This thesis has been submitted in fulfilment of the requirements for a postgraduate degree (e.g. PhD, MPhil, DClinPsychol) at the University of Edinburgh. Please note the following terms and conditions of use:

This work is protected by copyright and other intellectual property rights, which are retained by the thesis author, unless otherwise stated.
A copy can be downloaded for personal non-commercial research or study, without prior permission or charge.
This thesis cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author.
The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author.
When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given.
Apoptosis is promoted by unconventional FcγR-PI3K-Cdc42-Pak-Mek-Erk signalling in the human neutrophil

Ying Ying Julia Chu
MSc, BSc (Hons)

A thesis submitted for the degree of

Doctor of Philosophy

June 2017

School of Clinical Sciences
University of Edinburgh
Edinburgh, UK
# Table of contents

Table of contents .............................................................................................................................................. i  
Abstract ........................................................................................................................................................... v  
Lay Abstract .................................................................................................................................................... vii  
Declaration ...................................................................................................................................................... ix  
Acknowledgements .......................................................................................................................................... x  
List of publications ........................................................................................................................................... x  
List of Figures .................................................................................................................................................. xii  
List of tables .................................................................................................................................................... xvi  
List of abbreviations ....................................................................................................................................... xvii  
1 Introduction ................................................................................................................................................... 1  
  1.1 Neutrophil Biology ................................................................................................................................... 1  
    1.1.1 Neutrophil recruitment to inflammatory sites ....................................................................................... 2  
    1.1.2 Microbial killing of neutrophils ............................................................................................................ 6  
    1.1.3 Neutrophil apoptosis as a mechanism to resolve inflammation ....................................................... 16  
  1.2 Neutrophils in inflammatory disease ........................................................................................................ 22  
    1.2.1 Immune complexes in inflammation .................................................................................................. 22  
    1.2.2 Immune complex-driven neutrophilic inflammation in inflammatory diseases ...................................... 26  
  1.3 Signalling by Ras and Rho family small GTPases ....................................................................................... 27  
    1.3.1 Regulation of small G proteins ............................................................................................................. 27  
    1.3.2 Signalling through Ras family small GTPases ..................................................................................... 30  
    1.3.3 Signalling through Rho family small GTPases .................................................................................... 32  
  1.4 Phosphoinositide 3-kinases (PI3Ks) .......................................................................................................... 36  
    1.4.1 Class I PI3Ks ....................................................................................................................................... 38  
    1.4.2 Receptor activation of PI3Ks ............................................................................................................... 40  
    1.4.3 PI3K activation by small GTPases ........................................................................................................ 40  
    1.4.4 PI3K signalling in the neutrophil ......................................................................................................... 43  
    1.4.5 Signalling downstream of PI3K ........................................................................................................... 45  
  1.5 Hypotheses and Aims of thesis .................................................................................................................. 49  
  2 Materials and Methods ................................................................................................................................. 51  
    2.1 Isolation of primary neutrophils ............................................................................................................. 51
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1.1</td>
<td>Isolating human neutrophils from peripheral blood</td>
<td>51</td>
</tr>
<tr>
<td>2.1.2</td>
<td>Isolation of bone marrow-derived mouse neutrophils</td>
<td>52</td>
</tr>
<tr>
<td>2.2</td>
<td>Mammalian cell culture</td>
<td>53</td>
</tr>
<tr>
<td>2.2.1</td>
<td>Culture and granulocytic differentiation of PLB-985 cells</td>
<td>53</td>
</tr>
<tr>
<td>2.2.2</td>
<td>Culture of HEK-293ET cells</td>
<td>53</td>
</tr>
<tr>
<td>2.2.3</td>
<td>Generation of retrovirus</td>
<td>54</td>
</tr>
<tr>
<td>2.2.4</td>
<td>Spinoculation of PLB-985 cells</td>
<td>54</td>
</tr>
<tr>
<td>2.3</td>
<td>Preparation of plates for adhesion-dependent stimulation</td>
<td>55</td>
</tr>
<tr>
<td>2.3.1</td>
<td>Preparation of immobilised IgG-BSA immune complexes</td>
<td>55</td>
</tr>
<tr>
<td>2.3.2</td>
<td>Preparation of fibrinogen coated plates</td>
<td>55</td>
</tr>
<tr>
<td>2.3.3</td>
<td>Preparation of pRGD coated plates</td>
<td>56</td>
</tr>
<tr>
<td>2.3.4</td>
<td>Preparation of insoluble IgG-HSA immune complexes</td>
<td>56</td>
</tr>
<tr>
<td>2.4</td>
<td>Biochemical assays</td>
<td>57</td>
</tr>
<tr>
<td>2.4.1</td>
<td>Pre-treatment of primary neutrophils with DFP</td>
<td>57</td>
</tr>
<tr>
<td>2.4.2</td>
<td>Activation assays of primary neutrophils and differentiated PLB-985 cells</td>
<td>57</td>
</tr>
<tr>
<td>2.4.3</td>
<td>Preparation of protein gels for SDS-PAGE and protein transfer onto PDVF membrane</td>
<td>60</td>
</tr>
<tr>
<td>2.4.4</td>
<td>Western blot analysis</td>
<td>61</td>
</tr>
<tr>
<td>2.4.5</td>
<td>Pull down assays of small GTPases (Ras/Rac/Cdc42/RhoA) by GLISA</td>
<td>64</td>
</tr>
<tr>
<td>2.5</td>
<td>Analysis of neutrophil functions</td>
<td>64</td>
</tr>
<tr>
<td>2.5.1</td>
<td>L-selectin shedding</td>
<td>64</td>
</tr>
<tr>
<td>2.5.2</td>
<td>Measurement of ROS production assay (production of reactive oxygen species) using luminol chemiluminescence</td>
<td>65</td>
</tr>
<tr>
<td>2.5.3</td>
<td>Apoptosis assays</td>
<td>65</td>
</tr>
<tr>
<td>2.5.4</td>
<td>Internalisation assays</td>
<td>66</td>
</tr>
<tr>
<td>2.6</td>
<td>Analysis of cell surface integrins and Fcγ receptors</td>
<td>67</td>
</tr>
<tr>
<td>2.7</td>
<td>Molecular biology</td>
<td>69</td>
</tr>
<tr>
<td>2.7.1</td>
<td>Generation of a retroviral transfer vector expressing CD16</td>
<td>69</td>
</tr>
<tr>
<td>2.7.2</td>
<td>Making competent E.coli cells using rubidium chloride</td>
<td>69</td>
</tr>
<tr>
<td>2.7.3</td>
<td>Transformation of competent E.coli cells</td>
<td>70</td>
</tr>
<tr>
<td>2.7.4</td>
<td>Plasmid preparations</td>
<td>70</td>
</tr>
<tr>
<td>2.8</td>
<td>BCA protein assay</td>
<td>71</td>
</tr>
<tr>
<td>2.9</td>
<td>Statistical analysis</td>
<td>71</td>
</tr>
</tbody>
</table>
3 Results-A novel signalling pathway downstream of Fcγ receptors in human neutrophils ................................................................. 73
  3.1 Introduction .................................................................................. 73
  3.2 Results .......................................................................................... 75
    3.2.1 PI3K lies upstream of Erk in human neutrophils that have been stimulated with immune complexes or integrin ligands .................. 75
    3.2.2 Rapidly occurring protein degradation interferes with the analysis of signalling events in neutrophils stimulated in an adhesion-dependent fashion .. 77
    3.2.3 Erk activation is PI3K-dependent in human neutrophils following stimulation with insoluble immune complexes ....................... 81
    3.2.4 PI3K and Erk activation depend on Src kinases in insoluble IC-stimulated human neutrophils .................................................. 84
    3.2.5 The involvement of mTORC1 and 2 are ambiguous in insoluble IC-stimulated human neutrophils .................................................. 86
    3.2.6 PI3K activates Erk via an indirect mechanism that does not involve PKB and mTORC2 ................................................................. 91
    3.2.7 Ras is not downstream of PI3K following stimulation with insoluble ICs 93
    3.2.8 PI3K-dependent Erk activation is Raf-independent in insoluble IC-stimulated human neutrophils .................................................. 95
    3.2.9 PI3K does not activate Erk via Tpl2 ............................................. 99
    3.2.10 Pak acts as the MAP3K upstream of Mek and Erk in neutrophils following stimulation with insoluble ICs ............................... 103
    3.2.11 Cdc42, but not Rac, is regulated by PI3K in human neutrophils that have been stimulated with insoluble ICs ................................. 107
  3.3 Discussion .................................................................................... 111

4 Results-FcγR-PI3K-Cdc42-Pak-Mek-Erk signalling promotes apoptosis in human neutrophils ......................................................... 116
  4.1 Introduction .................................................................................. 116
  4.2 Results .......................................................................................... 118
    4.2.1 PI3K-activated Erk signalling does not regulate L-selectin shedding following stimulation of human neutrophils with insoluble ICs .......... 118
    4.2.2 Insoluble IC-induced ROS production requires PI3K but not Pak, Mek and Erk 120
    4.2.3 FcγR-PI3K-Cdc42-Pak-Mek-Erk signalling promotes apoptosis in human neutrophils ............................................................... 124
    4.2.4 FcγR-PI3K-Cdc42-Pak-Mek-Erk signalling promoted neutrophil apoptosis by altering the ratio of Mcl-1 and Bax ............................. 133
4.2.5. The size of neutrophils changed during the pro-longed incubation with insoluble ICs .............................................................. 135
4.2.6. Internalisation of insoluble ICs by neutrophils is independent of Pak/Mek/Erk ........................................................................ 137
4.3. Discussion ................................................................................. 140

5 Results- Signalling in mouse neutrophils ........................................ 144
5.1 Introduction ................................................................................ 144
5.2 Results ....................................................................................... 146
  5.2.1 Erk activation is PI3K-dependent in mouse neutrophils that were stimulated with immune complexes or by plating onto integrin ligands .... 146
  5.2.2 The contribution of individual PI3K isoforms to insoluble IC-induced ROS production differs between human and mouse neutrophils .......... 148
  5.2.3 Erk activation in insoluble IC-stimulated mouse neutrophils is dependent on Raf not Pak ...................................................................... 150
  5.2.4 PI3K regulated insoluble IC-induced apoptosis via an alternative pathway in mouse neutrophils .......................................................... 153
  5.2.5 Erk activation was PI3K- and Raf-dependent in fMLF-stimulated mouse neutrophils ......................................................................... 156
5.3 Conclusions ................................................................................ 159

6 Results- PLB-985 as a model system for elucidating FcγR signalling in human neutrophils........................................................................... 161
6.1 Introduction ................................................................................. 161
6.2 Results ....................................................................................... 163
  6.2.1 PLB-985 cells are morphologically and functionally similar to human neutrophils ............................................................................. 163
  6.2.2 PI3K/Erk signalling upon stimulation of G protein coupled receptors is conserved between primary human neutrophils and dPLBs.......................... 165
  6.2.3 Immune complexes and integrin ligation do not drive significant PI3K activation in dPLBs ........................................................................ 167
  6.2.4 PLB-985 cells lack CD16 ................................................................ 169
  6.2.5 Generation of PLB-985 cells expressing CD16 .................................. 171
  6.2.6 CD16-expressing PLB-985 cells still responded poorly to stimulation with immune complexes and integrin ligands ........................................ 173
6.3 Discussion ................................................................................. 175

7 Final discussions and future directions ............................................. 178
References.......................................................................................... 186
Abstract

Neutrophils form a first line of defence against infections. These short-lived, terminally differentiated cells perform many important functions, including chemotaxis, degranulation, reactive oxygen species (ROS) release and cytokine production. Whilst neutrophils are essential for host immunity, their inappropriate recruitment, activation and/or removal can contribute to excessive inflammation and host damage, as exemplified in autoimmune diseases such as rheumatoid arthritis. It is therefore essential that neutrophil function is tightly regulated.

Neutrophils are activated by a range of stimuli, including immune complexes. Neutrophil functions are tightly regulated by intracellular signalling events that are induced by the ligation of cell surface receptors, for example, the binding of immune complexes to Fc receptors. Phosphoinositide 3-kinase (PI3K) and extracellular signal-regulated kinase (Erk) are key signalling intermediates that act downstream of many cell surface receptors. They are involved in the regulation of numerous biological processes in the neutrophil.

Using pharmacological interventions, I analysed PI3K signalling in immune complex-stimulated human neutrophils and uncovered a previously uncharacterised, non-canonical signalling pathway, PI3K-Cdc42-Pak-Mek-Erk. This represents an unusual situation where Pak acts as the MAP3K downstream of Cdc42 in a PI3K-dependent fashion. By performing a range of functional experiments, I showed that this unconventional signalling pathway promotes apoptosis in human neutrophils by regulating the ratio between anti- and pro-apoptotic members of the Bcl-2 family.

Mouse knock-outs of all components of this signalling pathway have been described. Immune complex-induced apoptosis was also PI3K-dependent in mouse neutrophils, but experiments performed with inhibitors showed that, in contrast to human neutrophils, this was not dependent on PI3K-Cdc42-Pak-Mek-Erk signalling. The myeloid leukaemia cell line, PLB-985 is amenable to knock-down and can be differentiated to become neutrophil-like. These cells are not notably activated by immune complexes, perhaps because they do not express the major Fcγ receptor, CD16. Since retroviral expression of CD16 in PLB985 cells did not improve their response to activation by immune complexes, I was not able to confirm my observations with human neutrophils genetically.

Collectively, I showed that a novel, pro-apoptotic signalling pathway operates downstream of Fcγ receptors in the human neutrophil. The fact that this signalling pathway appears to regulate apoptosis specifically suggests uncoupling pro- and anti-inflammatory effects induced by immune complexes might be possible. This may be helpful in the design of improved therapies of autoimmune diseases such as rheumatoid arthritis, in which immune complex-driven neutrophilic inflammation contributes to disease pathogenesis and where neutrophil apoptosis is disturbed.
Lay Abstract

The immune system in our body comprises many types of white blood cells. When our body faces threats, the immune system is alerted and elicits a reaction termed ‘inflammation’, in which immune cells cooperate with each other to ensure protection from infections. Neutrophils are amongst the first immune cells to reach a site of inflammation, where they perform various destructive functions to remove threats. Neutrophils are essential in protecting the body from infections, but they can also damage the body if they generate inflammation indiscriminately. Such inappropriate behaviour of neutrophils is important in certain autoimmune diseases such as rheumatoid arthritis.

Neutrophils need to be stimulated, e.g. by antibody aggregates, before they can generate inflammation. Antibodies are important in fighting infections, but excessive antibody aggregates can drive inappropriate inflammation. Stimulating neutrophils activates intracellular communication within the neutrophil which regulates the neutrophil response. ‘Phosphoinositde 3-kinase’ (PI3K) and ‘extracellular signal-regulated kinase’ (Erk) are two key components of the intracellular communication network. I investigated here how these two regulators cross-talk in neutrophils. Understanding how the components of this communication network cooperates to regulate neutrophil functions may help with designing better treatment of autoimmune disease.

I found that PI3K activates Erk via an unconventional mechanism in human neutrophils that have been stimulated with antibody aggregates. Interestingly, this promoted neutrophil death in a process called ‘apoptosis’. Apoptosis is an important
mechanism by which neutrophilic inflammation is controlled. My finding is exciting as it suggests that this may be a mechanism by which excessive antibody aggregate-induced neutrophil activation can be counteracted. Given that antibody aggregates are critical in autoimmune diseases, it is possible that this communication pathway maybe disturbed. If so, activating this ‘pathway’ could improve therapy of autoimmune diseases.
Declaration

I declare that the work presented in this thesis is the work of my own, and the thesis is written by myself, with the support from my supervisors. All experiments described in this thesis were performed by myself (unless otherwise stated). This thesis has not been submitted for any other degree or professional qualification.

Ying Ying Julia Chu
Acknowledgements

Firstly, I would like to thank my primary supervisor, Dr. Sonja Vermeren, sincerely for her help and support (both technical and mental) throughout my PhD study, and also for her patience, inspiration and knowledge in time of conducting research (especially when my results seemed meaningless) and writing of this thesis. I would also like to express my gratitude to my other supervisors, Professor Adriano Rossi and Dr. Yi Feng, who have been showing their support by giving me advices and guidance throughout my PhD research and study. I also need to thank Professor Ian Dransfield for his generosity of gifting me with various agents and antibodies and advices to enable and improve the quality of my research.

Secondly, I would like to thank past and present members of the Vermeren lab, the lung lab and the pig lab, for creating a friendly and lively environment in which I enjoyed working immensely. I would also like to thank the staffs of the Flow cytometry facility, who provided technical help when necessary. I need to thank all my blood donors for their generosity of donating blood, I would never be able to conduct any research without their kindness.

Thirdly, I would like to express my sincere gratitude to my parents, my family and my friends, who have been supporting me (mentally and physically) throughout my PhD study by showing their love and patience, even though some of them are far away and do not have the slightest idea of what my PhD is about. I would not be able to come this far without their support.
Last but not least, I need to give thanks to my heavenly father, for his invisible presence at all times, and for being the one who gave me the spiritual power I needed to not just go through the entire PhD study, but also in many other aspects of life.
List of publications

URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5067281/

URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5136470/

URL: http://dx.doi.org/10.1080/21541248.2017.1304855

For copies of publications, please refer to the URL stated below the publications.
List of Figures

Figure 1.1.1 The neutrophil recruitment cascade .......................................................... 5
Figure 1.1.2 Neutrophil effector functions ................................................................. 7
Figure 1.1.2.2 Sequential formation of granules during granulopoiesis ................. 10
Figure 1.1.3.1 Extrinsic and intrinsic apoptosis pathways ......................................... 19
Figure 1.2.1.1 Structure of IgG .................................................................................. 24
Figure 1.3.1 Small GTPases cycle between an active and an inactive state ............... 29
Figure 1.4 Inositol phospholipids and the PI3K-dependent generation of PI(3,4,5)P3. ......................................................................................................................... 37
Figure 1.4.2 Regulation of PI3K-dependent signalling .............................................. 39
Figure 1.4.3 Regulation of Class I PI3Ks ................................................................. 42
Figure 1.4.5 The signalling network of Class I PI3Ks .................................................. 46
Figure 3.2.1 Erk activation was PI3K-dependent in human neutrophils that had been stimulated by plating onto immobilised ICs or Fgn&TNF. ................................. 76
Figure 3.2.2.1 Protein degradation occurred in human neutrophils that were stimulated by processes that required cell scraping ........................................ 78
Figure 3.2.2.2 DFP caused basal activation of PKB and Erk in human neutrophils. 80
Figure 3.2.3 Erk activation was PI3K dependent in human neutrophils stimulated with insoluble ICs or by plating onto immobilised ICs ............................... 83
Figure 3.2.4 SFK were upstream of PI3K and Erk in the insoluble IC-stimulated human neutrophil .......................................................... 85
Figure 3.2.5.1 Overview of mTORC1 and mTORC2 ............................................... 87
Figure 3.2.5.2 Regulation of mTORC1, mTORC2 and PKB by PI3K......................... 88
Figure 3.2.5.3 PI3K dependent Erk activation in the insoluble IC-stimulated human neutrophils does not involve TORC1 ......................................................... 90
Figure 3.2.6 PI3K-dependent Erk activation is an indirect event ............................. 92
Figure 3.2.7 Ras was not downstream of PI3K in insoluble IC-stimulated human neutrophils .......................................................... 94
Figure 3.2.8.1 Paradoxical Raf activation with the ATP competitive Raf inhibitor, ZM336372 .......................................................... 96
Figure 3.2.8.2 Erk activation does not depend on Raf in insoluble IC-induced signalling .......................................................... 98
Figure 3.2.9.1 Alternative MAP3Ks that can act upstream of Mek other than Raf. 100
Figure 3.2.9.2 PI3K activated Erk signalling did not involve Tpl2 in insoluble IC-stimulated human neutrophils. ................................................................. 102
Figure 3.2.10.1 PI3K dependent Erk activation involves Pak in insoluble IC-stimulated human neutrophils ................................................................. 104
Figure 3.2.10.2 Two possible mechanisms by which Pak regulated phosphorylation of Ser 217 and 221 on Mek. ................................................................. 106
Figure 3.2.11.1 Regulation of Pak by PI3K via Rac or Cdc42. .......................... 108
Figure 3.2.11.2 Activation of Cdc42 and Rho, but not Rac, was PI3K dependent in insoluble IC-stimulated human neutrophils. ................................. 110
......................................................................................................................................................................................... 112
Figure 3.3 The new non-canonical signalling pathway downstream of Fcγ receptors in the human neutrophil ................................................................. 112
Figure 4.2.1 Insoluble IC-induced L-selectin shedding is regulated by Src and PI3K, but not Pak, Mek and Erk in human neutrophils ........................................ 119
Figure 4.2.2.1 Insoluble IC-induced internal ROS production depends on PI3Kβ/δ but not Pak, Mek and Erk in human neutrophils ........................................ 122
Figure 4.2.2.2 Determination of the concentration at which BVD523 is effective. 123
Figure 4.2.3.1 Examples illustrating how insoluble IC-induced apoptosis were assessed in human neutrophils with and without inhibitor. ...................... 126
Figure 4.2.3.2 The unconventional signalling pathway regulated apoptosis and secondary necrosis in insoluble IC-stimulated human neutrophils. .............. 128
Figure 4.2.3.3 Insoluble IC-induced apoptosis and secondary necrosis were caspase-dependent. ................................................................. 129
Figure 4.2.3.4 AZD6244 but not tramatinib induces Raf activation during prolonged neutrophil incubation. ................................................................. 132
Figure 4.2.4 PI3K-activated Erk signalling alters the ratio of Mcl-1 and Bax expression in human neutrophils that had been stimulated with insoluble ICs. ............ 134
Figure 4.2.5 The Size of neutrophils changed during prolonged incubation with insoluble ICs. ................................................................. 136
Figure 4.2.6. PI3K activated Erk signalling did not regulate insoluble ICs internalisation by human neutrophils. ................................................................. 139
Figure 4.3 The non-canonical signalling pathway promotes apoptosis in insoluble IC-stimulated human neutrophils. ................................................................. 141
Figure 5.2.1 Erk activation was largely dependent on PI3Kβ rather than PI3Kδ in mouse neutrophils that had been stimulated with integrin ligands, immobilised ICs or insoluble ICs. ................................................................. 147
Figure 5.2.2 PI3Kβ played the larger role in insoluble IC-induced ROS production in mouse neutrophils, whilst it is PI3Kδ in humans. ......................................................... 149

Figure 5.2.3 Raf but not Pak acted upstream of Erk in insoluble IC-stimulated mouse neutrophils. ........................................................................................................ 152

Figure 5.2.4 Neutrophil apoptosis was dependent on PI3Kβ/δ but not Pak, Mek and Erk in mouse. ........................................................................................................ 155

Figure 5.2.5 Erk activation was dependent on PI3Kγ and Raf following fMLF stimulation in mouse neutrophils. .................................................................................. 158

Figure 6.2.1 Differentiated PLB-985 cells are a model for primary neutrophils.... 164

Figure 6.2.2 Erk activation does not depend on PI3K in fMLF-stimulated dPLBs. 166

Figure 6.2.3 Signalling events were conserved between dPLBs and human neutrophils. .................................................................................................................... 168

Figure 6.2.4 Differentiated PLBs do not express CD16. ................................. 170

Figure 6.2.5 Transduced PLB-985 cells express CD16. ................................. 172

Figure 6.2.6 CD16-expressing dPLBs responded poorly to stimulation with immune complexes and following plating onto integrin ligands. ............................... 174
List of tables

