inflammatory responses. This study utilised the cord blood derived model to generate large volumes of mast cells to allow the study of the function of CXCR3 on mast cells. The first challenge of this study was to generate large enough volumes of mature mast cells to determine if this cord blood derived model was appropriate. The results in this study have shown that it was possible to generate pure populations of mature mast cells. However the drawbacks to this model were the cost of maintaining the cells throughout the maturation and differentiation. Also lack of cord blood availability in the local area resulted in this study buying CD133+ precursors which further added to the cost of this cell model. As a result of the cost of this model slight limitations were placed on the amount of experiments that were able to be performed.

### 3.4.4 Evaluation of Cord Blood Derived Mast Cell Model

This investigation began by characterising the optimal concentration of antigen that induces the maximal degranulatory response from cord blood derived mast cells. IgE antibodies bind to the antigens of allergens. These allergen-bound IgE molecules interact with Fcε receptors on the surface of mast cells. Activation of mast cells following engagement of FcεRI results in degranulation of mast cells in a dose dependent manner.

The process of degranulation via aggregation of FcεRI involves many different signalling molecules. Many of the signalling networks identified as being involved in mast cell degranulation have been dervied from the analysis of mast cells from gene-targeted mice. Murine models have been instrumental in defining the basic mechanisms of anti-tumor immunity. Most of these mechanisms have since been shown to operate in humans as well. In common with other animal species murine models also have limitations. Mice do not spontaneously develop asthma and no model mimics the entire asthma phenotype making it critical in diseases linked to mast cells to also study human models (Kips et al., 2003).

### 3.4.5 Role of PI3K Isoforms in Mast Cell Degranulation

Mast cells express the class 1A PI3K, p85α, p85β and p50β regulatory subunit isoforms (Lu-Kuo et al., 2000; Tkaczyk et al., 2003). In addition to all three classes 1A PI3K catalytic subunit isoforms, p110α, p110β, and p110δ and the class 1B p110γ catalytic subunit (Ali et al., 2004; Okkenhaug et al., 2007). As c-Kit and the FcεRI initiate their signalling processes via activation of tyrosine kinases, either intrinsically or by recruitment of cytosolic kinases (Gilfillan and Tkaczyk, 2006; Roskoski, 2005). They use class 1A PI3Ks to mediate subsequent downstream signalling events. It is understood that GPCRs, such as adenosine, PGE2, S1P and complement component C3a, mediate their responses via class 1B PI3K (Gilfillan and Tkaczyk, 2006).

The activation of PI3K in mast cells by FcεRI aggregation induces the recruitment of the AKT to the plasma membrane resulting in phosphorylation of AKT by the serine/theronine kinase PDK-1 (Vanhaesebroeck and Alessi, 2000). Although activation of Akt seems to be crucial for mast cell function, the exact role of Akt in mast cells is not yet clear. Evidence suggests that PDK1-AKT may contribute to the PI3K-dependent signalling events regulating mast cell growth, homeostasis and cytokine production. This has been highlighted by the inhibition of PDK1 activation, resulting in the apoptosis of SCF-maintained mast cells (Sawamukai et al., 2007).

It is has been proved from gene targeting approaches in mice that gene knock-out of individual PI3K isoforms can alter the expression of other non-targeted isoforms and this is a likely complication of siRNA-based strategies. Knock-out mice lacking PI3K protein expression exhibit a different phenotype from knock-in mice displaying PI3K mutants that lack kinase activity. Such studies, in comparison of pharmacological and gene targeting of PI3K isoforms have revealed that PI3K isoforms have scaffolding roles in addition to their catalytic roles (Shaywitz et al., 2008; Vanhaesebroeck et al., 2004). Give that this thesis has used a range of novel inhibitors with differing selectivity profiles, the pharmacological analysis is actually more accurate and reliable that the siRNA strategy.

### 3.4.6 Role of Signalling PLC in Mast cell Degranulation by Aggregation of FcεRI

Upon cross-linking of FcεRI with IgE/cognate antigen complexes, the aggregation of multiple FcεRI complexes results in transphosphorylation of the ITAM regions of the β and γ-chains by Lyn, a src family protein tyrosine kinase (PTK) that is constitutively associated with the β-chain (Jouvin et al., 1994; Pribluda et al., 1994). After activation of mast cells by aggregation of FcεRI, this event recruits Lyn kinase to the phosphorylated β–chains and the phosphorylation of the ITAM regions present on the γ-chain resulting in a docking site for the spleen tyrosine kinase (SYK) (Kimura et al., 1996; Shiue et al., 1995). The activation of the SYK has been shown to be an indispensable initial signal for mast cell activation by FcεRI aggregation. Studies in SYK-deficient mast cell lines and primary mast cells taken from SYK-deficient fetal liver have lacked all the features of mast cell activation, including degranulation and leukotriene and cytokine release (Costello et al., 1996; Zhang et al., 1996).

Lyn is a kinase upstream of SYK activation. Lyn was the major src PTK that phosphorylates SYK and the FcεRI β and γ-chains. One particular study has demonstrated that in passive systemic anaphylaxis (PSA) in Lyn-deficient mice, PSA is severely diminished (Hibbs et al., 1995). This initial study has been contradicted by the findings in vitro that where mast cells were deficient of Lyn that degranulation was

unaffected or potentially enhanced in these cells (Hernandez-Hansen et al., 2004; Odom et al., 2004). The discrepancy in these studies was due to a difference in the age of mice used; leading to the conclusion that Lyn plays a key role in mast cells of the young but loses its importance as the mice age.

Activation of SYK results in the phosphorylation of several adaptor molecules. One of these is PLCγ, shown in this thesis and in other studies to be crucial for degranulation. The downstream phosphorylation of PLCγ and Vav was completely absent in Syk-deficient mast cells. Mast cells have been shown to express both isoforms of PLCγ (Wilde and Watson, 2001). The generation of IP3 by activation of PLC leads to mobilisation of intracellular $Ca^{2+}$, resulting in a sustained $Ca^{2+}$ flux response that is maintained by an influx of extracellular $Ca^{2+}$ (Hoth and Penner, 1992). This study has demonstrated the key role of IP3 in mast cell degranulation further unraveling the signalling profile of mast cell degranulation in cord blood derived mast cells. Activation of mast cells by aggregation of FcεRI it is known to, in conjunction with DAG, the increase in intracellular calcium levels causes downstream activation of PKC and Ras guanyl nucleotide- releasing protein (RasGRP), known to contribute to the release of cytokines from mast cells (Dower et al., 2000; Ebinu et al., 2000), further enhancing the important of PLC activation in mast cells demonstrated in this study.

Further proof PLC is a key player in mast cell degranulation was the findings that both PLCγ1 and PLCγ2 isoforms are phosphorylated in FcεRI aggregation. This is, however, drastically reduced in SLP-76 and LAT-deficient mast cells. The use of PLCγ1 deficient mast cells has not proved a successful model to date to investigate PLC's role in the mast cell due to the early embryonic lethality of PLCγ1-deficient mice, (Ji et al., 1997) mast cells from PLCγ2-deficient mice display reduced $Ca^{2+}$ flux and degranulation(Wen et al., 2002).

### 3.4.7 Reliance of Calcium Influx for Mast Cell Degranulation

This study highlighted the importance of calcium influx to the degranulatory response. Removal of calcium from the extracellular medium completely blocked the degranulation response. STIM1 has been demonstrated to be key in promoting the $Ca^{2+}$ influx that is essential for FcεRI-mediated mast cell activation and anaphylaxis (Baba et al., 2008). Mast cells lacking STIM1 also had much less degranulation and cytokine production after FcεRI I stimulation (Baba et al., 2008). Another key component that has been identified is the role CRAC channels in mast cell degranulation. Mast cells derived from CRACM1-deficient mice showed grossly defective degranulation and cytokine secretion, and the allergic reactions elicited in vivo were inhibited in CRACM1-deficient mice (Vig et al., 2008). These studies fit in with the data in this thesis, as activation of PLC drive STIM1 expression from the ER resulting in the opening on the CRAC allowing influx of $Ca^{2+}$ driving the degranulation response forward.

### 3.4.8 Rho Kinase is Crucial to Mast Cell Degranulation by FcεRI Aggregation

This thesis has shown the dependence on activation of Rho kinase in the induced degranulation of mature cord blood derived mast cells by FcεRI aggregation. It has also indicated a key role for the influx of calcium in mast cells. It has been demonstrated however that in studies of exocytosis of mast cells that members of the SNARE family, including SNAP-23, synaptotagmin, syntaxin (t-SNARE), and molecules of the VAMPs family (v-SNARE) regulate granule-to-plasma or granule-to-granule membrane fusion in response to elevated cytosolic calcium concentrations (Castle et al., 2002).

It has also been highlighted that calcium independent pathways are just as crucial to the degranulatory process. (Nishida et al., 2005) demonstrating a key involvement of the Fyn/Gab2/RhoA signalling and are required for granule translocation to the

plasma membrane. This study concluded that FcεRI stimulation induced the formation of microtubules and that this was independent of calcium; Fyn/Gab2/RhoA signalling was not only involved in microtubule formation but was also required for degranulation and the translocation of granules to the plasma membrane.

The presence of a calcium independent pathway has also been noted in studies with Fyn-deficient bone marrow derived mast cells in which degranulation after FcεRI aggregation was impaired, despite the presence of a functional calcium flux (Parravicini et al., 2002). It is believed that the requirement of 2 pathways, one dependent, the other independent of calcium, is required to mobilise the translocation of granules to the plasma membrane (calcium dependent pathway), where the calcium independent pathway is required for the fusions of the granules with the plasma membrane (Nishida et al., 2005). This study and others highlights the importance of Rho kinase in mast cell degranulation.

# Chapter 4 – Role of CXCR3 in Cord Blood Derived Mast Cells.

## 4.1 CXCR3 Function in Human Cord Blood Derived Mast Cells

### 4.1.1 Introduction to the Study

The findings so far in this study have demonstrated that primary human mast cells derived from cord blood create a suitable model to study CXCR3 responses in mast cells. It is already established that a range of mediators other than IgE cross-linking can activate mast cells (Kuehn and Gilfillan, 2007; Nishida et al., 2005; Tkaczyk et al., 2002), however the role of chemokines in mast cell activation has not yet been widely studied. Evidence has demonstrated that certain chemokines are capable of inducing augmentation of antigen-induced degranulation, for example CCL3 and CCL5 are capable of augmenting antigen-induced degranulation in a non-additive manner (Laffargue et al., 2002). However it has yet to be demonstrated that chemokines are capable of mediating granule release from mast cells via degranulation.Therefore it was of interest to discover the expression profile of chemokine receptors on the mast cell model utilised in this study and potential functions of chemokine receptors on mast cells.

### 4.1.2 Chemokine Expression Profile on Mature Human Cord Blood Derived Mast Cells

There is considerable heterogeneity in the chemokine receptor expression profile of mast cells according to species, how they are derived (cord-blood versus bone marrow) and their final anatomical location. It was therefore important to establish a comprehensive chemokine expression profile for the mature human cord blood derived mast cells.

CCR2 and CCR3 were found to be the most highly expressed chemokine receptors present on mature cord blood derived mast cells. CCR1, CXCR3 and CXCR6 were detected in much lower levels to those found previously with CCR2 and CCR3. CCR5, CXCR4 and CXCR7 were present in very low levels whereas CCR7 was not detectable at surface levels on mature cord blood derived mast cells (Figure 4.1).

The aim of this thesis was to investigate the role of CXCR3 agonists in degranulation and biochemical signalling responses in mature (approximately over 8 week old) human cord blood derived mast cells. In a physiological setting CXCR3 agonists have been implicated in a range of inflammatory diseases. It is possible therefore that CXCR3 plays a key role in amplification of mast cell responses in inflammatory diseases. Of interest was to investigate if the inflammatory CXCR3 chemokines were also capable of inducing this augmentation of IgE/Ag induced degranulation.

Figure 4.1 revealed that low levels of CXCR3 were expressed on the surface of mature mast cells but with currently 3 known variants of CXCR3 in existence it was imperative to try and decipher which variants were expressed on the primary mast cell model used in this study.

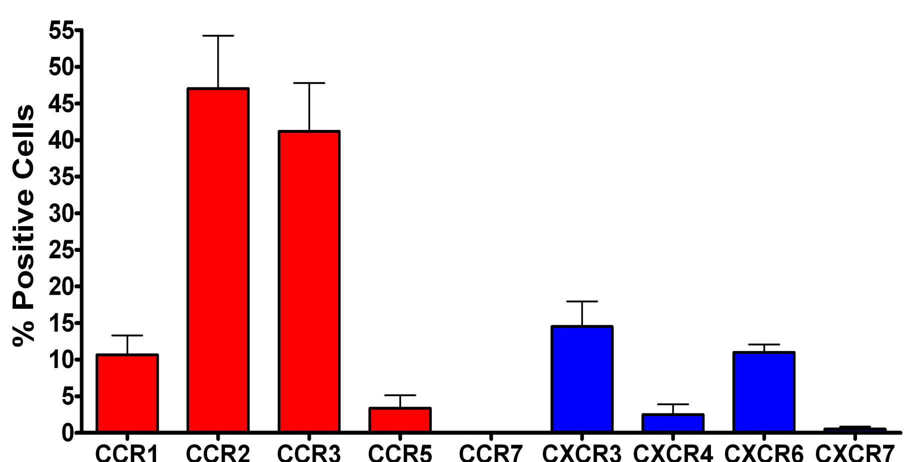



**Figure 4.1: Chemokine receptor expression profile on mature mast cells.** Mature mast cells (1 x $10^6$) were stained (40 min, 4 $^oC$) with a specific mouse PE or FITC-conjugated anti-chemokine mAb or with appropriately isotype matched PE or FITC-conjugated mouse IgG control at the same concentration Chemokine receptor expression was determined by flow cytometry as described in *Materials and Methods*. Data is presented as percentage of positive staining above isotype control from three different donors. Error bars are ± S.E.M

### 4.1.3 Expression of CXCR3 Variants and CXCR7 on CD133+ during Differentiation and Maturation

Analysis by RT-PCR of CXCR3A, CXCR3B and CXCR3-alt expression during differentiation/ maturation of CD133+ cells revealed that mRNA of all CXCR3 variants was detectable at every stage of differentiation (Fig 4.2). Analysis of surface protein expression of CXCR3 revealed low expression in all stages of differentiation and maturation of CD133+ cells. Due to the inability of commercially available antibodies to CXCR3 to reliably distinguish between the different forms of CXCR3, it is impossible to determine which variants are present at the surface. The THP-1 cell line was utilised as a comparison of CXCR3 variant expression (Figure 4.2). Analysis by RT-PCR of CXCR7 revealed only progenitor cells possess mRNA for CXCR7. Equal loading of mRNA was confirmed by the analysis of the “house-keeping” gene β-actin.

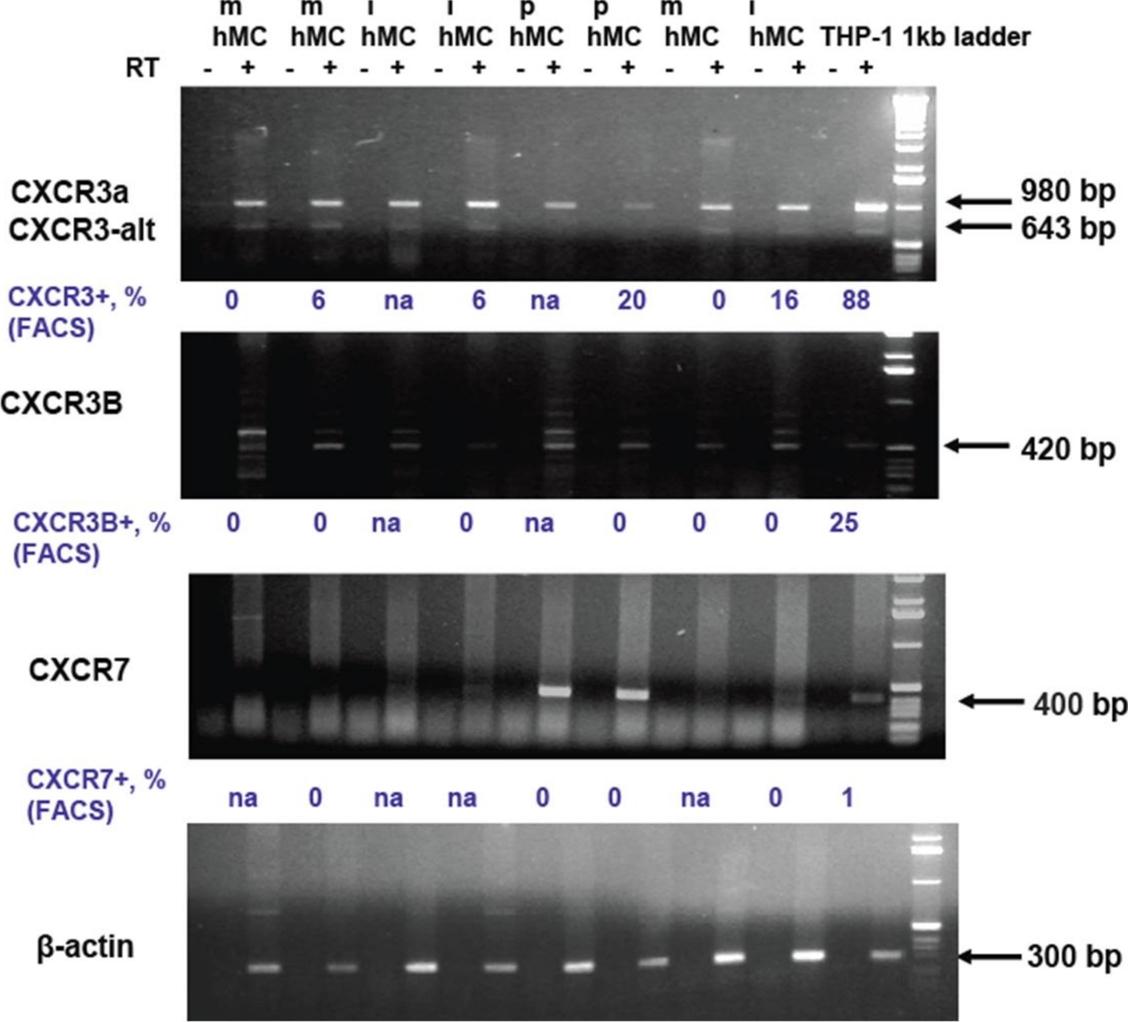



**Figure 4.2: Expression of CXCR3 variants and CXCR7 at the mRNA in different stages of mast cell development.** mRNA (5μg) from 5 x $10^6$ human mast cells or THP-1 was extracted and reverse transcribed in the presence (+) or the absence (–) of reverse transcriptase to ensure that samples were not contaminated with genomic DNA. mRNA of CXCR3 variants and CXCR7 was examined using specific primers as described in materials and methods. Arrows indicate mRNA expression for each CXCR3 variant or CXCR7 respectively. Each channel represents a different donor at different stages of mast cell maturation/development. Figure below each channel represents surface expression at time of extraction as measured using FACS. m ; mature, i ; immature, p ; precursor.

All variants of CXCR3 were expressed at the mRNA level but only low levels of protein were detected on the surface. Further investigation throughout the differentiation/maturation of CD133+ cells found protein expression of CXCR3 had robust intracellular expression, but much lower cell surface expression. However, intracellular levels of CXCR3 were markedly down-regulated during

the differentiation/maturation process, with little change in surface levels (Figure 4.3).

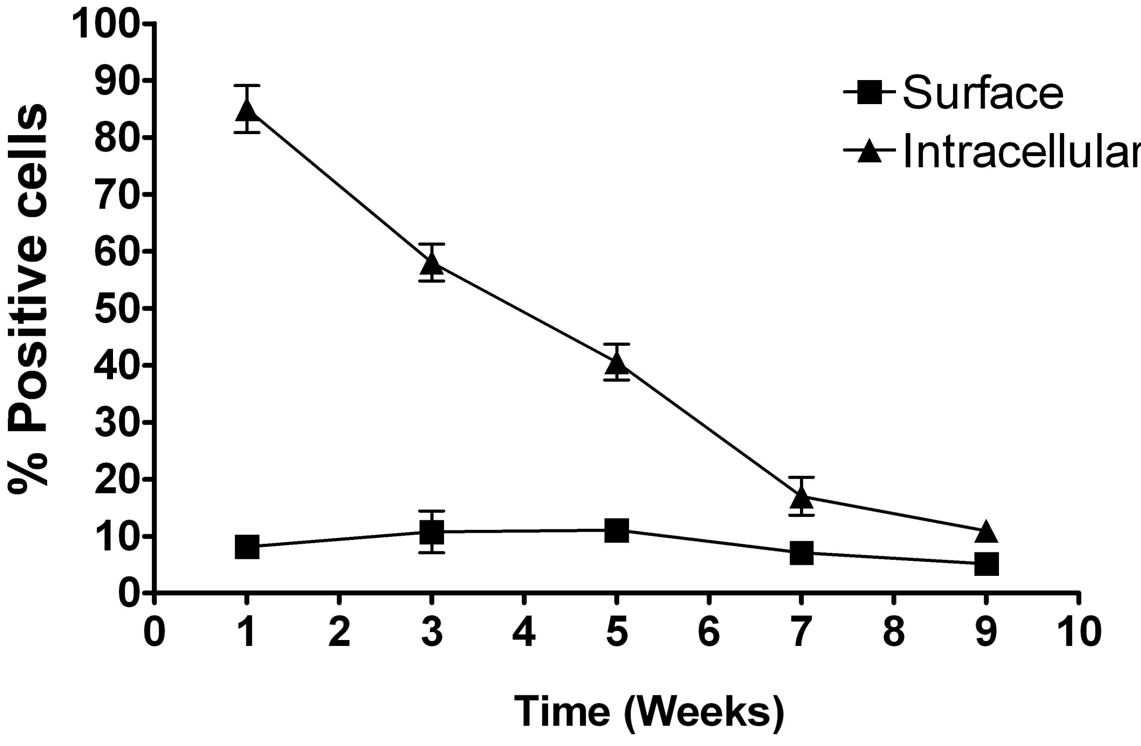



**Figure 4.3: Effect of differentiation/maturation on CXCR3 expression in mast cells.** Mast cells (1 x $10^6$) were sampled at weekly intervals over a specific time period of 9 weeks. Samples were stained with a specific mouse PE-conjugated anti-CXCR3 mAb or with isotype matched PE-conjugated mouse IgG control at the same concentration, and examined by flow cytometry as described in *Materials and Methods*. Data is presented as percentage of positive staining above isotype control from three different donors, mean ± S.E.M.

Further investigation of CXCR3 protein levels in different stages of CD133+ differentiation/maturation via western blotting reveal CXCR3 was clearly present at all stages of differentiation (Figure 4.4a). Surprisingly, the bands in more mature cells became more diffuse, suggesting the possibility of post-translational modification of CXCR3 by for example glycosylation.

Although current commercial antibodies do not reliably distinguish between variants of CXCR3B, one antibody claims to be specific to CXCR3B. Western blotting revealed that at any stage of differentiation, CD133+ cells do not express CXCR3B at the predicted molecular weight of ~44kDa (Figure 4.4b). In the precursor stage there was a clear band at ~65kDa suggesting that a modified form of CXCR3B may be present at this stage. The THP-1 cell line was used as a positive control and reveals two bands, one at the expected molecular weight for CXCR3B and a higher band that was also found in the precursors (Figure 4.4b). Probing for CXCR7 revealed no protein expression in either cell model (Figure 4.4c). The proposed molecular weight of CXCR7 is 41kDa, however as this is only one experiment, further experiments are needed to prove that the lack off expression is not only due to non binding of the anti-CXCR7 antibody.

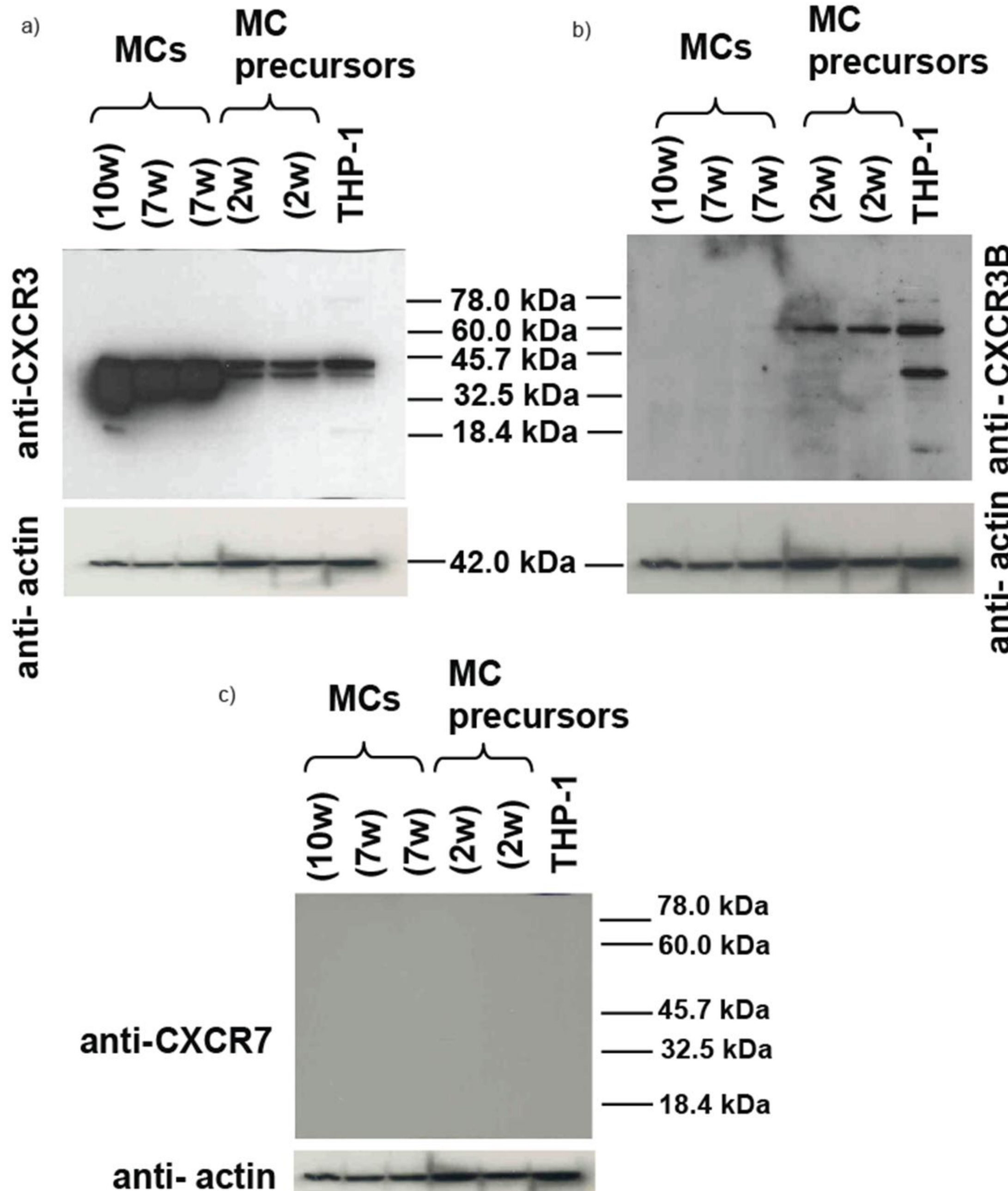



**Figure 4.4: Expression of CXCR3 variants and CXCR7 via western blots.** Mast cells or THP-1 ($1 \times 10^6$ cells/ml) taken from different stages of maturation/development were lysed by the addition of 1× sample buffer. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with antibodies specific for a) CXCR3, b) CXCR3-B and c) CXCR7. Proteins were visualised with ECL and blots were stripped and reprobed with anti-actin Ab to verify equal loading and efficiency of protein transfer (lower panel). Panels a and b are representative of three

other experiments, panal c is derived from a single experiment. W; no of weeks in culture.

To further explore the possible expression of CXCR3B, we utilised the specific CXCR3B antibody to investigate surface expression of CXCR3B. There was no detectable surface expression of CXCR3B through any stage of differentiation/maturation. THP-1 cells utilised as a positive control only demonstrated moderate levels of CXCR3B on the surface (Figure 4.5).

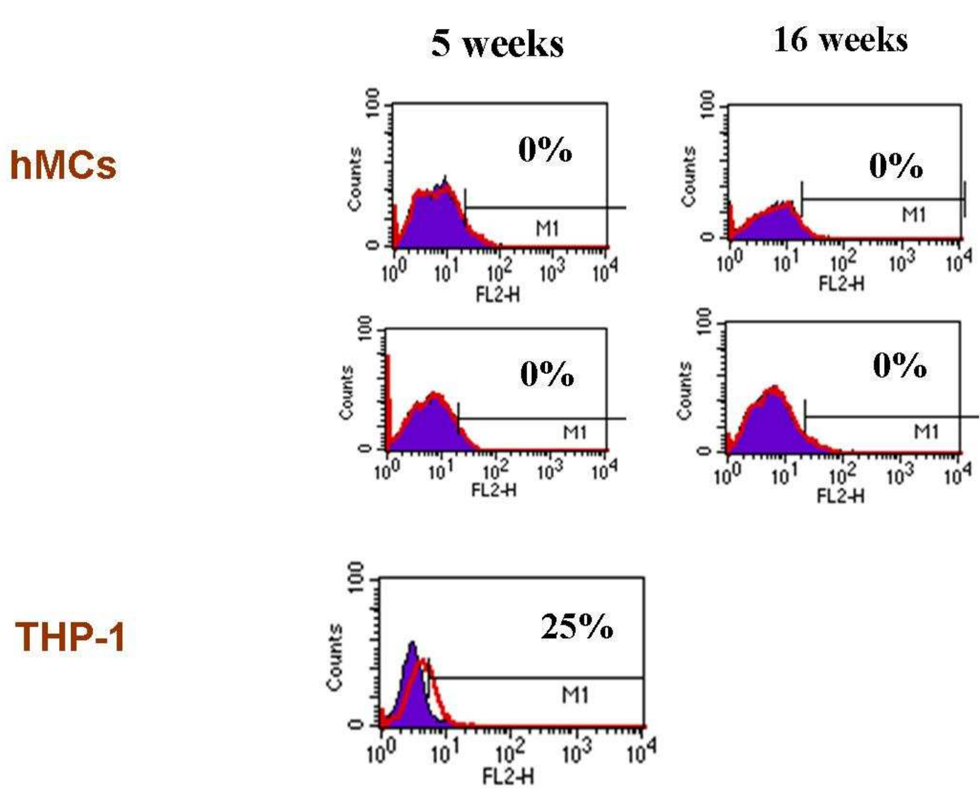



**Figure 4.5: FACS expression of CXCR3B.** Mast cells or THP-1 (1 x $10^6$) were stained with a specific mouse anti-CXCR3B mAb or with isotype matched PE-conjugated mouse IgG control at the same concentration for 30 mins. Excess primary antibody was washed off and incubated with an anti-mouse PE-conjugated secondary antibody and examined by flow cytometry as described in *Materials and Methods*. Data is representative of FACS staining from 2 different donors at different stages of maturation.

### 4.1.4 CXCR3 Agonists Induce Phosphorylation of the Protein Akt and ERK1/2

A range of biochemical signals are robustly induced by chemokine agonists; including the activation of PI3K and MAPK pathways. Therefore biochemical studies focused on establishing whether CXCR3 agonists could activate either of these pathways in the mature mast cells. To establish whether PI3K was activated in response to CXCR3 receptor stimulation, the phosphorylation of Akt was monitored. Akt is a serine/threonine kinase, whose recruitment; phosphorylation and subsequent activation are entirely dependent upon the lipid products produce by induction of the PI3K pathway. For MAPK activity the phosphorylation of ERK1/2 was monitored as this has been observed to be the major pathway preferentially activated by chemokines. All the CXCR3 agonists were observed to induce phosphorylation of Akt and ERK1/2 (Figure 4.6).

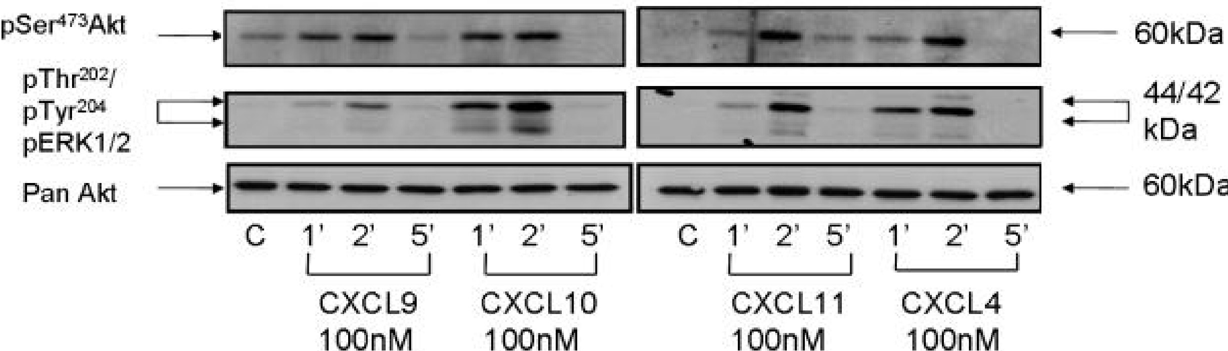



**Figure 4.6: CXCR3 agonists induce phosphorylation of the protein Akt and ERK1/2.** Mature mast cells ($1\times10^6$ cells/ml) were left untreated or stimulated at 37 °C with CXCR3 agonist for indicated time period and lysed by the addition of 1× sample buffer. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with a phospho-specific Erk or Akt Ab and proteins were visualised with ECL. The blots were stripped and reprobed with anti-Akt Ab to verify equal loading and efficiency of protein transfer (lower panel). The data is representative of three other experiments.

### 4.1.5 CXCR3 Induced Partial Degranulation of Cord Blood Derived Mast Cells

Before studying whether CXCR3 agonists were able to effect antigen induced mast cell degranulation, it was assessed whether treatment with CXCR3 agonists alone were able to induce degranulation of mature mast cells. Mast cell degranulation was assessed by measurement of β-hexosaminidase release. At concentrations <10nM, CXCR3 agonists were unable to induce release β-hexosaminidase above basal levels. However, at concentrations >100nM each agonist elicited partial degranulation, which represented around 40-45% of control ionomycin induced β-hexosaminidase release (Fig 4.7a). The peak response of degranulation mediated by all CXCR3 agonists was detected after 1 min post stimulation (Fig 4.7b). Interestingly, CXCL4 induced significant degranulation comparable to the classical CXCR3 agonists.

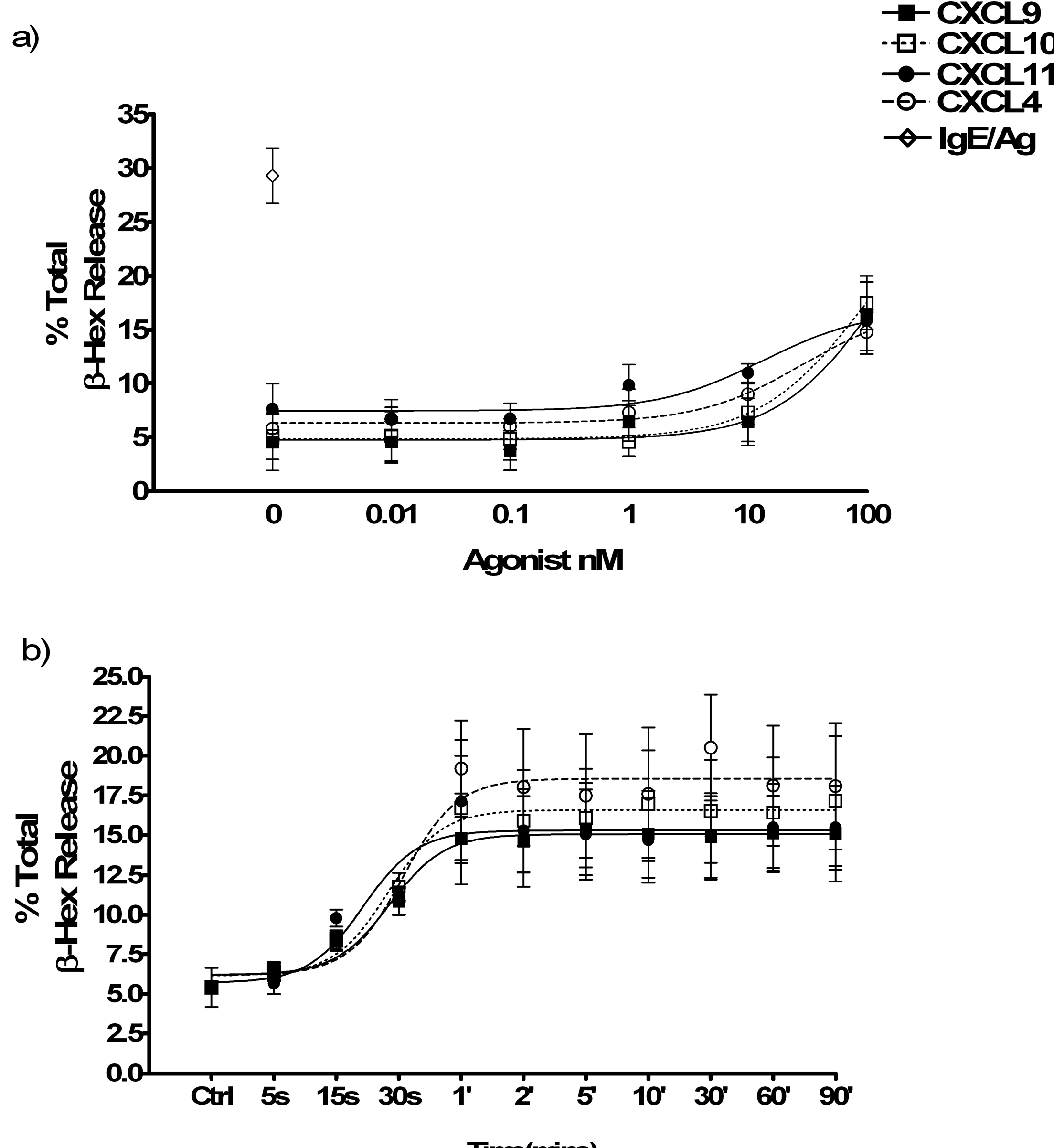



**Figure 4.7: Effect of CXCL9, CXCL10, CXCL11 and CXCL4 on mast cell degranulation.** Mature mast cells ($1x10^6$/ml) were treated overnight with chimeric human Fc anti-4-hydroxy-3-nitrophenylacetyl (NP)-specific IgE (1µg/ml). The cells were then treated with a) CXCL9, CXCL10, CXCL11 or CXCL4 at concentration indicated or 100ng/ml NP-BSA(IgE/Ag) for 1.5 hours or b) indicated time period at 100nM of agonist. Control in b) was treated for 1.5 hours. Samples were centrifuged for 10 mins and supernatant transferred to a new wells before addition of β-hexosaminidase substrate for 1.5 hours to measure β-hexosaminidase release. Data are expressed as % total β-hexosaminidase content. Results are expressed as mean ± SEM of 4 experiments performed in triplicate.

The induction of partial degranulation in the absence of antigen by CXCR3 agonists was a surprising finding. Therefore it was important to investigate the role of other chemokine receptors in this process. Using human cord blood derived mast cells as a model, it emerged that several chemokines operating through different chemokine receptors such as CCR3 and CCR2 can induce partial degranulation of mature mast cells in the absence of antigen (Fig 4.8).

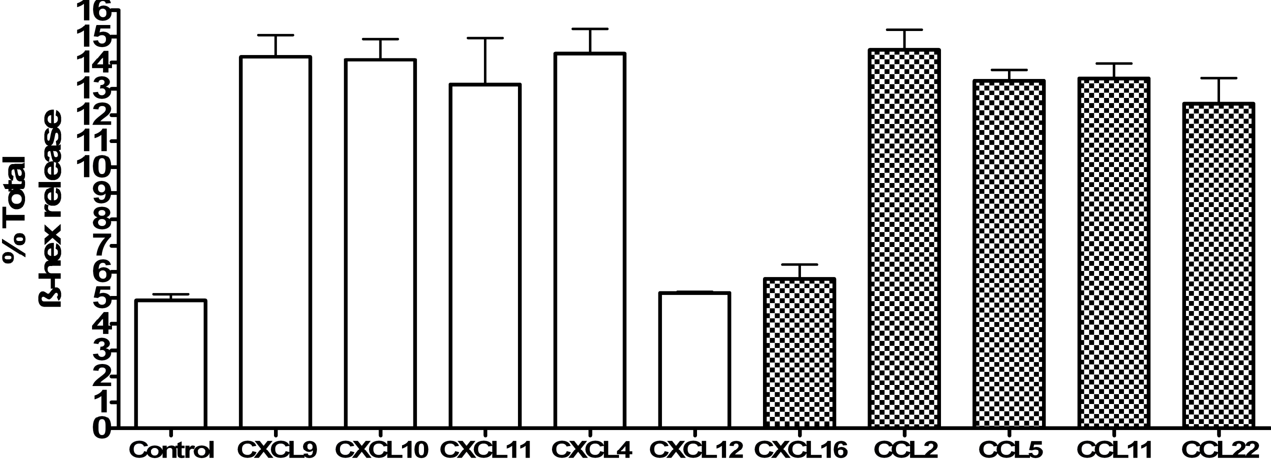



**Figure 4.8: Several chemokines induce partial degranulation of human mast cells.** Mature mast cells ($1x10^6$/ml) were treated overnight with chimeric human Fc anti-4-hydroxy-3-nitrophenylacetyl (NP)-specific IgE (1µg/ml). Cells were treated with specific chemokine at concentration of 100nM for 1.5hours. Samples were centrifuged for 10 mins and supernatants transferred to new wells before addition of β-hexosaminidase substrate for 1.5 hours to measure β-hexosaminidase release. Data are expressed as % total β-hexosaminidase content. Results are expressed as mean ± SEM of 4 experiments performed in triplicate.

### 4.1.6 CXCR3 Induced Partial Degranulation of Human Mast Cells is PTX Sensitive

Previous studies have found that CXCR3A and CXCR3-alt-mediated responses are PTX sensitive indicating that they couple to $G_{\alpha i/o}$, whereas CXCR3B responses are PTX insensitive. It was interesting that in the presented thesis observed a biochemical and functional response from CXCL4, despite no detectable expression of CXCR3B - the only known chemokine receptor for CXCL4 to date. Pre-treatment of mature mast cells for 16 hours with 10ng/ml PTX inhibited the partial degranulation observed in response to CXCL9, CXCL10 and CXCL11 (Figure 4.9). This suggests that the responses are mediated by a $G_{\alpha i/o}$-coupled form of CXCR3. However in direct conflict with the study by blah et al the partial degranulation induced by the CXCR3B agonist; CXCL4 was also inhibited by pre-treatment of cells with PTX (Figure 4.9).