Table 2.4.2 Inhibitors used in this thesis ................................................................. 59
Table 2.4.4.1 Primary antibodies used in western blot analysis. .......................... 62
Table 2.4.4.2 Secondary reagents used in western blot analysis. ....................... 63
Table 2.6 Antibodies used in FACS analysis of cell surface integrins and Fc gamma receptors. ........................................................................................................ 68
## List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>O</strong>₂</td>
<td>Oxygen radicals</td>
</tr>
<tr>
<td>ANCA</td>
<td>Anti-neutrophil cytoplasmic antibody</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium Persulphate</td>
</tr>
<tr>
<td>AS</td>
<td>AS252424 (PI3Kγ specific inhibitor)</td>
</tr>
<tr>
<td>AZ</td>
<td>AZ628 (Pan-Raf inhibitor)</td>
</tr>
<tr>
<td>AZD</td>
<td>AZD6244 (Mek inhibitor)</td>
</tr>
<tr>
<td>Bad</td>
<td>Bcl-2-associated death promoter</td>
</tr>
<tr>
<td>Bak</td>
<td>Bcl-2 homologous antagonist/killer</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-associated X protein</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>Bid</td>
<td>Bcl-2 homology-3 interacting domain</td>
</tr>
<tr>
<td>Bik</td>
<td>Bcl-2 interacting killer</td>
</tr>
<tr>
<td>Bim</td>
<td>Bcl-2 interacting protein</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Caspases</td>
<td>Cysteine-dependent aspartate-specific protease</td>
</tr>
<tr>
<td>CGD</td>
<td>Chronic granulomatous disease</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>Chloride ion</td>
</tr>
<tr>
<td>CRD</td>
<td>Cysteine-rich domain</td>
</tr>
<tr>
<td>CRIB</td>
<td>Cdc42- and Rac-interactive binding domain</td>
</tr>
<tr>
<td>dbcAMP</td>
<td>Dibutyryl-cAMP</td>
</tr>
<tr>
<td>DFP</td>
<td>Diisopropylfluorophosphate</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>dPLBs</td>
<td>Differentiated PLB-985 cells</td>
</tr>
<tr>
<td>Ecl</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>elf4E</td>
<td>The translation initiation factor 4E</td>
</tr>
<tr>
<td>Erk</td>
<td>Extracellular-regulated kinase</td>
</tr>
<tr>
<td>Fe²⁺</td>
<td>Iron ions</td>
</tr>
<tr>
<td>Fgn&amp;TNF</td>
<td>Fibrinogen and TNF</td>
</tr>
<tr>
<td>FPR</td>
<td>Formyl peptide receptor</td>
</tr>
<tr>
<td>FR</td>
<td>FR180204 (Erk inhibitor)</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward scatter</td>
</tr>
<tr>
<td>GAPs</td>
<td>GTPase-activating proteins</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanine diphosphate</td>
</tr>
<tr>
<td>GEFs</td>
<td>Guanine nucleotide exchange factors</td>
</tr>
<tr>
<td>GMP</td>
<td>Granulocyte/macrophage progenitor</td>
</tr>
<tr>
<td>GPCRs</td>
<td>G protein-coupled receptors</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanine triphosphate</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>HI-FBS</td>
<td>Heat-inactivated fetal bovine serum</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>HOCl</td>
<td>Hypochlorous acid</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IC</td>
<td>IC87114 (PI3Kδ specific inhibitor)</td>
</tr>
<tr>
<td>ICAM</td>
<td>Immunoglobulin-like cell adhesion molecules</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>Immobilised ICs</td>
<td>Immobilised immune complexes</td>
</tr>
<tr>
<td>Insoluble ICs</td>
<td>Insoluble immune complexes</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinases</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>Ly</td>
<td>Ly294002 (Pan-PI3K inhibitor)</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAPKK/MAP2K</td>
<td>Mitogen-activated protein kinase kinase</td>
</tr>
<tr>
<td>MAPKKK/MAP3K</td>
<td>Mitogen-activated protein kinase kinase</td>
</tr>
<tr>
<td>MB</td>
<td>Myoblast</td>
</tr>
<tr>
<td>MC</td>
<td>Myelocytes</td>
</tr>
<tr>
<td>Mcl-1</td>
<td>Myeloid cell leukaemia 1</td>
</tr>
<tr>
<td>MM</td>
<td>Metamyelocytes</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>mTOR</td>
<td>The mammalian target of rapamycin</td>
</tr>
<tr>
<td>NETs</td>
<td>Neutrophil extracellular traps</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen</td>
</tr>
<tr>
<td>O₂⁻</td>
<td>Superoxide ions</td>
</tr>
<tr>
<td>p38</td>
<td>p38 MAPK</td>
</tr>
<tr>
<td>P529</td>
<td>Palomid 529 (mTOR inhibitor)</td>
</tr>
<tr>
<td>P70S6K</td>
<td>The ribosomal p70S6 kinase</td>
</tr>
<tr>
<td>Pak</td>
<td>p21-activated kinase</td>
</tr>
<tr>
<td>PDK-1</td>
<td>Phosphoinositide-dependent kinase 1</td>
</tr>
<tr>
<td>PDVF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>Platelet/endothelial cell adhesion molecule 1</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>Penicillin-Streptomycin</td>
</tr>
<tr>
<td>PF</td>
<td>PF3758309 (Pan-Pak inhibitor)</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
</tr>
<tr>
<td>Phox</td>
<td>Phagocyte oxidase</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PI(3)P</td>
<td>Phosphatidylinositol-(3)-phosphate</td>
</tr>
<tr>
<td>PI(3,4)P2</td>
<td>Phosphatidylinositol-(3,4)-bisphosphate</td>
</tr>
<tr>
<td>PI(3,4,5)P3/PIP₃</td>
<td>Phosphatidylinositol-(3,4,5)-trisphosphate</td>
</tr>
<tr>
<td>PI(4)P</td>
<td>Phosphatidylinositol-(4)-phosphate</td>
</tr>
<tr>
<td>PI(4,5)P2</td>
<td>Phosphatidylinositol-(4,5)-bisphosphate</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PM</td>
<td>Promyelocytes</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>pRGD</td>
<td>Poly Arg-Gly-Asp</td>
</tr>
<tr>
<td>PRP</td>
<td>Platelet-rich plasma</td>
</tr>
<tr>
<td>PSGL-1</td>
<td>P-selectin glycoprotein ligand 1</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RalGEFs</td>
<td>Ral guanine nucleotide exchange factors</td>
</tr>
<tr>
<td>RBD</td>
<td>Ras-binding domain</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative light unit</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RTKs</td>
<td>Receptor tyrosine kinases</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>SFK</td>
<td>Src family kinases</td>
</tr>
<tr>
<td>SGK</td>
<td>Serum/glucocorticoid regulated kinase</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter</td>
</tr>
<tr>
<td>TGX</td>
<td>TGX221 (PI3Kβ specific inhibitor)</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>Tpl2</td>
<td>Tumour progression locus 2 kinase</td>
</tr>
<tr>
<td>Tra</td>
<td>Tramatinib (Mek inhibitor)</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing agent</td>
</tr>
<tr>
<td>Wo</td>
<td>Wortmannin (Pan-PI3K inhibitor)</td>
</tr>
<tr>
<td>ZM</td>
<td>ZM336372 (Pan-Raf inhibitor)</td>
</tr>
</tbody>
</table>
1 Introduction

1.1 Neutrophil Biology

Neutrophils are the most abundant circulating leukocytes in the blood stream in humans, accounting for approximately 60% of all white blood cells. They are amongst the first immune cells to be delivered to the site of infection or injury where they kill and clear any potentially harmful bacteria and fungi, thereby protecting the host from infections [1]. Circulating neutrophils are terminally differentiated cells that are relatively short-lived, estimated to remain in the circulation for between 4-8 hours and in tissues for over 24 hours, although this has been questioned recently [2]. Neutrophils are continuously produced in the bone marrow, which acts as a reservoir and releases neutrophils into the blood stream as replenishment of the ones that are cleared away or extravasated [3]. In the absence of activation, neutrophils have a half-life in the blood of approximately 8 hours, until they undergo constitutive apoptosis in tissues or home back to the bone marrow [4]. Clearance of apoptotic neutrophils (efferocytosis) by macrophages takes place following homing of the neutrophils to tissues such as liver, spleen and bone marrow [3] or at inflammatory site following neutrophil recruitment [5]. When neutrophils encounter inflammatory mediators that are released from damaged and infected tissues, they become activated and are recruited to sites of infection or insult. The lifespan of activated neutrophils is extended to ensure sufficient killing of pathogens [3, 6].
1.1.1 Neutrophil recruitment to inflammatory sites

The process by which neutrophils are recruited to an inflammatory site has been analysed in many tissues but more recently elegant studies have investigated neutrophil trafficking in depth in the mouse cremaster model system, which is particularly amenable to examination by intravital microscopy [7]. The findings from these models, especially the cremaster muscle preparation, are summarised below, and the process is often referred to as the ‘classical leukocyte/neutrophil recruitment cascade’.

This process is initiated by the release of pro-inflammatory mediators (e.g., chemokines and lipid mediators) from tissue sentinel leukocytes (e.g., dendritic cells and macrophages) when they recognise pathogens. Pro-inflammatory mediators remodel the endothelial lining, activating endothelial cells and upregulating adhesion molecules called selectins on their cell surface [8]. Selectins displayed on the surface of endothelial cells comprise P-selectin glycoprotein ligand 1 (PSGL-1) and P- and E-selectins. They bind to the L-selectins that are expressed on the surface of circulating neutrophils. This permits capturing of the neutrophils in the blood flow, and allows the neutrophils to loosely tether to and start rolling on the endothelium. The fact that PSGL-1 is also expressed on the surface of neutrophils leads to secondary capture of further neutrophils in the blood through neutrophil-neutrophil interactions [8]. Tethering of neutrophils causes the cross-linking of L-selectins, and this in turn elicits downstream signalling that activates another group of adhesion molecules called integrins [9]. As neutrophils roll along the endothelium, L-selectins are shed, which is usually regarded as a sign of activated neutrophils. Activated integrins and the upregulation of P and E selectins on the endothelium synergistically slow down
neutrophil rolling along the endothelium. Slow rolling is critical for increasing the
contact time of neutrophils with the inflamed endothelium, driving further neutrophil
activation and adhesion to the endothelium. When neutrophils come into contact with
the inflamed endothelium, chemokines (guidance molecules that direct neutrophils to
the inflammatory site) secreted by the endothelial cells induce re-localisation of certain
intracellular integrins [e.g. Mac-1(also known as αMβ2)] to the surface of neutrophils,
upregulating surface integrins on neutrophils [6]. Chemokines also induce integrin
conformational changes that shift integrins to a high affinity state for ligand binding.
Such ligands include immunoglobulin-like cell adhesion molecules (ICAM) 1 and 2
that are expressed on the endothelium, thereby further increasing neutrophil binding
to the endothelium and hence causing the rolling neutrophil to arrest. Ligation of
integrins with their ligands induces intracellular signalling that stabilises neutrophil
adhesion and initiates crawling on the endothelial lining. For example, binding of the
integrins LFA-1 (also known as αLβ2) and MAC-1 to ICAM1 and 2 are essential for
firm adhesion and subsequent neutrophil crawling respectively [10].

In order to reach the site of infection, neutrophils must leave the blood stream by
transmigrating through the endothelium and the basement membrane. The crawling of
neutrophils on the endothelial lining of the vessel permits them to search for junctions,
preferably ones that are low in matrix protein expression, through which they can
transmigrate. Transmigration can occur in two ways: paracellularly (between
endothelial cells) or transcellularly (through an endothelial cell). Neutrophils
preferentially transmigrate paracellularly, which is more efficient in comparison to
transcellular transmigration [11, 12]. In addition to integrins and ICAM1 and 2,
transmigration requires junctional proteins such as platelet/endothelial cell adhesion
molecule 1 (PECAM1) [13]. After extravasation, neutrophils migrate to the inflammatory site by a process called chemotaxis, following gradients of chemoattractants such as the chemokine IL-8. Directional movement of neutrophils is underpinned by intracellular signalling induced by chemoattractants, which regulates cytoskeletal organisation and drives persistent cell migration. The process of the neutrophil recruitment cascade is summarised in Fig 1.1.1.
Figure 1.1.1 The neutrophil recruitment cascade.

A diagram illustrating the neutrophil recruitment cascade during infection. The recruitment cascade involves several sequential stages: tethering, rolling, firm adhesion and transmigration. When there is an infection, endothelial cells express PSGL-1 and P and E selectins to capture the circulating neutrophils in the blood through the binding to the L-selectins that are expressed on the surface of neutrophils. Loosely tethered neutrophils then start rolling on the endothelium. During this process, neutrophils undergo molecular changes by which they upregulate surface integrin receptors such as MAC-1 on their surfaces. The interactions between the integrin receptors and their endothelial ligands are essential for the firm adhesion of neutrophils to the endothelium. In order to reach the site of infection, neutrophils need to transmigrate through the endothelium. This process also requires integrin-ligand binding as well as junctional proteins such as PECAM-1. Diagram adapted from [6].
Advances in intravital microscopy have enabled imaging of thicker tissues, such as the brain, the liver and the lung, with high resolution [7, 14]. These studies uncovered individual steps in the ‘classical neutrophil recruitment cascade’ apply in many sites, such as the skin and muscle, but not everywhere. For example, in the liver sinusoids, selectin-dependent tethering, rolling and slow rolling do not occur [15]. In the lung, pulmonary capillaries, which require deformation of the neutrophil to pass through, are thought to support selectin- and integrin-independent recruitment [16, 17].

1.1.2 Microbial killing of neutrophils

Neutrophils are highly specialised cells that engage in a number of specific effector functions, including phagocytosis, degranulation, reactive oxygen species (ROS) production, NET release and production of mediators including cytokines (Fig 1.2.1). These functions permit neutrophils to protect the host from pathogens as part of the innate immune system. Recent findings have moreover established that the neutrophil not only kills, but also engage in cross-talk with other immune cells, including those belonging to the adaptive immune system, and is therefore involved in orchestrating the immune response. The neutrophil is consequently an integral part of the body’s immune system.
Figure 1.1.2 Neutrophil effector functions.

A diagram illustrating the neutrophil effector functions that are specific for microbial killing or for the resolution of inflammation. Circulating neutrophils leave the blood stream and reach the inflammatory site by a process called chemotaxis, in which neutrophils migrate along gradients of chemoattractants/chemokines. At the inflammatory site, neutrophils are able to recognise and engulf small pathogens such as bacteria by performing phagocytosis. Neutrophils produce ROS (via the activation of NADPH oxidase) and release antimicrobial peptides and proteases (via degranulation) to kill the engulfed pathogens intracellularly. Neutrophils are also able to kill pathogens extracellularly by releasing NETs, which consist of decondensed chromatin and antibacterial proteins to trap and kill pathogens. To resolve inflammation, neutrophils undergo apoptosis where they display ‘eat me’ signals on their surfaces, to trigger their own uptake by macrophages. Diagram taken from [18].
1.1.2.1 Phagocytosis

As ‘professional’ phagocytes, neutrophils possess the capacity to engulf and phagocytose pathogens. The pro-inflammatory mediators released by tissue sentinel leukocytes increase vascular permeability of the endothelium, permitting an influx of plasma that contains antibodies and complement factors to the infected tissues. The surface of pathogens are ‘decorated’ with these antibodies and complement factors in a process termed opsonisation. These immunoglobulin and complement factors bind to the Fcγ receptors and complement receptors on the surface of neutrophils [19]. This facilitates recognition of pathogens and mediates efficient phagocytosis. Phagocytosis is a tightly regulated, actin-dependent process, in which receptor-mediated intracellular signalling leads to remodelling of actin. The neutrophil extends actin rich membrane around the particle, forming a phagocytic cup around it [20]. Fusion of these ‘arms’ effectively engulfs the particle, leaving a phagosome with its typical double membrane, which is plasma membrane-derived [21].

1.1.2.2 Degranulation

The internalisation of pathogens by neutrophils initiates the degradative machinery of neutrophils to kill these pathogens. Neutrophils contain several types of granules: azurophil (primary) granules, specific (secondary) granules, gelatinase (tertiary) granules and secretory vesicles. These granules are formed sequentially during granulopoiesis, different types of granules are therefore formed at a given stage of myelopoiesis (Fig 1.1.2.2) [22]. On encounter with pathogens, neutrophils degranulate by releasing the cytotoxic contents of these granules into the phagolysosome (fused phagosome and granule proteins) or to the exterior of the cell. Degranulation causes
pathogen killing via two major ways: release of antimicrobial proteins and myeloperoxidases. The release of antimicrobial proteins kills the pathogens by disrupting bacterial membranes or interfering with the bacterial metabolic pathways, whilst myeloperoxidases is required for the generation of reactive oxygen species (ROS). Excessive degranulation, as found for example in anti-neutrophil cytoplasmic antibody (ANCA) vasculitis, is a hallmark of inflammatory disorders [23].
Figure 1.1.2.2 Sequential formation of granules during granulopoiesis.

A diagram illustrating the formation of granules at specific stages of myelopoiesis and their contents. Several types of granules are formed during granulopoiesis: azurophil granules, specific granules, gelatinase granules and secretory vesicles. Each type of granule is packed with a specific set of granule proteins (black box). Granulopoiesis is initiated in promyelocytes (PM), which is derived from granulocyte/monocyte progenitor (GMP) derived-myeloblast (MB). Following the formation of azurophil granules in PM, specific granules are formed in myelocytes (MC) and metamyelocytes (MM), whilst gelatinase granules are formed in band cells and neutrophils. Secretory vesicles only appear in neutrophils. Diagram adapted from [22].
1.1.2.3 The NADPH oxidase and ROS production

In a reaction termed the ‘respiratory burst’, due to the incursion of oxygen consumption, the plasma membrane-associated NADPH oxidase catalyses the production of superoxide ions (O$_2^-$). This represents the first chemical reaction required for the generation of several microbicidal compounds. NADPH oxidase is composed of a number of cytosolic- and membrane-associated subunits, several of which are called phagocyte oxidase (phox) proteins [24]. Since the phagosomal membrane has an origin in the plasma membrane, assembly of the NADPH oxidase can occur in plasma membrane-derived phagosomal membrane as well.

In the resting state, the NADPH oxidase is disassembled, with two of its subunits, gp190$^{\text{phox}}$ and p22$^{\text{phox}}$, forming a membrane bound dimer. Three remaining subunits, p67$^{\text{phox}}$, p47$^{\text{phox}}$ and p40$^{\text{phox}}$ form a cytosolic complex [24]. The final subunit is the small GTPase Rac, which is also in the cytosol, being separate from the p67$^{\text{phox}}$/p47$^{\text{phox}}$/p40$^{\text{phox}}$ complex and is sequestered by Rho-GDI until activated (discussed in section 1.3.3.1). Activation of NADPH oxidase requires its own assembly, and this is dependent on the membrane translocation of the cytosolic complex and Rac2. Translocation of these proteins is mediated by phospholipids and protein phosphorylation events. Rac2 is activated by Rac GEFs (discussed in chapter 1.4.5), and recruitment to the plasma membrane [25, 26]. Phosphorylation of p47$^{\text{phox}}$ and PI3P binding by p40$^{\text{phox}}$ are also required for activation of the NADPH oxidase [27, 28]. The complex regulation of the NADPH oxidase suggests that correct regulation of this enzyme is critical for the well-being of the host.

Following the generation of superoxide anion by the NADPH oxidase, superoxide anion is converted into the more toxic hydrogen peroxide by superoxide dismutase.
Finally, hydrogen peroxide is converted into the potent microbicidal hypochlorous acid by myeloperoxidase (MPO), which is delivered and released by azurophil granules [29]. A diagram illustrating the intracellular killing of bacteria by neutrophils is shown in Fig 1.1.2.3.

The importance of NADPH oxidase is best demonstrated in chronic granulomatous disease (CGD) patients. Phagocytes of CGD patients are able to phagocytose, but lack the ability to produce ROS and kill the ingested pathogens due to genetic defects in one of the genes encoding the phox proteins that are required to assemble the NADPH oxidase. CGD patients therefore experience recurrent and potentially life-threatening bacterial and fungal infections [30].
Figure 1.1.2.3 Intracellular killing of bacteria by neutrophils.

A diagram illustrating the functions by which neutrophils perform intracellular killing of bacteria in a phagosome. Degranulation enables the release of antimicrobial proteins [from specific (yellow) and gelatinase (blue) granules] and myeloperoxidase [MPO, from azurophil (green) granules]. Antimicrobial proteins kill bacteria by disrupting bacteria membrane and metabolic pathways, whilst myeloperoxidase is required for the ROS production. Upon stimulation, ROS production is initiated with the assembly of NADPH oxidase at the phagosomal or the plasma membrane. Cytosolic complex (p47\textsubscript{phox}, p40\textsubscript{phox} and p67\textsubscript{phox}) and the small GTPase Rac translocate to the membrane, where they interact with the membrane-bound complex (gp190\textsubscript{phox} and p22\textsubscript{phox}), forming NADPH oxidase. This assembly activates the oxidase itself, allowing it to generate superoxide anions (O\textsuperscript{2-}) by oxidising oxygen (O\textsubscript{2}). These superoxide anions act as substrate for another enzyme, superoxide dismutase, generating hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}). The presence of hydrogen peroxide finally allows myeloperoxidase to oxidise chloride ions (Cl\textsuperscript{-}), generating hypochlorous acid (HOCl), which is a potent biocide. In addition, hydrogen peroxide also reacts with free iron ions (Fe\textsuperscript{2+}) to generate oxygen radicals (O\textsuperscript{2}).
1.1.2.4 Formation of neutrophil extracellular traps (NETs)

Phagocytosis of pathogens, and subsequent degranulation and ROS production are considered as the classical way by which neutrophils kill pathogens. However, a new concept has risen up over the past decade that suggests that neutrophils are capable of killing pathogens also via an alternative, phagocytosis-independent, extracellular mechanism by the formation of neutrophil extracellular traps (NETs) [31]. It is thought that phagocytosis and NETs work cooperatively to ensure efficient killing of pathogens. Whilst small pathogens such as bacteria or unicellular fungi (yeast) are killed following ingestion, those that are too large to be ingested such as parasites or multicellular fungi (hypae) may be killed by NETs. In addition, there is evidence suggesting that NET production can occur in the blood stream under conditions of life-threatening infections (e.g. sepsis) to capture bacteria [32].

NETs are prominent extracellular structures that consist of unfolded chromatin, histones, granular peptides and enzymes. In vitro, NET formation can be efficiently induced by non-physiological agents such as phorbol 12-myristate 13-acetate (PMA). In vitro induction of NET formation with physiological agents such as IL-8, lipopolysaccharide (LPS), bacteria (e.g. Staphylococcus aureus) and fungi is less efficient [33]. NETs are thought to amplify the effectiveness of antimicrobial protein released by neutrophils by forming a localised network that efficiently concentrates these proteins [33]. The histones that make up NETs also possess antimicrobial properties. For example, the histone-derived antimicrobial peptide, Buforin II, can penetrate bacterial cell membranes, bind to bacterial nucleic acids and disrupt their cellular functions, causing rapid cell death [34, 35]. Oxidation of H₂O₂ by myeloperoxidase is also required for NET formation [36], as demonstrated by the
observation that neutrophils of CGD patients, whose NADPH oxidase is defective, fail to make NETs when incubated with bacteria or stimulated with PMA. However, NET formation is restored when H$_2$O$_2$ is added exogenously [37].

1.1.2.5 Production of inflammatory mediators

Neutrophils have been shown to synthesise inflammatory mediators that are important for coordinating inflammatory and/or immune responses.

Neutrophil-derived inflammatory mediators can drive the recruitment of further neutrophils (as exemplified by neutrophil swarming induced by generation of the potent lipid mediator LTB$_4$ [38]). They can also mediate microvascular leakage to facilitate neutrophil transmigration at a site of infection; act as priming agents to potentiate anti-viral immune responses and modulate and shape immune responses of other immune cells such as macrophages and T cells [39-41]. Neutrophils can produce these mediators constitutively and/or upon activation; the pattern of mediators produced varies depending on the agonist; with LPS generally regarded as one of the most powerful inducer of cytokines [42].
1.1.3 Neutrophil apoptosis as a mechanism to resolve inflammation

Inflammation is an essential mechanism which organisms evolved to protect themselves from harmful pathogens. Since inflammation has potential to cause tissue damage following the destruction and clearance of pathogens, it needs to be resolved to protect the host. The resolution of inflammation is therefore a crucial phase of the inflammatory response. It involves several distinct processes: limitation of neutrophil infiltration, reduction of mediator release, induction of neutrophil apoptosis, uptake of apoptotic neutrophils by macrophages, and the initiation of healing. Disrupting any of these processes could result in chronic inflammation [43].

The induction of neutrophil apoptosis and their subsequent clearance by macrophages represent a major way by which neutrophil number and functions are limited at inflammatory sites. Neutrophil apoptosis ensures termination of neutrophil functions and induces the production of anti-inflammatory and pro-resolution mediators by macrophages [43].

Apoptosis (programmed cell death) acts as a homeostatic mechanism that maintains cell populations in tissues without generating inflammation. It is particularly important for maintaining the appropriate number of neutrophils in the circulation and at inflammatory sites. Excess or accelerated neutrophil death might lead to neutropenia, decreasing the efficiency by which invading pathogens can be killed and cleared. Conversely, insufficient or delayed neutrophil apoptosis might lead to neutrophilia, potentially increasing the chance of tissue injury caused by persistent neutrophil activities [44, 45].
Neutrophil apoptosis occurs in three main ways. Firstly, using the intrinsic pathway, where cytochrome c and pro-apoptotic factors are released into the cytosol due to damaged mitochondrial membrane [46]. Secondly, using the extrinsic pathway, which is activated by ligation of cell surface death receptors by tumour necrosis factor (TNF), Fas ligands and TNF-related apoptosis-inducing agent (TRAIL) [47, 48]. Thirdly, in phagocytosis-induced apoptosis, phagocytosis induces neutrophil apoptosis and ensures the safe disposal of ingested pathogens [49].

When cells undergo apoptosis, their cell bodies shrink, their cytoplasm becomes vacuolated, nuclear condensation occurs and DNA is fragmented. Cell surface receptors such as integrins are downregulated [50]. Instead, molecules like lysophosphatidylserine and phosphatidylserine are displayed. Lysophosphatidylserine acts as ‘find me’ signal and promotes migration of macrophages towards apoptotic neutrophils, whilst phosphatidylserine acts as ‘eat me’ signal and lead to efferocytosis through the binding of phosphatidylserine receptors on macrophages, thereby removing apoptotic neutrophils from inflammatory sites and preventing secondary necrosis and subsequent damages [51-53].

1.1.3.1 Regulation of neutrophil apoptosis

It is crucial that neutrophil apoptosis is well controlled, since dysregulation of neutrophil apoptosis can contribute to inflammatory diseases [54, 55]. Caspases and the B-cell lymphoma 2 (Bcl-2) family proteins are two key molecular components that regulate apoptosis.
Caspases (cysteine-dependent aspartate-specific protease) are cysteine proteases that coordinate efficient dismantling of dying cells in apoptosis. Caspases are synthesised as inactive zymogens (precursors) that contain a prodomain, which requires cleavage for caspase activation. Caspases can be generally divided into two groups: initiator caspases and effector caspases. Initiator caspases, include caspase-1, -2, -4, -5, -8, -9, -10, -11 and -12, and respond to external or internal stimuli. On activation, initiator caspases cleave and thereby activate effector caspases. Effector caspases, include caspase-3, -6 and -7, and act on a range of cellular substrates (e.g., regulators of transcription) to mediate apoptosis. An illustration of the process of extrinsic and intrinsic apoptosis is shown in Fig 1.1.3.1. Most apoptotic cell deaths are caspase-dependent. For example, in the human neutrophil, inhibition of caspases effectively blocked TNF-induced apoptosis [56].
Figure 1.1.3.1 Extrinsic and intrinsic apoptosis pathways.

A diagram demonstrating extrinsic and intrinsic apoptotic pathways in neutrophils. Different caspases are involved in these two pathways. Extrinsic apoptosis is initiated by the ligation of death receptors by death ligands such as the Fas ligand, inducing downstream signalling that leads to the activation of caspase 8. In contrast, intrinsic apoptosis is initiated in response to DNA damages and/or the presence of pro-apoptotic factors, which activates caspase 9. Both active caspases 8 and 9 can cleave pro-caspase 3 and 7, thereby turning them into active caspase 3 and 7, which then go on to cleave downstream effectors to mediate apoptosis.
Bcl-2 proteins, which govern life and death decisions in most cells, are important regulators of caspases. The Bcl-2 protein family has pro- and anti-apoptotic members; their balance determines whether or not a cell undergoes apoptosis. The major pro-apoptotic Bcl-2 family proteins expressed by neutrophils is Bcl-associated X protein (Bax). Other neutrophil pro-apoptotic Bcl-2 family proteins include Bcl-2 homologous antagonist/killer (Bak), Bcl-2-associated death promoter (Bad), Bcl-2 homology-3 interacting domain death agonist (Bid), Bcl-2 interacting protein (Bim) and Bcl-2 interacting killer (Bik) [57, 58]. These pro-apoptotic proteins normally exist in an inactive form, in which their transmembrane domains are enclosed in a hydrophobic groove. They undergo conformational changes and become active when cytotoxic signals are present [59]. Hence, Bax translocates to and inserts itself into the outer mitochondrial membrane where it oligomerises, forming pores in the membrane and allowing the release of apoptotic factors like cytochrome c into the cytosol [60]. This activates caspases and drives apoptosis. For example, the release of cytochrome c is crucial for the activation of apoptosome, through which caspase 9 activity is promoted and thereby inducing the intrinsic apoptosis [61].

Anti-apoptotic members expressed in neutrophils are myeloid cell leukaemia 1 (Mcl-1) and A1, with Mcl-1 being the major regulator [59, 62]. Anti-apoptotic proteins delay apoptosis by binding to pro-apoptotic proteins, thereby restraining their pro-apoptotic activities. Expression of anti-apoptotic proteins is low in circulating neutrophils. They undergo rapid degradation and are short-lived, with a half-life of approximately 2-3 hours, whilst pro-apoptotic proteins are expressed at relatively high levels with long half-lives [63]. The dominance of pro-apoptotic over anti-apoptotic proteins explains the short half-lives of circulating neutrophils. Expression of anti-apoptotic proteins is
upregulated upon neutrophil activation, increasing binding of anti-apoptotic Bcl family proteins to their pro-apoptotic cousins and thereby dramatically extending the lifespan of activated neutrophils [64, 65]. It is worth noting that apart from apoptosis, other forms of programmed cell death also exist, such as autophagic degeneration, calpains- and cathepsin B-dependent cell deaths [66], but for the purpose of this thesis, only apoptosis is explored here.
1.2 Neutrophils in inflammatory disease

By destroying and clearing pathogens, neutrophils play an essential part in host defence. If the regulatory mechanisms that control neutrophils are impaired, they can drive excessive inflammation, and contribute to considerable host damage. Indeed, dysregulated neutrophil infiltration and neutrophilic inflammation are hallmarks of many inflammatory and autoimmune diseases. Antibody- and antigen-, as well as usually complement-containing immune complexes are activators of neutrophils; they are frequently involved in the aberrant activation of neutrophils in the context of autoimmune disease. Immune complexes are formed in the circulation as part of the normal physiological mechanism for antigenic clearance. Usually they are rapidly cleared from the circulation in the liver and/or spleen, but in some autoimmune conditions soluble and insoluble free immune complexes persist, such as those that are found in the synovial fluid in the joints of rheumatoid arthritis patients [67].

Alternatively, immune complexes can be deposited on biological surfaces, where they trigger neutrophil activation. Frequent sites include vessel walls (in ANCA vasculitis) or the renal glomerulus (in glomerulonephritis) [68].

1.2.1 Immune complexes in inflammation

Antibodies (immunoglobulins; Ig) are specialised protein complexes made up from at least two heavy and two light chains. Several classes exist (IgM, IgD, IgG, IgA and IgE), which are generated in a process called ‘class switching’ [69]. Different antibody classes vary with regards to their protein structure, expression and function. IgG is the best understood and most common antibody (Fig 1.2.1.1). Antibodies have a variable
region that forms the antigen binding site, and a constant region, that comprises the Fc region of the antibody. The Fc portion binds to Fc receptors (with IgG binding to Fcγ receptors, IgA binding to Fcα receptors etc) that are expressed on a number of immune cells, including neutrophils.
Figure 1.2.1.1 Structure of IgG.

A simplified diagram showing the structure of the IgG as an example to illustrate the general features of immunoglobulins. Monomeric immunoglobulins resemble a ‘Y’ shape structure and are made up of two heavy chains (blue) and two light chains (green). The Ig chains are linked with disulphide bonds (black lines), and can be divided up into the constant region and the variable region. Immunoglobulins bind to antigen via their variable regions, whilst the type of heavy chain they contain determines their isotype. The Fc region of immunoglobulins is responsible for binding to Fc receptors that are expressed by many cell types.
B cells produce a large repertoire of antibodies (initially IgM) in a process involving recurrent somatic DNA recombination events. During ‘clonal deletion’, a stringent negative selection process, immature B cells producing antibodies that recognise ‘self’ antigens are eliminated [70]. This aims to protect the host from autoantibodies, however, occasionally a B cell producing a self-antigen recognising-antibody survives. This can occur when the self antigen is not present in the tissues through which immature B cells pass, for example, when it localises to the inside of another cell type (as is the case in ANCA vasculitis) [71].

When antibodies bind to their specific antigens, they form immune complexes. In the healthy state, this occurs during the opsonisation of a pathogen. Neutrophil Fcγ receptors engagement with antibody Fc regions drives multiple intracellular signalling events, and triggers the activation of a number of cellular regulators, inducing a series of neutrophil functions, including phagocytosis and killing of opsonised pathogens as detailed in section 1.1.2.1.

Excessive production of self-recognising antibodies or new self epitopes are apparent in immune complex-driven autoimmune disease. In these diseases, self-antigen is recognised, abundant immune complexes are formed, circulate and deposit in tissues. It has been suggested that the inflammatory mediators secreted by local mast cells or basophils during inflammatory responses increase the permeability of the vascular wall, permitting the escape of immune complexes from the circulation and enabling deposition in tissues [72, 73]. Immune complexes themselves can also promote vascular leakage in susceptible tissues such as joint tissue. Properties of antigens and antibodies that make up the complexes can modulate the extent and location of immune complex deposition [74].
1.2.2 Immune complex-driven neutrophilic inflammation in inflammatory diseases

Immune complex-driven neutrophilic inflammation is implicated in many inflammatory diseases, including ANCA vasculitis, rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and certain types of glomerulonephritis. The pathogenesis of these diseases shares common features with excessive neutrophil recruitment and dysregulated neutrophilic inflammation playing important roles. Autoimmune diseases are often associated with, and exacerbated by NET release. NETs are highly immunogenic as they expose intracellular antigens, spurring on the development of autoantibodies [75]. The activation of neutrophils by immune complexes that have been deposited on a large surface might lead to ‘frustrated’ phagocytosis, since the target is too large for neutrophils to engulf [67]. As a result, the cytotoxic proteins released during degranulation and ROS contribute to host tissue destruction. At the same time, oxygen radicals released cause DNA damage, as well as oxidation of lipids, proteins and lipoproteins, contributing further to excessive inflammation. Finally, immune complexes induce neutrophilic chemokine production, thereby promoting renewed neutrophil influx [76]. In fact, immune complexes themselves may facilitate neutrophil recruitment by tethering to circulating neutrophils [77].
1.3 Signalling by Ras and Rho family small GTPases

Neutrophil behaviour in response to external changes is tightly regulated by a complex network of intracellular signalling. Small GTPases (also known as small GTP binding proteins or small G proteins) are a crucial component of this network. Small GTPases are monomeric proteins that have a conserved guanine nucleotide binding (G) domain, with which can bind to and hydrolyse guanine triphosphate (GTP) to guanine diphosphate (GDP) \[78\]. Small GTPases act as molecular switches that cycle between active (GTP bound) and inactive (GDP bound) states. They regulate many cellular processes ranging from cell migration, cell growth and differentiation to vesicular transport \[79-81\]. Small GTPases are classified into several families, Ras, Rho, Arf, Ran and Rab. In this thesis, I will focus on Ras and Rho family small GTPases.

1.3.1 Regulation of small G proteins

Small GTPases alternate between two different states, GTP-bound (active) and GDP-bound (inactive). Small GTPases are able to bind GTP and GDP at low picomolar concentrations. Since cellular concentrations of GTP and GDP are in the millimolar range (with an approximate 10-fold excess of GTP over GDP), small GTPases are almost always nucleotide-bound. The intrinsic rate of GTP hydrolysis is very slow, therefore, regulators are required to accelerate GDP dissociation and GTP hydrolysis \[82\].

The exchange of GDP to GTP in GDP-bound small GTPases is facilitated by guanine nucleotide exchange factors (GEFs). GEFs bind to GDP-bound small GTPases and induce conformational changes that result in lowered affinity for GDP. This catalyses
the dissociation of GDP, and stabilises the nucleotide-free small GTPases. The binding of GTP to the complex displaces the GEF, leaving the active GTPase to interact with its effectors to promote cellular responses [82]. To terminate the effects of active small GTPases, they need to return to their inactive state by hydrolysing GTP back to GDP. Hydrolysis of GTP is facilitated by another group of regulators called GTPase-activating proteins (GAPs). The binding of GAPs to GTP-bound GTPases induces conformational changes that increase the rate of hydrolysis of GTP, resulting in GDP-bound GTPases [82]. Each small GTPase has its own set of GEFs and GAPs that are specific to it. A summary of the small G protein cycle is illustrated in Fig 1.3.1.
Small GTPases cycle between an active and an inactive state. Small GTPases are molecular switches that regulate many cellular functions. They are active when they are bound to GTP and inactive when they are bound to GDP. The intrinsic rates of nucleotide exchange and hydrolysis are very slow, therefore these processes are accelerated by two groups of regulators called GEFs and GAPs.
1.3.2 Signalling through Ras family small GTPases

Ras family small GTPases comprise at least 21 members, with H-Ras, N-Ras and K-Ras being the best characterised. Some Ras proteins are oncogenic, constitutively active mutation (e.g. G12V, which locks them in the GTP-bound state) of which are key contributors to the development of cancer. Although best known for their role in cancer, Ras-induced signalling also regulates inflammation. Once activated, Ras proteins can interact with downstream effectors. These include Raf kinases, phosphoinositide 3-kinases (PI3Ks) and Ral guanine nucleotide exchange factors (RalGEFs), which in turn regulate numerous signalling pathways and ultimately coordinate a host of physiological responses.

1.3.2.1 The Ras-Raf-Mek-Erk signalling cascade

Raf kinases are arguably the best characterised downstream effectors of Ras. Their activation elicits the canonical Ras-Raf-Mek-Erk signalling pathway. There are 3 Raf proteins: A-Raf, B-Raf and C-Raf (also known as Raf-1), all of which can bind to GTP-bound Ras. A-Raf is expressed highest in urogenital tissues and B-Raf is expressed in neuronal tissues, testis and hematopoietic cells. C-Raf is ubiquitously expressed and has been studied most extensively. With the help of other proteins (e.g. 14-3-3), Raf proteins are kept inactive in the cytoplasm in resting cells [83].

The effector domain of GTP-bound Ras is exposed, and permits effector binding. Ras binds to two sites within Raf, the Ras binding domain (RBD) and the cysteine-rich domain (CRD). Binding of Raf to GTP-Ras is essential for initiating Raf activation [84, 85]; it drives Raf translocation from the cytoplasm to the plasma membrane,
where Raf becomes activated after a series of dimerization and phosphorylation events [86].

Activated Raf kinases directly interact with and phosphorylate Mek-1 and -2, dual specificity protein kinases that in turn phosphorylate and thereby activate Erk-1 and -2. Activated Erk kinases phosphorylate a number of downstream cytoplasmic and nuclear effectors that include transcription factors, leading to changes in gene expression and thereby bringing about physiological changes [86].

1.3.2.2. Mitogen activated protein (MAP) kinases including extracellular regulated kinase (Erk)

Erk belongs to a family of protein kinases called mitogen-activated protein kinase (MAPK). This family comprises protein kinases that are involved in the regulation of numerous biological processes, ranging from gene expression to cellular morphology. c-Jun N-terminal kinases (JNK), p38 MAPK (p38) and Erk1/2 (also known as p44/p42) are the best characterised MAPKs [87]. p38 and JNK are generally activated in response to stress stimuli such as ionising radiation and cytokine stimulation, whilst Erk1/2 is activated in response to growth factor stimulation. Although each MAPK has unique characteristics, they all share common features. As exemplified by the Ras-Raf-Mek-Erk signalling cascade mentioned above, their activation always involves a MAP Kinase Kinase Kinase (MAPKKK or MAP3K; e.g. Raf), which activates a MAP Kinase Kinase (MAPKK or MAP2K; e.g. Mek), which in turn activates the MAPK (e.g. Erk). Once activated, the MAPK can act on a range of downstream substrates, which include phospholipases, transcription factors and cytoskeletal proteins [87].
this introduction, I will focus on Erk1/2; the functions of p38 and JNK will not be discussed.

Erk is a crucial regulator of inflammation. In hematopoietic cells, Erk has been shown to regulate several processes, including cell differentiation, proliferation and apoptosis, by phosphorylating transcription factors, thereby altering expression of genes that are important for these processes [88, 89]. Although well known for its anti-apoptotic effect, under certain circumstances, the Ras-Raf-Mek-Erk signalling can be pro-apoptotic [89].

1.3.3 Signalling through Rho family small GTPases
Rho family small GTPases comprise Rac, Cdc42 and RhoA as the three best characterised family members. Rho family small GTPases are well-known for controlling dynamic actin rearrangements [90], where Rac regulates lamellipodia (membrane ruffles); Cdc42 regulates filopodia (microspikes); and RhoA regulates stress fibres (actin cables) [80, 91, 92]. By signalling through numerous effector proteins, which include protein kinases and scaffold proteins, Rho small GTPases regulate a diversity of biological processes, particularly involving cell migration and morphological changes [93]. Rho family small GTPases (and their GEFs and GAPs) represent key regulators of inflammation.

1.3.3.1 Signalling through Rac
Based on sequence homology, Rac1, Rac2, Rac3 and RhoG form a subfamily of Rac GTPases, with Rac1, Rac2 and RhoG expressed by neutrophils [94, 95]. Rac1 and
Rac2 have been studied extensively, and are known to be critical regulators of lamellipodium formation and migration in neutrophils [90]. Despite their high sequence similarity, Rac1 and Rac2 have distinct roles in the regulation of these processes. Rac2 is predominantly expressed in hematopoietic cells, and has been shown to have an essential role in various stages of the neutrophil recruitment cascade. Under normal physiological flow conditions, neutrophils derived from Rac2−/− mice showed defective tethering to the L-selectin ligand, GlyCAM-1. These neutrophils also showed impaired chemotaxis and F-actin polymerisation in response to fMLF and IL-8 stimulation [96]. In contrast to Rac2, Rac1 is not essential for efficient neutrophil migration per se, but it is required for the directionality of cells migrating towards fMLF [97]. Rac1 and Rac2 have also been shown to contribute to phagocytosis of apoptotic cells and to Fc receptor-mediated engulfment of particles, as demonstrated with neutrophils isolated from Rac2-null mice and from patients with dominant-negative mutation in Rac2 [98-100]. Finally, Rac2 is also required for ROS production in neutrophils, where Rac1 activity does not rescue the impaired ROS production observed with Rac2-deficient neutrophils [101, 102]. RhoG has not been as extensively studied as Rac1 and Rac2, but has been suggested to act upstream of Rac, and is also important for Rac-dependent NADPH oxidase activity [103, 104].

1.3.3.2 Signalling through Cdc42

Cdc42 has a conserved role in the regulation of cell polarity in many biological systems. In the neutrophil, Rac was originally thought to be a critical regulator of cell polarity, but the essential role of Cdc42 in the regulation of this process has since been revealed. In neutrophil-like differentiated HL-60 cells, Rac was demonstrated to be
crucial for persistent movement at the leading edge, whilst Cdc42 was required for stabilising polarity and motility [105]. This function of Cdc42 was supported and further investigated by a recent study, where the authors showed that Cdc42 activity is localised at the leading edge of (fMLF-stimulated) neutrophil-like differentiated PLB-985 cells, where it regulates cellular turning and steering towards chemoattractant [106]. The important function of Cdc42 is also exemplified in neutrophils derived from Cdc42-null or Cdc42GAP-null mice, where loss of Cdc42 or too much Cdc42 activity alike were shown to impair leukocyte recruitment to inflammatory sites [107]. Apart from regulating cell polarity, Cdc42 is also an important regulator of cell differentiation and cell fate in a number of cell types, including hematopoietic stem cells [108, 109].

1.3.3.3 **Signalling through Rho**

The subfamily of Rho GTPases consists of three Rho isoforms: RhoA, RhoB and RhoC. All three Rho isoforms exert different effects in cells [110]. In the neutrophil, RhoA is the major Rho isoform that is expressed, and like Rac and Cdc42, RhoA is an important regulator of neutrophil migration. In contrast to Rac and Cdc42, RhoA is best known for its activity at the rear end of the chemotaxing cell [95, 111]. Unlike other cell types (e.g. fibroblasts and T cells), where RhoA-GTP has been shown to localise to the leading edge and to the trailing end, in neutrophil-like cells, it has so far only been detected at the rear of the migrating cell [95, 112, 113]. Although RhoA-FRET mice have been generated, no RhoA FRET probe expressing primary neutrophils have yet been described [114]. RhoA activity is associated with cell contractility, which is required for rear end retraction and maintains persistent
movement of the migrating cell. It has been demonstrated that migrating neutrophils with lack of RhoA activity exhibited defective detachment of cell at the rear end, resulting in impaired migration [115]. However, a more recent report suggested that RhoA can act as a positive as well as a negative regulator of neutrophil recruitment, depending on the stimulus [116].
1.4 Phosphoinositide 3-kinases (PI3Ks)

PI3Ks comprise a family of enzymes that catalyse the phosphorylation of inositol phospholipids at the 3-position of the inositol ring. Both substrates and products for this reaction are resident in cellular membranes. Some PI3K lipid products serve as molecular messengers in the membrane in which they reside, thereby bringing about different cellular effects [117]. To date, eight mammalian PI3K isoforms have been identified, and based on their structural features and substrate preferences, these isoforms are classified into three classes. Class I PI3Ks are the most extensively investigated and best understood. They use the plasma membrane localised phosphatidylinositol-(4,5)-bisphosphate [PI(4,5)P₂] as substrate, generating the lipid second messenger phosphatidylinositol-(3,4,5)-trisphosphate [PI(3,4,5)P₃] (Fig 1.4) [117]. The somewhat enigmatic class II PI3Ks convert their substrate PI or PI4P, generating PI3P and PI(3,4)P₂, respectively, and are thought to act at the plasma membrane and/or endosomal membranes. Finally, class III PI3K consists of a single enzyme, Vps34, which uses as substrate endosomal PI, generating PI3P [118].

Signalling downstream of PI3K occurs through effectors that are capable of binding to specific PI3K lipid products via specialised binding domains, such as PH domains for PI(3,4,5)P₃ (for short, PIP₃) and sometimes PI(3,4)P₂, and FYVE or PX domains to PI3P [119]. This permits spatiotemporal regulation of signalling in the cells, by driving the translocation of PI3K effector proteins (from the cytosol to a membrane compartment).

Since the focus of this thesis is on the signal transduction from extracellular cues to cellular responses, only class I PI3Ks will be discussed here; please note that in chapters other than the introduction, the term PI3K is used to refer to class I PI3Ks.
Figure 1.4 Inositol phospholipids and the PI3K-dependent generation of PI(3,4,5)P$_3$.

Inositol phospholipids contain an inositol ring and a diacylglycerol backbone. They further comprise two fatty acid moieties that bind to the SN1 and SN2 positions on the diacylglycerol backbone. Class I PI3Ks phosphorylate phosphatidylinositol (4,5) bisphosphate [PI(4,5)P$_2$] at the D3 position of the inositol ring, thereby generating phosphatidylinositol (3,4,5) triphosphate [PI(3,4,5)P$_3$]. Diagram adapted from [117].
1.4.1 Class I PI3Ks

Class I PI3Ks, also known as agonist-activated PI3Ks, are active at the plasma membrane; they can be activated downstream of a range of cell surface receptors either directly or indirectly via an adaptor [120]. Their major lipid product, PIP₃ is a lipid second messenger (it relays signals from cell surface receptors to many intracellular targets). Numerous effector proteins, many of which contain Pleckstrin homology (PH) domains, have been identified to respond to PIP₃. To terminate the effects of PIP₃, PIP₃ can be dephosphorylated by PTEN at the 3-position, returning it to PI(4,5)P₂, or by phosphatases of the SHIP family (SHIP1/2) at the 5-position, forming PI(3,4)P₂ (Figure 1.4.1) [121, 122]. Only a small number of studies have been carried out to address the roles of PTEN and SHIP in neutrophils. As expected, both PTEN and SHIP are regulators of PI3K-regulated neutrophil functions. Both PTEN and SHIP have been shown to regulate adhesion, chemotaxis and cell polarity in mouse and also in zebrafish neutrophils [123-126]. However, the underlying mechanisms remain controversial. Owing to their ability of limiting PI3K-induced neutrophil functions, PTEN and SHIP, especially SHIP1, represent attractive therapeutic targets for diseases that are dependent on neutrophilic inflammation. For example, a SHIP1 activator is being tested alongside PI3Kγ/δ specific inhibitors for treatment of lung inflammation [127, 128].
Figure 1.4.2 Regulation of PI3K-dependent signalling.

Receptor activation drives recruitment of PI3K to the plasma membrane. PI3K causes phosphorylation of PI(4,5)P_2 at the D3 position of the inositol ring to generate PI(3,4,5)P_3 (PIP_3). To terminate effects induced by PIP_3, PTEN dephosphorylates PIP_3 at the 3-position, generating PI(4,5)P_2, whereas SHIP dephosphorylates the 5-position and generates PI(3,4)P_2.
Class I PI3Ks are heterodimeric enzymes that contain a regulatory subunit and a catalytic subunit. Based on their structural features and mode of regulation, class I PI3Ks are subdivided into class IA and class IB. Class IA PI3Ks contain a p85α, p85β, p55α, p55γ or p50α regulatory subunit, and a p110α, p110β or p110δ catalytic subunit. Any of these regulatory subunits can partner with any of the catalytic subunits, resulting in fifteen possible combinations of heterodimers for class IA PI3Ks. Class IB PI3Ks contain a p101 or a p84 regulatory subunit, which specifically bind to p110γ catalytic subunit. PI3K isoforms are usually named after the catalytic subunit they contain. For example, any PI3K that contains p110β is called ‘PI3Kβ’, regardless of its regulatory subunit [117].

1.4.2 Receptor activation of PI3Ks

Class IA PI3Ks are activated downstream of receptor tyrosine kinases (RTKs), where the p85-type adaptor binds to phosphotyrosine motifs contained in the cytoplasmic tail of the activated RTK or in an adaptor. Phosphotyrosine binding drives recruitment of the PI3K to the plasma membrane. In addition, PI3Kβ was shown to be activated also (synergistically with phosphopeptide) by G protein βγ subunits after activation of G protein coupled receptors (GPCRs). Class IB PI3Ks are activated by G protein βγ subunits that interact with the regulatory subunit [120, 129].

1.4.3 PI3K activation by small GTPases

In addition to being activated following receptor ligation, class I PI3Ks are also activated by small GTPases. Hence, p110α, δ and γ are activated by Ras whilst p110β
is activated by Rac/Cdc42 [130]. In either case, the p110 catalytic subunits bind to the Ras/Rho effector domain using its RBD, which shares its tertiary structure with the Raf RBD [131, 132]. Ras-GTP binding to the PI3K RBD activates PI3K together with another activating event. Hence, phosphopeptide and Ras together activate PI3Kα, and δ. Gβγ and Ras together activate PI3Kγ, whilst in the case of PI3Kβ, phosphopeptide, Gβγ and Rac/Cdc42 are required for its full activation. It is thought that PI3K is activated catalytically by Ras/Rac/Cdc42 (and Gβγ) binding, rather than being entirely dependent on plasma membrane translocation of the PI3K [133]. A diagram illustrating the regulation of Class I PI3K is shown in Figure 1.4.3.
Figure 1.4.3 Regulation of Class I PI3Ks.

A diagram illustrating the regulation of Class I PI3Ks by receptor ligation and small GTPases. PI3Kγ is activated by GPCRs [e.g. formyl peptide receptor (FPR)] via the Gβγ subunits, whilst PI3Kα and PI3Kδ are activated by receptors such as FcγRs, via binding to an adapter protein (e.g. Syk). PI3Kβ is activated by Gβγ subunits and RTK. In addition, all Class I PI3Ks can be activated by small GTPases, with Ras, or Rac/Cdc42 binding to the Ras binding domain (RBD) of the PI3K catalytic subunits.
1.4.4 PI3K signalling in the neutrophil

All Class PI3Ks are expressed in the neutrophil; PI3Kα and PI3Kβ are ubiquitously expressed in all cell types, whereas PI3Kγ and PI3Kδ were found to be preferentially expressed in leukocytes [118]. Based on their differential expression pattern, class I PI3K isoforms have been proposed to exert different cellular effects.

Since neutrophils are not amenable to long term culture, transfection or transduction, genetically modified mice offer a unique opportunity to study PI3K signalling in primary neutrophils. A variety of genetically modified mice have been reported, in which individual PI3K subunits were (sometimes conditionally) knocked out; there are also reports of point mutation knock-ins (e.g. kinase dead or those in which residues enabling individual activating events, such as small GTPase binding, have been mutated). Comparatively, fewer experiments have been carried out with human neutrophils, where PI3K inhibitors are employed to understand PI3K isotype contribution to individual effector functions. These were made possible by a formidable collection of PI3K inhibitors that are available for use in research (some are being trialled for use in the clinic), thanks to concerted efforts by academia and pharma who have synthesized compounds for use in treatment of the many pathological conditions that PI3K isoforms regulate (especially cancer) [134, 135]. Inhibitors range from pan-PI3K (e.g. wortmannin) over pan-class I PI3K (e.g. PI103) to isoform-specific inhibitors. Selectivity of these inhibitors have been studied extensively; pan-PI3K inhibitors such as Ly294002 and wortmannin are shown to be able to inhibit other intracellular targets (e.g. PLK1) at concentrations similar to those inhibit PI3Ks, and isoform-specific inhibitors also have the potency to inhibit other PI3K isoforms apart from the one they are designed to target [134, 136, 137]. However,
the concentrations of inhibitors used in this thesis have been described to only affect the target(s) they are designed to (please see table 2.4.2) [117]. Interestingly, the relatively small number of studies that have compared mouse and human cells are suggestive of substantive differences in signalling between mouse and human neutrophils [138, 139]. These observations are in-line with observations made elsewhere in the immune system [140], and reinforce the view that the mouse’s immune system faces very different challenges to the human one. In other words, the mouse is a useful genetic model system, but as any model system, it has limitations.