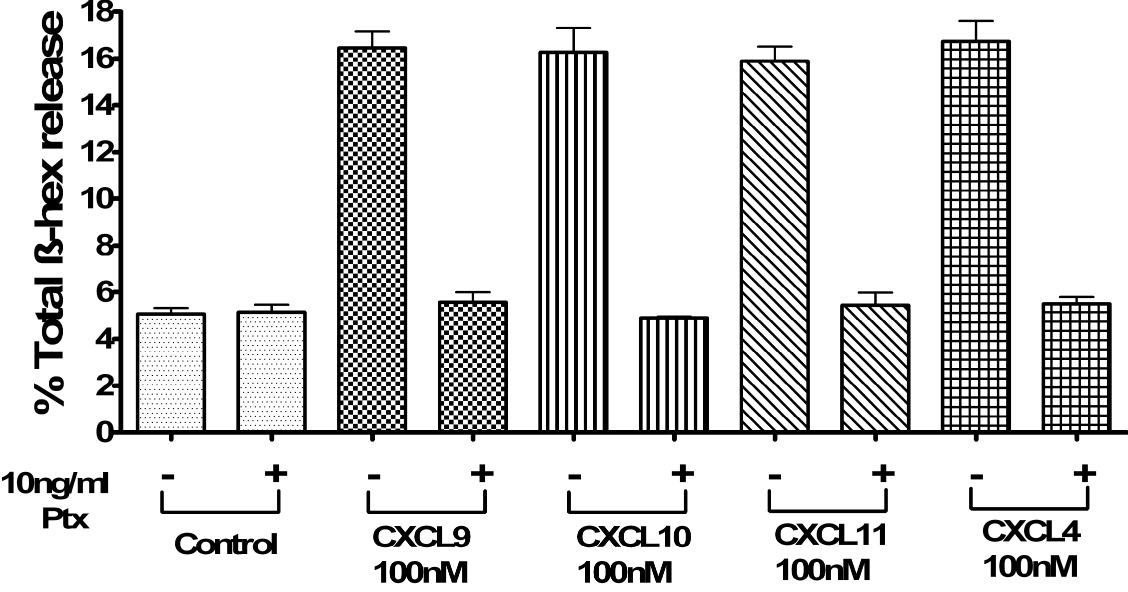



**Figure 4.9: Effect of PTX treatment on CXCR3 agonist induced partial degranulation.** Mature mast cells ($1x10^6$/ml) were treated overnight with chimeric human Fc anti-4-hydroxy-3-nitrophenylacetyl (NP)-specific IgE (1µg/ml). Cells were stimulated with 100nM of appropriate CXCR3 agonist for 1 hour in presence or absence of PTX 10ng/ml pre-treatment for 16hrs. Samples were centrifuged for 10mins and supernatants transferred to new wells before addition of β-hexosaminidase substrate for 1.5 hours to measure β-hexosaminidase release. Data are expressed as % total β-hexosaminidase content. Results are expressed as mean ± SEM of 4 experiments performed in triplicate.

#### 4.1.7 CXCR3 Agonist- Induced Degranulation is CXCR3 Dependent.

With the surprising finding that chemokines could induce partial degranulation of cord blood mature mast cells, it was important to verify that the partial degranulation induced by CXCL9 CXCL10, CXCL11 and CXCL4 was indeed mediated by CXCR3. It was possible that the response observed was due to potential contamination of the CXCR3 ligands with LPS, or that the ligands could be potentially mediating the effect via another chemokine receptor. Therefore it was important to clarify that CXCR3 was responsible for activating these responses.

In recent years several classes of small-molecule compounds targeting CXCR3 have recently been described, including 4-*N*-aryl- [1,4] diazepane ureas (Cole et al., 2006), 1-aryl-3-piperidin- 4-yl-urea derivatives (Allen et al., 2007), quinazolin-4-one, 3*H*-pyrido[2,3-*d*]pyrimidin-4-one derivatives (Heise et al., 2005; Storelli et al., 2005) (Johnson et al., 2007; Storelli et al., 2007), and quaternary ammonium anilide TAK-779 which also shares affinity for CCR5, CCR2b, (Gao et al., 2003).

This study utilised three of these small non-peptide, non-competitive CXCR3 antagonists namely, T487 (reported $IC_{50}$ – 8nM), NBI-74330 (reported $IC_{50}$ 7–18 nM) and N-Oxide metabolite NBI-74330. Accordingly, both T487 (Fig 4.10a) and NBI-74330 (Fig 4.10b) potently inhibited the degranulation induced by CXCR3 agonists. N-Oxide metabolite NBI-74330 was less potent than the other compounds but still demonstrated clear inhibition of CXCR3 induced degranulation (Figure 4.10c) In contrast, all three compounds were unable to inhibit partial degranulation that was induced by CCL2, a known agonist for CCR2 (Fig 4.10 a, b and c).

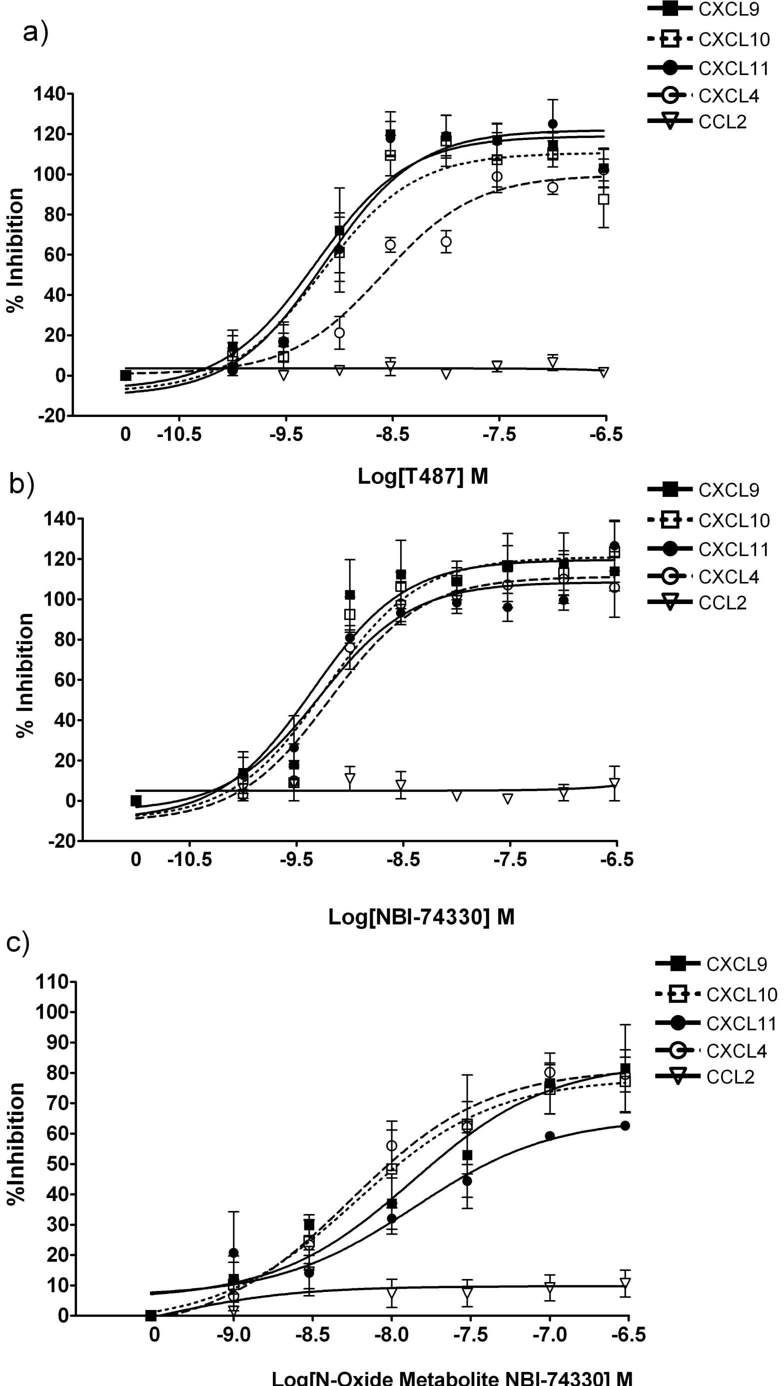



**Figure 4.10: Effect of CXCR3 antagonist on CXCR3 and CCR2 induced partial degranulation.** Mature mast cells ($1x10^6$/ml) were treated overnight with chimeric human Fc anti-4-hydroxy-3-nitrophenylacetyl (NP)-specific IgE (1µg/ml). Mast cells were treated with a selective CXCR3 antagonist at concentration indicated for 30 mins before stimulation with CXCR3 or CCR2 agonist for 1 hour. Samples were centrifuged for 10 mins and supernatants transferred to new wells before addition of β-hexosaminidase substrate for 1.5 hours to measure β-hexosaminidase release. Data are expressed as % inhibition of chemokine agonist response. Results are expressed as mean ± SEM of 4 experiments performed in triplicate.

### 4.1.8 CXCR3 Induced Biochemical ignalling are CXCR3 Dependent

As demonstrated previously, CXCR3 agonists are able to induce robust biochemical signalling responses as demonstrated by the phosphorylation of ERK1/2 and Akt. To determine that these responses are mediated by activation of CXCR3; mast cells were pre-treated with 300 nM of either T487 or NBI-74330 for 30 mins before stimulation with agonists. Both compounds were able to block phosphorylation of ERK1/2 and Akt induced by CXCL9, CXCL10, CXCL11 and CXCL4 (Fig 4.11). However Akt and ERK1/2 phosphorylation induced by the CCR2 and CCR4 ligand CCL2 was unaffected by pre-treatment with either inhibitor.

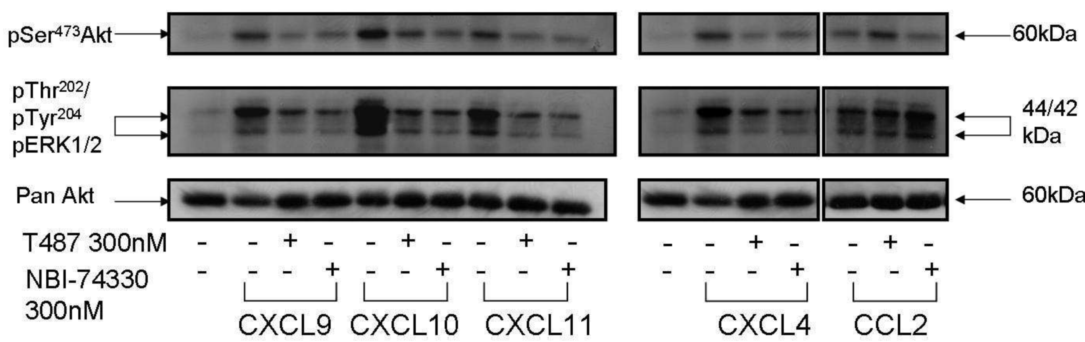



**Figure 4.11: CXCR3 agonist dependent signalling**. Mature mast cells ($1\times10^6$ cells/ml) were treated with a selective CXCR3 antagonist at concentration indicated for 30 mins before stimulation with CXCR3 agonist for 2 min and lysed by the addition of 1× sample buffer. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with a phospho-specific Erk1/2 or Akt Ab and proteins were visualised with ECL. The blots were stripped and reprobed with anti-Akt Ab to verify equal loading and efficiency of protein transfer (lowest panel). The data is representative of three other experiments.

#### 4.1.9 CCL2 Induced Partial Degranulation is CCR2 Dependent.

CCL2 induced degranulation of mature cord blood derived mast cells was not inhibited by any of the CXCR3 antagonists utilised in this study. It was therefore important to investigate which chemokine receptor that this response was mediated by as CCL2 is a known agonist for CCR2. Accordingly this study utilised the CCR2 antagonist, RS102895 to investigate the CCL2 induced degranulation response. Figure 3.24 reveals that CCL2 induced degranulation of mature cord blood mast cells was inhibited by pre-treatment with RS102895. This compound was unable to inhibit CXCR3 agonist induced degranulation indicating that the CXCR3 induced responses are not dependent on CCR2 (Figure 4.12).

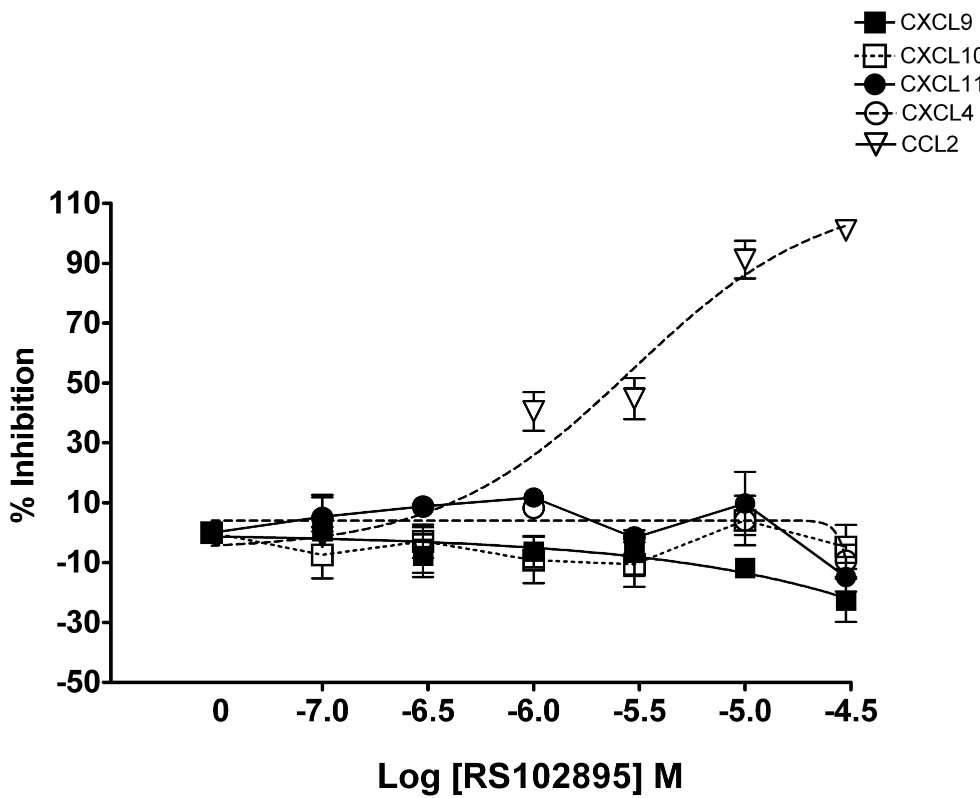



**Figure 4.12: Effect of CCR2 antagonist on CXCR3 and CCR2 agonist induced partial degranulation.** Mature mast cells ($1x10^6$/ml) were treated overnight with chimeric human Fc anti-4-hydroxy-3-nitrophenylacetyl (NP)-specific IgE (1µg/ml). Mast cells were treated with a selective CCR2 antagonist, RO102895, at concentration indicated for 30 mins before stimulation with CXCR3 or CCR2 agonist for 1 hour. Samples were centrifuged for 10 mins and supernatant transferred to new well before addition of β-hexosaminidase substrate for 1.5 hours to measure β-hexosaminidase release. Data is expressed as % inhibition compared to each agonist response. Results are expressed as mean ± SEM of 4 experiments performed in triplicate.

#### 4.1.10 Other Chemokines unable to Elicit Degranulation Responses are still able to Stimulate Biochemical Responses.

The induction of partial degranulation in the absence of antigen by a range of different chemokine receptor agonists was a surprising finding. Although not all the chemokine agonists utilised in this study were capable of inducing the partial degranulation. CXCL12 and CXCL16, agonists for CXCR4 and CXCR6 respectively did not induce partial degranulation of human mature cord blood derived mast cells (Figure 4.8). This led to the question can chemokines induce biochemical signals such as PI3K and MAPK, without inducing partial degranulation. CXCL16 did not induce activation of Akt or ERK1/2 in mature mast cells. However CXCL12 did induce phosphorylation of ERK1/2 and Akt (Figure 4.13) indicating that activation of these pathways alone is not sufficient to induce degranulation. Therefore other signalling pathways would be hypothesised to be involved in chemokine receptor induced degranulation.

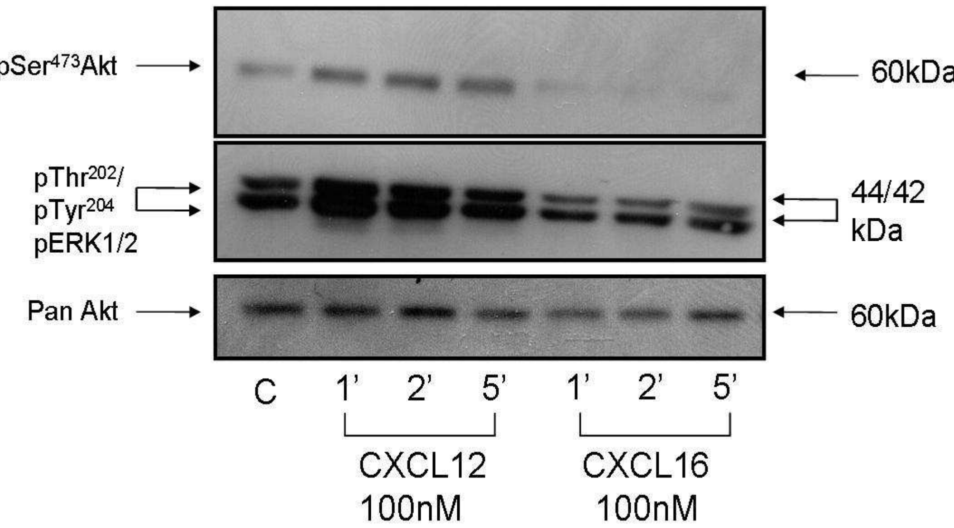



**Figure 4.13: CXCL12 elicits biochemical responses in mast cells.** Mature mast cells ($1\times10^6$ cells/ml) were left untreated or stimulated at 37 °C with CXCL12 or CXCL16 for indicated time period and lysed by the addition of 1× sample buffer. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with a phospho-specific Erk or Akt Ab and proteins were visualised with ECL. The blots were stripped and reprobed with anti-Akt Ab to verify equal loading and efficiency of protein transfer (lower panel). The data is representative of three other experiments.

### 4.1.11 CXCR3 Agonists Augment Sub-Optimal Ag Induced Degranulation

The surprising finding that CXCR3 agonists induce partial degranulation of mature human cord blood derived mast cells made it crucial to re-visit the original hypothesis and idea behind this study that CXCR3 agonists could induce augmentation of sub-optimal Ag induced degranulation.

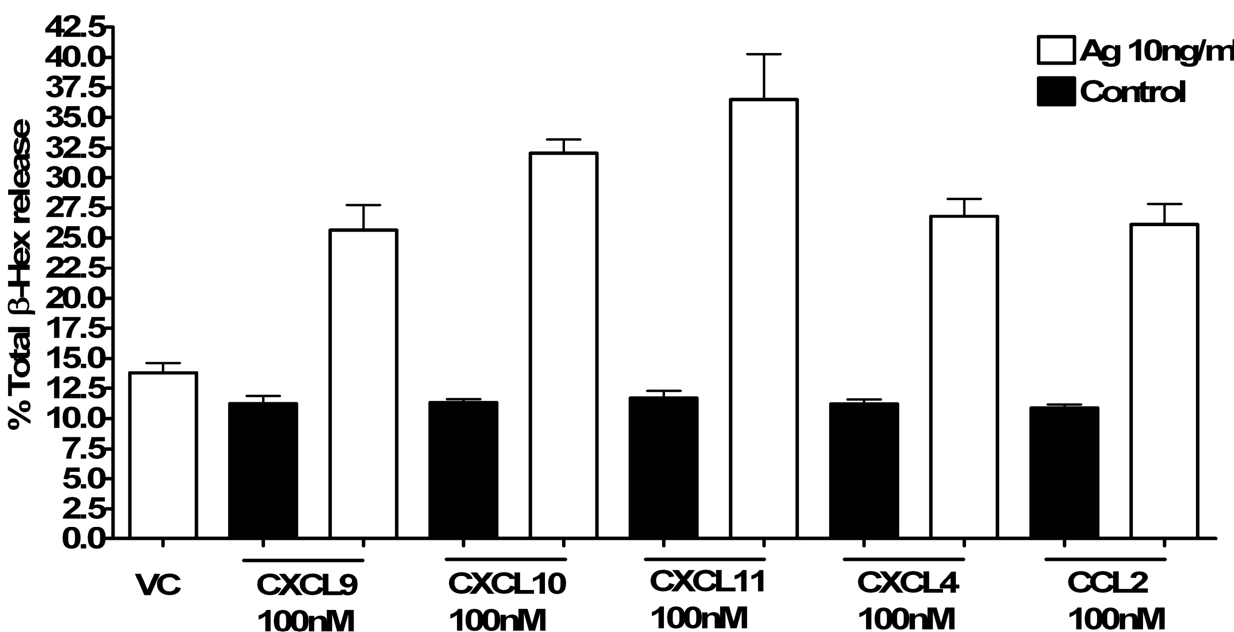
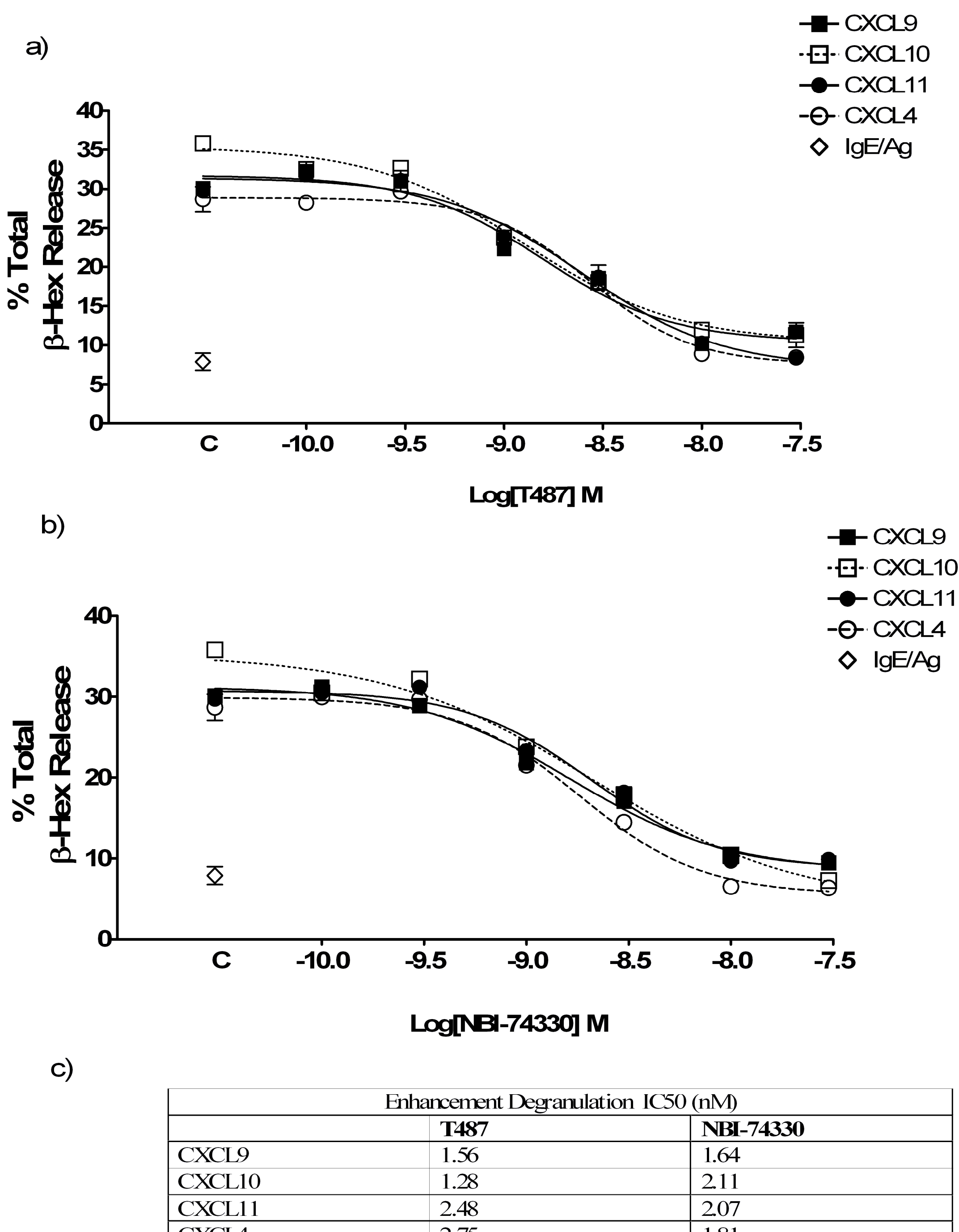



**Figure 4.14: CXCR3 augmentation of sub-optimal IgE/Ag.** Mature mast cells ($1x10^6$/ml) were treated overnight with chimeric human Fc anti-4-hydroxy-3-nitrophenylacetyl (NP)-specific IgE (1µg/ml). Mature mast cells were treated overnight with chimeric human Fc anti-4-hydroxy-3-nitrophenylacetyl (NP)-specific IgE (1µg/ml). Cells were stimulated with 10ng/ml Ag for 1 min before stimulation with CXCR3 or CCR2 agonist for 1 hour. Samples were centrifuged for 10mins and supernatants transferred to new wells before addition of β-hexosaminidase substrate for 1.5 hours to measure β-hexosaminidase release. Data are expressed as % total β-hexosaminidase release. Results are expressed as mean ± SEM of 4 experiments performed in triplicate.