In the neutrophil, the leukocyte-enriched PI3Kγ and PI3Kδ are thought to be particularly important. PI3Kγ was shown to play an essential role in signalling downstream of chemoattractant receptors (e.g. fMLF, IL-8 and LTB4) regulating neutrophil recruitment to the sites of inflammation [141]. Sequential activation of PI3Kγ and PI3Kδ was shown to be required for the respiratory burst in TNF-primed human neutrophils in response to fMLF stimulation [138]. This temporal dependency on PI3Kγ and PI3Kδ is also observed in the neutrophil recruitment in response to MIP-2, where PI3Kγ is responsible for early response, whilst PI3Kδ is responsible for more prolonged response [142]. Due to their involvement in the regulation of neutrophil functions (and those of other immune cells), PI3Kγ and PI3Kδ represent attractive therapeutic targets for autoimmune diseases such as rheumatoid arthritis, allergic inflammation (e.g. asthma) as well as chronic diseases with a strong inflammatory component (e.g. chronic obstructive pulmonary disease) [143, 144]. Genetic and pharmacological interventions have demonstrated convincingly that inhibition of PI3Kδ and PI3Kγ induce protective effects in several disease models (e.g., rheumatoid arthritis [145], immune complex-induced lung injury [146] and in B-cell malignancies.
PI3Kβ was shown genetically to play a critical role downstream of integrins and Fcγ receptors in the mouse neutrophil, and is important in regulating downstream functions elicited by these receptors [148, 149]. In contrast to the other three Class I PI3Ks, the role of PI3Kα in the neutrophil remains to be investigated.

1.4.5 Signalling downstream of PI3K

PI3K signals through multiple effectors. An average cell is thought to contain approximately 25 PI3K effectors. Although many proteins have been isolated in PIP₃ binding screens (Figure 1.4.5), many PIP₃ binding proteins remain comparatively under investigated [150].
Figure 1.4.5 The signalling network of Class I PI3Ks.

A diagram illustrating the regulation of some cellular functions by class I PI3Ks through some downstream effectors. PI3Ks effectors are recruited to the plasma membrane via binding of their PH domain to PIP$_3$. These downstream effectors include serine/threonine (Ser/Thr) kinases, tyrosine kinases, adaptors proteins and GEFs and GAPs of Ras/Rho/Arf GTPases. Through these effectors, Class I PI3Ks are able to control a host of diverse cellular functions (some examples are shown in purple).
Interestingly, a large proportion of PIP₃ binding proteins were shown to be GEFs and GAPs for small GTPases (Ras, Rho, and Arf families), suggestive of a large amount of cross-talk between PI3Ks and small GTPases. Here, I discuss cross-talk between PI3K and Rho family small GTPases as an example of this principle.

In particular, the association between PI3K and Rac activity is very well established in neutrophils and in other cell types. Several PIP₃ regulated Rac GEFs have been identified in the neutrophil: Tiam1/2, Vav1/3, P-REX1 and DOCK2/5 [26, 151, 152]. P-REX1 was shown to control a subset of Rac-dependent neutrophil functions, in particular ROS production in response to GPCRs stimulation [153]. Vav proteins (Vav1/3) are mainly responsible for the regulation of neutrophil functions induced by integrin stimulation [154]. Although these two GEF families do not have any major role in regulating chemotaxis individually, they control chemotaxis and recruitment when they act together [155]. DOCK-2 deficient neutrophils exhibit loss of cell polarity [156]. In addition, PIP₃ also regulates Rac activity by regulating Rac GAPs, regulating chemotaxis, recruitment, and phagocytosis.

In contrast, no PIP₃-regulated RhoA GEF has yet been identified. However, ARAP3, a dual RhoA/Arf6 GAP regulated by PIP₃ and Rap, was originally isolated from neutrophils [150]. Mouse Arap3⁺⁻ neutrophils or those in which ARAP3 was uncoupled from activation by PI3K, showed upregulated neutrophil functions upon adhesion-dependent stimulation, likely as a result of increased β2 integrin activity [157, 158].

Similarly, no PI3K-regulated Cdc42 activator has yet been described. However, Cdc42GAP, which has been shown to regulate neutrophil chemotaxis, was isolated in
a screen for PIP3 binding proteins from neutrophils [107, 150]. A recent report also suggests the capacity of PI3K to regulate Cdc42 activity by regulating additional Cdc42GAPs, at least in macrophages [159].
1.5 Hypotheses and Aims of thesis

Given that immune complex-driven neutrophilic inflammation is implicated in many inflammatory diseases and PI3K signalling plays an important role in orchestrating neutrophil response, it is therefore of great interest to investigate about immune complex-induced PI3K signalling in neutrophils and how it regulates their functions, as this may provide new insights into new therapeutic targets or treatment design. The main hypothesis of this thesis is PI3K signalling induced by immune complexes is crucial in the regulation of neutrophil functions, and this can be subdivided into the following hypotheses:

i) Immune complexes induce PI3K signalling in neutrophils

ii) Immune complex-induced PI3K signalling is an important regulator of neutrophil function(s)

iii) Immune complex-induced PI3K signalling in neutrophils is conserved between species (human and mouse)
To address the above hypotheses, the aim of this thesis was to investigate PI3K signalling by elucidating the pathway by which PI3K activates Erk in immune complex-stimulated neutrophils, and which neutrophil function this pathway regulates. Signalling events and functional responses were analysed pharmacologically in primary human and mouse neutrophils. Moreover, I aimed to confirm my results genetically, by using genetically modified mice and neutrophil-like cells.
2 Materials and Methods

2.1 Isolation of primary neutrophils

2.1.1 Isolating human neutrophils from peripheral blood

Aliquots of 40 mL peripheral venous blood from healthy donors was drawn into 50 mL Falcon tubes (BD Sciences) already containing 4 mL of sodium citrate [0.38% (final concentration)] to prevent clotting. After careful and gentle mixing, the blood was centrifuged at 350 g for 20 minutes at room temperature (RT), with low acceleration and without brake, for the separation of platelet-rich plasma (PRP) from the remaining leukocytes and erythrocytes. After centrifugation, the PRP layer was collected into 10 mL glass tubes. Clotting was induced by adding CaCl$_2$[20 mM (final concentration)] into 10 mL of PRP, followed by 1 hour incubation at 37°C to generate autologous serum. The platelet plug was removed from the serum, and the serum was retained for use in subsequent assays. Dextran (Sigma; 12% of final volume) was added to the remaining blood cell layer and the tube topped up with NaCl (0.9%, Baxter) that had been pre-warmed at 37 °C to a final volume of 50 mL; the tube was allowed to stand to allow sedimentation of erythrocytes. After approximately 30 minutes, the leukocyte rich, upper layer was collected into a new 50 mL Falcon tube and topped up with NaCl (0.9%, Baxter), followed by 6 minutes centrifugation at 350 g at RT. A discontinuous Percoll (GE Health)/ PBS (-CaCl$_2$, -MgCl$_2$, Gibco) cation gradient was made by gently layering 4 mL 63% Percoll onto 4 mL 72% Percoll. This was overlaid with the leukocytes that had been resuspended in 3 mL 49.5% Percoll (all in PBS). The discontinuous Percoll gradient was subject to centrifugation at 720 g for 20 minutes at RT, with low acceleration and without brake. Granulocytes were
collected at the interface between 72% and 63%, and were washed twice with 50 mL of PBS (-CaCl₂, -MgCl₂, Gibco) by centrifugation at 230 g for 6 minutes at RT. For use in assays, cells were suspended in PBS++ [Dulbecco’s PBS (+CaCl₂, +MgCl₂), supplemented with 1 g/L glucose and 4 mM sodium bicarbonate]. Neutrophil purity and viability were >95% as assessed by cytocentrifuge preparations.

### 2.1.2 Isolation of bone marrow-derived mouse neutrophils

Hind femurs and tibiae were collected from 10-14 weeks old C57B1/6 mice. Bone marrow (BM) was flushed into HBSS++/-- [Hank’s buffered saline solution without Ca++ and Mg++, supplemented with 0.25% fatty acid, endotoxin-free bovine serum albumin (BSA) and 14 mM Hepes (pH 7.4 at RT)] using a 1 mL syringe (BD Plastipak) and a 25 g needle (TERUMO). BM cells were resuspended in HBSS++/--, followed by centrifugation at 300 g for 10 minutes. During this centrifugation, a discontinuous Percoll gradient was made by under-lying 6 mL of 62% underneath 4 mL of 55% Percoll. BM cells were resuspended in 3 mL of HBSS++/--, overlayed on top of the discontinuous Percoll gradient, and subjected to centrifugation at 1300 g for 30 minutes, with low acceleration and without brake. Neutrophils were collected at the interface between the 55% and 62% Percoll layers and placed into a new 50 mL Falcon tube, for washing with 50 mL HBSS++/--. After centrifugation at 300 g for 10 minutes. The erythrocytes were lysed in Geyes solution [70% dH₂O, 20% Geyes A (1 M NH₄Cl, 1M KCl, 1 M Na₂PO₄, 1 M KH₂PO₄ and glucose), 5% Geyes B (1 M MgCl₂.6H₂O, 196 mM MgSO₄.7H₂O and 1 M CaCl₂.2H₂O) and 5% Geyes C (1M NaHCO₃)], followed by two further washes in 50 mL HBSS++/--. The purity of the preparation
was assessed by cytocentrifuge preparation stained using Reastain Quick Diff reagents (Reagena).

2.2 Mammalian cell culture

2.2.1 Culture and granulocytic differentiation of PLB-985 cells

PLB-985 cells were cultured in complete RPMI medium [RPMI 1640 (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS, Gibco), 5% glutamine (Gibco) and 5% Penicillin-Streptomycin (Pen/Strep, Gibco)], and were maintained at a cell density of 2x10⁵ to 8x10⁵ cells per mL at 37 °C in a humidified atmosphere at 5% CO₂ in a tissue culture incubator. PLB-985 cells were cultured for at least 1 week before induction of differentiation. Granulocytic differentiation of PLB-985 cells was induced by culturing the cells (at a starting cell density of 2x10⁵ per mL) in complete RPMI supplemented with 1.25% DMSO (Sigma) for 5 days. Differentiated PLB-985 cells were washed twice with PBS by centrifugation at 230 g for 5 minutes at RT prior to use. The morphology of the differentiated PLB-985 cells was assessed by cytocentrifuge preparations that had been stained using Reastain Quick Diff reagents.

2.2.2 Culture of HEK-293ET cells

HEK-293ET cells were cultured in complete DMEM medium [DMEM (Gibco) supplemented with 10% HI-FBS (Gibco), 5% glutamine (Gibco) and 5%
Penicillin/Streptomycin (Gibco)] at 37°C in a humidified atmosphere at 5% CO₂ in a tissue culture incubator. They were passaged regularly to avoid cells reaching confluency. HEK-293 cells were cultured for at least 1 week prior to transfection for generation of retrovirus.

2.2.3 Generation of retrovirus

2.5x10⁶/mL HEK293ET cells were seeded in a T25 tissue culture flask containing 3.3 mL complete DMEM medium the day before transfection took place. At least 30 minutes prior to transfection, fresh culture medium was added to the cells. To perform transfection, medium was aspirated and cells were incubated with a total of 3 mL medium containing plasmids [packaging (psPAX2), envelop plasmids (pMD2.G) and the bicistronic pQXCIH-CD16 vector (which conferred hygromycin resistance and CD16 expression)] as well as 5μl polyethylenimine (1 mg/mL, Alfa Aesar). After incubation for 48 hours, supernatant containing retrovirus was harvested, 0.4 μm filtered and stored at -70°C. 3 mL of medium was applied to the cells for the second harvest, supernatant of which was collected, filtered and stored at -70°C the following day.

2.2.4 Spinoculation of PLB-985 cells

2x10⁵/mL PLB-985 cells in 2 mL complete RPMI medium supplemented with 10 μg/mL polybrene were gently mixed with 1 mL lentivirus containing cell culture medium in a 15 mL Falcon tube and subjected to centrifugation at 800 g for 30 minutes
at 32°C. Supernatant was then removed, the cell pellet was resuspended in 2mL fresh, complete RPMI medium (without polybrene) and plated into a 6-well plate. After incubation at 37°C for 2 days, cells were put onto appropriate antibiotic selection (e.g. 1 μg/mL puromycin; effective antibiotic concentration had been identified by performing a killing curve). After 5 to 6 days of selection, growing PLB-985 cells were subjected to analysis of the expression of the protein of interest.

2.3 Preparation of plates for adhesion-dependent stimulation

2.3.1 Preparation of immobilised IgG-BSA immune complexes

6cm tissue culture plates (Corning) were coated with 100 μg/mL BSA made up in PBS++ overnight at 4°C. Aspirated plates were blocked with 1% non-fat milk powder in PBS++ for 45 minutes at RT, and then were washed extensively with PBS++. For the formation of immune complexes, the plates were then incubated with rabbit anti-BSA IgG in PBS++ (1/2000, Sigma Aldrich) for 1 hour at RT. Plates were again washed extensively with PBS++ prior to plating neutrophils for stimulation. For controls, plates were treated identically but not incubated with anti-BSA antibody.

2.3.2 Preparation of fibrinogen coated plates

6cm tissue culture plates were coated with 150 μg/mL human fibrinogen (Sigma) in PBS++ overnight at 4°C. The plates were washed 3 times with PBS++ prior to
stimulating neutrophils by plating in the presence of TNF (20 ng/mL); for control stimulations, neutrophils were plated onto fibrinogen-coated plates in the absence of co-stimulation with TNF.

2.3.3 Preparation of pRGD coated plates

6 cm tissue culture plates were coated with poly Arg-Gly-Asp (pRGD; 20μg/mL) in PBS++ for 3 hours at RT. Control plates were coated with HI-FBS. Plates were washed with PBS++ 3 times prior to plating neutrophils for activation.

2.3.4 Preparation of insoluble IgG-HSA immune complexes

IgG-human serum albumin (HSA) insoluble immune complexes (insoluble ICs) were prepared in batches. Insoluble ICs were made up following the identification of the point of equivalence between antigen and antibody for each individual batch of antibody. The protocol for preparing insoluble ICs was kindly provided by Professor Steve Edwards, University of Liverpool [160]. In brief, anti-HSA rabbit IgG and HSA (both from Sigma) were made up in PBS to a final concentration of 5 mg/mL, and loaded in different ratios into wells of a 96 well plate. The plate was incubated with gentle agitation for 1 hour at 37°C, followed by measurement of absorbance at 450 nm. The highest reading corresponded to the point of equivalence, and this ratio of HSA and anti-HSA antibody is required for the formation of insoluble ICs. A batch of insoluble ICs was then prepared based on this ratio and incubated with gentle agitation for 1 hour at 37°C. Insoluble ICs were then washed thoroughly with PBS followed by centrifugation at 1734 g for 5 minutes at RT to discard any soluble immune complexes.
present. Following extensive washing, insoluble ICs were resuspended in PBS and stored at 4°C.

2.4 Biochemical assays

2.4.1 Pre-treatment of primary neutrophils with DFP

All work with diisopropylfluorophosphate (DFP) was performed in a Class II safety cabinet. Primary neutrophils that have been isolated from peripheral blood of healthy donors was incubated with DFP (7mM) for 10 minutes at RT. At the end of the incubation, to remove DFP, cells were washed with PBS twice by centrifugation at 8000g for 1 minute at RT. All DFP waste, including tips that have been in contact with DFP, were disposed into 2% aqueous sodium hydroxide.

2.4.2 Activation assays of primary neutrophils and differentiated PLB-985 cells

Freshly prepared neutrophils or differentiated PLB-985 cells were pre-warmed for 5 minutes, followed by incubation with the inhibitor(s) of choice (Table 2.4.2) or vehicle for 10 minutes at 37°C. Cells were then stimulated with fMLF (1 μM), insoluble ICs (10 μg/mL), or as a control, PBS++ (refer as assay buffer thereafter). After stimulation for an appropriate time, cells were pelleted and lysed in 100 μl ice-cold lysis buffer [20 mM Hepes; pH7.4, 1 mM Na$_3$VO$_4$, 1% Triton X-100 (w/v), 3 mM β-glycerol phosphate, 30 mM NaF, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1 mM PMSF,
10 μg/mL of each antipain, aprotinin, pepstatin A and leupeptin] for 1 minute at 4°C. For adhesion-dependent stimulations, neutrophils were pre-warmed, incubated with inhibitors, plated onto carefully aspirated coated dishes (as described in 2.3.1 to 2.3.3) and placed into a humidified tissue-culture incubator at 37°C. After 12 minutes, supernatants were carefully harvested and non-adherent cells were collected by centrifugation. Adherent cells were collected by scraping into 100 μl ice cold lysis buffer and combined with the non-adherent, carefully aspirated cell pellets on ice for lysis. Cell lysates were collected by centrifugation at 8000 g for 10 minutes at 4°C, boiled in sample, and subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for protein separation (see 2.4.3).
<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Target</th>
<th>Concentration used</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP1</td>
<td>Src family kinases</td>
<td>10μM</td>
<td>Cayman</td>
</tr>
<tr>
<td>PI103</td>
<td>Pan-PI3K</td>
<td>10μM</td>
<td>Selleckchem</td>
</tr>
<tr>
<td>Ly294002</td>
<td>Pan-PI3K</td>
<td>10μM</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Wortmannin</td>
<td>Pan-PI3K</td>
<td>50μM</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>TGX221</td>
<td>PI3Kβ</td>
<td>40μM</td>
<td>Abcam</td>
</tr>
<tr>
<td>IC87114</td>
<td>PI3Kδ</td>
<td>1μM</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>A66</td>
<td>PI3Kα</td>
<td>10μM</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>AS252424</td>
<td>PI3Kγ</td>
<td>30μM</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>CZC24832</td>
<td>PI3Kγ</td>
<td>10μM</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>PF3758309</td>
<td>Pan-Pak</td>
<td>5μM</td>
<td>Selleckchem</td>
</tr>
<tr>
<td>IPA3</td>
<td>Pan-Pak</td>
<td>10μM</td>
<td>Selleckchem</td>
</tr>
<tr>
<td>ZM336372</td>
<td>Pan-Raf</td>
<td>10μM</td>
<td>Cayman</td>
</tr>
<tr>
<td>AZ628</td>
<td>Pan-Raf</td>
<td>5μM</td>
<td>Selleckchem</td>
</tr>
<tr>
<td>Tpl2 inhibitor II</td>
<td>Tpl2</td>
<td>5μM</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>AZD6244</td>
<td>Mek</td>
<td>1μM</td>
<td>Selleckchem</td>
</tr>
<tr>
<td>Tramatinib</td>
<td>Mek</td>
<td>1μM</td>
<td>Selleckchem</td>
</tr>
<tr>
<td>FR180204</td>
<td>Erk</td>
<td>10μM</td>
<td>Selleckchem</td>
</tr>
<tr>
<td>BVD523</td>
<td>Erk</td>
<td>5μM</td>
<td>Selleckchem</td>
</tr>
<tr>
<td>Z-VAD-FMK</td>
<td>Pan-Caspases</td>
<td>10μM</td>
<td>Cayman</td>
</tr>
<tr>
<td>Palomid 529</td>
<td>mTORC</td>
<td>20μM</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>mTORC1</td>
<td>20μM</td>
<td>Sigma Aldrich</td>
</tr>
</tbody>
</table>

**Table 2.4.2 Inhibitors used in this thesis.**

A table showing all inhibitors used in this thesis, and their respective protein targets, final concentrations and suppliers.
2.4.3 Preparation of protein gels for SDS-PAGE and protein transfer onto PDVF membrane

Protein gels were cast between glass plates at 1-1.5 mm depth, with a resolving gel (typically 8% polyacrylamide) overlaid by a stacking gel. The resolving gel was made by mixing acrylamide (ProtoFLOWgel, 30% stock), 2 x resolving buffer (90.86 g/L Tris-HCl; pH8.8, 0.2% SDS, 42% H₂O) and H₂O in appropriate ratios. Polymerisation was induced by adding TEMED (0.1%) and ammonium persulphate (APS, 0.5%, Sigma). The polymerising resolving gel was overlaid with water-saturated butanol to ensure a flush finish. Once the resolving gel had fully polymerised, it was overlaid with the stacking gel [made with 2 x stacker buffer (30.28 g/L Tris-HCl; pH6.8, 0.2% SDS, 42% H₂O) and prepared as described for the resolving gel to a final concentration of 3.75% polyacrylamide]. Wells for loading samples (15-20) were formed by inserting a 1-1.5 mm thick Teflon comb into the stacking gel prior to polymerisation.

SDS-PAGE was performed by loading protein samples that had been boiled in sample buffer [45.53% glycerol, 10% β-mercaptoethanol, 2% SDS, 4% 1 M Tris (pH6.8), 0.01% bromophenolblue] onto the protein gel, and current was applied until the dye front reached the bottom of the gel. Proteins were wet transferred onto polyvinylidene difluoride (PDVF) membrane (Millipore) in ice-cold transfer buffer [2.9 g/L Tris (Sigma), 14.5 g/L glycine (Sigma) and 10% methanol (Fisher Scientific)] under cooling for 1 hour at 100V in a transfer tank (Bio-Rad).
2.4.4 Western blot analysis

Membranes were blocked with 5% non-fat milk powder in PBS supplemented with 0.1% Tween-20 (PBST) for 2 hours at RT with gentle shaking, followed by incubation with primary antibodies (Table 2.4.4.1) in PBST supplemented with 1% BSA, typically for 2 hours at RT under gentle rocking. After 3 washes in PBST, membranes were incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Table 2.4.4.2; for detection of phospho-PKB Ser473, a biotinylated primary rabbit antibody was employed in combination with streptavidin-HRP to avoid background with the antibody heavy chain in insoluble ICs) for 30 minutes at RT. Membranes were washed 3 times with PBST, followed by incubation with an enhanced chemiluminescence (ECL) substrate (Bløk-CH buffer, Millipore) for 5 minutes at RT. Chemiluminescence was detected using x-ray films that were developed in an automated developer (Kodak). For analysis of western blots, numerical values of bands were generated by performing densitometry analysis using a plug-in in ImageJ (NIH).
<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospho-Erk (Thr202/Tyr204)</td>
<td>1/1000</td>
<td>Cell Signalling Technology</td>
</tr>
<tr>
<td>Phospho-PKB (Thr 308)</td>
<td>1/500</td>
<td>Cell Signalling Technology</td>
</tr>
<tr>
<td>Phospho-PKB (Ser 473)</td>
<td>1/3000</td>
<td>Cell Signalling Technology</td>
</tr>
<tr>
<td>Phospho-Mek (Ser217/221)</td>
<td>1/1000</td>
<td>Cell Signalling Technology</td>
</tr>
<tr>
<td>Phospho-p38 (Thr180/Tyr182)</td>
<td>1/1000</td>
<td>Cell Signalling Technology</td>
</tr>
<tr>
<td>Phospho-Pak (Ser144)</td>
<td>1/1000</td>
<td>Cell Signalling Technology</td>
</tr>
<tr>
<td>Phospho-Tpl2 (Thr 290)</td>
<td>1/1000</td>
<td>Abcam</td>
</tr>
<tr>
<td>Phospho-S6 (Ser235/236)</td>
<td>1/1000</td>
<td>Cell Signalling Technology</td>
</tr>
<tr>
<td>β-actin</td>
<td>1/3000</td>
<td>Abcam</td>
</tr>
<tr>
<td>β-COP</td>
<td>1/90</td>
<td>Gift from Dr. Nick Kistakis, Babraham Institute</td>
</tr>
<tr>
<td>Mcl-1</td>
<td>1/1000</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Bax</td>
<td>1/1000</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Ras</td>
<td>1/1000</td>
<td>Cell Signalling Technology</td>
</tr>
<tr>
<td>RhoA</td>
<td>1/1000</td>
<td>Cell Signalling Technology</td>
</tr>
</tbody>
</table>

Table 2.4.4.1 Primary antibodies used in western blot analysis.

A table showing all the primary antibodies used in western blot analysis, and their respective dilutions and suppliers, and the species from which they are produced.
<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Dilutions</th>
<th>Suppliers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-mouse-HRP conjugated</td>
<td>1/2000</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td>Anti-rabbit-HRP conjugated</td>
<td>1/3000</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Biotin-HRP conjugated</td>
<td>1/3000</td>
<td>Biolegend</td>
</tr>
</tbody>
</table>

Table 2.4.4.2 Secondary reagents used in western blot analysis.

A table showing the secondary reagents used in western analysis, and their respective dilutions and suppliers.
2.4.5 Pull down assays of small GTPases (Ras/Rac/Cdc42/RhoA) by GLISA

Small GTPases activity assays were performed by G-LISA assay (cytoskeleton) basically as per manufacturer’s instructions. However, as additional precaution against the significant proteases freed upon neutrophil lysis, the lysis buffer that had been supplied by the manufacturer was supplemented with DFP (7 mM), in addition to the supplied anti-protease inhibitors.

2.5 Analysis of neutrophil functions

2.5.1 L-selectin shedding

Neutrophils were stimulated with insoluble immune complexes or vehicle. Following 30 minutes of incubation at 37°C, cells were pelleted by centrifugation at 8000g for 1 minute at 4°C, and resuspended in 50μl of ice-cold PBS++. To detect cell surface L-selectin, cells were incubated with PC5-conjugated anti-CD62L (Immunotech) or isotype control for 30 minutes on ice. Cells were then washed twice with 180μl of PBS++. Expression of L-selectin was analysed by subjecting cells to flow cytometry (FACS Calibur, BD) and subsequent data analysis using FlowJo software. Neutrophils were gated by relative size (FSC, forward scatter) and granularity (SSC, side scatter), and data were expressed as histograms.
2.5.2 Measurement of ROS production assay (production of reactive oxygen species) using luminol chemiluminescence

A white polystyrene 96 well plate (Nunc) was used for measuring ROS production in a chemiluminescence-based assay. Neutrophils (5x10^5 per well in PBS++) were pre-warmed with luminol (150 μM), isoluminol (150μM) and HRP (18.75 U/mL; all from Sigma and made up in PBS++) or luminol and HRP for measurement of internal, external or total ROS production, respectively. The mixture was added to wells that had or had not been coated as described in 2.3.1., containing buffer or stimuli (e.g. insoluble ICs; final concentration 10 μg/mL). For certain stimulation conditions (e.g. fMLF; 1μM), cells were TNF (4.54ng/mL) primed or mock primed with buffer for one hour at 37°C prior to measuring ROS production. Measurements of light emission started immediately after thorough mixing of cells and stimuli. Data were expressed in relative light units (RLU) over a measured period of time.