Figure 4.14 revealed that all CXCR3 agonists are capable of augmenting sub-optimal degranulation induced by sub-optimal concentrations of antigen (as determined in Figure 3.7). Sub-optimal concentrations of Ag or treatment with CXCR3 agonists alone induced partial degranulation; this ranged from 25-35% of total β-hexosaminidase release (Figure 4.14). This could imply that the augmentation observed is more than a purely additive effect of the two individual stimulations.

The augmentation of sub-optimal Ag induced degranulation by CXCR3 agonists was CXCR3 dependent. Pre-treatment of mature human cord blood derived mast cells with either T487 or NBI-74330 inhibited the potentiation in degranulation seen with CXCR3 ligand treatment (Figure 4.15 a and b). The CXCR3 antagonists were unable to fully inhibit the degranulation responses indicating that these antagonists are unable to inhibit degranulation induced by sub-optimal concentrations of Ag (Figure 4.15 a and b). The respective $IC_{50}$s for each antagonist to each CXCR3 agonist are shown in figure 4.15c.




| Enhancement Degranulation IC50 (nM) | | |
|---|---|---|
| | T487 | NBI-74330 |
| CXCL9 | 1.56 | 1.64 |
| CXCL10 | 1.28 | 2.11 |
| CXCL11 | 2.48 | 2.07 |
| CXCL4 | 2.75 | 1.81 |

**Figure 4.15: CXCR3 augmentation of sub-optimal IgE/Ag is CXCR3 dependent.** Mature mast cells ($1x10^6$/ml) were treated overnight with chimeric human Fc anti-4-hydroxy-3-nitrophenylacetyl (NP)-specific IgE (1μg/ml). Cells were treated with a selective CXCR3 antagonist at concentrations indicated for 30 mins before stimulation with 10ng/ml Ag for 1 min followed by stimulation with a CXCR3 or CCR2 agonist for 1 hour. Samples were centrifuged for 10mins and supernatants transferred to new wells before addition of β-hexosaminidase substrate for 1.5 hours to measure β-hexosaminidase release. Data are expressed as % of the total β-hexosaminidase release. Results are expressed as mean ± SEM of 4 experiments performed in triplicate.

### 4.1.12 Up-regulation of Mast Cell Markers Stored within the Granules

Granule translocation to the plasma membrane is a key process for mast cells to degranulate. Two such markers that are utilised to investigate activation of myeloid linage cells are CD63 and CD203c; which are both upregulated in response to allergen challenge. CD63 is a 53-kDa glycoprotein present on the lysosome membrane of various cells. It has been reported to be able to translocate to the plasma membrane after activation in a wide variety of cell types (Jaiswal et al., 2002) and plays an important role in intracellular trafficking, recycling of plasma membrane components and integrin-dependent post-adhesion functions. It has been previously demonstrated to be expressed at a high density on activated basophil membrane (Metzelaar et al., 1991). Furthermore in the mast cell RBL model, CD63 has been shown to colocalise with SNARE proteins syntaxin 3 and VAMP7, which are involved in fusion of secretary granules with plasma membranes (Puri et al., 2003). CD203c expression among hematopoietic cells is restricted to basophils, mast cells and their precursors and has been described as specific for this lineage (Bühring et al., 1999). Due to its restricted expression pattern, CD203c is used as a specific marker to monitor the allergen-induced activation of mast cells.

Previously reported in this study was the induction of partial degranulation by CXCR3 agonists. By inducing degranulation it was possible that the fusing of the membrane and the granules could lead to an upregulation of the markers CD63 and CD203c. Figure 4.16 demonstrates that both CD63 and CD203c are up-regulated after activation of mature mast cells derived from cord blood in response to CXCR3 ligands. The number of cells expressing positive staining for each of these molecules after stimulation by CXCR3 agonists was approximately 50% of the levels induced by treatment with the calcium ionophores; ionomycin. This finding is consistent with previous data in this study; demonstrating that CXCR3 induced degranulation is only half the level observed with ionomycin treatment.

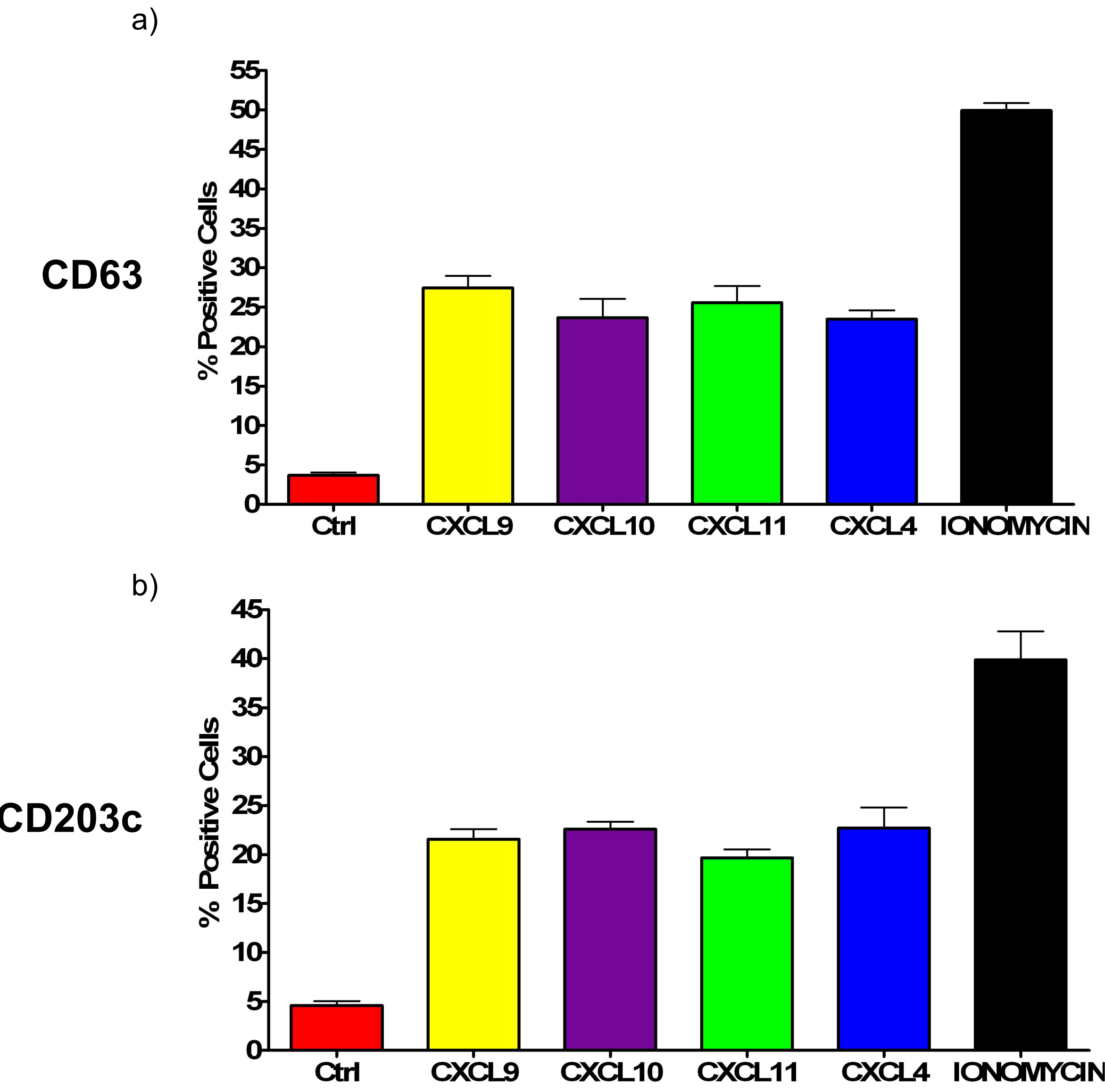



**Figure 4.16: Expression of CD63 and CD203c after CXCR3 activation-** Mature mast cells ($1x10^6$/ml) were treated with CXCR3 specific agonists (100nM) or ionomycin (1μM) for 1.5 hours. Samples were centrifuged for 10 mins and supernatant removed and remaining cells were collected. Mature mast cells ($1 \times 10^6$) were stained with a specific mouse-conjugated anti-CD63 (a) or CD203c (b) mAb or with appropriately isotype matched PE or FITC-conjugated mouse IgG control at the same concentration. Antibodies were incubated for 40 mins at 4$^o$C and staining examined by flow cytometry as described in *Materials and Methods*. Data is presented as percentage of positive staining above isotype control from three different donors. *N* = 3, mean ± S.E.M

### 4.1.13 Reorganisation of the cell membrane is critical for degranulation

Several studies have suggested the involvement of cytoskeletal rearrangements in mast cell degranulation. Actin polymerisation-inhibiting agents increase both the rate and extent of FcεRI-induced degranulation (Frigeri and Apgar, 1999; Oka et al., 2002) and biochemical studies indicated that FcεRI stimulation causes a rapid increase in the level of F-actin in RBL mast cells (Frigeri and Apgar, 1999; Pfeiffer et al., 1985). Furthermore, activation-induced rearrangement of microtubules is observed in rat peritoneal mast cells while tubulin polymerisation inhibiting agents block degranulation (Martin-Verdeaux et al., 2003; Nielsen and Johansen, 1986; Tasaka et al., 1991). The precise roles of the cytoskeletal rearrangements in the mast cell degranulation process have not been established.

With the finding in this study that CXCR3 agonists can induce the partial degranulation of mature cord blood derived mast cells, it was therefore likely CXCR3 agonists were inducing the re-organisation of the cytoskeleton in order to mediate the release of granules from mature mast cells. The three “classical” CXCR3 ligands previously have been shown to induce actin reorganization in intestinal myofibroblasts (Kouroumalis et al., 2005).

Figure 4.17 demonstrates that all four CXCR3 agonists can induce an increase in phalloidin binding indicative of actin polymerisation. Maximal actin polymerisation was observed for each agonist around 1-2 mins after stimulation with the levels of F-actin present decreasing back to basal after a 10 min stimulation (Fig 4.17).

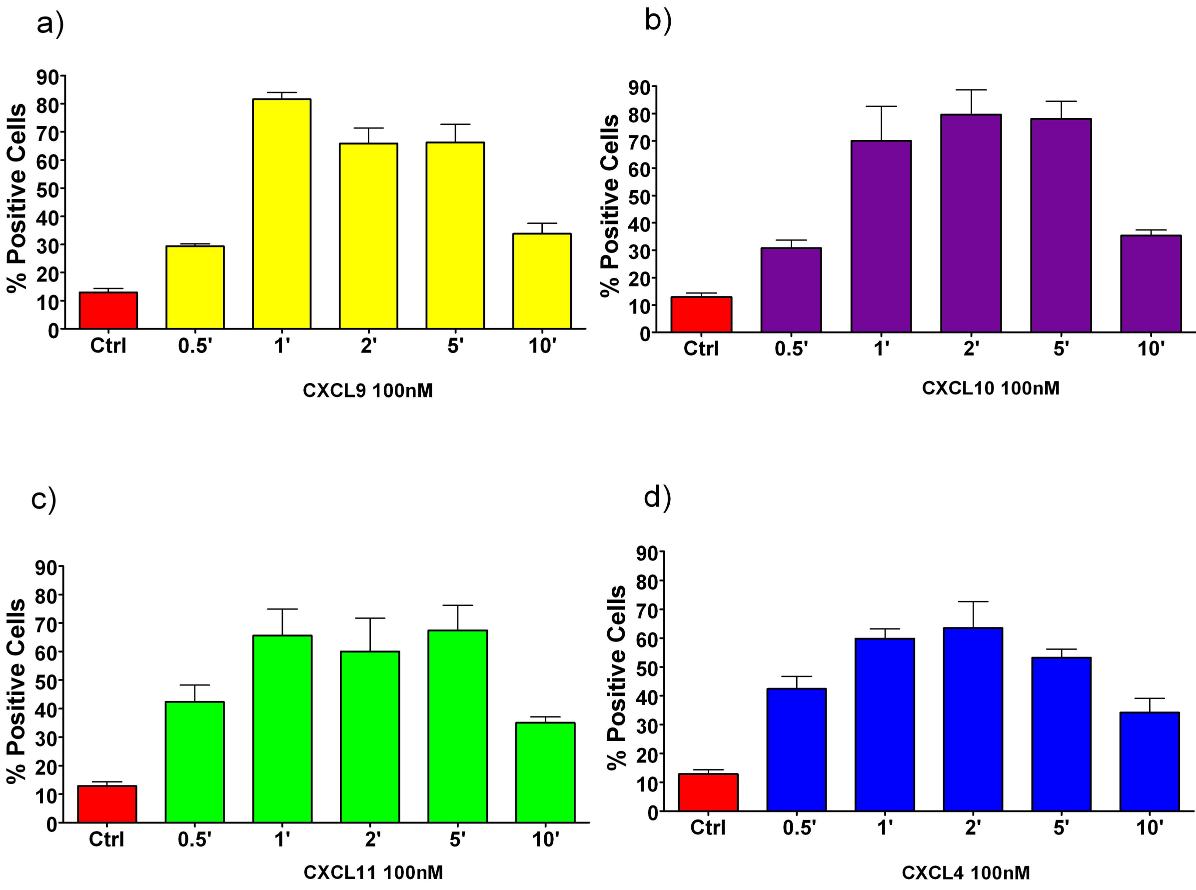



**Figure 4.17: CXCR3 agonists induce actin polymerisation in mature cord blood derived mast cells.** Human mature cord blood derived mast cells ($1x10^6$) were stimulated with CXCR3 agonists (100 nM) for the time periods indicated. Cells were fixed in 4% paraformaldehyde and permeabilised using 1% TRITON-X. Cells were stained with 0.3μM phallodin for 40mins at 4$^o$C. Samples were washed and re-suspended and analysed using a FACs canto. Data is presented as percentage of positive staining above negative control from three different donors. *N* = 3, mean ± S.E.M.

#### 4.1.14 Polymerisation of Actin by CXCR3 Agonists is CXCR3 Dependent

The reorganisation of actin by CXCR3 agonists further supported the evidence that CXCR3 agonists are capable of releasing mediators from mature mast cells. Figure 4.18 demonstrates that pre-treatment of human cord blood derived mast cells with the CXCR3 antagonists; T487 or NBI-74330 blocked CXCR3 but not CCR2 agonist induced actin reorganisation. This indicates that the CXCR3 agonists are only utilising a form of CXCR3 able to induce the reorganisation of the cellular membrane.

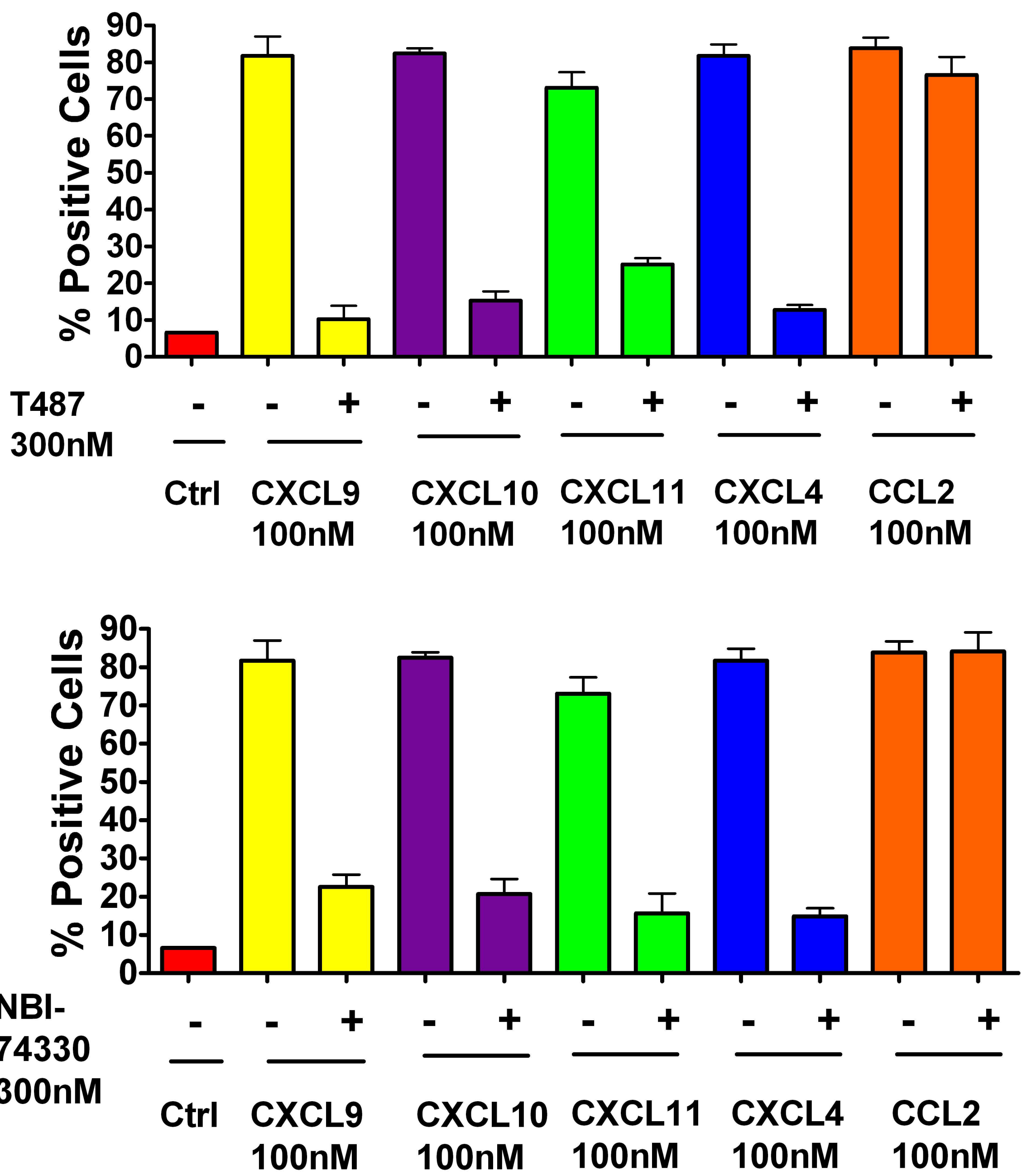



**Figure 4.18: CXCR3 agonists reorganise actin filaments in mature cord blood derived mast cells in a CXCR3 dependent manner.** Human mature cord blood derived mast cells ($1x10^6$) were incubated in the presence or absence of either T487 (300 nM, 30mins) or NBI-74330 (300 nM, 30mins) before stimulation with CXCR3 agonists (100nM, 2 min) Cells were fixed in 4% paraformaldehyde and permeabilised using 1% TRITONX. Cells were stained with 0.3μM phallodin for 40mins at 4°C. Samples were washed and re-suspended and analysed using a FACs canto. Data is presented as percentage of positive staining above negative control ± S.E.M. taken from 3 different donors.

### 4.1.15 Involvement of Rho kinase in Actin Reorganisation

Localised disassembly of cortical F-actin has long been considered necessary for facilitation of exocytosis. Exposure of permeabilised mast cells to calcium/ATP induces cortical F-actin disassembly (calmodulin-dependent) and secretion (calmodulin-independent) (Sullivan et al., 1999). The signals that generate the actin filament reorganization are often mediated by several theronine kinases such as Rho kinase (ROCK). Pre-treatment of human mast cells with the ROCK inhibitor Y27632 completely inhibited the partial degranulatory response induced by all of the CXCR3 agonists (Figure 4.19).

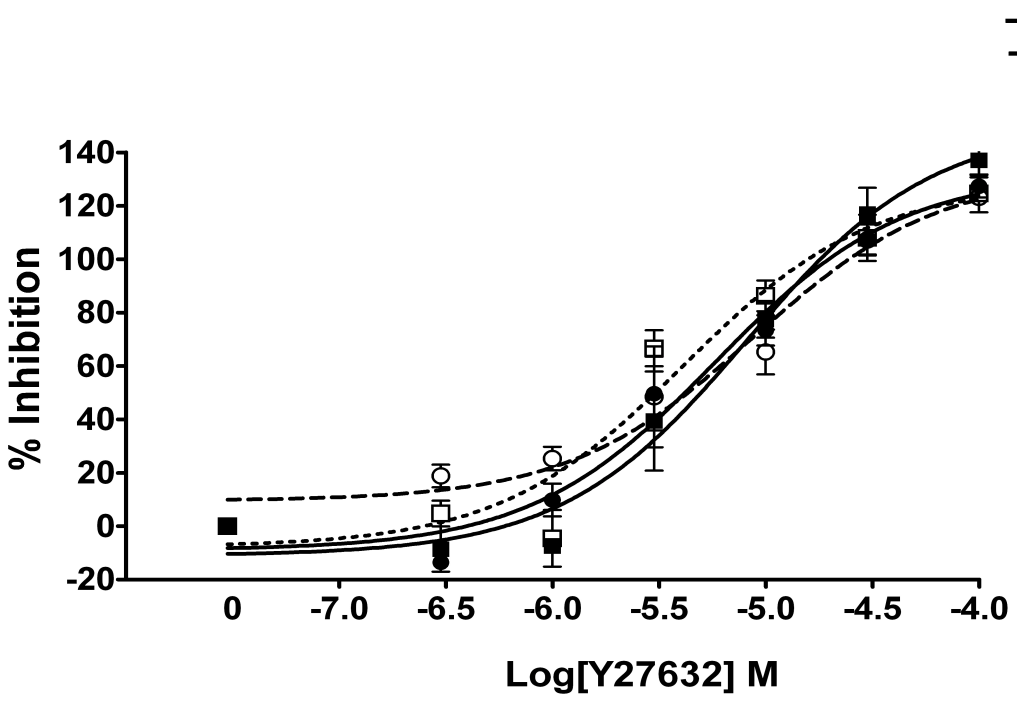



**Figure 4.19 – Inhibitory effect of Rho kinase inhibitor on CXCR3 induced partial degranulation.** Human mast cells ($1x10^6$) were pre-treated with Y27632 (30 min) at the concentrations indicated, before stimulation with CXCR3 agonists (100nM, 1hr). Samples were centrifuged for 10mins and supernatant transferred to new wells before addition of β-hexosaminidase substrate for 1.5 hours to measure β-hexosaminidase release. Data are expressed as % inhibition of agonist response. Results are expressed as mean ± SEM of 4 experiments performed in triplicate.

There is an overlap in cellular functions regulated by Rac and class I PI3K. CXCR3 can activate Rac via $PIP_3$-dependent Rac guanine nucleotide exchange factors (GEFS) (Wang et al., 2002). With the discovery that both Rho kinase and PI3K were involved in the mechanism behind CXCR3 induced partial degranulation, it was important to determine if both kinases were activated at different times. The reorganisation of actin occurs in the latter stages of the degranulation process in order to mediate the release of the mast cell mediators. Figure 4.20 reveals that ROCK but not PI3K is involved in the polymerisation of actin filaments suggesting that PI3K is involved in a much earlier stage of the degranulation mechanism, possibly in the mobilisation, but not the release of mast cell mediators.

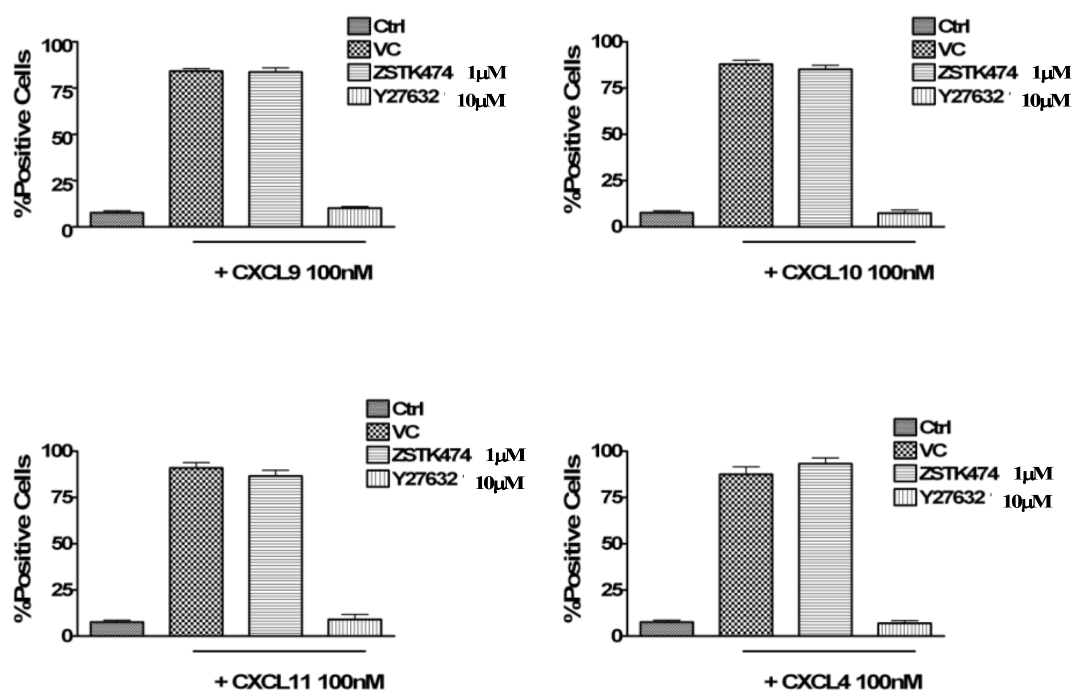



**Figure 4.20 – Reorganisation of actin filaments in mature cord blood derived mast cells is RHO kinase but not PI3K dependent.** Human mature cord blood derived mast cells ($1x10^6$) were incubated in the presence or absence of either a PI3K (ZSTK474, 1 μM, 30mins) or ROCK (Y27632, 10 μM, 30mins) inhibitor before stimulation with CXCR3 agonists (100nM, 2 mins). Cells were fixed in 4% paraformaldehyde and permeabilised using 1% TRITONX. Cells were stained with 0.3μM phallodin for 40mins at $4^oC$. Samples were washed and re-suspended and analysed using a FACs canto. Results are expressed as mean ± SEM of 4 experiments performed in triplicate.

### 4.1.16 CXCR3 Agonists do not Induce Calcium Mobilisation in Mature Mast Cells

Elevation of cytosolic calcium levels is one of the most robust biochemical signals elicited by chemokine agonists (Heise et al., 2005). Interestingly, treatment of mature mast cells with all of the CXCR3 agonists at a concentration of 100nM failed to induce an increase in cytosolic calcium (Figure 4.21). Treatment of mature mast cells with 100ng/ml Ag did induced prolonged increases in cytosolic calcium concentrations in mature mast cells.

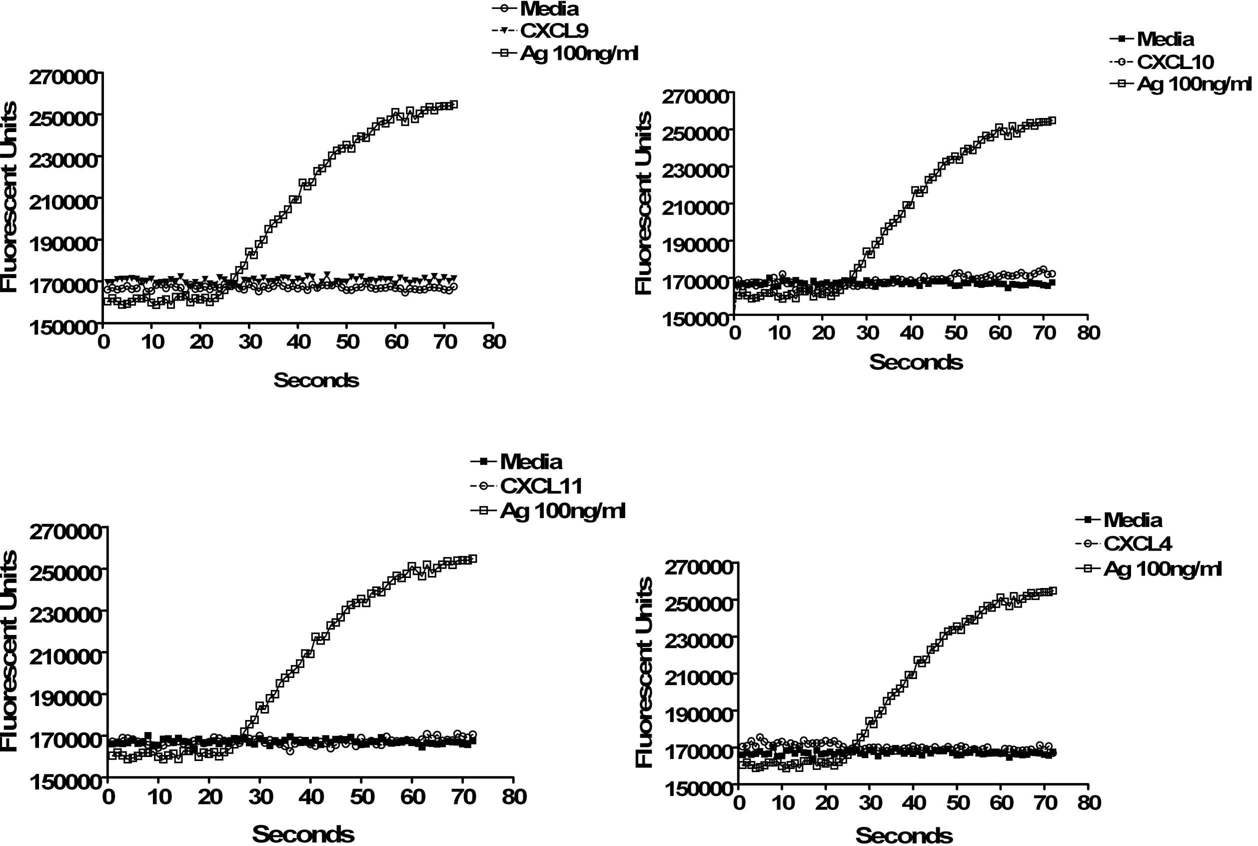



**Figure 4.21: Cytosolic $Ca^{2+}$ levels do not increase in mast cells stimulated with CXCR3 agonists.** Mature mast cells (50µl; 350,000 cells) were prepared and treated as described in the Material\Methods section. CXCR3 agonist (100 nM) or Ag (NP-BSA) (100 ng/ml) were applied to FLUO-4 AM loaded mast cells and fluorescence levels were recorded using a FLEXstation. Traces are representative of 3 different experiments.

Although no increases in intracellular $Ca^{2+}$ was detectable in mast cells, figure 4.22 clearly demonstrates that $Ca^{2+}$ is required for induction of mast cell degranulation. Treatment of mature mast cell with CXCR3 agonists in either $Ca^{2+}$ free buffer or in the presence of 1mM EGTA failed to induce degranulation of mature cord blood derived mast cells.

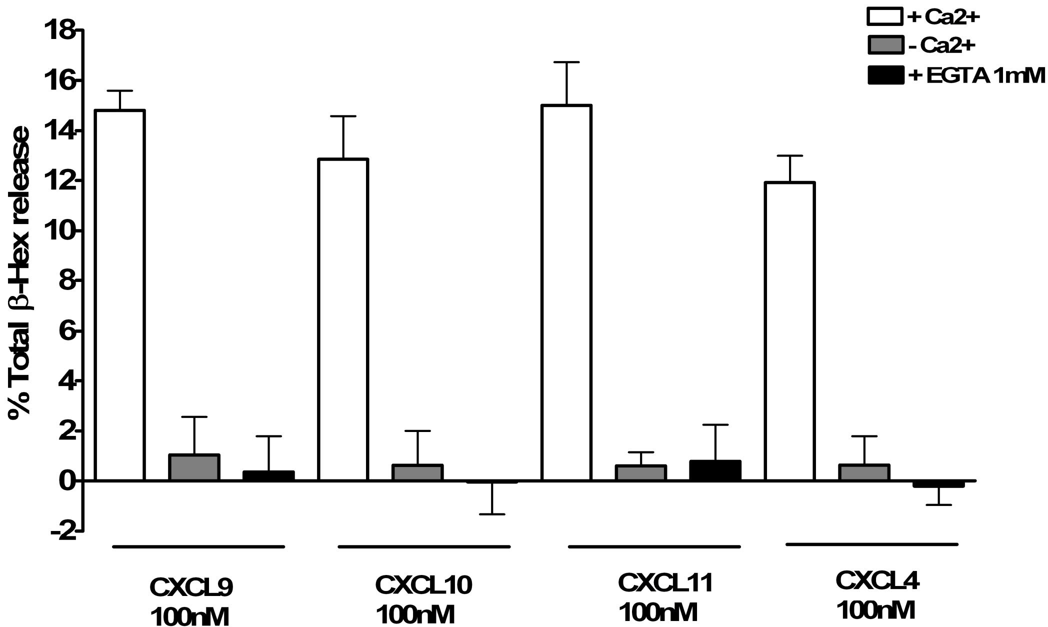


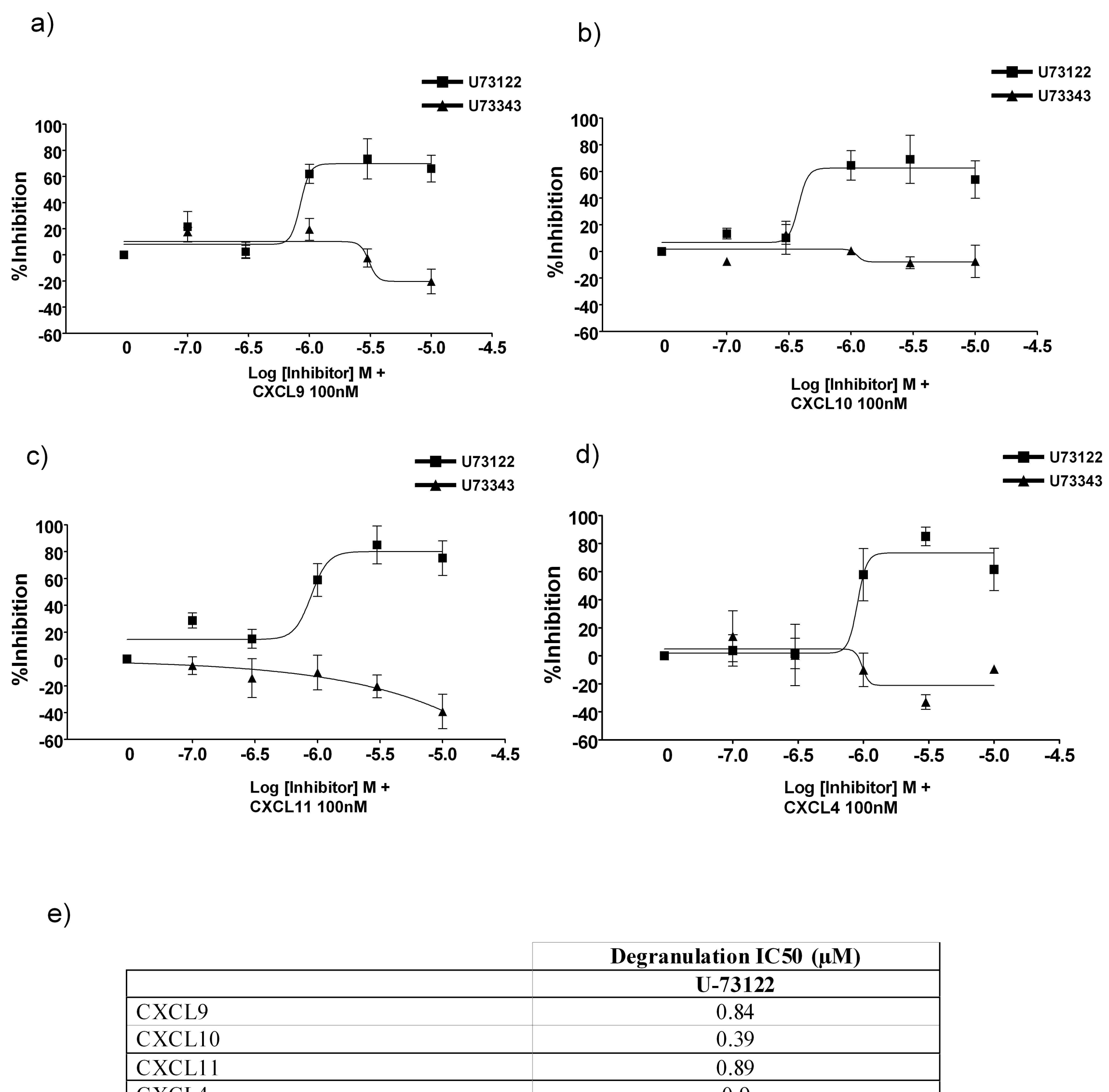

**Figure 4.22: Effect of different extracellular $Ca^{2+}$ conditions on mast cell degranulation.** Human mature cord blood derived mast cells ($1x10^6$) in indicated buffers (See Materials and Methods) were stimulated CXCR3 agonists (100 nM, 1hr). Samples were centrifuged for 10mins and supernatant transferred to new wells before addition of β-hexosaminidase substrate for 1.5 hours to measure β-hexosaminidase release. Data are expressed as % total β-hexosaminidase release. Results are expressed as mean ± SEM of 4 experiments performed in triplicate.

### 4.1.17 The Role of PLC of CXCR3 Induced Mast Cell Degranulation.

The signalling cascade involved in inducing mast cell degranulation requires the recruitment and activation of many different kinases and other signalling proteins. One pivotal step is the activation of phospholipase Cγ1 and 2 (PLCγ1/2), leading to hydrolysis of the lipid substrate $PIP_2$ resulting in the formation of two key signalling intermediates, diacylgylercol (DAG) and inositol 1,4,5-triphosphate ($IP_3$). DAG is the physiological activator of PKC and $IP_3$ the ligand for the $IP_3$ channel receptor (Berridge et al., 2003).

One of the major mechanisms by which calcium is mobilised from intracellular stores is via the binding of $IP_3$ on to its receptor located on the endoplasmic reticulum (ER) resulting in the release of $Ca^{2+}$ from the ER into the cytoplasm. PLCß can be activated by the Gßγ sub-units after chemokine receptor ligation (Jiang et al., 1997; Wu et al., 1992). To determine whether the increase in $[Ca^{2+}]_i$ following ligation of the CXCR3 receptor requires the enzymatic function of PLC isoforms, the use of U73122 a broad spectrum pharmacological inhibitor of PLC isoforms was emplyed. U73122 is reported to have an $IC_{50}$ between 500 nM and 2.1 μM (Smith et al., 1996).

CXCR3-agonist induced degranulation was abolished by U73122 but not by its inactive derivative U73343 indicating that PLC plays a role in the induction of CXCR3 induced partial degranulation of human cord blood derived mast cells (Figure 4.23 a-d). The respective $IC_{50}$'s for each agonist are shown in Figure 4.23e.

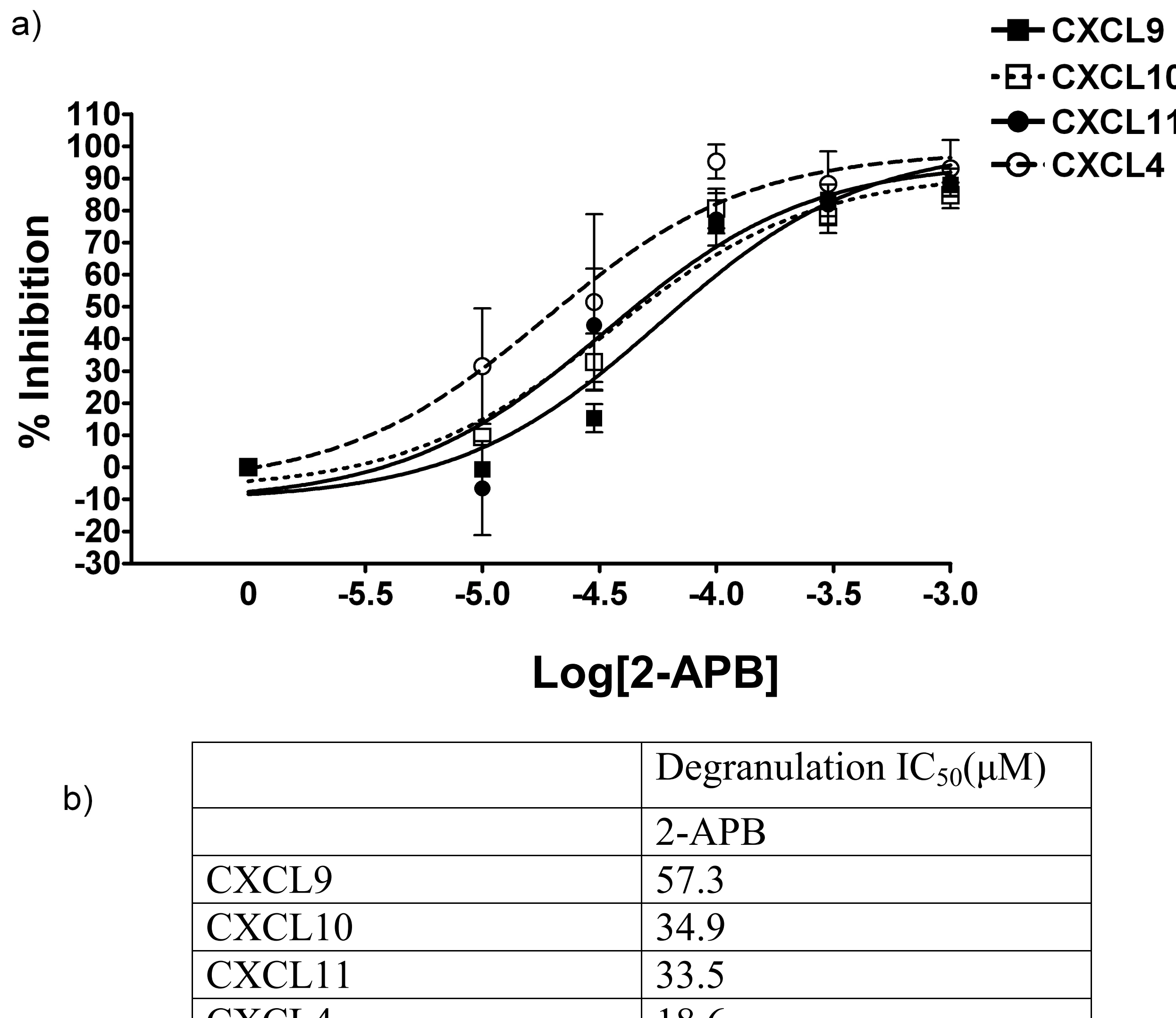



| | Degranulation IC50 (μM) |
|---|---|
| | U-73122 |
| CXCL9 | 0.84 |
| CXCL10 | 0.39 |
| CXCL11 | 0.89 |
| CXCL4 | 0.9 |

**Figure 4.23: Effect of PLC inhibitors on CXCR3 mediated degranulation.** Human mast cells ($1x10^6$) were treated with either U73122 or U73343 (30 min) at concentrations indicated before stimulation with CXCR3 agonists (100 nM, 1hr). Samples were centrifuged for 10mins and supernatant transferred to new wells before addition of β-hexosaminidase substrate for 1.5 hours to measure β-hexosaminidase release. Data are expressed as % inhibition of agonist response**.** Results are expressed as mean ± SEM of 4 experiments performed in triplicate.