2.5.3 Apoptosis assays

Neutrophils at 1x10^7/mL in IMDM medium (Gibco) supplemented with 10% autologous serum were pre-incubated with inhibitors of choice for a range of time points at 37°C followed by stimulation with insoluble ICs (10 μg/mL) or with vehicle. At indicated timepoints, 30 μl of cells were mixed with FITC-conjugated Annexin V (1:500 dilution from stock FITC-conjugated Annexin V antibody, Roche) in a FACS tube for a 30 minutes incubation on ice. 1 μl of propidium iodide (PI) was added to cells just prior to analysis by flow cytometry (FACS Calibur, BD).
Subsequent data analysis was performed using the FlowJo software. Neutrophils were gated by relative size and granularity. Double negative cells were defined as viable; Annexin V-positive, PI-negative cells were defined as apoptotic; double positive cells were defined as necrotic. The percentage of apoptotic and necrotic cells were analysed by One Way ANOVA for statistical analysis.

2.5.4 Internalisation assays

Insoluble ICs were pre-stained with Alexa 488-conjugated goat anti rabbit (1:800 dilution; Invitrogen) for 30 minutes on ice. Neutrophils (1x10⁶ per condition) were pre-incubated with inhibitors of choice for 10 minutes at 37°C prior to incubation with insoluble ICs (1μg/mL). After incubation for 10 minutes at 37°C, cells were pelleted by centrifugation at 4°C, followed by resuspension in 100 μl of ice-cold PBS. Cells were then allowed to adhere to a cover slip for 3 minutes, and washed three times with PBS prior to fixation with 4% paraformaldehyde (PFA) for 5 minutes at RT, followed by 3 further washes with PBS. Cell-surface bound insoluble ICs were then stained with Alex-568-conjugated goat anti rabbit antibody (1:800 dilution, Invitrogen) for 30 minutes on ice, followed by 3 washes with PBS. Coverslips were mounted to slides using the anti-fade reagent (Gold antifade mountant, Life technologies), and internalised (green) and bound (green and red) insoluble ICs were viewed by indirect immunofluorescence (20x objective; EVOS microscope).
2.6 Analysis of cell surface integrins and Fcγ receptors

Human neutrophils isolated from peripheral blood of healthy donors or PLB-985 cells that had been differentiated to become neutrophil-like were stained with primary antibodies or isotype controls (Table 2.6 for clones, suppliers and concentrations employed) directed against integrins and Fcγ receptors for 30 minutes on ice, followed by three washes with PBS++. Secondary PE-conjugated goat anti-mouse antibody (Immunotech) or vehicle was then applied for 30 minutes on ice. Cells were washed 3 times with PBS before being subjected to analysis by flow cytometry (FACS Calibur; BD). Subsequent data analysis was performed using the FlowJo software. Neutrophils and differentiated PLB-985 cells were gated by FSC and SSC, and data were expressed as histograms.
<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Target</th>
<th>Isotype</th>
<th>Dilution/concentration used</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>LM2</td>
<td>CD11b (αM)</td>
<td>Mouse IgG1</td>
<td>10μg/ml</td>
<td>Provided by Professor Ian Dransfield</td>
</tr>
<tr>
<td>TS1/18</td>
<td>CD18 (β2)</td>
<td>Mouse IgG1</td>
<td>10μg/ml</td>
<td>Provided by Professor Ian Dransfield</td>
</tr>
<tr>
<td>WAC70</td>
<td>CD11e (αL)</td>
<td>Mouse IgG2A</td>
<td>1/500</td>
<td>Provided by Professor Ian Dransfield</td>
</tr>
<tr>
<td>CD49F</td>
<td>VLA6 (α6, β1)</td>
<td>Mouse IgG1</td>
<td>1/5</td>
<td>Sero-technology</td>
</tr>
<tr>
<td>Anti-CD49B</td>
<td>CD49B (α2)</td>
<td>Mouse IgG1</td>
<td>1/5</td>
<td>Sero-technology</td>
</tr>
<tr>
<td>Anti-CD16 (FITC conjugated)</td>
<td>CD16 (FcyRIlb)</td>
<td>FITC IgG</td>
<td>1/10</td>
<td>Dako</td>
</tr>
<tr>
<td>Anti-CD32 (FITC conjugated)</td>
<td>CD32 (FcyIIA)</td>
<td>FITC IgG</td>
<td>1/10</td>
<td>Dako</td>
</tr>
<tr>
<td>Anti-CD64 (FITC conjugated)</td>
<td>CD64 (FcyI)</td>
<td>FITC IgG</td>
<td>1/10</td>
<td>Dako</td>
</tr>
</tbody>
</table>

Table 2.6 Antibodies used in FACS analysis of cell surface integrins and Fc gamma receptors.

A table showing the antibodies used in FACS analysis of cell surface integrins and Fc gamma receptors, and their respective targets, isotypes, dilutions or concentrations used and suppliers.
2.7 Molecular biology

2.7.1 Generation of a retroviral transfer vector expressing CD16

The human CD16 cDNA (OriGene) was amplified by PCR, using primers designed to insert AgeI and BamHI restriction sites, as well as an ATG start and a TAA stop codon (this was done by Dr. Sonja Vermeren). The PCR reaction employed Phusion polymerase (Invitrogen), a proof-reading polymerase which generates a high yield of PCR product, as per manufacturer’s instructions. The PCR product was gel purified using a gel extraction kit (Quiagen) as per manufacturer’s instructions, digested with AgeI and BamHI (Promega). The retroviral vector pQCXIH (Clontech) was prepared by restriction digest using AgeI and BamHI and dephosphorylated using Antarctic phosphatase (NEB) according to manufacturer’s instructions. Both insert and vector were subjected to phenol:chloroform extraction followed by ethanol precipitation prior to setting up a ligation using a rapid ligation kit (Roche) as per manufacturer’s instructions. Ligation products were transformed into rubidium chloride competent DH5α cells.

2.7.2 Making competent E.coli cells using rubidium chloride

Competent cells were made using a method adopted from the rubidium chloride method developed by Professor Douglas Hanahan. An overnight culture of DH5α was diluted 1:100 and grown in 5 mL Luria Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl and 1 mM NaOH) at 37°C for 3.5 hours, inoculated into 100 mL LB and grown until an OD600 of 0.45 to 0.55 was reached. The culture was then incubated on ice for 5 minutes, followed by centrifugation at 1700 g for 15 minutes at
4°C. Each bacterial pellet was resuspended in 10 mL of ice-cold TFBI (30 mM CH₃COOH, 50 mM MnCl₂, 100 mM RbCl, 10 mM CaCl₂, 15% glycerol, pH5.8) and incubated on ice for 10 minutes. Following centrifugation as above, bacteria were resuspended in 2 mL of ice-cold TFBII (10 mM Na-MOPS pH7.0, 75 mM CaCl₂, 10 mM RbCl, 15% glycerol). Cells were quickly frozen in 100 μl aliquots using a dry ice ethanol bath and stored at -80°C.

2.7.3 Transformation of competent E.coli cells
Ligation products were transformed into competent E.coli cells by mixing 5μl ligation products and 100μl competent E.coli cells immediately after they had thawed on ice. After 30 minutes incubation on ice, cells were then heat shocked (42°C for 45 seconds), incubated on ice for 2 minutes and diluted into 900 μl of SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄ and 20 mM glucose). After 1 hour incubation in a bacterial shaker at 37°C, cells were plated onto LB agar plates supplemented with antibiotic for selection (e.g. 100 μg/mL carbenicillin). The LB agar plates were incubated overnight at 37°C for growth of colonies.

2.7.4 Plasmid preparations
Individual colonies were picked and inoculated into 5 mL LB containing appropriate antibiotic for selection (e.g. 100 μg/mL ampicillin), for overnight shaking at 37°C. Plasmid DNA was purified using a plasmid miniprep (Thermo Scientific) or plasmid midi- or maxiprep kits (Qiagen). The procedure of purification was performed
according to the instructions supplied by the manufacturers. Concentrations of purified plasmids were measured using a Nanodrop spectrophotometer (Thermo Scientific). Correct plasmids were also identified by performing test digests with the appropriate restriction enzymes (Promega), followed by analysis of restriction fragments of GelRed (Roche) stained DNA following agarose gel electrophoresis with DNA stained using Gel Red (Biotinium).

2.8 BCA protein assay

BCA protein assays were performed to determine the protein concentrations. The assay was performed according to the instructions supplied by the manufacturer (Thermo Scientific), employing a standard curve of BSA with analysis of OD at 562 nm. In brief, the assay utilise the peptide bonds in protein to reduce the Cu$^{2+}$ ions in the solution supplied by the manufacturer to Cu$^{+}$, the amount of Cu$^{2+}$ ions reduced is proportional to the amount of protein present in the solution. Addition of bicinchoninic acid, which chelates with Cu$^{+}$ ions, gives a purple coloured complex that strongly absorbs at wavelength of 562nm [161].

2.9 Statistical analysis

Where data met assumption for a parametric test, the two-tailed Student’s t-test was applied for pairwise comparisons. Otherwise, the non-parametric Mann Whitney test
was used. For kinetic analyses (e.g. ROS assays), the area under the graph was used for analysis. Data for multiple comparisons, such as those obtained from analysis of neutrophil apoptosis were analysed by ONE WAY ANOVA. A p-value of 0.05 was considered to be statistically significant.
3 Results-A novel signalling pathway downstream of Fcγ receptors in human neutrophils

3.1 Introduction

Under normal physiological conditions, circulating neutrophils are kept inactive; they only become active in response to a variety of extracellular stimuli, one of which is immune complexes. Immune complexes bind to Fc receptors on the surface of neutrophils, thereby eliciting intracellular signalling cascades. Class I PI3Ks and Erk are key signalling intermediates that transduce signals from extracellular cues and ultimately regulate numerous physiological responses in neutrophils. PI3K and Erk are generally thought to lie on separate pathways in this signalling network, where they follow the conventional PI3K-PKB-mTORC1 and Ras-Raf-Mek-Erk cascades. However, a number of studies have indicated crosstalk between the two signalling pathways, and in some rare situations, PI3K was even shown to lie upstream of Ras [162-164]. Previous unpublished observations in the laboratory suggest that Erk activation is dependent on PI3K in human neutrophils that have been stimulated by being plated onto immobilised immune complexes or onto integrin ligands. However, the mechanism by which PI3K activates Erk remained elusive.
Hypothesis and aims

The aim of this chapter was to uncover the mechanism by which PI3K regulated Erk activation in immune complex-stimulated human neutrophils. It was hypothesised that PI3K regulates Erk activation via canonical Ras-Raf-Mek-Erk signalling. To address this, the following aims were investigated:

i) determine which PI3K isoform(s) were involved in the regulation of Erk activation

ii) determine whether PI3K activated Erk via Ras-Raf-Mek-Erk signalling or via a novel signalling pathway
3.2 Results

3.2.1 PI3K lies upstream of Erk in human neutrophils that have been stimulated with immune complexes or integrin ligands

Unpublished observations in the laboratory suggested that Erk activation was dependent on PI3K in human neutrophils that had been stimulated by being plated onto immobilised immune complexes (immobilised ICs) or fibrinogen and TNF (Fgn&TNF). I first determined I could reproduce these findings. For this, I examined Erk activation in human neutrophils that had been plated onto immobilised ICs or onto Fgn&TNF, in the presence or absence of the pan-PI3K inhibitor wortmannin by analysing Erk phosphorylation using a phosphospecific antibody by western blotting. In parallel, phosho-PKB was detected as an indirect readout of PI3K activity.

As shown in Fig 3.2.1, PKB (Thr 308 and Ser 473) and Erk (Thr202/Tyr204) were phosphorylated upon the stimulation of neutrophils following plating onto immobilised ICs or Fgn&TNF. Inhibition of PI3K with wortmannin interfered with both PKB and Erk phosphorylation, indicating that PI3K was not only upstream of PKB, but was also upstream of Erk. These results were in-line with the previous unpublished observations in the laboratory.
Figure 3.2.1 Erk activation was PI3K-dependent in human neutrophils that had been stimulated by plating onto immobilised ICs or Fgn&TNF.

Neutrophils that had been isolated from peripheral blood of healthy donors were pre-incubated with wortmannin (Wo; pan-PI3K inhibitor, 50nM) for 10 minutes at 37°C prior to stimulation by plating onto BSA (as a control), immobilised ICs, or onto fibrinogen in the presence or absence of TNF (20ng/ml). After 12 minutes of stimulation, adherent cells were scraped into ice-cold lysis buffer and combined with non-adherent, pelleted neutrophils for lysis. Lysates were collected after centrifugation, and subjected to SDS-PAGE for protein separation. Phosphorylated kinases were detected by western blotting using phosphospecific antibodies. β-COP served as a loading control. Blots are representative of at least 3 separately conducted experiments. Graphs include data obtained from a minimum of 3 independent experiments. Error bars show SEM. **=p<0.01, ***=p<0.005. Figure adapted from [139].
3.2.2 Rapidly occurring protein degradation interferes with the analysis of signalling events in neutrophils stimulated in an adhesion-dependent fashion

I next set out to determine which PI3K isoform(s) acted upstream of Erk in human neutrophils that had been stimulated with immobilised ICs or Fgn&TNF. These stimulations required scraping adherent cells into lysis buffer. This took approximately 1 minute for each sample in the experiment, increasing the opportunity for protein degradation within the lysates. Fig 3.2.2.1 shows that, as the number of conditions tested increased (to greater than five), protein degradation became problematic. The severity of degradation worsened over time, i.e. the cells that were scraped first exhibited the worst protein degradation.
Figure 3.2.2.1 Protein degradation occurred in human neutrophils that were stimulated by processes that required cell scraping.

Neutrophils that had been isolated from peripheral blood of healthy donors were pre-incubated with or without small molecules inhibitors prior to stimulation by plating onto immobilised ICs or fibrinogen in the presence and absence of TNF (20ng/ml). After 12 minutes of stimulation, adherent cells were scraped into ice-cold lysis buffer and combined with non-adherent, pelleted neutrophils for lysis. Cells lysates were collected after centrifugation and subjected to SDS-PAGE for protein separation. β-COP was detected by western blotting. The blot above is representative of at least 3 separately conducted experiments. Numbers 1 to 6 indicate the order in which the assay dishes were scraped.
In order to overcome this problem, I pre-incubated the neutrophils with diisopropyl fluorophosphate (DFP), a powerful, cell permeable serine protease inhibitor, prior to stimulation of the cells. DFP has been used to limit proteolysis during the isolation of proteins from human neutrophils [165]. However, due to its toxicity, it is desirable from a health and safety perspective to minimise the treatment of neutrophils with DFP; for this reason I had not used DFP in the first instance. Initially, I carried out a control experiment to test the unlikely possibility that DFP might interfere with the activation status of the proteins of interest. For this, I stimulated human neutrophils that had or had not been pre-treated with DFP with fMLF. PKB and ERK were phosphorylated following fMLF stimulation. As expected, inhibition of PI3K abolished PKB, but not of Erk activation (Fig 3.2.2.2 A-C). Unexpectedly, however, on pre-incubation with DFP, both PKB and Erk were activated even in the absence of any stimulation (Fig 3.2.2.2 D). To investigate why this had happened, I tested whether the solvent (isopropanol) in which DFP was dissolved might have caused this unexpected activation. As shown in Fig 3.2.2.2 E, following pre-incubation with isopropanol, PKB and Erk were activated only after fMLF stimulation. This indicated that DFP itself causes activation of PKB and Erk. Since DFP interfered with the activation status of the proteins of interest, it was not suitable for use in my assays. I therefore sought an alternative method to activate Fcγ receptors in human neutrophils.
Figure 3.2.2.2 DFP caused basal activation of PKB and Erk in human neutrophils.

Neutrophils that had been isolated from peripheral blood of healthy donors were pre-treated with A-C) assay buffer, D) DFP (7mM) or E) isopropanol (1μl) prior to incubation with wortmannin (Wo; pan-PI3K, 50nM). Cells were then stimulated with fMLF (1μM) for 1 minute, pelleted, carefully aspirated, and assays were terminated by the addition of ice-cold lysis buffer. Cell lysates were collected after centrifugation and were subjected to SDS-PAGE for protein separation. Phosphorylated kinases were detected by western blotting using phosphospecific antibodies. β-actin served as loading control. Blots are representative of 3 separately conducted experiments. Graphs include data obtained from 3 independent experiments. Error bar show SEM. NS=not significant, **=p<0.005. Figure adapted from [139].
3.2.3 Erk activation is PI3K-dependent in human neutrophils following stimulation with insoluble immune complexes

An alternative way to activate Fcγ receptors in human neutrophils is to use insoluble immune complexes (insoluble ICs). Soluble and insoluble ICs have been described and characterised in depth, and can easily be prepared and separated from one another [160]. It has been shown that neutrophil stimulation with insoluble ICs causes the same neutrophil functions and signalling events as stimulation by plating neutrophils onto immobilised ICs, whilst soluble ICs induced quite different events [160, 166]. Given that insoluble ICs allow cells to be stimulated in suspension, this made insoluble ICs a promising alternative to immobilised ICs. I first investigated whether PI3K acted upstream of Erk in human neutrophils that had been stimulated with insoluble ICs.

As shown in Fig 3.2.3A-C, PKB, Mek, Erk and p38 MAPK (p38) were significantly phosphorylated upon neutrophil stimulation with insoluble ICs. The dose of insoluble ICs used in the assay has been tested to be the minimum dosage to elicit the maximum response of cells (data not shown), therefore this dose of insoluble ICs was used for all subsequent assays. Inhibition of PI3K abolished PKB, Mek and Erk activation, whilst p38 activation remained unaffected. This indicated that PI3K was upstream of Mek and Erk but not of p38, demonstrating that PI3K was not upstream of all members of the MAPK family. Inhibition of Mek abolished Mek and Erk, but not PKB activation, indicating that PI3K was not downstream of Mek (Fig 3.2.3 A-C). Taken together, these results showed activation of Mek and Erk, but not p38, depended on PI3K following stimulation of human neutrophils with insoluble ICs. Moreover, signalling events were demonstrated to be the same in insoluble IC- and in
immobilised IC-stimulated human neutrophils (Fig 3.2.3 A-C and G-I), indicating that insoluble ICs can be used as an alternative stimulus to immobilised ICs. Importantly, no significant protein degradation occurred upon stimulation of neutrophils with insoluble ICs (not shown). Insoluble ICs were therefore used in all subsequent assays.

I next sought to determine which PI3K isoform(s) were involved upstream of Erk in insoluble IC-stimulated human neutrophils. Erk and PKB phosphorylation was abolished when PI3Kβ and PI3Kδ had been inhibited together with inhibitors directed specifically against PI3Kβ or PI3Kδ (Fig 3.2.3 D-F). Contrasting with previous finding in the mouse neutrophil [148], I found that PI3Kδ, rather than PI3Kβ, played the critical role in insoluble IC-induced signalling in the human neutrophil (Fig 3.2.3 D-F). Collectively, these results suggested that PI3Kβ/δ were upstream of Erk in human neutrophils following stimulation with insoluble ICs.
Figure 3.2.3 Erk activation was PI3K dependent in human neutrophils stimulated with insoluble ICs or by plating onto immobilised ICs.

Neutrophils that had been isolated from peripheral blood of healthy donors were pre-incubated with PI3K inhibitors [wortmannin (Wo, 50nM); pan PI3K, TGX221 (TGX, 40nM); PI3Kβ, IC87114 (IC, 1μM); PI3Kδ; AZD6244 (AZD, 1μM) and Tramatinib (Tra, 1μM); Mek] prior to stimulation (A-F) with insoluble ICs (HSAαHSA, 10μg/ml) or assay buffer as a control, or (G-I) by plating onto immobilised ICs or BSA as a control. Following stimulation, cells were lysed in ice-cold lysis buffer and lysates were collected after centrifugation. Cell lysates were subjected to SDS-PAGE and western blotting for detection of phosphorylated kinases using phosphospecific antibodies. β-actin served as a loading control. Blots are representative of a minimum of 3 experiments. For ease of viewing, densitometry data were normalised to activated control and graphs include data obtained from at least 3 independent experiments. Error bars show SEM. NS=not significant, *=p<0.05, **=p<0.01, ***=p<0.005. Figure adapted from [139].
3.2.4 PI3K and Erk activation depend on Src kinases in insoluble IC-stimulated human neutrophils

I next set out to dissect the mechanism by which PI3K activated Erk in the insoluble IC-stimulated human neutrophil. Src family kinases (SFK) are known to play a role immediately downstream of activated Fcγ receptors where they phosphorylate phosphotyrosine motifs to activate downstream signalling events [167]. I therefore investigated the involvement of SFKs in the activation of PI3K and Erk in insoluble IC-stimulated human neutrophils. As expected, inhibiting SFK by using the pan-Src inhibitor, PP1, abolished PKB and Erk activation following insoluble ICs stimulation (Fig 3.2.4). This confirmed that SFKs were upstream of PKB and Erk in insoluble IC-induced signalling in human neutrophils.
Figure 3.2.4 SFK were upstream of PI3K and Erk in the insoluble IC-stimulated human neutrophil.

Neutrophils that had been isolated from peripheral blood of healthy donors were pre-incubated with a pan-SFK inhibitor (PP1, 10μM) prior to stimulation with insoluble ICs (HSAαHSA, 10μg/ml) or assay buffer as a control. To terminate the assay, cells were lysed in ice-cold lysis buffer and lysates were collected after centrifugation. Cell lysates were subjected to SDS-PAGE and western blotting for detection of phosphorylated kinases using phosphospecific antibodies. β-actin acted as loading control. For ease of viewing, densitometry data were normalised to the activated control. Graphs include data obtained from 3 separately conducted experiments. Blots are representative of 3 experiments. Error bars show SEM. NS=not significant, *=p<0.05
3.2.5 The involvement of mTORC1 and 2 are ambiguous in insoluble IC-stimulated human neutrophils

The mammalian target of rapamycin (mTOR) family consists of PI3K related protein kinases that function as multi-protein complexes. Two mTOR complexes, mTORC1 and mTORC2, with distinct composition of proteins and functions have been studied extensively (Fig 3.2.5.1). mTOR signalling is known to regulate a variety of biological processes, including transcription, translation, cell survival and differentiation etc., in response to growth factors, nutrients and stress [168, 169]. Activation of both mTORC1 and mTORC2 is regulated by PI3K. Although the mechanism by which PI3K activates mTORC2 remains unclear, this event was shown to be essential for PKB activation [170]. PI3K activates PKB by regulating two phosphorylation sites: Ser473 and Thr308, both of which need to be phosphorylated for the full activation of PKB. Ser473 phosphorylation is mTORC2-dependent, and this is a slow process that is regulated by PI3K indirectly. Thr308 is phosphorylated directly by phosphoinositide-dependent kinase 1 (PDK-1) when PDK-1 and PKB are brought in close proximity following the binding of PIP₃ at the plasma membrane. This is a rapid process, occurring within seconds of PI3K activation [171]. Activated PKB permits downstream signalling that is crucial for the activation of mTORC1 [172]. A diagram illustrating the regulation of mTORC1, mTORC2 and PKB by PI3K is shown in Fig 3.2.5.2. Since several studies have shown that crosstalk occurs between the PI3K-mTOR and Ras-Raf-Mek-Erk signalling pathways [173], I investigated whether mTORC1 and/or mTORC2 might be involved in the activation of Erk by PI3K.
mTORC1 and mTORC2 are two well characterised mTOR complexes that are known to involve in processes including transcription, translation and cell survival. They have different composition of proteins; mTORC1 consists of mTOR, PRAS40, Deptor, Raptor and mLST, whilst mTORC2 consists of mTOR, Sin1, mLST8, Deptor, Rictor and Protor 1/2. mTORC1 and mTORC2 act on different downstream effectors in response to different stimuli. Downstream effectors of mTORC1 include the ribosomal p70S6 kinase (p70S6K) and the translation initiation factor 4E (eIF4E); whilst mTORC2 is upstream of protein kinase C (PKC), serum/glucocorticoid regulated kinase (SGK) and PKB Ser473.
Figure 3.2.5.2 Regulation of mTORC1, mTORC2 and PKB by PI3K.

PI3K activates PKB by regulating two phosphorylation sites on PKB: Thr308 and Ser 473. Activated PI3K generates PIP₃ from PIP₂ following insoluble ICs stimulation, and thereby recruiting PDK-1 and PKB to the plasma membrane where PDK-1 phosphorylates Thr308 on PKB. The other phosphorylation site, Ser 473, is phosphorylated by mTORC2, which is activated by PI3K via an as-yet unknown mechanism. Activated PKB then permits downstream signalling that is crucial for mTORC1 activation. Palomid 529 (P529) is a poorly characterised mTOR inhibitor that inhibits mTORC1 and mTORC2, whilst rapamycin is an mTORC1 specific inhibitor.
Inhibition of both mTORC1 and 2 by Palomid 529 (P529), a relatively poorly defined PKB/mTOR inhibitor [174], following neutrophil stimulation with insoluble ICs abolished Erk activation, and attenuated P-S6 and PKB Ser473 phosphorylation but had no effect on PKB Thr308 phosphorylation (Fig 3.2.5.3). This observation is in agreement with the literature, where P529 has been described to inhibit activation of mTORC1 and 2, but not PDK [175]. Rapamycin interacts with and specifically inhibits mTORC1 when bound to FKBP12 (a 12 kDa protein that acts as a receptor for the immunosuppressant drug FK506, and is a regulator of the cell cycle [176]). Since mTORC2 does not interact with the FKBP12-rapamycin complex, it was originally thought to be not rapamycin sensitive. However, it has been demonstrated that prolonged treatment (at least 24 hours) with rapamycin reduces the assembly of mTORC2, and thereby also inhibits mTORC2 and its downstream signalling [177]. In this assay, the incubation time of cells with rapamycin was 10 minutes, which is a short incubation, therefore it can be assumed that only mTORC1 was sensitive to rapamycin treatment. Use of rapamycin showed that the inhibition of mTORC1 abolished P-S6 activation but it did not affect PKB nor Erk activation, suggesting that mTORC1 did not act upstream of PKB nor Erk. The fact that Erk activation was abolished in the presence of P529 but not rapamycin, suggested that mTORC2 might potentially be involved in the activation of Erk by PI3K. However, given the relatively poor credentials of P529, any potential involvement of mTORC2 upstream of Erk in this context would need to be confirmed by a separate experimental approach.
Figure 3.2.5.3 PI3K dependent Erk activation in the insoluble IC-stimulated human neutrophils does not involve TORC1.

Neutrophils that had been isolated from peripheral blood of healthy donors were pre-incubated with pan-PI3K inhibitor (Wortmannin, Wo, 50nM) or mTOR inhibitors [Palomid 529 (P529, 20μM); mTORC1 and mTORC2, Rapamycin (20nM); mTORC1] prior to stimulation with insoluble ICs (HSAαHSA, 10μg/ml) or assay buffer. Following stimulation, cells were lysed in ice-cold lysis buffer and lysates were collected after centrifugation. Cell lysates were subjected to SDS-PAGE and western blotting for detection of phosphorylated proteins using phosphospecific antibodies. β-actin acted as loading control. For ease of viewing, densitometry data were normalised to the activated control. Graphs include data from at least 3 separately conducted experiments. Blots are representative of a minimum of 3 experiments. Error bars show SEM. NS=not significant, *=p<0.05, **=p<0.01.
3.2.6 PI3K activates Erk via an indirect mechanism that does not involve PKB and mTORC2

Since I had no access to mTORC2 specific inhibitors, any potential involvement of mTORC2 in PI3K-activated Erk signalling needed to be addressed using a different approach. I investigated this by performing an activation time course. As already discussed, PI3K regulates two phosphorylation sites on PKB via two different mechanisms: directly via PDK-1, and indirectly via mTORC2 (Fig 3.2.5.2). The timing at which the two phosphorylation events occur can provide an indication of any potential involvement of upstream regulators, such as mTORC2. If PI3K-activated Erk phosphorylation induced by insoluble ICs involves mTORC2, then Erk activation should coincide with the phosphorylation of PKB Ser473, but not of Thr308.

As shown in Fig 3.2.6, PKB Thr308 was phosphorylated within 15 seconds of neutrophil stimulation with insoluble ICs, whilst phosphorylation of PKB Ser473 appeared after 10 minutes of stimulation and peaked at 30 minutes. In contrast, Erk phosphorylation was apparent after 5 minutes of stimulation and peaked at 10 minutes. The fact that Erk activation coincided neither with the phosphorylation of PKB Thr308 nor of Ser473 suggested that PI3K-dependent Erk activation following stimulation with insoluble ICs is an indirect event, which is regulated separately to activation of PKB.
Figure 3.2.6 PI3K-dependent Erk activation is an indirect event.

Neutrophils that had been isolated from peripheral blood of healthy donors were subjected to stimulation with insoluble ICs (HSAαHSA, 10μg/ml) or assay buffer at indicated time points. To terminate the assay, cells were lysed in ice-cold lysis buffer and lysates were collected after centrifugation. Cell lysates were subjected to SDS-PAGE, and phosphorylated kinases were detected by western blotting using phosphospecific antibodies. β-actin acted as loading control. For ease of viewing, densitometry data were normalised to activated control at 600 seconds. Graphs include data obtained from 3 separately conducted experiments. Blots are representative of 3 independent experiments. Error bars show SEM. Figure adapted from [139].
3.2.7 Ras is not downstream of PI3K following stimulation with insoluble ICs

I further characterised the mechanism by which PI3K activated Erk following stimulation with insoluble ICs. In canonical signalling, Erk is activated through the Ras-Raf-Mek-Erk signalling pathway; PI3K does not play a role in this (Fig 3.2.7 A). Ras is a small GTPase that acts upstream of the PI3K-PKB-mTORC1 and Raf-Mek-Erk signalling pathway (please see section 1.3.2 for a discussion of Ras family small GTPases). Only when Ras is GTP-bound, can it trigger activation of any downstream effectors. Ras can activate all Class I PI3Ks except for PI3Kβ [130], but in some rare situations, PI3K can act upstream of Ras [163]. I therefore asked whether PI3K activates Erk by acting upstream of Ras in insoluble IC-stimulated human neutrophils. Since I was not aware of any Ras specific inhibitor, the potential role of PI3K in Ras activation was examined indirectly by measuring Ras activity in insoluble IC-stimulated neutrophils in the presence and absence of wortmannin.

As shown in Fig 3.2.7 B, Ras was significantly activated upon neutrophil stimulation with insoluble ICs; Ras activation was not affected by the presence of wortmannin. This indicated that in this context PI3K was not upstream of Ras.
Figure 3.2.7 Ras was not downstream of PI3K in insoluble IC-stimulated human neutrophils.

A) A schematic diagram to illustrate canonical activation of PI3K and Erk signalling by Ras. B) Neutrophils that had been isolated from peripheral blood of healthy donors were pre-incubated with pan-PI3K inhibitor (wortmannin, 50nM) prior to stimulation with insoluble ICs (HSAαHSA, 10μg/ml) or assay buffer. To terminate the assay, cells were lysed in ice-cold lysis buffer and lysates were collected after centrifugation. For the analysis of Ras activity, cell lysates were assessed for the presence of Ras-GTP as described in Chapter 2.4.5. Graph shown includes data obtained from 3 separately conducted experiments. Error show SEM. NS=not significant. Figure adapted from [139].
3.2.8 PI3K-dependent Erk activation is Raf-independent in insoluble IC-stimulated human neutrophils

Following on from the previous observation, I further characterised the mechanism of PI3K-dependent Erk activation by analysing the involvement of Raf in insoluble IC-induced signalling. Raf is the MAP3K that canonically activates Mek and Erk. Extensive crosstalk has been described between the PI3K-PKB and Ras-Raf-Mek-Erk signalling pathways [173]. I therefore asked whether Raf was involved in PI3K-activated Erk signalling following stimulation with insoluble ICs.

PKB, Mek and Erk were significantly activated in neutrophils that had been stimulated with insoluble ICs. As expected, inhibiting Raf by using a pan-Raf inhibitor, ZM336372, did not affect PKB activation. Surprisingly, Mek and Erk phosphorylation were also not affected by this inhibitor (Fig 3.2.8.2 A-D), suggesting that Raf was not upstream of Mek and Erk. ZM336372 was originally described as a potent and specific inhibitor for Raf in vitro, but it has been since shown to drive paradoxical Mek and Erk activation on prolonged use in vivo [178]. Paradoxical Erk activation occurs due to the initiation of an Erk-induced negative feedback loop upon Raf activation, and this serves to avoid hyperactivation of the pathway [179, 180]. Raf also suppresses its own activation, so that activation is always counterbalanced by inhibition. These negative feedback loops regulating Raf signalling are lifted and the counterbalance is lost when ZM336372 is applied, as a result, ZM336372 drives paradoxical Mek and Erk activation (Fig 3.2.8.1). This paradoxical activation of Mek and Erk have also been observed with other competitive Raf inhibitors [181, 182]. I therefore employed an alternative Raf inhibitor, AZ628. AZ628 is a covalent Raf inhibitor that belongs to a
Figure 3.2.8.1 Paradoxical Raf activation with the ATP competitive Raf inhibitor, ZM336372.

A schematic diagram showing the mechanism by which ZM336372 paradoxically induces Mek and Erk activation. Inhibition of Raf by ZM336372 relieves Raf auto-inhibition and also the negative feedback from Erk, thereby inducing activation of Mek and Erk despite Raf inhibition.
new generation of Raf inhibitors that is termed ‘paradox breakers’. These inhibitors do not support any paradoxical Mek and Erk activation, even when used for a prolonged period of time. In the case of AZ628, it binds to the ATP-binding pocket of inactive Raf irreversibly, thereby preventing Raf to be activated [182]. As shown in Fig 3.2.8.2 A-D, inhibition of Raf by AZ628 also did not affect Mek, Erk and indeed PKB activation in insoluble IC-stimulated human neutrophils, conforming that Raf really is not involved in the regulation of Erk activation in insoluble IC-induced signalling. Raf inhibitors, including ZM336372 and AZ628, are mostly described in literature relating to cancer. Cancer cells are transformed cells that undergo rapid cell division, and grow well in culture. Treatment of cancer cells with inhibitors usually lasts for a prolonged period of time (e.g. days). I wondered whether findings obtained with prolonged treatments of cancer cells were applicable to short term treatment of primary, non-transformed neutrophils. Therefore, as a control, I also analysed fMLF-stimulated neutrophils, where Erk activation is known to follow canonical Ras-Raf-Mek-Erk signalling and is PI3K-independent (Fig 3.2.2.2 A-C). In fMLF-stimulated human neutrophils, PKB and Erk were significantly activated upon fMLF stimulation; inhibition of PI3K abolished PKB activation but had no effect on Erk activation (Fig 3.2.2.2 A-C). In contrast, inhibition of Raf by either ZM336372 or AZ628 inhibited Mek and Erk phosphorylation but did not affect PKB (Fig 3.2.8.2 E-H). This confirmed first, that Raf did indeed lie upstream of Mek and Erk in fMLF-stimulated neutrophils, and second, that both Raf inhibitors worked when used in neutrophils for a short time. Collectively, these results indicated insoluble IC-induced PI3K-dependent Erk activation did not involve Raf.
Neutrophils that had been isolated from peripheral blood of healthy donors were pre-incubated with a pan-PI3K inhibitor (wortmannin, Wo, 50nM) or Raf inhibitors [ZM336372 (ZM, 10μM) and AZ628 (AZ, 5μM)] prior to stimulation with insoluble ICs (HSAαHSA, 10μg/ml) or fMLF (1μM) or assay buffer. Following stimulation, cells were lysed in ice-cold lysis buffer and lysates were collected after centrifugation. Cell lysates were subjected to SDS-PAGE and western blotting for detection of phosphorylated kinases using phosphospecific antibodies. β-actin was detected as loading control. For ease of viewing, densitometry data are normalised to activated control. Graphs shown include data obtained from at least 3 separately conducted experiments. Blots shown are representative of a minimum of 3 independent experiments. Error bar show SEM. NS=not significant, **=p<0.01. Figure adapted from [139].
3.2.9 PI3K does not activate Erk viaTpl2

The irrelevance of Ras and Raf observed in insoluble IC-induced Erk activation prompted me to consider the possibility that an alternative MAP3K other than Raf might play a role in this context. I therefore sought candidates that could act as alternative MAP3K. A list of alternative MAP3Ks candidates is shown in Fig 3.2.9.1.
<table>
<thead>
<tr>
<th>Candidates</th>
<th>Why are they good candidates?</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tpl2 (MAP3K8 or Cot)</td>
<td>Play a dominant role as MAP3K in TLR, TNFR and IL1R-induced signalling in myeloid cells</td>
<td>[183], [184], [185], [186]</td>
</tr>
<tr>
<td>MOS</td>
<td>Activates Erk in Oocyte</td>
<td>[187]</td>
</tr>
<tr>
<td>TAK1</td>
<td>Activates Mek in response to chemotactic and growth factors stimulations in human neutrophils</td>
<td>[188]</td>
</tr>
<tr>
<td>PAK1,2 and 4</td>
<td>Rho family kinases regulate MAPK family via Pak signalling</td>
<td>[189], [190], [88]</td>
</tr>
</tbody>
</table>

**Figure 3.2.9.1 Alternative MAP3Ks that can act upstream of Mek other than Raf.**

A table showing some candidates that have been shown to act as a MAP3K upstream of Erk. Tpl2, MOS, TAK1 and Pak have all been shown to be important in the regulation of MAPK family, and they all are able to act upstream of Mek in some context.
I first determined whether Tumour progression locus 2 kinase (Tpl2), an alternative serine/threonine kinase that can act immediately upstream of Mek and plays a dominant role in innate immune responses [191, 192], is involved in PI3K-dependent Erk activation in insoluble IC-stimulated human neutrophils.

As shown in Fig 3.2.9.2 A-C, both PKB and Erk were activated upon neutrophil stimulation with insoluble ICs. Inhibition of Tpl2 did not affect this, suggesting that Tpl2 was not upstream of PKB nor Erk. In a separate experiment, I determined whether Tpl2 lay upstream of Mek. Like Erk, Mek phosphorylation remained unaffected by the inhibition of Tpl2, suggesting that Tpl2 was not upstream of Mek either (Fig 3.2.9.2 D and F). In contrast, the Tpl2 inhibitor interfered with activation of Tpl2, as assessed by Tpl2 phosphorylation on Thr 290 (Fig 3.2.9.2 D and E). Moreover, inhibition of PI3K did not affect Tpl2 phosphorylation, indicating that PI3K was not upstream of Tpl2 (Fig 3.2.9.2 D and E). Collectively, this showed that PI3K does not activate Erk via Tpl2 in insoluble IC-stimulated human neutrophils.
Figure 3.2.9.2 PI3K activated Erk signalling did not involve Tpl2 in insoluble IC-stimulated human neutrophils.

Neutrophils that had been isolated from peripheral blood of healthy donors were pre-incubated with pan-PI3K inhibitor (Wortmannin, Wo, 50nM) or Tpl2 inhibitor (Tpl2 II inhibitor, 5μM) prior to stimulation with insoluble ICs (HSAαHSA, 10μg/ml) or assay buffer. To terminate the assay, cells were lysed in ice-cold lysis buffer and lysates were collected after centrifugation. Cell lysates were subjected to SDS-PAGE and western blotting for detection of phosphorylated proteins using phosphospecific antibodies. β-actin was detected as loading control. For ease of viewing, densitometry data were normalised to activated control. Graphs include data obtained from 3 separately conducted experiments. Blots were representative of 3 independent experiments. Error bars show SEM. NS=not significant, **=p<0.01
3.2.10 Pak acts as the MAP3K upstream of Mek and Erk in neutrophils following stimulation with insoluble ICs

Under certain circumstances, p21 activated kinase (Pak) has been shown to signal via Erk and it can be regulated by PI3K [88, 190]. I therefore determined next whether PI3K activated Erk via Pak in human neutrophils that had been stimulated with insoluble ICs.

Insoluble ICs significantly induced Pak phosphorylation on Ser144. Inhibition of PI3Ks using wortmannin or of PI3Kβ and δ together using isoform specific inhibitors attenuated Pak phosphorylation significantly (Fig 3.2.10.1), indicating that PI3Kβ/δ were upstream of Pak. Inhibition of Pak by two pan-Pak inhibitors, PF3758309 and IPA3, significantly attenuated Pak, Mek and Erk activation, indicating that Pak was upstream of Mek and Erk (Fig 3.2.10.1). PF3758309 and IPA3 were originally designed to target at Pak4 and Pak1 respectively, but act as pan-Pak inhibitors at the concentrations they were used in this assay [193]. Despite the fact that the two inhibitors have different mechanisms of action and different off-target effects, data obtained with both inhibitors showed the same trend, making the results more reliable.

Inhibition of Pak abolished Mek phosphorylation at sites Ser217 and 221 (Fig 3.2.10.1 A, D-E). This was surprising since these residues correspond to Raf activation, whilst Pak has been reported to phosphorylate Ser298 on Mek [194]. I therefore looked into the literature for clarification. There are several possibilities why which Pak might regulate phosphorylation of Ser217 and 221 on Mek (Fig 3.2.10.2). In principle, phosphorylation of Ser298 on Mek by Pak might sensitise Mek for Raf-dependent phosphorylation on Ser217 and 221 as has been previously described [195, 196].
Figure 3.2.10.1 PI3K dependent Erk activation involves Pak in insoluble IC-stimulated human neutrophils.

Neutrophils that had been isolated from peripheral blood of healthy donors were pre-incubated with pan-PI3K inhibitor [Wortmannin; Wo (50nM), TGX221; TGX (40nM), IC87114; IC (1μM)] or Pak inhibitors [PF3758309; PF, IPA3 (10μM)] prior to stimulation with insoluble ICs (HSAαHSA, 10μg/ml) or assay buffer. Following stimulation, cells were lysed in ice-cold lysis buffer and lysates were collected after centrifugation. Cell lysates were subjected to SDS-PAGE and western blotting for detection of phosphorylated proteins using phosphospecific antibodies. β-actin was detected as loading control. For ease of viewing, densitometry data were normalised to activated control. Graphs include densitometry data from at least 3 separately conducted experiments. Blots were representative of a minimum of 3 separately conducted experiments. Please note data obtained from the use of IPA3 was not included in the blots shown, but was included in the densitometry data. Error bars show SEM. NS=not significant, *=p<0.05, **=p<0.01. Figure adapted from [139].
However, my previous results indicated that Raf is not involved in Mek and Erk activation following stimulation with insoluble ICs (Fig 3.2.8.2 A-D). I therefore ruled this possibility out. Pak has also been shown to directly activate Mek via a mechanism that does not involve Ras and Raf. Phosphorylation of Ser 298 on Mek by Pak led to a Raf-independent autophosphorylation of Ser 217 and 221 on the activation loop of Mek, which in turn induced Mek activity towards Erk [197]. My results were in agreement with this possibility. I therefore concluded that Pak might regulate Mek autophosphorylation of Ser 217 and 221. Collectively, my results showed that Pak acts as a MAP3K in PI3K-activated Erk activation following stimulation of human neutrophils with insoluble ICs.

Phosphorylation of Ser 298 on Mek by Pak led to a Raf-independent autophosphorylation of Ser 217 and 221 on the activation loop of Mek, which in turn induced Mek activity towards Erk [197]. My results were in agreement with this possibility. I therefore concluded that Pak might regulate Mek autophosphorylation of Ser 217 and 221. Collectively, my results showed that Pak acts as a MAP3K in PI3K-activated Erk activation following stimulation of human neutrophils with insoluble ICs.
A schematic diagram illustrating two possible mechanisms by which Pak has been suggested to regulate phosphorylation of Ser 217 and 221 on Mek. 

A) Pak phosphorylates Ser 298 on Mek, which sensitises Mek for phosphorylation on Ser 217 and 221 by Raf. Mutation of Ser 298 has been shown to almost completely abolished Raf phosphorylation on Mek Ser 217 and 221 [195]. B) Pak phosphorylates Ser 298 on Mek, which induces autophosphorylation on Ser 217 and 221 independent of Raf [197].
3.2.11 Cdc42, but not Rac, is regulated by PI3K in human neutrophils that have been stimulated with insoluble ICs

Pak can lie downstream of either Rac or Cdc42, two Rho family small GTPases. GTP-bound, active Rac and Cdc42 can both bind to Pak’s Cdc42/Rac interactive binding (CRIB) motif, thereby inducing a conformational change within Pak and priming it to autophosphorylate, a pre-requisite for subsequent full Pak activation [198, 199]. As discussed in sections 1.4.3 and 1.4.5 of the introduction, the link between PI3K and Rac is well established, with several PIP₃ regulated Rac GEFs identified in neutrophils [26, 200]. In contrast, little evidence indicates any link between PI3K and Cdc42 GEFs (Fig 3.2.11.1). Given that no specific inhibitors for Rho family small GTPases have been described, I investigated whether PI3K regulated Rac and/or Cdc42 activation in insoluble IC-stimulated human neutrophils by directly measuring Rac and Cdc42 activity in the presence and absence of wortmannin.
Figure 3.2.11.1 Regulation of Pak by PI3K via Rac or Cdc42.

A schematic diagram showing the mechanism by which PI3K regulates Pak. The PI3K-dependent Rac activation is well established with several PIP3 regulated Rac GEFs identified [26, 200], whilst no PIP3-regulated Cdc42 (or RhoA) GEFs have yet been described.
Both Rac and Cdc42 activities were significantly increased upon neutrophil stimulation with insoluble ICs (Fig 3.2.11.2 A and C). Unexpectedly, inhibition of PI3K reduced activation of Cdc42 but not of Rac. As a control, I also analysed Rac and Cdc42 activity upon stimulation of neutrophils with fMLF in the presence and absence of wortmannin, where it has already been demonstrated that PI3K lies upstream of Rac [26, 200]. Both Rac and Cdc42 were also significantly activated upon fMLF stimulation of human neutrophils (Fig 3.2.11.2 B and D). In contrast to my observation with insoluble ICs stimulation but in-line with previous observations [26, 200], inhibition of PI3K interfered with fMLF-induced Rac but not Cdc42 activation. Collectively, these results suggest that PI3K-mediated Pak activation via Cdc42 in neutrophils that had been stimulated with insoluble ICs, and describe an unusual situation where PI3K acts upstream of Cdc42 but not Rac.

Since there are three well-characterised members of the Rho family of small GTPases, I also measured activity of RhoA, in the presence and absence of wortmannin following stimulation with insoluble ICs or with fMLF. Like Rac and Cdc42, RhoA was significantly activated upon stimulation with insoluble ICs or fMLF (Fig 3.2.11.2 E and F). Inhibition of PI3K attenuated RhoA activation induced by insoluble ICs but by fMLF, suggesting that under some conditions, RhoA, like Cdc42, can also be activated in a PI3K-dependent manner in the neutrophil.
Neutrophils that had been isolated from peripheral blood of healthy donors were pre-incubated with pan-PI3K inhibitor (Wortmannin, Wo, 50nM) prior to stimulation with insoluble ICs (HSAαHSA, 10μg/ml), fMLF or assay buffer. To terminate the assay, cells were lysed in ice-cold lysis buffer and lysates were collected after centrifugation. For the analysis of Rac, Cdc42 or Rho activity, cell lysates were assayed for the presence of Rac-GTP, Cdc42-GTP and RhoA-GTP as detailed in chapter 2.4.4. Graphs shown include data from at least 3 separately conducted experiments. Error bars show SEM. NS=not significant, **=p<0.01. Figure adapted from [139].
3.3 Discussion

The work presented in this chapter describes a previously uncharacterised signalling pathway downstream of Fcγ receptors in human neutrophils (Fig 3.3). In this situation, Erk activation is entirely PI3K dependent. My results suggest that PI3K-dependent Erk activation involves Pak acting as the MAP3K upstream of Mek and Erk, rather than involving Ras and Raf. My results are moreover suggestive of an unusual situation in which Cdc42 rather than Rac is regulated in a PI3K-dependent fashion.

Early on in my project, I stimulated neutrophils by plating them onto immobilised ICs, which required scraping cells into lysis buffer. When doing this with several dishes, I encountered problems with protein degradation. To overcome this problem, I attempted to use DFP, a powerful cell permeable protease inhibitor, and discovered inadvertently that pre-treating neutrophils with DFP affects both PKB and Erk activity status. I managed to overcome this problem by changing the mode of activation, so that cell scraping was no longer required, speeding up the process dramatically. In addition, I was very careful and cautious with the number of samples used in any one experiment, no more than eight samples were used at any one time. Where lysates were required to persist for any extended length of time, as was the case in the small GTPase activity assays I performed, I included DFP into the lysis buffer rather than pre-treating the cells with it.

More broadly, my findings suggest that the use of DFP must be considered with caution when included in assays performed with neutrophils. Many biochemical studies involve immunoprecipitations of signalling proteins (e.g. SFKs, which are very susceptible to proteolysis); when doing these with neutrophil lysates, cells are
Figure 3.3 The new non-canonical signalling pathway downstream of Fcγ receptors in the human neutrophil.

A schematic diagram illustrating the non-canonical signalling pathway found in insoluble IC-stimulated human neutrophils, where PI3K activates Erk via a Ras and Raf independent mechanism. In this unusual pathway, activated Src family kinases (SFK) and PI3Kβ/δ induce Cdc42, rather than Rac, activation. Cdc42 activation in turn activates Pak, Mek and Erk subsequently, providing a mechanism to regulate Mek and Erk independently of Ras and Raf.
customarily pre-incubated with DFP [165], yet I am not aware of controls being
carried out routinely that analyse any potential side-effects of DFP. My results
suggest that it is essential to include such controls to avoid any misinterpretations
due to other potential DFP-induced protein phosphorylation events.

Since neutrophils are not amenable to culture and transfection or transduction, I
characterised this signalling pathway using a combination of activity assays (in most
cases by assessing the phosphorylation status of proteins of interest using
phosphospecific antibodies) and by employing inhibitors directed against candidates
suspected to be involved in the pathway. This strategy is valid as long as the inhibitors
employed are well characterised, as is the case with PI3K isoforms, Raf, Mek and Erk.
Although I had some misleading results with the extremely well characterised Raf
inhibitors, thanks to the cancer literature I was able to learn about the paradoxical
activation induced by some Raf inhibitors. I therefore employed ‘paradox breakers’ to
circumvent these problems. Unfortunately, there were no such good reagents available
for other components of the pathway I have uncovered. mTOR complexes, Tpl2 and
Pak are examples of kinases where the off-target effects of the inhibitors used
(palomid-529, PF3758309 and IPA3 respectively) were not very well characterised. In
the first instance, I tried to reduce the likelihood of drawing incorrect conclusions by
performing additional controls. I performed a time-course of PKB Ser 473/Thr308 and
Erk phosphorylation to test any potential involvement of mTORC2 in this pathway.
Despite the inconclusive results obtained initially with Palomid 529, this strategy
provided clear and strong evidence against any involvement of mTORC2.

Small GTPases are famously ‘un-druggable’, I therefore assessed Ras and Rho family
small GTPase activation with activity assays alone. In the case of Ras, I was able to
exclude any involvement downstream of PI3K. The situation was less clear-cut with Cdc42. Whilst I could clearly show that Cdc42 (but not Rac) is activated in a PI3K-dependent fashion, I could not actually show Cdc42 lies upstream of Pak. Since Pak is usually activated by either Cdc42 or Rac, I concluded that Cdc42 was the likely small GTPase involved here. Clearly, Cdc42 and Pak are the ‘weakest links’ of the pathway I described. Ideally, their involvement should be demonstrated in a separate fashion. A very convincing way would be to use genetics, which would not only permit me to confirm the involvement of these two proteins, but moreover enable the identification of the Pak protein involved (neutrophils express Pak 1,2 And 4). Both Cdc42 and Pak isoform specific knock-out mice have been described [201, 202], opening up the possibility of analysing primary neutrophils from such knock-outs. This approach is attempted in Chapter 5.

An alternative strategy would be to employ a cell-based model that is amenable to transfection or transduction. This would permit the analysis of knock-downs, as well as constitutively active and dominant negative constructs for ‘pathway walking’. Several promyelocytic leukemia cell lines exist and can be induced to become neutrophil-like. An example of such a cell line that has previously been used to analyse neutrophil signalling pathways is PLB-985. My attempts of testing this signalling pathway in PLB-985 are described in chapter 6.