The compound 2-aminoethyl diphenylborate (2-APB: also blocks TRP channels), an inhibitor of $IP_3$ receptors in some cell types, has been used to assess the role of IP3R mediated increases in intracellular $Ca^{2+}$ (Ma et al., 2001). 2-APB has been described as an inhibitor of calcium release from $IP_3$ stores without affecting binding of IP3 to $IP_3R$ and has a reported $IC_{50}$ of 42 μM (Ascher-Landsberg et al., 1999). The data presented in this study suggests that the mobilisation of intracellular $Ca^{2+}$ is required in the degranulation process. Figure 4.24a reveals that pre-treatment with the $IP_3R$ inhibitor 2-APB strongly inhibits CXCR3 induced degranulation. The respective $IC_{50}$s for each agonist are shown in Figure 4.24b.

a)




b)

| | Degranulation $IC_{50}$(μM) |
|---|---|
| | 2-APB |
| CXCL9 | 57.3 |
| CXCL10 | 34.9 |
| CXCL11 | 33.5 |
| CXCL4 | 18.6 |

**Figure 4.24: Effect of $IP_3$ inhibition on CXCR3 induced degranulation.** Human mast cells ($1x10^6$) were treated with 2-APB (30 min) at concentrations indicated before stimulation with CXCR3 agonist (100nM, 1 hr). Samples were centrifuged for 10mins and supernatant transferred to new wells before addition of β-hexosaminidase substrate for 1.5 hours to measure β-hexosaminidase release. Data are expressed as % inhibition of agonist response**.** Results are expressed as mean ± SEM of 4 experiments performed in triplicate.

## 4.2 The Role of PI3K in CXCR3 Induced Degranulation

The signalling mechanism that induces the degranulation of mature mast cells is a complex process involving a wide range of different signalling molecules. With the reported role of PI3K isoforms in IgE/Ag-mediated mast activation and degranulation, the role of PI3K in the degranulation of human cord blood derived mast cells by CXCR3 agonists was assessed. This was evaluated by inducing degranulation by CXCR3 agonists in the presence of increasing concentrations of the PI3K inhibitor ZSTK474. Pre-treatment of mast cells with ZSTK474 was observed to induce dose-dependent inhibition of CXCR3 agonist induced-degranulation of mast cells (Fig 4.25).

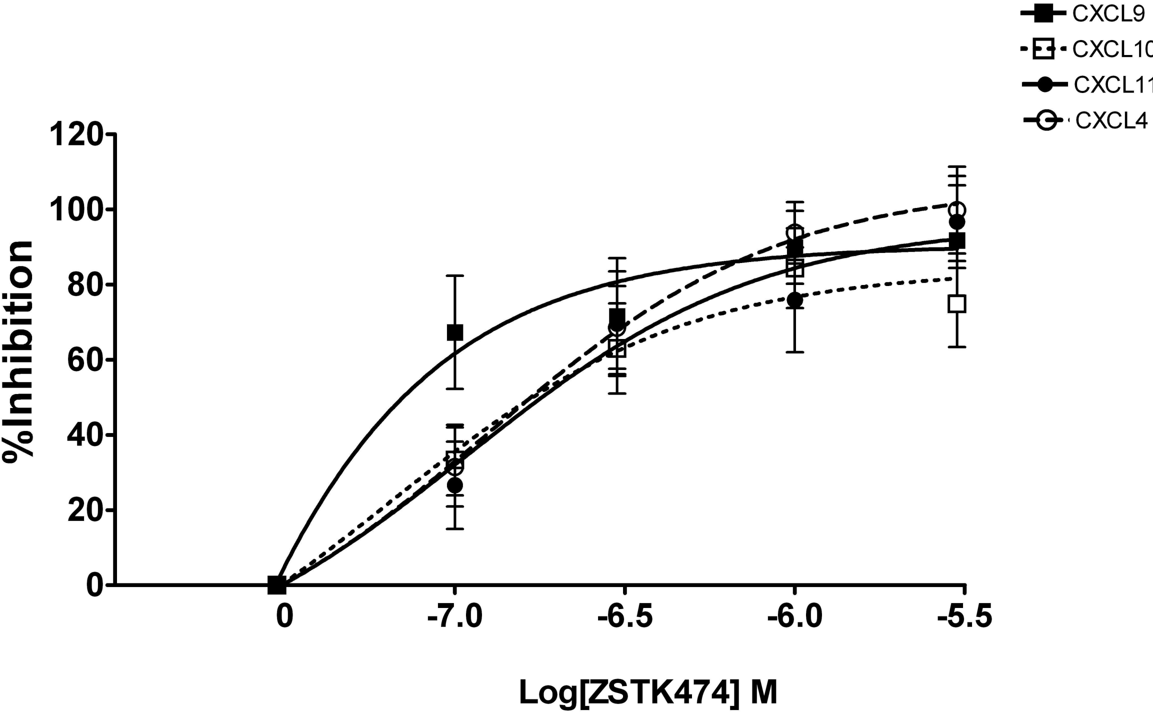



**Figure 4.25: Effect of PI3K inhibitor on CXCR3 agonist degranulation.** Human mast cells ($1x10^6$) were pre-treated with increasing concentrations of ZSTK474 (30mins) followed by stimulation with CXCR3 agonists (conc, 1hr). Samples were centrifuged for 10mins and supernatant transferred to new wells before addition of β-hexosaminidase substrate for 1.5 hours to measure β-hexosaminidase release. Data are expressed as % inhibition of agonist response. Results are expressed as mean ± SEM of 4 experiments performed in triplicate.

In the last decade, evidence has suggested that the PI3K catalytic subunit p110γ may play a role in mast cell hyperactivation. This is thought to occur via an auto/paracrine mechanism, whereby GPCR agonists released FcεRI activated mast cells can relieve PI3K negative regulation by antagonising the lipid phosphatases SHIP and PTEN (Harris et al., 2008). Recent studies employing the use of murine models demonstrated that both the PI3K isoforms p110γ and p110δ are required for FcεRI-driven mast cell degranulation in vitro, however in vivo p110 γ is required whereas p110δ is dispensable for allergic responsiveness (Ali et al., 2004; Ali et al., 2008; Laffargue et al., 2002).

Isoform-specific PI3K inhibitors were employed to investigate the role of individual PI3K catalytic isoforms in CXCR3-induced degranulation. The inhibitors utilised were IC87114 for PI3Kδ, TGX-221 for p110β , TGX-121 for PI3Kβ/PI3Kδ, PIK75 for PI3Kα and AS605240 for PI3Kγ.

PIK75, which displays around 116-fold selectivity against PI3Kα versus the δ isoform, was observed at concentrations around the predicted $IC_{50}$ to have no effect on mast cell degranulation induced by CXCR3 agonists (Figure 4.26a). Figure 4.26b reveals that at the predicted $IC_{50}$ for p110β inhibitor TGX-221 there is no inhibition of CXCR3 agonist induced degranulation. Whereas the equipotent inhibitor of p110β and p110δ; TGX-121 was observed to have a strong inhibitory effect on CXCR3 induced degranulation (Figure 4.26c). The $IC_{50}$ for TGX-121 on CXCR3 induced degranulation is well below the predicted impingement of the next PI3K isoform. This would suggest that p110δ is the dominant isoform involved in CXCR3 induced degranulation however the p110δ -discriminating inhibitor IC87114 only partially inhibits CXCR3 agonist induced degranulation at the quoted $IC_{50}$ for the p110δ isoform (Figure 4.26d). Furthermore there is only any notable inhibition of CXCR3 agonist induced degranulation when the inhibitor concentration reaches levels expected to impinge on p110β. This therefore suggests a synergy between the p110β and p110δ catalytic isoforms of PI3K in promoting

CXCR3 evoked mast cell degranulation. These findings compares to my previous observation that IgE/Ag induced degranulation that reported a good correlation between $IC_{50}$'s obtained for IC87114 against p110δ using purified enzyme versus $IC_{50}$'s derived from cell based assays (Crabbe et al., 2007; Knight et al., 2006; Smith et al., 2007).

AS605240 displays around 10-fold selectivity against p110γ versus α isoforms but approximately 35-fold selectivity vs δ/β. AS605240 inhibited CXCR3 agonist induced β-hexosaminidase release with an $IC_{50}$ comparable to its reported $IC_{50}$ for p110γ (Figure 4.26e) and possible concerns about off-target effects of AS606240 on p110α at concentrations >10 nM seem unfounded, as the p110α-targeting inhibitor PIK75 had no effect on degranulation (Figure 4.26e). Table 4.1 lists the $IC_{50}$ values of each PI3K isoform inhibitor against degranulation induced by each CXCR3 agonist. Similar $IC_{50}$'s for each inhibitor where observed against each CXCR3 agonist.

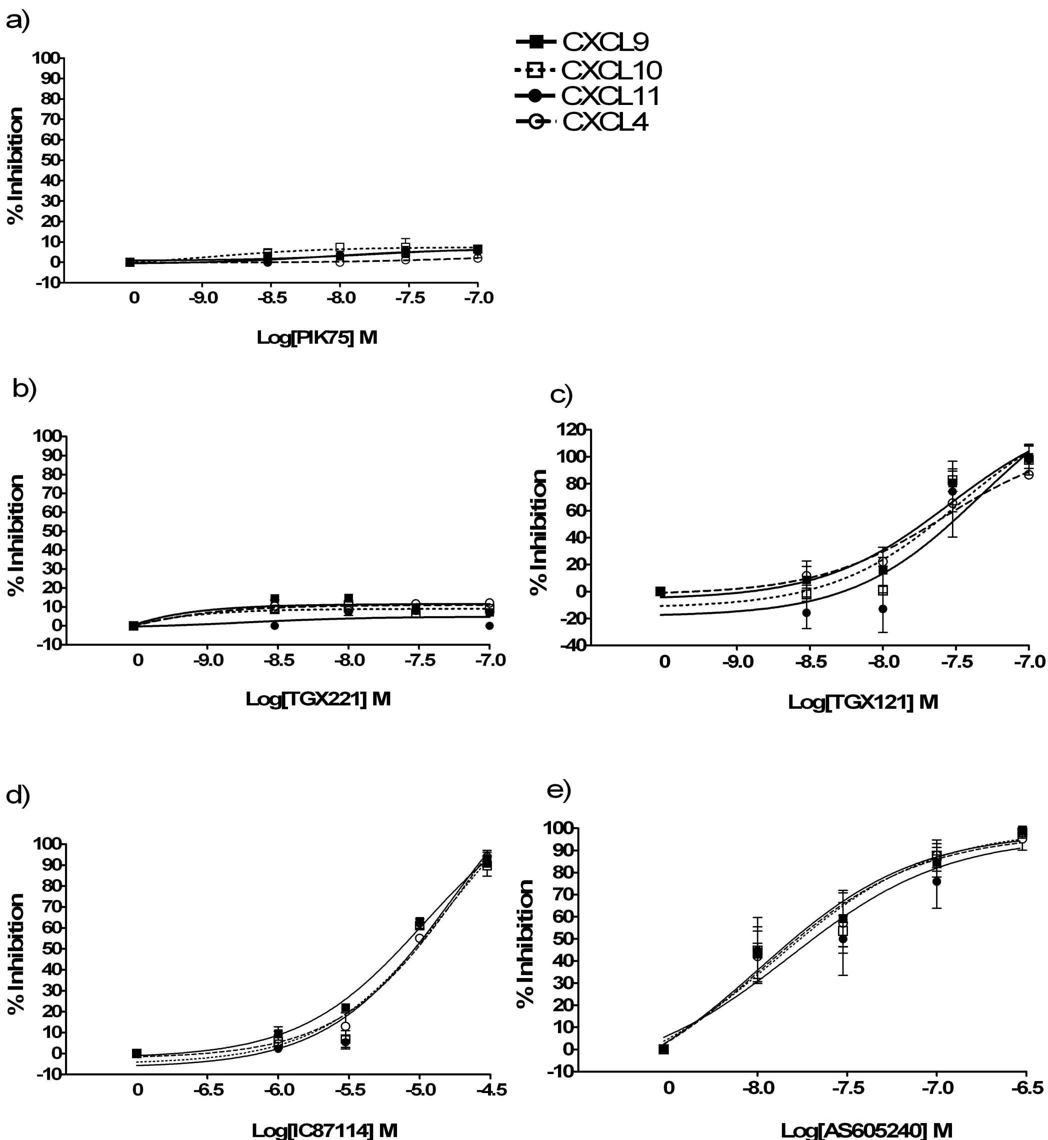



**Figure 4.26: Effect of PI3K isoform specific inhibitors on CXCR3 agonist induced degranulation.** Human mast cells ($1x10^6$) were pre-treated with increasing concentrations of PI3K isoform-discriminating inhibitors (30 min) before stimulation with CXCR3 agonist (100nM, 1hour). Samples were centrifuged for 10mins and supernatant transferred to new wells before addition of β-hexosaminidase substrate for 1.5 hours to measure β-hexosaminidase release. Data are expressed as % inhibition of chemokine agonist responses. Results are expressed as mean ± SEM of 4 experiments performed in triplicate.

| | **$IC_{50}$ (μM)** | | | | |
|---|---|---|---|---|---|
| | **CXCL9** | **CXCL10** | **CXCL11** | **CXCL4** | **IgE/Ag** |
| PIK75 | >0.1 | >0.1 | >0.1 | >0.1 | 0.65 |
| TGX-221 | >0.1 | >0.1 | >0.1 | >0.1 | 0.44 |
| TGX-121 | 0.046 | 0.028 | 0.033 | 0.026 | 0.031 |
| IC87114 | 10.66 | 15.49 | 16.65 | 19.92 | 4.81 |
| AS605240 | 0.0101 | 0.0124 | 0.0151 | 0.0105 | 0.0228 |
| ZSTK474 | 0.003 | 0.064 | 0.108 | 0.124 | 0.637 |

**Table 4.1 -** $IC_{50}$ values for inhibition of degranulation induced by CXCR3 agonists and IgE/Ag were obtained by comparing concentration-dependent effects of individual inhibitors against optimal degranulation in response to 100 nM of specific chemokines or 100ng/ml NP-BSA(Ag). *N = 4.*

### 4.2.1 Further Assessing the Role of p110δ and p110β in Mast Cell Degranulation

The finding that IC87114 and TGX-221 did not inhibit degranulation induced by both IgE/Antigen and CXCR3 agonists at their predicted $IC_{50}$ values, yet TGX-121 inhibited CXCR3 induced degranulation responses, suggested the potential synergy of the delta and beta isoforms of PI3K. To evaluate this hypothesis mast cells were treated with approximate $IC_{50}$ concentrations of IC87114 and TGX-221 in combination. A combined treatment of TGX-221 and IC87114 resulted in full inhibition of CXCR3 induced degranulation (Fig 4.27). This observation further supports the idea that the p110β and p110δ catalytic isoforms of PI3K act in synergy to promoting CXCR3 evoked mast cell degranulation.

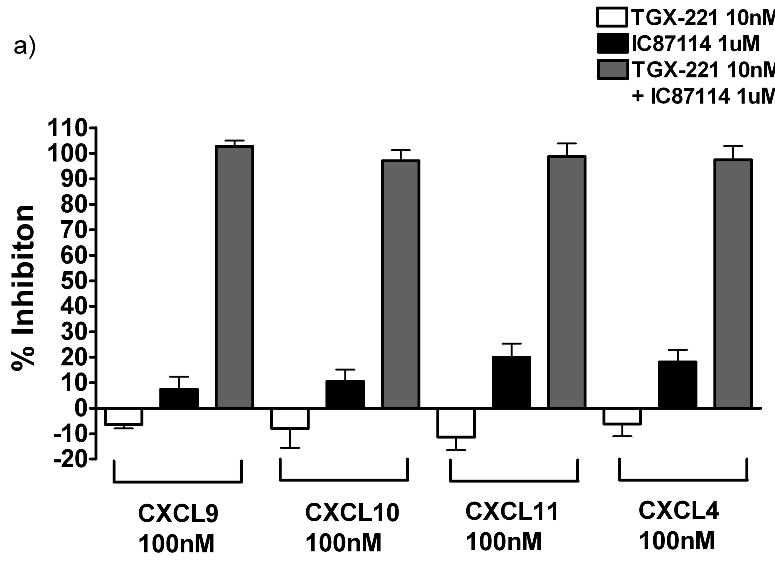



**Figure 4.27 – Combination of PI3K isoform inhibitors on CXCR3 degranulation.** Human mast cells ($1x10^6$) were pre-treated with either TGX-221 (10 nM, 30 min) and/or IC87114 (1μM, 30 min) before stimulation with CXCR3 agonists (1 hr). Samples were centrifuged for 10mins and supernatant transferred to new wells before addition of β-hexosaminidase substrate for 1.5 hours to measure β-hexosaminidase release. Data are expressed as % inhibition of control agonist responses. Results are expressed as mean ± SEM of 4 experiments performed in triplicate.

Activation of PI3K leads to the formation of 3- phosphorylated phosphoinositides which are able to recruit various signalling molecules to cellular membranes, once located at the membrane these signalling molecules can be phosphorylated by there activating kinases. Therefore measurement of the phosphorylation of these signalling molecules is a useful method to assess PI3K activity. One such signalling molecule is the protein AKT which is phosphorylated by PDK1 (T308) and MTORC2 (S473).

In order to determine which PI3K isoforms are activated downstream of CXCR3; the effect of the isoform specific PI3K inhibitors on AKT phosphorylation was assessed by western blot. Stimulating mast cells with CXCL9 in the presence of the class I PI3K inhibitor ZSTK474 resulted in a loss of AKT phosphorylation (Figure 4.28a). The p110α inhibitor PIK75 had no effect on CXCL9-stimulated Akt phosphorylation (Fig 4.28b). The p110-γ targeting AS605240 resulted in partial inhibition of Akt phosphorylation (Figure 4.28b). The inhibition by AS605240 was incomplete even at concentrations well above the reported $IC_{50}$ values (Figure 4.28b). Similar to the results obtained for degranulation responses, the dual p110β/δ-targeting TGX-121 completely inhibited Akt phosphorylation (Fig 4.28e). Furthermore the combined (but not individual) treatment of TGX-221 and IC87114, at concentrations predicted to be selective for β and δ isoforms respectively, also inhibited CXCL9-stimulated Akt phosphorylation (Fig 4.28c-e).

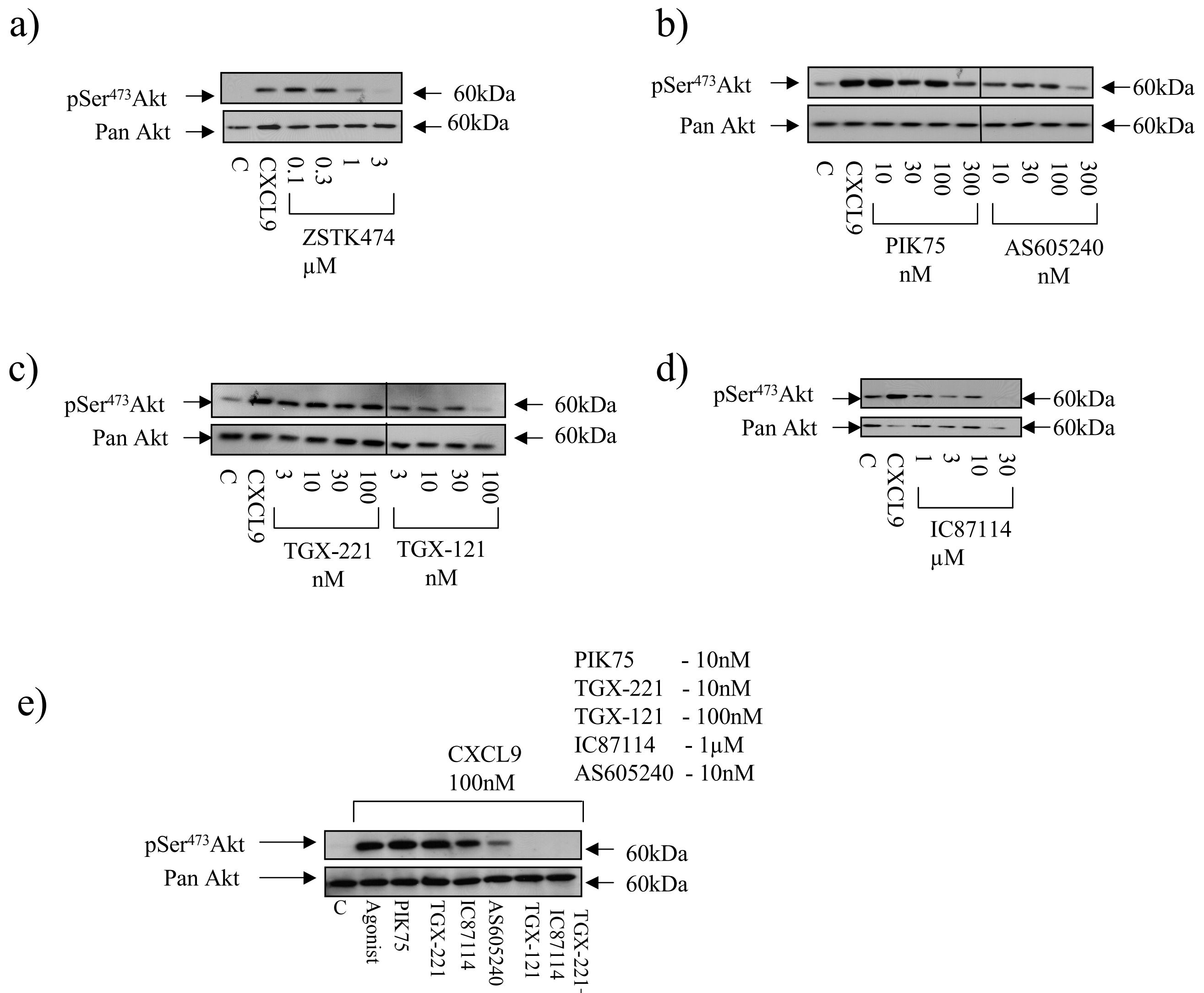



**Figure 4.28**: **Effect of PI3K inhibitors on CXCL9, CXCL10, CXCL11, and CXCL4 induced phosphorylation of Akt.** (*A-E*) Mature mast cells ($1 \times 10^6$ cells/ml) were pre-treated with vehicle or PI3K isoform-discriminating inhibitor at concentrations indicated (30 mins) before stimulation with CXCL9 (100 nM, 2 min) and lysed by the addition of 1× sample buffer. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with a phospho-specific Akt Ab with affinity for the active Ser473-phosphorylated form of Akt and proteins were visualised with ECL. The blots were stripped and reprobed with anti-Akt Ab to verify equal loading and efficiency of protein transfer (*lower panel*). The data are representative of two experiments.

### 4.2.2 Summary

- CXCR3 agonists induced partial degranulation of cord blood derived mast cells in a CXCR3 and PTX dependent manner.
- CXCL4 induced responses were PTX insensitive therefore suggesting that it is not limited to mediating its effect via the PTX insensitive CXCR3-B receptor.
- Other inflammatory chemokines induce partial degranulation of mature mast cells.
- CXCR3 agonists are capable of inducing the phosphorylation of ERK and Akt in mast cells.
- The phosphorylation of ERK and AKT by all tested CXCR3 agonists was PTX sensitive.
- CXCR3 mediated effects in mast cells are PI3K dependent.
- The PI3K isoforms suggested to be involved in these responses are p110γ and p110β in synergy with p110δ isoforms.

## 4.3 Discussion

### 4.3.1 Expression of CXCR3 Variants in Cord Blood Derived Mast Cells

The cord blood derived mast cell model appeared to be an appropriate model to study CXCR3 expression and function on mast cells. All variants of CXCR3 were expressed at the mRNA level and proteins expression of at least one CXCR3 variant was detectable upon the surface. It was notable that protein levels of CXCR3 decrease as CD133+ precursors differentiate and mature into mast cells. This down regulation of CXCR3 was surprising as all variants of CXCR3 were detectable at the mRNA throughout the maturation process. There are several possible explanations for the apparent disparity between detection of CXCR3 mRNA and lack of detectable protein expression.

Firstly, the CXCR3 receptor could become post translationally modified which interferes with the ability of commercial available antibodies to recognise this receptor. Indeed, previous reports have shown that some human chemokine receptors are alleged to be sulphated and/or glycosylated at their N-terminal extracellular domains, (Colvin et al., 2006; Neel et al., 2005). Secondly, CXCR3 variants maybe expressed at the mRNA level but may either not be translated to protein or rapidly degraded post-translationally. In this instance, a novel receptor expressed on human cord blood derived mast cells may be responsible for the biochemical and functional responses observed.

### 4.3.2 Up-Regulation of Chemokine Receptors after Mast Cell activation.

The presented study reveals that levels of CXCR3 were highly expressed intracellularly at the progenitor stage. Despite degradation of CXCR3 as the cells mature, intracellular levels of CXCR3 were equal to or higher, than was expressed on the surface. The chemokine receptor expression profile expressed on mat cell is thought to be mainly expressed within cytoplasmic granules. Consequently it is believed upon allergic activation, chemokine

receptors, especially CCR3 expression is increased on the cell surface (Juremalm and Nilsson, 2005). It is possible that this could apply to CXCR3.

The CXCR3 expression data presented in this thesis is in contrast to other studies of CXCR3 in mast cells. Other investigations have reported that in human bone marrow-derived precursors, expression of CXCR3 at a protein level is low and upon maturation, protein levels of CXCR3 are up-regulated (Brightling et al., 2005). Brightling et al, (2005) reports a receptor profile on cord blood derived mast cells including CCR1, CCR2, CCR3, CCR7, CXCR1, CXCR4, with CXCR3 being the most highly expressed. The differences between these studies and the work presented in this thesis highlight that mast cells derived from different sources have differing chemokine receptor expression profile.

Human mast cells differentiated from cord blood developed by cytokine control have found to be comparable to human mast cells in the lung and gut mucosa by similarities found in the sub-structural granule patterns of the cells and that the mature cells contained only tryptase (Mitsui et al., 1993). There appears to be a consider heterogeneity in the chemokine receptor expression profile of mast cells according to species. This is dependent on whether they are cord blood derived or bone marrow derived and their final anatomical location (Juremalm and Nilsson, 2005). Hence, a mast cells chemokine receptor profile is most likely chaped by the cytokine milieu present at the relevant progenitor stage. The chemokine receptor profile will be crucial in determining the homing progenitor and mature mast cells.

### 4.3.3 Chemokine Agonists Induce Partial Degranulation of Human Mature Mast Cells

To investigate the functional role of CXCR3 agonists within mature mast cell degranulation and potential mechanisms behind this degranulation process, functional and biochemical studies were performed following stimulation with CXCR3 agonists. The work began by carrying out the control experiments with the intention of studying the potential of CXCR3 augmentation of sub-optimal antigen induced degranulation of mature mast cells. Previous studies

in mast cells have shown that GPCR agonists such as CCL5 and CCL3 are capable of augmenting antigen-induced degranulation in a non-additive manner (Laffargue et al., 2002). The evidence presented in this study using human cord blood derived mast cells as a model indicates that several chemokines operating through different chemokine receptors such as CXCR3, CCR3 and CCR2 can induce partial degranulation of mature mast cells in the absence of antigen.

Evidence presented in this thesis highlights that not all chemokines are capable of inducing partial degranulation of human mast cells but are still able to induce biochemical signals. The data demonstrated that other chemokines, for example, CXCL12, can elicit signalling events but this is not sufficient to elicit partial degranulation. This suggests that inflammatory chemokines, and not homeostatic chemokines, are capable of inducing the partial degranulation observed. CXCR3 agonists are classed as inflammatory chemokines due to their nature of being all released after IFN-γ stimulation. With the inflammatory nature of CXCR3 agonists, they are implicated in a wide range of inflammatory diseases.

One of the hallmarks of allergic inflammatory disorders is the accumulation of an abnormally large number of leukocytes including eosinophils, neutrophils, lymphocytes, basophils and macrophages in the lung (Bousquet et al., 1990). Another distinguishing feature that defines allergic inflammatory disorders is the activation of leukocytes by the release of biologically active mediators from such cells as mast cells (Rothenberg et al., 1999). Chemokines are key mediators in the recruitment of leukocytes to the site of inflammation and the subsequent release of a further wide range of mediators. Consequently chemokines are a key component in the pathogenesis of inflammatory responses in many allergic diseases such as asthma (Baggiolini and Dahinden, 1994). The data presented here could provide new insight in to the role of chemokines in inflammatory disease.

All the chemokines observed in this study induced the partial degranualtion of mature mast cells. To date they have been named “inflammatory” chemokines. The only chemokine not to induce degranulation, yet still able to elicit biochemical signals in this mast cell model was CXCL12, a so- called “homeostatic” chemokine. This revelation could provide a new role for chemokines in inflammatory disorders. If chemokines are capable of inducing the release of mediators from mature mast cells, this in turn will trigger further immune responses, initating an immune cascade that could result in damage to the host.

The data presented in this study is in contrast to other research using murine bone marrow-derived mast cells, where it was found several chemokines (CCL2, CCL5, CXCL10 and CXCL4), are incapable of inducing partial degranulation of mast cells (Taub et al., 1995). This serves to underline the view that there is considerable heterogeneity in mast cell responsiveness and phenotype according to species. The purpose of this chemokine-mediated partial degranulation in response to higher concentrations of chemokine agonists is likely to function to optimise mast cell activation and tailor it according to the local inflammatory context. Since CXCR3 agonists have been reported to be present in high levels of allegic inflammatory diseases (Widney et al., 2000; Woodman et al., 2006),the data portrayed here indicates that CXCR3 agonists can contribute to, and possibly perpetuate, the allergic response by inducing partial mast cell degranulation.

### 4.3.4 The Role of CXCL4 in Mast Cells

CXCL4 was previously reported to bind with high affinity to the receptor CXCR3B, the first known chemokine receptor to be accredited to CXCL4, despite being the first chemokine discovered. Lasagni et al., (2003) demonstrated that CXCL4 binds with low nanomolar affinty to CXCR3 B but not CXCR3A. This study also demonstrated that this was a functional receptor capable of intracellular signalling but was unable to induce migratory responses. Recent reports challenge the findings of this particular study. Mueller et al., (2008) demonstrated that in L1.2 cell expressing individual

isoforms of CXCR3 A or B, CXCL4 was able to bind both isoforms with low affinty and was able to induce chemotactic responses via both receptors. This highlights the possiblity that CXCL4 can mediate effects through other forms of CXCR3 similar to the findings observed in the work presented here.

The original study that discovered CXCR3B reported microvascular endothelial transfectants expressing CXCR3B were coupled to Gαs proteins. This is contrast to the other isoforms which are reported to bind to the Gαi protein. Unlike Gαi, Gαs is not reported to be sensitive to PTX. Mueller et al., (2008) reports that CXCL4 mediates response in CXCR3B transfectants in a PTX sensitive manner indicating the presence of a form of CXCR3 that is Gαi/o bound. It is understood that CXCL4 can play a wide role in inflammatory responses. Recent data has suggested that it can contribute to immune activation and T cell trafficking as part of the pathogenesis of ECM in experimental cerebral malaria models (Srivastava et al., 2008). The high concentrations of CXCL4 reported to be released by activated platelets might suggest that CXCL4 could play a role in the subsequent recruitment of lymphocytes into the tissues. Based on the data in this thesis the activation of mast cells by CXCL4 could result in the large amplification of the immune response suggesting it is possible that the blockade of the CXCR3/CXCL4 axis could be a good target in atherosclerosis (Mueller et al., 2008).

This investigation only detected the presence of CXCR3B at an mRNA level with no detectable presence of protein suggesting that CXCR3B did not play a role in the responses observed. The roles of CXCR3A and CXCR3B have posed interesting questions. One proposal is that CXCR3A and CXCR3B have opposite regulatory effects. This model was proposed by Romagnani et al., (2005) who examined the opposite regulatory effects of CXCL10 and CXCL4 on human Th1/Th2 polarisation. Luster and Ravetch, (1987) demonstrated the Th1-polarising activity of CXCL10 providing a positive amplification loop with IFN-γ which is its major inducer. A line of thought developed whereby the generation of CXCR3 agonists could help in the protection of Th2-mediated allergic inflammatory responses by amplifying the TH1 mediated responses against environmental allergens (Gangur et al.,

1998). CXCL4, however, in its physiological role, is a cationic protein stored in the granules and secreted upon platelet activation (McLaren et al.1993). With regard to allergic disease increased expression of CXCL4 has been detected in human asthmatic subjects (Averill et al., 1992; Yamamoto et al., 1993), indicating a role of CXCL4 in Th2 diseases.

The data in this thesis provides a new avenue to be explored in examining the role of CXCL4 in allergic disease. The release of CXCL4 by platelets is providing key signalling in the amplification of inflammatory diseases by mediating granule release from mature mast cells present in the airways.

#### 4.3.5 CXCR3 Agonists Induce a Short Signalling Profile

This study investigated biochemical signalling of CXCR3 agonists on mature mast cells concentrating specifically on the phosphorylation of the protein Akt and Erk1/2. The signalling profile of all CXCR3 agonists is fairly similar in length. The signalling profile of CXCR3 agonists varies depending on cell type. It has been observed that in intestinal myofibroblasts the signalling profile of CXCR3 agonists is longer than observed in this particular study, whereas the signalling profile in peripheral blood mononuclear cells is quite similar to the signalling prolife in mast cells observed in this thesis (Kouroumalis et al., 2005). This may reflect differences in the expression of regulatory components such as phosphatases between the different cell types. The robustness and duration of the activation of a given signalling pathway have far-reaching biological consequences.

Studies of CXCR3 activation by CXCL11 leads to sustained phosphorylation of Akt in T cells that is similar to that previously observed for CXCL12 and CXCR4 but distinct from other chemokine receptors (Smit et al., 2003). This is in contrast to the data presented in this thesis, which demonstrates that in mast cells activation is similar for each ligand and not sustained. Persistent activation of Akt by CXCL12 has been explained by the fact CXCR4 is involved in homeostasis rather than inflammation. Therefore sustained activation could protect $CXCR4^+$ cells from undergoing apoptosis- a process that is critical for the activation of T cells (Tilton et al., 2000).

In the case of CXCR3, sustained activation of Akt has only been observed at low concentrations, whereas higher concentrations of the activation of Akt is transient (Smit et al., 2003). It is proposed that the chemokine signalling profile can induce different properties in different cell types. Vlahakis et al., (2002) demonstrated that CXCR4 activation in CD4 T cells by CXCL12 led to the activation of the prosurvival secondary messengers, Akt and extracellular signal-regulated protein kinase. In contrast Akt activation through CXCR4 by SDF1alpha interactions is necessary to confer resistance to apoptosis.

#### 4.3.6 CXCR3 Specificity of the CXCR3 Agonists

The recent discovery of CXCL11 binding to another chemokine receptor CXCR7 raised the question of the specificity (is this a word) of the CXCL11 responses noted in this study. CXCR7 is another high affinity receptor for the inflammatory chemokine CXCL11 and the homestatic chemokine CXCL12. Despite binding these two chemokines, it is thought that the CXCR7 signalling pathway is distinct from the typical GPCR mechanism of other CXC chemokine receptors (Burns et al., 2006). It is still believed that CXCR7 provides an advantage in growth and survival and evidence suggests CXCR7 is constitutively active in tumour cells (Burns et al., 2006; Meijer et al., 2008; Miao et al., 2007). These studies demonstrate that CXCR7 is a key factor in the growth and expression of tumour cells in breast and lung cancer and could play a role in other malignancies. Treatment with a small molecule weight antagonist against CXCR7 limits growth in syngenic and xenograft models thus highlighting further a key role in potential cancer diseases (Burns et al., 2006).

Many atypical receptors exist in the chemokine system. The most studied are DARC and D6 whose defined roles are the scavenging of chemokines from the system. It has recently been proven that CXCR7 can scavenge the chemokine CXCL12. Investigations have suggested a regulated expression of CXCR7 and CXCR4 in migrating cells of the primordium,CXCR7 is found predominantly in trailing cells and CXCR4 in the front cells (Dambly-Chaudière et al., 2007; Valentin et al., 2007). This evidence suggests that

CXCR7 is an atypical receptor. CXCR7 shares many features similar to DARC and D6. Mutations in the DRYLAIV motif and the glycine associated with the NxxPY motif is much further down stream than in other chemokine receptors similar to D6.

A more recent investigation has discovered further evidence of CXCR7 as a decoy receptor (Boldajipour et al., 2008). This study discovered the enhanced internalisation of CXCR7 in somatic cells suggesting that CXCR7 acts as a sink for SDF-1a, thus allowing the dynamic changes in the transcription of SDF-1a to be mirrored by similar dynamics at the protein level.

Based on recent research it is now understood that the differential expression of CXCR4 and CXCR7 might provide a fine-tuning of CXCL12-migration. This view is also supported by the concept that CXCR7 acts as a scavenger receptor but does not exclude the possibility that the receptor also activates signalling pathways. It is possible that this role of CXCR7 and CXCL12 also applies for CXCL11 with CXCR7 tailoring CXCL11 responses to specific situations. No obvious or visible protein levels of CXCR7 were detected in this study suggesting that other receptors must act as scavenger receptors of CXCL11 induced responses.

### 4.3.7 CXCR3 Augmentation of Sub-Optimal Degranulatory Responses.

This research has proved that all CXCR3 agonists are capable of inducing augmentation of sub-optimal Antigen induced degranulation of human mast cells. This augmentation process has been noted with other GPCRs. The degranulation of mast cells by GPCRs in the absence of antigen was first observed by (Tilley et al., 2000). It was then subsequently reported by (Laffargue et al., 2002) that CCL5 and CCL3 as well as other GPCR agonists like ATP, ADP, inosine and LPA were capable of augmenting antigen-induced calcium influx and mast cell granule release. All these investigations, including this one, highlighted the role of GPCR agonists in mast cell activation and involvement in many immune reactions: for example- system

anaphylaxis. A variety of research models has shown the activation of mast cells by chemokines both in vitro and in vivo (Alam et al., 1992; Alam et al., 1994; Conti et al., 1995) further emphasising a key role of a wide range of chemokines in mast cell activation. This further endorses the role of chemokines in many allergic and inflammatory diseases by the accumulation of chemokines at many disease sites (Alam et al., 1996; Gerard and Rollins, 2001; Gordon, 2000; Luster and Rothenberg, 1997).

### 4.3.8 Role of Activation Markers in Mast Cells

It was clearly demonstrated in this thesis that upon activation of mast cells by CXCR3 agonists, the activation markers CD63 and CD203c were up-regulated. It is understood that membrane complexes containing CD63 and CD203c control mast cell adhesion and induced degranulation by a shared pathway. The translocation of granules to the cell membrane is vital to the exocytosis of mast cell granules. (Nishida et al., 2005) observed the translocation of CD63-containing granules to the plasma membrane in the absence of calcium. In these particular conditions they did not observe FcåRI-induced increase of cell surface expression of CD63 by FACS analysis. It is considered that the membrane fusion is calcium dependent in general (Lin and Scheller, 2000). (Kraft et al., 2005) demonstrated that CD63, is associated to signalling components that are key to the formation of the membrane complexes containing CD63. They also established that inhibiting CD63 impairs the Gab2–PI3K pathway known to be essential for both degranulation and adhesion (Kraft et al., 2005).

### 4.3.9 Pharmacological Investigations into the PI3K Family

Following clarification of the function and biochemical effects of the CXCR3 agonists, attention was then focused on investigating the mechanism of CXCR3 induced degranulation. Initial interest was on the PI3K family utilising inhibitors to dissect the role of the class 1 isoforms on degranulation and signalling. Cross-linking of the FcεRI by antigen is known to activate a Tyr kinase signalling cascade which provides a direct molecular link to class IA PI3K signalling (Deane and Fruman, 2004; Okkenhaug et al., 2007). Genetic or pharmacological inactivation of p110δ has been shown to lead to a

substantial, but not complete, block in the allergic responses in mice (Ali et al., 2004; Lee et al., 2006; Nashed et al., 2007). Worthy of note is the fact that genetic inactivation of p110γ in mice has been reported to lead to a complete block in passive cutaneous and systemic anaphylaxis responses in vivo (Laffargue et al., 2002).

This is quite remarkable given that the FcεRI Tyr kinase signalling pathway does not appear to provide a direct molecular link to this GPCR coupled PI3K. Evidence based largely on the use of in vitro cultured mast cells suggested that p110γ may be part of an auto/paracrine mechanism whereby exocytosed mast cell-derived GPCR agonists, initially released by an FcεRI-dependent pathway, promote hyperactivation of mast cells through GPCR signalling to overcome inhibition by lipid phosphatases such as SHIP and PTEN, which antagonize PI3K signalling (Harris et al., 2008). Recent experiments in murine models have shown that *in vitro* both p110δ and p110γ are required for FcεRI-driven mast cell degranulation, whereas in vivo, p110γ (but not p110δ) is dispensable for allergic responsiveness (Ali et al., 2004; Ali et al., 2008; Laffargue et al., 2002).

### 4.3.10 A Critical Role for PI3Kγ in CXCR3 Agonists Induced Partial Degranulation

In common with all pharmacological tools to unequivocally attribute function to a particular isoform, it was important to establish the respective selectivity for these compounds. The PI3K inhibitors utilised in this study, exhibit selectivity between their primary isoform target and the other membranes of the family (Table 1). As anticipated, the data presented in this study using the p110γ-discriminating PI3K inhibitor AS605240 indicates that p110γ makes a significant contribution to the signalling events that underpin CXCR3 agonist-induced mast cell degranulation. This was also observed in signalling through inhibition of Akt in responses to CXCR3 agonists. The discovery that p110γ plays a key role in mast cell responses is supported in both in vivo and in vitro studies (Laffargue et al., 2002; Wymann et al., 2003). The inhibition by

AS605240 recorded in this study was incomplete even at concentrations well above the reported IC50 values, suggesting the presence of other PI3K isoforms.

### 4.3.11 What is the contribution of other Class 1 PI3K isoforms to CXCR3 Agonist-Induced Mast Cell Degranulation?

The inhibitor PIK75 was utilised in this thesis to investigate the role of PI3K class 1A. This inhibitor is selective for the α member of the PI3K family with an $IC_{50}$ for this isoform at approximately 8nM and had no effect on CXCR3 induced degranulation, even at concentrations above its $IC_{50}$, especially at the concentrations it is predicted to impinge on p110γ. This implies reduced cell permeability and bioavailability of this compound in comparison to the other PI3K isoform-discriminating inhibitors undertaken in this thesis

Other kinases (most notably DNA-PK) have been reported to be inhibited by PIK75. This occurs at an $IC_{50,}$ at an even lower concentration, than that predicted for p110α. The evidence presented in this study would suggest that this kinase has no role to play in mast cells degranulation by CXCR3 agonists. The role of PI3K has become clearer over the last few years Recent genetic knock-in and pharmacological approaches have suggested that, of class IA, it is the p110*α* isoform (PIK3CA) that plays the predominant role in insulin signalling (Chaussade et al., 2007).