A third way to test the pathway lies in the identification of an effector function that is regulated by it. If the pathway does indeed exist in neutrophils, inhibiting all of its components will interfere with this function. Since a signalling pathway that is known to regulate a specific function is more interesting than one that has been described in the absence of such a function, I next set out to identify which neutrophil function was
regulated by FcγR-PI3K-Cdc42-Pak-Mek-Erk signalling. This is described in chapter 4.
4 Results-FcγR-PI3K-Cdc42-Pak-Mek-Erk

signalling promotes apoptosis in human neutrophils

4.1 Introduction

In the previous chapter, I described the elucidation of an unconventional signalling pathway downstream of Fcγ receptors in human neutrophils: PI3K-Cdc42-Pak-Mek-Erk. I was interested to identify which neutrophil effector function(s) were regulated by this pathway. Immune complexes are known to mediate neutrophil recruitment and contribute to tissue injury by inducing a range of neutrophil effector functions including ROS production, degranulation and cytokine production [74, 160, 166]. Neutrophilic inflammation driven by immune complexes is therefore implicated in inflammatory diseases such as rheumatoid arthritis. Identifying which neutrophil effector function(s) are regulated by the FcγR-PI3K-Cdc42-Pak-Mek-Erk signalling pathway might provide new insight for therapeutic targets or design of disease treatment.
Hypothesis and aims

If the FcγR-PI3K-Cdc42-Pak-Mek-Erk signalling pathway identified in the previous chapter indeed exists in neutrophils, it is likely that the pathway will regulate some neutrophil functions. The aim of this chapter was to identify which neutrophil effector function(s) are regulated by this pathway. This was determined by assessing the following neutrophil functions in the presence and absence of small molecule inhibitors that are directed against the components of the pathway:

i) L-selectin shedding
ii) Production of reactive oxygen species
iii) Apoptosis
iv) Internalisation
4.2 Results

4.2.1 PI3K-activated Erk signalling does not regulate L-selectin shedding following stimulation of human neutrophils with insoluble ICs

L-selectin plays an essential role in the initial rolling during the neutrophil recruitment cascade. Rapid proteolytic cleavage (shedding) of L-selectin is induced by the release of metalloproteinases from intracellular granules to the plasma membrane [22]. This cleavage is crucial to elicit downstream signalling for subsequent stages in the neutrophil recruitment cascade (see section 1.1.1). I determined whether PI3K-activated signalling regulated L-selectin shedding in human neutrophils that had been stimulated with insoluble ICs.

Insoluble ICs efficiently induced L-selectin shedding (Fig 4.2.1 A); this shedding was inhibited by the presence of the Src and PI3K inhibitors (Fig 4.2.1 B-D), but not by Pak, Mek and Erk inhibitors (Fig 4.2.1 E-G). This indicated that in the insoluble IC-stimulated human neutrophil L-selectin shedding was Src and PI3Kβ/δ dependent but Pak, Mek and Erk independent. Hence, Src and PI3K regulated L-selectin shedding via alternative signalling pathways to the non-canonical signalling I described in the previous chapter.
Figure 4.2.1 Insoluble IC-induced L-selectin shedding is regulated by SFK and PI3K, but not Pak, Mek and Erk in human neutrophils.

Neutrophil that had been isolated from peripheral blood of healthy individuals were pre-incubated with PP1 (pan-SFK inhibitor, 10μM), wortmannin (Wo; pan-PI3K, 50nM), TGX221 (TGX; PI3Kβ, 40nM), IC87114 (IC; PI3Kδ, 1μM), PF3758309 (PF; pan-Pak inhibitor, 5μM), Mek inhibitor (AZD; AZD6244, 1μM) or Erk inhibitor (FR; FR180204, 10μM) prior to stimulation with insoluble ICs (10μg/ml) or assay buffer. For the analysis of cell surface L-selectin, cells were stained with anti-CD62L antibody and analysed by flow cytometry as detailed in section 2.5.1. For comparison, histograms of cells that had been mock (black) and insoluble IC-stimulated (blue) were copied into each inhibitor treatment (red). Figure adapted from [139].
4.2.2. Insoluble IC-induced ROS production requires PI3K but not Pak, Mek and Erk

Neutrophils kill pathogens by producing internal and external ROS. Several methods have been developed to detect and distinguish between these processes, one of which is by using luminol chemiluminescence based assays. Luminol can cross cell membranes and emits light when oxidised by peroxidases. In principle, incubation of neutrophils with luminol permits measurement of both intra- and extracellular ROS production, but owing to the fact that little myeloperoxidase is released in the extracellular space, incubating cells with luminol alone mostly measures intracellular ROS production. For the measurement of both intra- and extracellular ROS production, cells are incubated with luminol supplemented by horseradish peroxidase (HRP), where HRP is used to enhance extracellular signal. Isoluminol is a derivative of luminol that cannot cross cell membranes due to the different position of an amino group in its aromatic ring, making it hydrophilic. Therefore, detection of light emitted from cells that have been incubated with isoluminol and HRP permits the measurement of extracellular ROS. Both soluble and insoluble ICs have been shown to drive ROS production in neutrophils. Using the method described above, I determined which type of ROS production insoluble ICs induced in human neutrophils and whether PI3K-activated Erk signalling regulated this process.

Insoluble ICs induced significant internal ROS production in human neutrophils. Contrary to this observation, fMLF stimulation of human neutrophils induced primarily external ROS production (Fig 4.2.2.1 A-B). Inhibition of PI3Kβ and δ and of all PI3Ks significantly reduced insoluble IC-induced internal ROS production, but
inhibition of Pak, Mek and Erk had no effect on this process. Please note that a second Erk inhibitor (BVD523) was used here (the concentration at which BVD523 was effective had been determined by carrying out an apoptosis assay as shown in Fig 4.2.2.2). This was done since FR180204 is a bright yellow compound, which interfered with the luminescence-based read-out of this assay. Other yellow compounds are also known to interfere with luminescence based ROS assays (Su Kulkarni & Phil Hawkins, The Babraham Institute, personal communication). Together, these results demonstrated that PI3K-activated Erk signalling did not regulate insoluble IC-induced internal ROS production in human neutrophils. Instead, other PI3K regulated pathway(s) are likely to regulate this process.
Figure 4.2.2.1 Insoluble IC-induced internal ROS production depends on PI3Kβ/δ but not Pak, Mek and Erk in human neutrophils.

Neutrophils that had been isolated from peripheral blood of healthy donors were pre-incubated with Ly294002 (pan-PI3K, 10μM), TGX221 (PI3Kβ, 40nM), IC87114 (PI3Kδ, 1μM), PF3758309 (Pak, 5μM), tramatinib (Mek, 1μM), FR180204 (Erk, 10μM) and BVD523 (Erk, 5μM) prior to stimulation with insoluble ICs (10μg/ml) or fMLF (1μM) or assay buffer. A and B) Internal and external ROS production were analysed by measuring light emission from cells incubated with luminol alone or from cells incubated with isoluminol and HRP respectively. Graphs are representative of 3 independent experiments. C) Internal ROS production was measured as in A and B. For ease of viewing, graphs shown are normalised to activated control and include data from a minimum of 3 separately conducted experiments. Error bar show SEM. NS=not significant, *=p<0.05, **=p<0.01. Figure adapted from [139].
Figure 4.2.2.2 Determination of the concentration at which BVD523 is effective.

Neutrophils that had been isolated from peripheral blood of healthy human donors were pre-incubated with Ly294002 (Ly; pan-PI3K inhibitor, 10μM) or BVD523 (Erk inhibitor) at indicated concentrations prior to incubation with insoluble ICs (HSAαHSA, 10μg/ml) or assay buffer. After 12 hours of incubation, apoptosis was assessed by flow cytometry of Annexin V and PI stained cells. Graphs shown include data from 2 separately conducted experiments. Error show SEM.
4.2.3. FcγR-PI3K-Cdc42-Pak-Mek-Erk signalling promotes apoptosis in human neutrophils

I continued to investigate which neutrophil effector function was regulated by FcγR-PI3K-Cdc42-Pak-Mek-Erk signalling. Another neutrophil function immune complexes can induce is apoptosis [203]. Cell viability is commonly assessed by the flow cytometric analysis of Annexin V and propidium iodide (PI) stained cells, where Annexin V binds to phosphatidylserine displayed on the surface of apoptotic cells and PI binds to the DNA exposed by apoptotic cells. Double negative cells are defined as viable; Annexin V positive, PI negative cells are apoptotic; double positive cells are necrotic. Using this method in conjunction with cytocentrifuge preparations (Fig 4.2.3.1 as an example), insoluble IC-induced neutrophil apoptosis was assessed in the presence and absence of inhibitors.

Insoluble ICs accelerated apoptosis at least two-fold in neutrophils compare to cells that had been treated with vehicle at 6 hours, 9 hours and 12 hours. Treatment with inhibitors (PP1, Ly294002, TGX221, IC87114, PF3758309, Tramatinib and FR180204) significantly reduced the apoptosis induced by insoluble ICs, indicating that FcγR-PI3K-Cdc42-Pak-Mek-Erk signalling regulated apoptosis in human neutrophils (Fig 4.2.3.2 A). The same trend was also observed with secondary neutrophil necrosis (Fig 4.2.3.2 B). As a control, apoptosis was assessed in human neutrophils that had been incubated with inhibitors in the absence of insoluble ICs. As shown in Fig 4.2.3.2 C, cells that had been treated only with inhibitors behaved like the vehicle-treated cells at all time points tested, indicating that the inhibitors used had no effect on neutrophil apoptosis in the context of this assay. The involvement of
caspases in insoluble IC-induced apoptosis was also assessed. Inhibition of caspases with the pan-caspase inhibitor, Z-VAD-FMK, significantly reduced insoluble IC-induced apoptosis and necrosis, demonstrating that these processes were caspase-dependent (Fig 4.2.3.3). Collectively, these results showed that FcγR-PI3K-Cdc42-Pak-Mek-Erk signalling promoted apoptosis in human neutrophils.
Figure 4.2.3.1 Examples illustrating how insoluble IC-induced apoptosis were assessed in human neutrophils with and without inhibitor.

Neutrophils that had been isolated from peripheral blood of healthy donors were pre-incubated with tramatinib (Tra; Mek inhibitor, 1 μM) prior to incubation with insoluble ICs (HSαHSA, 10 μg/ml) or vehicle. After 12 hours of incubation, apoptosis was assessed by flow cytometry analysis of Annexin V and PI stained cells in conjunction with cytocentrifuge preparations. Double negative cells were defined as viable (green arrow); Annexin V positive, PI negative cells as apoptotic (red arrow); double positive cells as necrotic (black arrow). FACS plots and images of cytocentrifuge preparations shown above were representative of at least 3 independent experiments.
A

% apoptotic cells

0 hours 6 hours 9 hours 12 hours

PP1
Ly294002
TGX221
IC87114
PF3758309
Tramatinib
FR180204
HSAαHSA

B

% necrotic cells

0 hours 6 hours 9 hours 12 hours

PP1
Ly294002
TGX221
IC87114
PF3758309
Tramatinib
FR180204
HSAαHSA
Figure 4.2.3.2 The unconventional signalling pathway regulated apoptosis and secondary necrosis in insoluble IC-stimulated human neutrophils.

Neutrophils that had been isolated from peripheral blood of healthy donors were pre-incubated with small molecule inhibitors [PP1; pan-SFK (10μM), Ly294002; pan-PI3K (10μM), TGX221; PI3Kβ (40nM), IC87114; PI3Kδ (1μM), PF3758309; pan-Pak (5μM), Tramatinib; Mek (1μM), FR180204; Erk (10μM)] prior to incubation with insoluble ICs (HSAαHSA, 10μg/ml) or vehicle. A and C) Apoptosis and B) Necrosis were assessed by flow cytometry of Annexin V and PI stained cells in conjunction with cytocentrifuge preparations at indicated time points, as described in Fig 4.2.4.1. Graphs shown above include data obtained from at least 3 independent experiments. Error show SEM. NS=not significant, *=p<0.05. Figure adapted from [139].
Figure 4.2.3.3 insoluble IC-induced apoptosis and secondary necrosis were caspases-dependent.

Neutrophils that had been isolated from peripheral blood of healthy human donors were pre-incubated with Z-VAD-FMK (pan-caspases inhibitor, 10μM) prior to incubation with insoluble ICs (HSAαHSA, 10μg/ml) or vehicle. Apoptosis and necrosis were assessed by flow cytometry of Annexin V and PI stained cells in conjunction with cytocentrifuge preparations at indicated time points, as described in Fig 4.2.4.1. Graphs shown above include data obtained from 3 independent experiments. Error show SEM. NS=not significant, *p<0.05
During the course of the apoptosis assay, I noticed that the Mek inhibitor tramatinib significantly reduced the apoptosis induced by insoluble ICs, whilst a second Mek inhibitor, AZD6244, did not. I explored why the results brought by these two Mek inhibitors were so different. According to the (cancer) literature, Raf and Mek signalling are inhibited by Erk induced-negative feedback as a mechanism to avoid over-activation of the pathway in wild-type cells [204-206]. Studies with Mek inhibitors showed that the loss of Erk signalling following long-term Mek inhibition by AZD6244 caused the Erk induced-negative feedback on Raf and Mek signalling to be relieved, thereby reactivating Mek and Erk [207]. This reactivation of Mek and Erk was not observed with tramatinib, which belongs to a class of Mek inhibitors called ‘feedback busters’. Tramatinib preferentially binds to unphosphorylated Mek and prevents activation of Mek mediated by upstream signalling (Fig 4.2.3.4 A) [208, 209]. Since studies of Mek inhibitors are mostly based on prolonged use in cancer cells, I therefore determined whether AZD6244-induced reactivation also occurred on prolonged use in neutrophils (this AZD6244-induced reactivation did not occur when used for short inhibition in neutrophils, please see chapter 3). If this was the case, inhibition of Mek and Raf by the use of AZD6244 and AZ628 synergistically should reduce the apoptosis induced by insoluble ICs, as observed with tramatinib.

As shown in Fig 4.2.3.4 B, neither inhibition of Raf nor Mek by AZ628 or AZD6244 individually affected insoluble IC-induced apoptosis, but inhibition of both Raf and Mek by AZ628 and AZD6244 together reduced insoluble IC-induced apoptosis almost as efficiently as tramatinib.

Although no significance was reached, this trend suggested that the Erk-induced negative feedback on Raf and Mek signalling also occurs in neutrophils (Fig 4.2.3.4
B). It is interesting that although the Ras-Raf-Mek pathway does not operate downstream of Fcγ receptors in the human neutrophil, the Erk-dependent feedback loop is still functioning. One possibility might be that during prolonged stimulation, a Raf-dependent input is triggered because of the negative feedback mediated by Erk inhibition with AZD6244. Together this suggests that, in neutrophils as in cancer cells, the mechanisms by which Mek inhibitors inhibit Mek can affect their efficacy and ultimately cellular responses. Interestingly, whilst negative feedback of Erk on Raf and Mek signalling has only been shown to occur on prolonged incubation (at least 24 hours) of cancer cells [208], I observed this feedback in neutrophils at only 12 hours. This difference may be due to the different nature of cancer cells and neutrophils: cancer cells are transformed cells, whereas neutrophils are short-lived, terminally differentiated cells.
Figure 4.2.3.4 AZD6244 but not tramatinib induces Raf activation during prolonged neutrophil incubation.

A) A schematic diagram describing the mechanism by which AZD6244, but not tramatinib induces reactivation of Raf and Mek signalling. Mek inhibition by AZD6244 inhibits Erk signalling which abolishes Erk-induced negative feedback on Raf and Mek, therefore ultimately resulting in reactivation of Raf and Mek signalling. Tramatinib preferentially binds irreversibly to unphosphorylated Mek, thereby also preventing re-activation of Mek by upstream signalling. B) Neutrophils that had been isolated from peripheral blood of healthy donors were pre-incubated with Mek inhibitors [AZD6244 (1μM), Tramatinib (1μM)] or Raf inhibitor (AZ628, 5μM) prior to incubation with insoluble ICs (HSAαHSA, 10μg/ml) or vehicle. After 12 hours incubation, cells were stained with Annexin V and PI for flow cytometry analysis. The Graph shown above includes data obtained from 3 separately conducted experiments. Error bars show SEM. The p values stated above the columns show the statistical significance between insoluble IC-treated cells in the presence and absence of tramatinib or AZD6244 and AZ628. Figure adapted from [139].
4.2.4. FcγR-PI3K-Cdc42-Pak-Mek-Erk signalling promoted neutrophil apoptosis by altering the ratio of Mcl-1 and Bax

I next set out to dissect the mechanism by which FcγR-PI3K-Cdc42-Pak-Mek-Erk signalling regulated insoluble IC-induced apoptosis in human neutrophils. Neutrophil apoptosis is regulated by Bcl-2 family proteins; the balance between the expression of the pro- and anti-apoptotic members of this family determine the ultimate cell fate. As discussed in chapter 1, two Bcl-2 family members, Mcl-1 and Bax are particularly important Bcl-2 family proteins in determining cell fate in neutrophils. Mcl-1 is anti-apoptotic whereas Bax is pro-apoptotic, and the effect of one counteracts the effect of the other one. I therefore analysed expression of Mcl-1 and Bax in insoluble IC-stimulated human neutrophils.

Both Mcl-1 and Bax were induced upon neutrophil stimulation with insoluble ICs. Inhibitor treatment had no effect on Bax expression but further increased expression of Mcl-1, thereby altering their ratio, and having an anti-apoptotic effect between them (Fig 4.2.4). As a control, I analysed whether inhibitor treatment alone affected Mcl-1 and Bax expression. Both Mcl-1 and Bax expression remained constant in cells that had been treated with inhibitors in the absence of stimulation with insoluble ICs. This showed that the inhibitors did not have any effects on apoptosis per se. I concluded that FcγR-PI3K-Cdc42-Pak-Mek-Erk signalling promoted apoptosis by altering the expression ratio of Mcl-1 and Bax.
Figure 4.2.4 PI3K-activated Erk signalling alters the ratio of Mcl-1 and Bax expression in human neutrophils that had been stimulated with insoluble ICs.

Neutrophils that had been isolated from peripheral blood of healthy donors were pre-incubated with small molecule inhibitors [PP1; pan-SFK (10μM), Ly294002; pan-PI3K (10μM), TGX221; PI3Kβ (40nM), IC87114; PI3Kδ (1μM), PF3758309; pan-Pak (5μM), Tramatinib; Mek (1μM), FR180204; Erk (10μM)] prior to incubation with insoluble ICs (HSAαHSA, 10μg/ml) or vehicle. After 3 hours of incubation, cells were lysed with ice-cold lysis buffer and lysates were collected, clarified by centrifugation and subjected to SDS-PAGE. Cellular Mcl-1 and Bax were detected by western blot. βactin acted as loading control. The blot shown is representative of 3 independent experiments. For ease of viewing, densitometry data was normalised to β-actin. The graph shown includes data obtained from 3 separately conducted experiments. For statistical analysis, data was analysed by ONE-WAY ANOVA. *=p<0.05. Figure adapted from [139].
4.2.5. The size of neutrophils changed during the prolonged incubation with insoluble ICs

I noticed that the position of neutrophils within the FACS plots changed after prolonged incubation with insoluble ICs in the course of the apoptosis assay. The forward scatter (FSC) and the side scatter (SSC) parameters give an indication of the relative size of cells and of cell granularity, respectively. I explored the change of position within the FACS plots by plotting the forward scatter (FSC) and side scatter (SSC) parameters for the different cell populations. As shown in Fig 4.2.5 A-H, there was no difference in the size of neutrophils that had been treated with vehicle, insoluble ICs alone or insoluble ICs and inhibitors at timepoint 0. However, cells that had been treated with insoluble ICs had increased in size after 12 hours of incubation whilst the size of vehicle-treated neutrophils remained constant. Neutrophils that had been treated with insoluble ICs and inhibitors showed variable phenotypes: PP1, Ly294002, TGX221 and IC87114 treated cells were no different to cells that had been treated with vehicle, whilst PF3758309, tramatinib, FR180204 and Z-VAD-FMK treated cells increased in size (Fig 4.2.5 I-P). This curious observation prompted me to explore whether insoluble ICs might be internalised by neutrophils.
Figure 4.2.5 The Size of neutrophils changed during pro-longed incubation with insoluble ICs.

Neutrophil size was determined by re-analysing the flow cytometry data shown in Figure 4.2.4. FSC and SSC graphs were plotted. For ease of viewing, FSC and SSC plots of cells that had been vehicle treated (red) were copied into insoluble IC-treated cells (blue) and into each inhibitor treatment (blue with the name of the inhibitor used in the assay stated in the top right corner). Plots shown are representative of at least 3 independent experiments. A-H) FSC and SSC plots of cells incubated with or without insoluble ICs in the presence or absence of inhibitors for 0 hour. I-P) FSC and SSC plots of cells incubated with or without insoluble ICs in the presence or absence of inhibitors for 12 hours.
4.2.6. Internalisation of insoluble ICs by neutrophils is independent of Pak/Mek/Erk

The increased size of insoluble IC-treated neutrophils and the fact that insoluble IC-treatment triggered internal ROS production prompted me to wonder insoluble ICs may be internalised by neutrophils. I therefore determined whether human neutrophils internalised insoluble ICs when they were incubated with insoluble ICs, and whether the non-canonical PI3K-activated Erk signalling regulated this process.

As shown in Fig 4.2.6, insoluble IC-treated neutrophils did indeed internalise insoluble ICs. Inhibition of SFK and PI3K inhibited this internalisation. In contrast, inhibition of Pak, Mek and Erk did not interfere with internalisation of insoluble ICs. Interestingly, and contrary to treatment with a pan-PI3K inhibitor, inhibition of PI3Kβ/δ did not completely inhibit internalisation of insoluble ICs, suggesting that a PI3K other than Class I may regulate this process [210, 211]. These results suggest that different pathways are used to regulate internalisation of insoluble ICs and insoluble IC-induced apoptosis.
<table>
<thead>
<tr>
<th></th>
<th>Neutrophils</th>
<th>HSAαHSA on cell surface</th>
<th>Total HSAαHSA</th>
<th>Overlay</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSAαHSA</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
<td>HSAαHSA and PP1</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
<td>HSAαHSA and Wo</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
<td>HSAαHSA and TGX &amp; IC</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
<td>HSAαHSA and FF</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
<td>HSAαHSA and Tra</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
<td>HSAαHSA and FR</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
</tbody>
</table>
Figure 4.2.6. PI3K activated Erk signalling did not regulate insoluble ICs internalisation by human neutrophils.

Neutrophils that had been isolated from peripheral blood of healthy donors were pre-incubated with small molecule inhibitors [PP1; pan-SFK (10μM), Ly294002; pan-PI3K (10μM), TGX221; PI3Kβ (40nM), IC87114; PI3Kδ (1μM), PF3758309; pan-Pak (5μM), Tramatinib; Mek (1μM), FR180204; Erk (10μM)] prior to incubation with pre-stained insoluble ICs [HSAαHSA (10μg/ml), green] or buffer. Cells were fixed following adhesion to coverslips. Insoluble ICs bound to cell surface were stained (red). A) All structures were viewed under a microscope. Scale Bar (white) represents 5μm. B and C) Total number of cells viewed under the microscope were counted, and the number of cells that had internalised insoluble ICs were expressed as a percentage. Graphs shown above include data obtained from one experiment.
4.3. Discussion

In this chapter, I identified the neutrophil function that was regulated by the unconventional FcγR-PI3K-Cdc42-Pak-Mek-Erk signalling pathway in human neutrophils (Fig 4.3), thus supporting the biochemical approach used to identify the pathway as laid out in chapter 3. In-line with previous observations, insoluble ICs were shown to induce neutrophil apoptosis [203, 212]. Insoluble ICs induced a range of functions in human neutrophils. Although PI3K was required for all of these functions, the unconventional signalling pathway regulated only apoptosis specifically. This confirms that PI3K is upstream of many signalling cascades, and regulates neutrophil functions via more than one signalling cascade. I showed further that mechanistically, insoluble IC-induced apoptosis was due to altered expression of Mcl-1 and Bax, which are both important Bcl-2 family proteins that determine cell fate in neutrophils.

The pro-apoptotic function of PI3K and Erk in this pathway is surprising. Both proteins are well known for their anti-apoptotic function [213, 214]. However, some pro-apoptotic effects of PI3K and Erk have been reported. For example, class IA PI3Ks have been shown to transduce pro-apoptotic signals mediated by TNFα in neutrophils [215]. Pro-apoptotic Erk (Ras-Raf-Mek-Erk) signalling can be induced by DNA damaging agents, and this Erk activity seems to be involved in activation of both the extrinsic and intrinsic death pathways. The mechanism by which Erk exerts its pro-apoptotic activity is suggested to act via modulation of Bcl-2 family proteins, where Erk signalling upregulates pro-apoptotic proteins (e.g. Bax) and downregulates anti-apoptotic proteins (e.g. Bcl-2) [216]. In agreement with these observations, my results also showed PI3K/Erk-regulated modulation of Bcl-2 family proteins. Interestingly,
Figure 4.3 The non-canonical signalling pathway promotes apoptosis in insoluble IC-stimulated human neutrophils.