### 4.3.12 A Potential Synergy between p110δ and p110β

Combined (but not individual) treatment of both p110β and p110δ discriminating inhibitors attenuated degranulation responses and phosphorylation of Akt in response to CXCR3 agonists. Compounds that inhibit p110β tend also to inhibit p110δ isoforms (Crabbe et al., 2007; Knight et al., 2006; Smith et al., 2007). Consequently these isoforms have been termed pharmalogs. Carefully controlled experiments with a panel of PI3K isoform-discriminating inhibitors with differing selectivities suggest a hitherto unrecognised functional relationship between p110β and p110δ.

Evidence has previously been presented for the coupling of p110β to GPCRs either by *in vitro* studies that documented activation of p110β by Gβγ subunits (Kurosu et al., 1997; Maier et al., 1999) or in cellular experiments where p110β function was probed by microinjection of neutralising Ab to p110β (Graness et al., 1998; Roche et al., 1998), RNAi against p110β (Graness et al., 1998) or expression of p110β (Kubo et al., 2005; Yart et al., 2002), all highlight activation of p110β by GPCRs. Recent evidence using cells derived from mice with conditional genetic inactivation of p110β has revealed that p110β and p110γ can couple redundantly to the same GPCRs including some chemokines receptors (Guillermet-Guibert et al., 2008). The work presented in this thesis would suggest that, at least in human mast cells, p110β operates in tandem with p110δ and provides a conduit for agonist-induced degranulation.

#### 4.3.13 Class1A and 1B PI3K dependent degranulation

This thesis implicates a key role for the p110γ as well as p110delta in combination with p110β in CXCR3 mediated partial degranulation of human mast cells. Other studies have highlighted a role for both the class 1A and 1B PI3Ks. Dependence on PI3K has been listed in a number of systems with migratory processes being the most widely studied.

Throughout the presented thesis, it was highlighted that p110γ played a key role in chemokine-mediated degranulation. The importance of 110 γ in other studies has also been emphasised) These studies focused particular attention on the role of p110γ with neutrophils (Hannigan et al., 2002; Hirsch et al., 2000; Sasaki et al., 2000). The p110γ knock out murine models also revealed that in neutrophils, no PtdIns(3,4,5)$P_3$ production was detected, therefore suggesting a role for the class 1B PI3Ks within the generation of PtdIns(3,4,5)$P_3$ in these knockout murine models (Li et al., 2000).

The biphasic generation of PtdIns(3,4,5)$P_3$ has been shown to be dependent on both classes of PI3K. The first phase is dependent on p110γ, whereas the second is determined largely by the class1A, mostly through p110δ, but to

some extent influenced by p110α and p110β. This points to a potential dual role of the distinct classes of PI3K as both classes are seen to be crucial to the induction of mast cell degranulation. It is possible that the two activation stages of PtdIns(3,4,5)$P_3$ by the classes of PI3K are critical to the activation of mast cells whereas one activation is not enough to generate degranulation.

#### 4.3.14 CXCR3 Reorganises the Actin Cytoskeleton.

Initial studies on the adrenal chromaffin cell highlight that reorganisation of the cortical actin network is necessary to allow granules to reach exocytotic sites in stimulated cells. This reorganisation may involve changes in actin filament cross-linking, assembly and interactions with secretory granules and plasma membranes (Burgoyne) et al., 1987). Localised disassembly of cortical F-actin has long been considered necessary for the facilitation of exocytosis. Several studies have proved that the reorganisation of actin increases both the rate and extent of FcεRI-induced degranulation (Frigeri and Apgar, 1999; Oka et al., 2002). Biochemical studies indicate that FcεRI stimulation causes a rapid increase in the level of F-actin in RBL cells (Frigeri and Apgar, 1999; Pfeiffer et al., 1985). The precise roles of the cytoskeletal re-arrangements in the mast cell degranulation process have not been established.

Chemokine signalling induces re-modelling of cytoskeleton such as the reorganisation of F-actin filaments that allows the cells to flatten and attain cellular polarisation. The classical CXCR3 agonists, CXCL9, CXCL10 and CXCL11 have been previously shown to promote actin reorganization (Kouroumalis et al., 2005). It is now well established that the dynamics of actin cytoskeleton are closely regulated by the activation of members of the Rho GTPase family including RhoA and Rac1 and their activities control mast cell degranulation. Rho, in particular, has been associated with stress fibre formation and cell contractility (Ridley and Hall, 1992). Several proteins have been identified as putative Rho effectors on the basis of their selective interaction with GTP-bound Rho. The ROCK family of kinases has been shown to be involved in Rho-induced formation of actin stress fibres and focal adhesions (Amano et al., 1997; Leung et al., 1996; Leung et al., 1995). In

association with this pre-treatment of human mast cells with Y27632 abolished CXCR3 agonist-induced actin polymerisation.

Regulation of actin polymerization, downstream from G protein-coupled receptors, was originally thought to depend on PI3K activation and involves the activation of PKB and the GTPases Cdc42 and Rac2 (Pollard et al., 2000). Recently an alternative PI3K-independent mechanism for actin polymerization in human neutrophils has been proposed (Chodniewicz and Zhelev, 2003). This PI3K-independent pathway in neutrophils has been shown to be dependent on Src tyrosine kinases, NADPH oxidase and protein kinase A, as well as RhoA and ROCK. Further evidence supports the existence of a PI3K-independent pathway. The discovery that LY294002 did not appear to have any effect on CXCL9-, CXCL10-, or CXCL11-induced actin polymerisation (Kouroumalis et al., 2005) supports the presence of a PI3K independent pathway of the reorganisation actin by CXCR3 agonists is PI3K independent.

### 4.3.15 Clinical importance of CXCR3 Agonists

CXCR3 and its four agonists, CXCL9, CXCL10, CXCL11 and CXCL4 play a role in a wide range of cellular functions and diseases. Considerable interest in this chemokine receptor and its agonists has raised many questions about its importance in a clinical setting. Clinical and pre-clinical studies suggest that CXCR3 and its ligands significantly contribute to the cellular infiltration that result in heart, lung and skin allograft rejection (Agostini et al., 2001; Belperio et al., 2002; Belperio et al., 2003; Hancock et al., 2000; Koga et al., 1999; Miura et al., 2001).

The role of CXCR3 agonists is dependent on the diseases setting, or in many cases, the experimental model used (Belperio et al., 2002; Fahmy et al., 2003; Kao et al., 2003; Zhao et al., 2002). One example of this is the role of CXCL9 and CXCL10 having an involvement in bronchiolitis obliterans syndrome after lung transplantation whereas CXCL11 plays no part (Belperio et al., 2003). This pattern has also been observed in acute cardiac allograft rejection (Zhang et al., 2004; Zhao et al., 2002).

CXCR3 is known to be a key target in the treatment against graft rejection. High levels of CXCL10 have been shown to directly correlate with instances of heart and lung rejection in human tissue (Agostini et al., 2001; Melter et al., 2001). CXCL9 has been identified as the dominant factor directing CXCR3$^{+}$ T cells into both cardiac and tracheal allografts in murine models (Belperio et al., 2003; Miura et al., 2001). The data presented in this thesis has further highlighted the potential clinical importance of CXCR3 by presenting evidence that CXCR3 agonists are important in any immune response involving mast cells. Inhibition of the amplification of degranulation reponses could help reduce many recognised diseases such as rheumatoid arthritis, allowing easier resolution of imflammatory responses.

#### 4.3.16 Cytosolic $Ca^{2+}$ not require for CXCR3 Function in Mast Cells

In most, but not all cells, activation of chemokine receptors induces an increase in cytosolic calcium. In this thesis, not all CXCR3 agonists changed cytosolic calcium levels in mature mast cells. These results differ from studies in other mast cell models where CXCR3 agonists increased $Ca^{2+}$(Brightling et al., 2005). Of notable interest is the absence of a $Ca^{2+}$ which has been associated with other chemokine receptors and their agonists. For example, RANTES/CCL5 induces CCR5-mediated T-lymphocyte chemotaxis without changing cytosolic $Ca^{2+}$ (Turner et al., 1995). Furthermore, IL-8/CXCL2 induces CXCR2-mediated neutrophil migration without an increase in $Ca^{2+}$ in cells from PLC-β2/β3 knockout mice (Li et al., 2000). The absence of calcium mobilisation in response to chemokine agonists ,despite the ability to induce a functional response, has also been highlighted by CXCR3 agonists. CXCR3 agonists induced chemotaxis in human airway epithelial cells without mobilising cytosolic calcium (Shahabuddin et al., 2006). Coupled with the data presented in this study, this suggests that changes in $Ca^{2+}$ are not always necessary for directional sensing and cell shape change.

### 4.3.17 Role of PLC in Degranulation

This study and many other studies have shown the dependence of PI3K in mast cell degranulation. It has been recently highlighted that the presence of a PLC dependent but PI3K independent pathway leads to mast cell activation (Kuehn and Gilfillan, 2007). PLC-mediated hydrolysis is required for mast cell exocytosis. On the onset of exocytosis, PtdIns(4,5)$P_2$ is eliminated from the extra cellular membrane. PLC is essential to the process of removal of PtdIns(4,5)$P_2$ from the plasma membrane. PLC is required to initiate the $Ca^{2+}$ signal at the onset of exocytosis yet $Ca^{2+}$ is necessary for PLC activity. PLC depletes PtdIns(4,5)$P_2$ from incipient phagosomes causing breakdown of the surrounding F-actin (Scott et al., 2005). It has also been proven that mast cells containing an F-actin cortex  break down at the onset of degranulation (Nishida et al., 2005; Price et al., 1995). Disruption of this cortex is seen to enhance degranulation (Borovikov et al., 1995; Martin-Verdeaux et al., 2003). A fair conclusion would be that PtdIns(4,5)$P_2$ breakdown is associated with the breakdown of the cortical actin cytoskeleton. Hammond et al., (2006) discovered that PLC is required for exocytosis, independent of the Ins(1,4,5)-*P*3–$Ca^{2+}$ pathway by eliminating PtdIns(4,5)P2 from the plasma membrane.

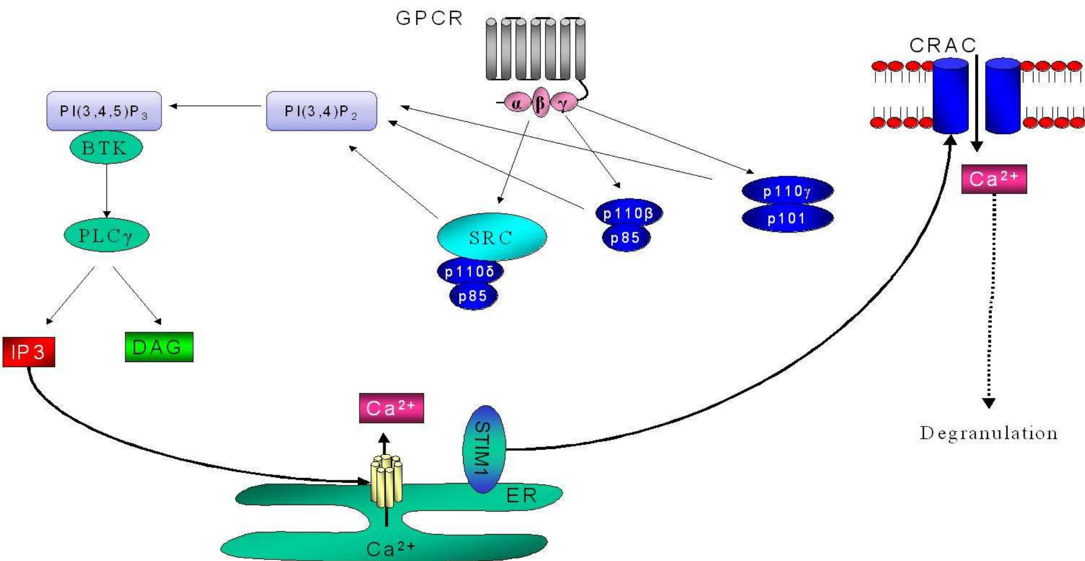



**Figure 4.29: Proposed model for PI3K signalling role in cord blood mast cell degranulation.** Stimulation of CXCR3 by its agonists leads predominantly to activation of p110γ leading to the accumulation of $PI(3,4,5)P_3$ at levels sufficient to initiate activation of downstream effectors such PLC and drive degranulation. CXCR3 agonists also lead to the activation of the G protein coupled p110βisoform, though the levels of $PI(3,4,5)P_3$ formed by activation of this PI3K isoform alone are insufficient to promote downstream effectors and initiate degranulation and/or redundant with p110γ-derived $PI(3,4,5)P_3$. However, the $PI(3,4,5)P_3$ formed by p110β leads instead to the recruitment of PH domain-containing adaptor proteins which, in turn, lead to the recruitment and activation of p110δ, via SRC by GPCRs, contributing to the formation of $PI(3,4,5)P_3$. The pool of $PI(3,4,5)P_3$ which is formed by the combined actions of p110β and p110δ is sufficient to evoke PI3K effectors and elicit degranulation responses. This model allows for discrete and critical differences in the spatio-temporal regulation of $PI(3,4,5)P_3$ accumulation by distinct PI3Ks that ensure optimal mast cell responses to activating stimuli.

## 4.4 Future Work

### 4.4.1 The Role of CXCR3 in Precursor and Immature Mast Cell Stages.

As this study demonstrated in immature mast cells, CXCR3 agonists are functional at least at a biochemical signalling level. With the reported down-regulation of CXCR3 in this investigation it would be of interest to further investigate the function of CXCR3 at earlier stages than the mature mast cell stage explored here. This study has focussed on the potential role of CXCR3 in the migration of precursors to blood and the migration of immature mast cells from the blood to the tissues. It would be of considerable interest to further investigate the migratory role of CXCR3 agonist in these stages of mast cell development.

#### 4.4.1.1 Clarifying the Synergy of the PI3K Isoforms.

This study highlighted the importance of PI3K isoforms in the degranulatory mechanism of mast cells by both CXCR3 and FcεRI induced degranulation, focusing essentially on the role of the synergy between the β and δ isoforms. With the emerging role of PI3K isoforms in mast cell degranulation it would be prudent to further investigate the potential synergy between the β and δ isoforms of PI3K. The data demonstrates the activation of signalling and degranulation.

The evidence for the PI3K isoforms however is only based on the use of inhibitors. Therefore utilising other technologies such as siRNA interference to provide further evidence of the synergy of the PI3K isoforms involved in mast cell degranulation would be useful

#### 4.4.1.2 Other Mast Cell Models

The evidence presented in this study looked specifically at a mast cell model derived from cord blood precursor cells. It is widely known that the differences in mast cells are dependent upon their final anatomical location. It would

therefore be of considerable interest to investigate if the key findings in this study are mirrored in other primary human mast cell models.

# Chapter Five

# Summary

## 5. Summary

The presented work characterises the role of the chemokine receptor CXCR3 and its spliced variants, namely CXCR3B and CXCR3-alt, in different human mast cell model. In the investigation of CXCR3 in HMC-1 cell line, only variants CXCR3A and CXCR3B were detected while on the primary mast cell model all variants of CXCR3 were detected at the mRNA level. This thesis concludes that the HMC-1 cell line make a poor model for CXCR3 studies in mast cells. This is due to the mutation in the SCF receptor resulting in the constitutive activation of downstream signalling proteins, making signalling events induces by CXCR3 impossible to determine. No functional responses by any of the CXCR3 agonist were detected in HMC-1 in this thesis.

The work presented in this thesis highlights the downregulation CXCR3 in the differentiation and maturation of CD133+ precursors into mature mast cells. This is in direct contrast to other studies which report the upregulation of CXCR3 in mast cell maturation process. It is proposed in this thesis that the cytokine mileu that the precursors are exposed to will determine the subtype of mast cells that they will eventually differentiate into. Therefore this thesis has also proposed that the role of CXCR3 in mast cell precursors derived from cord blood is to induce the migration into the tissues, the place where mast cells will mature.

This thesis reports for the first time the induction of degranulation, albeit partial, of human mast cells by CXCR3 agonists. It has previously been shown that GPCRs can augment the sub-optimal antigen/FcεRI induced release, but never the direct release of mediators from mast cells after CXCR3 activation. This response was further surprising with respect to CXCL4, as the only current known receptor for CXCR3B which was undetectable at a protein level in cord blood derived mast cells. Therefore it is proposed in this thesis that CXCL4 could bind isoforms other than CXCR3 or

that CXCR3B becomes post-translationally modified making it undetectable by currently available commercial antibodies.

This result highlights a new role for chemokines in mast cells never before reported. With this finding of direct induced degranulation of human mast cells by CXCR3, this study focused on how closely this responses mirrored antigen/FcεRI responses.

This thesis demonstrated that pathways involved in both antigen degranulation and CXCR3 degranulation were identical. The pathways highlighted in this thesis that are critical to the degranulation response were the PLC and PI3K pathways.

It is presented in this thesis that activation of individual PI3K isoforms are key to the degranulation response. All CXCR3 agonists induced degranulation that was dependent on the p110γ isoform of PI3K. However, for the first time, it is also reported in this thesis that p110δ isoform in synergy with p110β isoform are also key to induction of the degranulation response. This finding was observed utilising a of wide range of PI3K isoform specific inhibitors. This synergy was not only observed in degranulation responses but also in Akt phosphorylation where both p110δ and p110β isoforms were needed to be inhibited before complete attenuation of Akt responses.

In summary, despite only low levels of detectable CXCR3 expression, I have demonstrated biochemical and functional mast cell responses to CXCR3 agonists that are inhibited by PTX and selective CXCR3 antagonists. I provide the first evidence that chemokine induced partial degranulation of human cord blood derived mast cells responses are dependent not only on p110γ, but also on an unexpected p110δ and p110β synergy. These findings provide a novel insight into problems and opportunities to selective target of PI3K isoforms in allergic disease settings and general manipulation of the immune system with such tools.

# 6. Appendix

## 6.1 Buffers and Solutions

### 6.1.1 Solutions and buffers for SDS-PAGE and western blotting

| Lysis Buffer | 4x Resolving Gel Buffer |
|---|---|
| 1% (v/v) Nonidet P-40 | 1.5M Trizma base pH 8.8 |
| 150nM NaCl | 0.4 % (w/v) SDS |
| 50mM Tris pH 7.5 | MilliQ Water |
| 5mM EDTA | |
| 10mM Sodium Fluoride* | |
| 1mM Phenylmethylsulfonyl fluoride* | |
| 10µg ml-1Leupeptin* | **4 x Stacking Gel Buffer** |
| 10µg ml-1Aprtinin* | 0.5 M Trizma Base pH 6.8 |
| 1µg ml-1Soybean Trypsin Inhibitor* | 0.4% (w/v) SDS |
| 1µg ml-1Pepstatin A* | MilliQ Water |
| 1mM Sodium Orthovanadate* | |
| 1mM Sodium Molybdate | |
| MilliQ Water | |

N.B * Denotes added on the day of use

| SDS-PAGE Running Buffer | 5 x SDS-Sample Buffer |
|---|---|
| 25mM Trizma Base | 5% SDS |
| 192mM Glycine | 50% Glycerol |
| 0.1%(w/v) SDS | 200mM Tris-HCl pH 6.8 |
| MilliQ Water | MilliQ Water |
| | Bromophenol Blue |
| | 5% 2-mercaptoethanol |
| **Semi-Dry Transfer Buffer** | **Tris-Buffered Saline(TBS)** |
| 29mM Glycine | 20mM Tris-HCl pH 7.5 |
| 48mM Trizma Base | 150mM NaCl |
| 0.0375% SDS | MilliQ Water |
| 20% (v/v) Methanol | |
| MilliQ Water | |
| **Tris-Buffered Saline-Tween (TBST)** | **Stripping Buffer** |
| TBS + 0.1% (v/v) Tween-20 | 62.5mM Tris-HCL pH 6.8 |
| | 2% (w/v) SDS |
| | 100mM 2-mercaptoethanol |
| | MilliQ Water |

### 6.1.2 Recipes for SDS-PAGE Gels

| | Resolving Gel | Stacking Gel |
|---|---|---|
| | 10% | 4X |
| 4x Resolving Buffer or Stacking Buffer (ml) | 5 | 5 |
| MilliQ Water (ml) | 8.17 | 6.85 |
| Acrylamide (ml) | 6.67 | 2.0 |
| APS(10%) (µl) | 150 | 150 |
| TEMED (µl) | 15 | 15 |

### 6.1.3 Immunoblotting Conditions for Specific Primary Antibodies

| Molecule Targeted By Primary Antibody | Primary Antibody Species | Molecular Weight (kDa) | Blocking Buffer[1] | Primary Antibody Concentration[2] | Secondary Antibody Concentration[3] |
|---|---|---|---|---|---|
| PhosphorSer473-Akt | Rabbit | 60 | 5% | 1:1000 | 1:10000 |
| Akt1 | Goat | 60 | 5% | 1:1000 | 1:10000 |
| phosphoThr402/Tyr404 ERK1/2 | Rabbit | 42/44 | 5% | 1:1000 | 1:10000 |
| phosphoSer236/236S6 | Rabbit | 32 | 5% | 1:1000 | 1:10000 |
| CXCR3 | Mouse | 42 | 5% | 1:100 | 1:10000 |
| CXCR7 | Mouse | 40 | 5% | 1:100 | 1:10000 |

[1] Expressed as percentage (w/v) non-fat milk diluted w/v in TBST
[2] Diluted v:v in 0.01%(w/v) Sodium Azide TBST
[3] Dilution v:v in 1% (w/v) non-fat milk in TBST

### 6.1.4 Recipes for Buffers in Flexstation $Ca^{2+}$ Readings

| Master Buffer | Assay Buffer |
|---|---|
| 500ml HPSS (w/o $Ca^{2+}$) | 198mls Master Buffer |
| 750µl $CaCl_2$ 1M | 2mls Probenecid (250mM) |
| 10ml HEPES 1M (pH 7.3) | |

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