A schematic diagram illustrating the non-canonical PI3K-activated Erk signalling regulates apoptosis in insoluble IC-stimulated human neutrophils. Although PI3Kβ/δ are involved in the regulation of neutrophil functions such as L-selectin shedding, internal ROS production, cytokine production and internalisation, the FcγR-PI3K-Cdc42-Pak-Mek-Erk signalling only regulates apoptosis in the human neutrophil.
instead of modulating both anti- and pro-apoptotic Bcl-2 proteins, PI3K/Erk signalling only downregulated Mc1-1, whilst Bax was unaffected (Fig 4.2.4). These results further suggest PI3K and Erk can exert opposing effects on a cell depending on the upstream signal (and stimulation) they receive. The finding of insoluble IC-induced apoptosis being dependent on PI3K-activated Erk signalling opens up many questions. Perhaps, the most burning question lies in the underlying mechanism by which insoluble ICs induce apoptosis. As mentioned in the introduction, apoptosis can occur via three ways: intrinsic, extrinsic or phagocytosis-induced (see section 1.1.3). The observations of insoluble ICs being internalised by neutrophils and induce internal ROS production prompted me to wonder whether insoluble IC-induced apoptosis could be a form of phagocytosis-induced apoptosis. Phagocytosis-induced apoptosis is a mechanism by which phagocytes use to clear and destroy small pathogens such as bacteria and fungi [217-219]. The engulfed pathogens induce internal ROS production, which in turn mediates apoptosis, linking phagocytosis and apoptosis together. Internal ROS production is therefore a feature of phagocytosis-induced apoptosis [217]. Mek-Erk signalling has been reported to be anti-apoptotic in phagocytosis-induced apoptosis [220], whilst my results showed PI3K/Erk signalling promoted apoptosis. This suggests that the two processes may be distinct. This is an interesting point that remains to be addressed in the future. Interestingly, internalisation of insoluble ICs by neutrophils was dependent on PI3K but not Pak, Mek and Erk (Fig 4.2.6). It will therefore be interesting to examine whether internalisation of insoluble ICs might be required, but not sufficient to induce apoptosis, or whether these might be two separate processes.
Cytokine release by neutrophils has also been investigated in the laboratory. The release of IL-8, but not TNFα or IL-1ra, was induced in human neutrophils stimulated with insoluble ICs. By using the panels of inhibitors, it was shown that this release of IL-8 depended on PI3K, but not on other components of the new signalling pathway [139]. This suggested indirectly, that insoluble IC-induced apoptosis is unlikely to be due to a feedback loop that is dependent on TNF production.
5 Results- Signalling in mouse neutrophils

5.1 Introduction

Primary neutrophils are short-lived and terminally differentiated, rendering them near impossible to genetic modifications including transfection or transduction. Studies of biochemical cascades that regulate functional responses in response to stimuli in primary neutrophils are therefore frequently analysed using pharmacological inhibitors. Whilst inhibitors directed against specific proteins are useful tools for studying signalling events, any off-target effects can lead to misleading results. Moreover, biochemical studies in vitro do not always replicate events that occur in the more complex environment in vivo. Therefore, it is good practice to verify and consolidate results obtained with pharmacological inhibitors by analysing genetically modified cells.

Mice are widely used as a biological model of human biology. Mouse and human genes are characterised by significant genetic conservation [221-225]. It is commonly assumed that pathways and physiological processes are also conserved between these two species. Genetically modified mice are therefore very popular for studying signalling cascades and the underlying mechanisms that govern cellular responses.

The data obtained with human neutrophils (see chapter 3 and 4) were based on inhibitors in combination with activity assays. Whilst the isoform-specific PI3K, Mek, Erk and Raf inhibitors used for this study are very well characterised and substrate specific at the concentrations employed, the two Pak inhibitors used are known to have non-overlapping off-target effects [226]. Since no good inhibitors are currently available for small GTPases, inhibitors were not used to confirm the role of
Cdc42 in the activation of Pak. Knowing that these limitations are relevant to my work with human neutrophils, I was keen to address and confirm the involvement of Pak and Cdc42 using a different experimental approach. Both Pak1 and Cdc42 knockout mice are available [109, 227], I therefore intended to import suitable mouse models for analysis of the signalling cascade in neutrophils derived from these mice. Since importing a mouse strain is a lengthy and expensive undertaking, it was addressed in the first instance, whether mouse neutrophils replicated my findings with human neutrophils.

Hypothesis and aims

Signalling events downstream of Fcγ receptors were hypothesised to be conserved between human and mouse neutrophils, and therefore the role of Pak and Cdc42 in PI3K-activated Erk signalling can be confirmed with neutrophils derived from genetically modified mice. To address this question, there were specifically two aims for this chapter:

i) test whether the signalling events induced by insoluble ICs were the same between human and mouse neutrophils

ii) confirm the role of Pak and Cdc42 genetically
5.2 Results

5.2.1 Erk activation is PI3K-dependent in mouse neutrophils that were stimulated with immune complexes or by plating onto integrin ligands

Previously it was shown that PI3K lies upstream of Erk in human neutrophils that were stimulated with insoluble ICs, or by being plated onto immobilised ICs or onto integrin ligands (see chapter 3). To test whether this was conserved in mouse neutrophils, it was initially determined whether PI3K was also upstream of Erk in mouse neutrophils under these conditions.

As shown in Fig 5.2.1, both PKB and Erk were activated upon plating neutrophils onto surfaces coated with poly Arg-Gly-Asp (pRGD, a synthetic pan-integrin ligand), immobilised ICs and by stimulation with insoluble ICs. Inhibition of PI3Kβ/δ using isoform specific inhibitors attenuated Erk activation to a similar extent as inhibition of all PI3Ks with a pan-PI3K inhibitor. This indicated that, as with human neutrophils, PI3Kβ/δ acted upstream of Erk in mouse neutrophils upon these stimulation conditions. In-line with published work [148], but in contrast to the observations with human neutrophils (Fig 3.2.3 D-F), inhibition of PI3Kβ attenuated both PKB and Erk activation to a greater extent than inhibition of PI3Kδ. This suggested that PI3Kβ played a more important role than PI3Kδ in mouse neutrophils, whilst in human neutrophils, the opposite was true (as already discussed in chapter 3).
Figure 5.2.1 Erk activation was largely dependent on PI3Kβ rather than PI3Kδ in mouse neutrophils that had been stimulated with integrin ligands, immobilised ICs or insoluble ICs.

Neutrophils that had been freshly isolated from mouse bone marrow were pre-incubated with PI3K inhibitors [wortmannin (Wo, 50nM); pan-PI3K, TGX221 (TGX, 40nM); PI3Kβ, IC87114 (IC, 1μM); PI3Kδ] prior to stimulation by plating onto (A-C) HI-FBS or pRGD; (D-F) BSA or BSAαBSA (immobilised ICs) or with insoluble ICs (HSAαHSA, 10μg/ml) or assay buffer. (A-F) After 12 minutes incubation, adherent and non-adherent cells were scraped into ice-cold lysis buffer. (G-I) After 10 minutes incubation, cells were pelleted for lysis in ice-cold buffer. Cell lysates were collected after centrifugation and were then subjected to SDS-PAGE followed by western blotting. Activated kinases were detected using phosphospecific antibodies. β-actin or β-COP served as loading control. Graphs shown include data from at least 3 independent experiments and were normalised to activated control. Blots shown were representative of a minimum of 3 separately conducted experiments. Error bars show SEM. *=p<0.05, **=p<0.01, ***=p<0.005. Figure adapted from [139].
5.2.2 The contribution of individual PI3K isoforms to insoluble IC-induced ROS production differs between human and mouse neutrophils

The fact that individual PI3K isoforms contributed differently to PI3K-activated signalling in human as opposed to mouse neutrophils prompted me to explore whether this difference extended to PI3K-regulated neutrophil functions. I therefore examined the roles PI3Kβ and PI3Kδ played in insoluble IC-induced ROS production in human and mouse neutrophils.

Insoluble or immobilised ICs induced ROS production in both human and mouse neutrophils; a response that was greatly reduced by PI3Kβ and δ inhibition (Fig 5.2.2). The use of substrate specific PI3Kβ and δ inhibitors showed that ROS production was largely dependent on PI3Kβ in mouse neutrophils (Fig 5.2.2 B), whereas PI3Kδ played the major role in humans (Fig 5.2.2 A). This backed up the previous observations, where PI3Kβ contributed most to immobilised IC-induced PI3K signalling in mouse neutrophils (Fig 5.2.1), whilst it was mainly PI3Kδ dependent in humans (Fig 3.2.3).
Figure 5.2.2 PI3Kβ played the larger role in insoluble IC-induced ROS production in mouse neutrophils, whilst it is PI3Kδ in humans.

Neutrophils that had been isolated from peripheral blood of healthy donors (A) or mouse bone marrow (B) were pre-incubated with PI3K inhibitors [wortmannin (50nM); pan-PI3K, TGX221 (40nM); PI3Kβ, IC87114 (1μM); PI3Kδ] prior to stimulation (A) with insoluble ICs (HSAαHSA, 10μg/ml) or buffer or (B) with immobilised ICs (BSAαBSA). To analyse ROS production, total light emission was measured. Graphs shown are representative of at least 3 independent experiments.
5.2.3 Erk activation in insoluble IC-stimulated mouse neutrophils is dependent on Raf not Pak

Although I did not encounter any problems with protein degradation in lysates from scraped samples of mouse neutrophils, in order to be consistent across the work, I focused on the stimulation with insoluble ICs. We next investigated signalling events upstream of Erk. In insoluble IC-stimulated human neutrophils, Erk activation was dependent on Pak, but not Raf (Fig 3.2.8.2 and Fig 3.2.10.1). We therefore determined the role of Pak and Raf in Erk activation in mouse neutrophils that had been stimulated with insoluble ICs.

Inhibiting Mek with AZD6244 abolished Erk phosphorylation without affecting PKB phosphorylation, suggesting that Mek lies upstream of Erk as expected (Fig 5.2.3 A-C). To address the role of Pak upstream of Mek, the Pak inhibitor PF3758309 was used. To our surprise, Erk activation induced by insoluble ICs remained unaffected by the presence of PF3758309 (Fig 5.2.3 A and C), indicating that Pak might not be upstream of Erk in mouse neutrophils that had been stimulated with insoluble ICs.

AZ628 was used to test the potential involvement of Raf upstream of Mek and Erk. As with human neutrophils, AZ628 did not affect PKB phosphorylation (Fig 5.2.3 D and E), but in contrast to the observation with human neutrophils (section 3.2.8), it significantly reduced Erk activation (Fig 5.2.3 D and F). This suggested that Erk activation in insoluble IC-stimulated mouse neutrophils was at least in part regulated by Raf. It was then attempted to confirm these data by using another Raf inhibitor, ZM336372, which is an older generation Raf inhibitor and is known to induce paradoxical Mek and Erk activation (discussed in section 3.2.8) [178]. However, Erk
activation was not reduced in ZM336372-treated neutrophils upon stimulation with insoluble ICs. Whilst we cannot explain the discrepancy between the results obtained with these two Raf inhibitors, one explanation might be that this was due to ZM335372-induced paradoxical Mek and Erk activation.

In conclusion, in contrast to human neutrophils, PI3K-activated Erk signalling in insoluble IC-stimulated mouse neutrophils did not involve Pak. Rather, the data suggest a partial involvement of Raf in mouse neutrophils. Since the involvement of Pak was not conserved between the two species, neutrophils derived from Pak knockout mouse did not present to be a good model for testing the involvement of Pak genetically.
Figure 5.2.3 Raf but not Pak acted upstream of Erk in insoluble IC-stimulated mouse neutrophils.

Neutrophils that had been isolated from mouse bone marrow were pre-incubated with PI3K inhibitors [wortmannin (Wo, 50nM); pan-PI3K, TGX221 (TGX, 40nM); PI3Kβ, IC87114 (IC, 1μM); PI3Kδ], Mek inhibitor (AZD, AZD6244, 1μM) or Pak inhibitor (PF, PF3758309, 5μM) prior to stimulation with insoluble ICs (HSAαHSA, 10μg/ml) or buffer. To terminate the assay, cells were lysed in ice-cold lysis buffer, and lysates were collected after centrifugation. Soluble proteins were subjected to SDS-PAGE followed by western blotting. Activated kinases were detected using phosphospecific antibodies. β-actin served as loading control. Blots shown are representative of at least 3 independent experiments. Graphs shown include data from a minimum of 3 experiments and were normalised to activated control. Error bars show SEM. NS= not significant, *=p<0.05. Figure adapted from [139].
5.2.4 PI3K regulated insoluble IC-induced apoptosis via an alternative pathway in mouse neutrophils

The observation that human and mouse neutrophils employ different signalling pathways downstream of Fcγ receptors was surprising. I therefore examined whether insoluble ICs induced apoptosis in mouse neutrophils.

As with human neutrophils, significant increase in neutrophil apoptosis was observed at 9 hours and 12 hours after stimulation with insoluble ICs (Figure 5.2.4). Inhibition of PI3Kβ/δ significantly reduced the apoptosis induced by insoluble ICs. However, in-line with the differential signalling in mouse neutrophils, inhibition of PAK, Mek and Erk did not affect insoluble IC-induced apoptosis in mouse neutrophils.

Collectively, these results showed that PI3Kβ/δ regulates insoluble IC-induced apoptosis in both human and mouse neutrophils, but the signalling events regulating this process differ between the two species.
0 hour

A Cells only  B HSAαHSA  C HSAαHSA+ PI103  D HSAαHSA+ TGX and IC

E HSAαHS+ PF  F HSAαHSA+ Tra  G HSAαHSA+ FR

12 hours

H Cells only  I HSAαHSA  J HSAαHSA+ PI103  K HSAαHSA+ TGX and IC

L HSAαHS+ PF  M HSAαHSA+ Tra  N HSAαHSA+ FR
Figure 5.2.4 Neutrophil apoptosis was dependent on PI3Kβ/δ but not Pak, Mek and Erk in mouse.

Neutrophils that had been freshly isolated from bone marrow were pre-incubated with PI3K inhibitors [PI103 (10μM); pan-PI3K, TGX221 (TGX, 40nM); PI3Kβ, IC87114 (IC, 1μM); PI3Kδ], Mek inhibitor (Tra, tramatinib, 1μM), Pak inhibitor (PF, PF3758309, 5μM) or Erk inhibitor (FR, FR180204, 10μM) prior to incubation with insoluble ICs (HSAαHSA, 10μg/ml) or assay buffer for A-N 0 or 12 hours or O at indicated time points. At the end of the incubation, cells were (A-N) subjected to cytocentrifugation (scale bar, 5μm) or (O) stained with Annexin V and propidium iodide (PI) and subjected to flow cytometry for the analysis of apoptosis. (O) Data shown include data from 3 independent experiments. Error bars show SEM. *=p<0.05
5.2.5 Erk activation was PI3K- and Raf-dependent in fMLF-stimulated mouse neutrophils

We next wondered whether the difference in signalling cascades used by human and mouse neutrophils was specific to stimulation with insoluble ICs, or is part of a wider phenomenon. As shown in chapter 3, in fMLF-stimulated human neutrophils Erk activation was independent of PI3K (Fig 3.2.8.2). I therefore analysed fMLF-induced signalling in mouse neutrophils to test whether this was conserved between the two species.

As shown in Fig 5.2.5, pre-treatment with wortmannin, a pan-PI3K inhibitor, abolished both PKB and Erk phosphorylation induced by fMLF stimulation of mouse neutrophils. This indicated that PI3K was upstream of PKB and Erk following fMLF stimulation in mouse neutrophils. Using a panel of PI3K isoform-specific inhibitors, PKB and Erk phosphorylation were found to be dependent on the Gβγ-activated PI3Kγ (Fig 5.2.5 A-C). It was next analysed whether this PI3K-dependent Erk activation followed canonical Raf-Mek-Erk signalling. Inhibiting Raf with AZ628 (but not with ZM336372) partially interfered with, but did not abolish Erk activation (Fig 5.2.5 D and F). PKB phosphorylation was not affected by the presence of AZ628 (and ZM336372) (Fig 5.2.5 D and E). This indicated that Raf was upstream of Erk, but not of PKB, in fMLF-stimulated mouse neutrophils. We attributed the different results obtained with AZ628 and ZM336372 to ZM336372-induced paradoxical Mek and Erk activation, as already discussed in section 3.2.8. Hence, in contrast to the situation observed in human neutrophils, Erk activation following fMLF stimulation of mouse neutrophils was PI3K- and Raf-dependent. It would be interesting to address, whether
this might be a rare situation where PI3K lies upstream of Ras, but due to time limitation, we was unable to test this possibility.
Figure 5.2.5 Erk activation was dependent on PI3Kγ and Raf following fMLF stimulation in mouse neutrophils.

Neutrophils that had been isolated from bone marrow were pre-incubated with PI3K inhibitors [wortmannin (Wo, 50nM); pan-PI3K, TGX221 (TGX, 40nM); PI3Kβ, IC87114 (IC, 1μM); PI3Kδ, A66 (10μM); PI3Kα, AS252424 (AS, 30μM); PI3Kγ], or Raf inhibitor [ZM336372 (ZM, 10μM) and AZ628 (AZ, 5μM)] prior to stimulation with fMLF (1μM) or vehicle. Cells were lysed in ice-cold lysis buffer and cell lysates were collected after centrifugation. Soluble proteins were subjected to SDS-PAGE followed by western blotting. Activated kinases were detected using phosphospecific antibodies. β-actin or βCop acted as loading control. Densitometry data (B,C,E,F) were integrated from at least 3 independent experiments and are accompanied by representative blots (A and D). Error bars show SEM. NS= not significant, *=p<0.05, **=p<0.01. Figure adapted from [139].
5.3 Conclusions

The results obtained in this chapter have revealed that, despite significant genetic conversion between the two species, PI3K signalling is not conserved between human and mouse neutrophils, at least upon stimulation with immune complexes and fMLF. My findings indicate that PI3K regulates Erk activation in both insoluble IC-stimulated human and mouse neutrophils, but the mechanism by which this occurs is not conserved. Insoluble immune complexes caused PI3K-dependent apoptosis in neutrophils from both species. In human neutrophils, Pak acts as the MAP3K in immune-complex induced, PI3K-activated Erk activation, whereas Raf appears to play this role in mouse neutrophils. Different signalling events were not only observed following neutrophil stimulation with insoluble ICs, but also upon stimulation with fMLF. This suggests that the differences in pathways utilised downstream of PI3K in human and mouse neutrophils observed are unlikely to be unique examples, but may be rather commonplace. Indeed, an unrelated study previously identified differences in PI3K isoform usage in fMLF-stimulated ROS generation in human and mouse neutrophils [138].

In the present work, mouse neutrophils were isolated from bone marrow, whereas human neutrophils were prepared from peripheral blood of healthy individuals. Since the number of cells isolated from mouse blood is very limited, it is a common practice to prepare mouse neutrophils from bone marrow. It is possible, but not likely that the differences observed were due to the different preparation of neutrophils from human and mouse. Bone marrow-derived neutrophil preparations harbour cells of more variable maturity than peripheral blood-derived neutrophils. Interestingly though,
whilst neutrophils derived from human bone marrow were shown to be functionally immature, mouse bone marrow has been reported to serve as a large reservoir of functionally mature neutrophils, with regard to their morphology and functional responses to fMLF stimulation [228]. It is likely, therefore, that the signalling pathways employed downstream of PI3K in human and mouse neutrophils are genuinely different.

The original aim was to use neutrophils derived from genetically modified mice to confirm and extend the findings with human neutrophils. However, the lack of conservation of signalling pathways between the two species rendered this aim futile. Although Cdc42 knock-out mice and Pak1 mice have all been reported [229, 230], this approach was not pursued any further. Instead, it was decided to identify and use a cell-line based model system that would be amenable to genetic manipulation, whilst being representative of human neutrophils.
6 Results- PLB-985 as a model system for elucidating FcγR signalling in human neutrophils

6.1 Introduction

Several human cell lines with phagocytic capacity are being used as alternative models to primary human neutrophils. A human promyelocytic leukaemia cell line called PLB-985 is one such model that has been used in functional studies of neutrophils. PLB-985 cells have the capacity to become granulocytic or monocytic depending on the inducing agent used [231]. PLB-985 cells that are differentiated to become neutrophil-like display similar morphology as primary human neutrophils and have been described as functionally competent [232, 233]. In contrast to terminally differentiated neutrophils, PLB-985 cells are amenable to transfection and (lentiviral) transduction prior to being induced to become neutrophil-like, making them a popular model for elucidating signalling processes. Following on from the previous chapter where it was demonstrated that signalling events are not well conserved between human and mouse neutrophils, we set out to knock down the signalling components of interest (Cdc42, Pak) in differentiated PLB-985 cells in order to confirm the signalling cascade that had been elucidated in primary neutrophils.
Hypothesis and aims

Signalling events were hypothesised to be the same in differentiated PLB-985 cells as in human neutrophils, and therefore genetically modified differentiated PLB-985 cells can be used to confirm the findings obtained with human neutrophils. To address this hypothesis, the main aim for this chapter was to knock down proteins of interest to confirm the signalling cascade I described in chapter 3. This was assessed by:

i) testing whether differentiated PLB-985 looked and behaved like primary human neutrophils

ii) testing whether signalling events were conserved between differentiated PLB-985 cells and primary human neutrophils

iii) performing knock down experiments using shRNA to test the validity of the signalling cascade, with a particular focus on Cdc42 and Pak1/4.
6.2 Results

6.2.1 PLB-985 cells are morphologically and functionally similar to human neutrophils

It was first determined how much DMSO-differentiated PLB-985 cells (dPLBs) resembled primary human neutrophils with regards to their morphology and functional competence. As shown in Fig 6.2.1 A, whilst growing (in suspension), PLB-985 were large and round cells with a big, round nucleus in the centre of the cell. After 5 days of culture in growth medium supplemented with 1.25% DMSO, dPLBs had arrested growth and were characterised by a banded or multilobular nucleus; they were smaller in size compared to growing PLB-985 cells that had not undergone differentiation (Fig 6.2.1 B). The nuclear morphology displayed by dPLBs somewhat resembled the morphology of primary human neutrophils (Fig 6.2.1 B-C). As published in the literature [232], dPLB-985 cells were able to produce ROS upon stimulation with fMLF, indicating that dPLBs had indeed become neutrophil-like (Fig 6.2.1 D-E).
Primary human neutrophils were freshly isolated from peripheral blood of healthy donors. PLB-985 cells were cultured as detailed in chapter 2; for differentiation, PLB-985 cells were cultured in growth medium that had been supplemented with 1.25% DMSO for 5 days. (A-C) Primary human neutrophils (C) and PLB-985 cells that had (B) or had not been differentiated (A) were subjected to cytocentrifuge preparation, followed by Reastain Quick Diff staining. Whilst peripheral blood-derived primary neutrophils had multilobular nuclei throughout, dPLBs were characterised by a varied nuclear morphology with some multilobular nuclei (black arrows), some banded-like nuclei (red arrows) and some cells with rounded nuclei (green arrow). Scale bars, 5μm. (D-E) Primary human neutrophils and dPLBs were stimulated with fMLF (1μM) and total ROS production was assayed as detailed in section 2.5.2. Representative images and graphs from 3 separate experiments are shown.
6.2.2 PI3K/Erk signalling upon stimulation of G protein coupled receptors is conserved between primary human neutrophils and dPLBs

It was next asked whether the signalling events were conserved between human neutrophils and dPLBs. To address this, PKB and Erk activation upon stimulation of dPLBs with fMLF was assessed. As shown in Fig 6.2.2, fMLF stimulation induced PKB and Erk phosphorylation in dPLBs. As with human neutrophils (Fig 3.2.2.2 A-C), inhibition of PI3K attenuated PKB activation but had no effect on Erk phosphorylation in dPLBs, indicating that PI3K was upstream of PKB but not of Erk (Fig 6.2.2). In these assays, there was a noticeable amount of basal Erk phosphorylation; this was somewhat variable between experiments. Since fMLF is a very strong activating signal, this did not pose any problem. It was concluded that dPLBs were a useful alternative model system for primary human neutrophils.
Figure 6.2.2 Erk activation does not depend on PI3K in fMLF-stimulated dPLBs.

For differentiation, PLB-985 cells were cultured in complete RPMI medium supplemented with 1.25% DMSO for 5 days. dPLBs were pre-incubated with the pan PI3K inhibitor wortmannin (50nM) prior to 1 minute stimulation with fMLF (1μM). At the end of the stimulation, cells were lysed in ice-cold lysis buffer, followed by centrifugation to clear detergent insoluble fractions. Soluble proteins were separated by SDS-PAGE. Western blotting was performed to detect phosphorylated proteins as indicated or β-COP as loading control. For ease of viewing, densitometry data were normalised to activated control. Graphs shown include data from 3 independent experiments. Blots were representative of a minimum of 3 separately conducted experiments. Error bars show SEM. NS=not significant, *=p<0.05
6.2.3 Immune complexes and integrin ligation do not drive significant PI3K activation in dPLBs

Adhesion-dependent stimulation of dPLBs is much more subtle than stimulation with fMLF. When performing adhesion-dependent stimulation, the significant basal activation status of PI3K/PKB and of Erk interfered with identifying any additional stimulation dependent phosphorylation (data not shown). In an attempt to improve the signal to noise ratio, dPLB cells were primed. By doing this, a small, but reproducible signal for both PKB and Erk activation upon plating dPLB-985 onto fibrinogen in the presence of TNF (Fgn&TNF) (Fig 6.2.3.A-C) was obtained. Although PKB activation was very weak, and there was significant basal Erk activation, inhibition of PI3K significantly reduced Erk activation and attenuated PKB activation. In line with the observation in primary human neutrophils, this suggested that PI3K was upstream of PKB and Erk. Unfortunately, plating dPLBs onto immobilised ICs (Fig 6.2.3 D-F) or stimulation of dPLBs with insoluble ICs (Fig 6.2.3 G-I), caused a near negligible activation of PKB and Erk. Together with the somewhat variable background Erk phosphorylation, there was no sufficiently large window to detect any potentially decreased responses.
For differentiation, PLB-985 cells were cultured in complete RPMI medium supplemented with 1.25% DMSO for 5 days. dPLBs were primed with 250pM GM-CSF for 1 hour at 37°C, primed dPLBs were then incubated with the pan-PI3K inhibitor wortmannin (50nM) for a further 10 minutes. Following incubation with wortmannin, dPLBs were stimulated by being plated onto dishes that had been coated with fibrinogen in the presence or absence of TNF [Fgn & TNF (20ng/ml)] (A-C), or that were coated with BSA (as a control) or BSAαBSA (immobilised ICs) (D-F) or were kept in suspension and stimulated with insoluble ICs (HSAαHSA, 10μg/ml) (G-I). For stimulation with immobilised ICs and Fgn&TNF, cells were incubated for 12 minutes at 37°C, adherent cells were then combined with non-adherent cells by scraping into ice-cold lysis buffer. For stimulation with insoluble ICs, cells were incubated for 10 minutes at 37°C, followed by cell lysis in ice-cold lysis buffer. Detergent insoluble fractions in the cell lysates were cleared by centrifugation and proteins were separated by SDS-PAGE. Western blotting was performed to detect phosphorylated proteins as indicated or β-COP served as loading control. For ease of viewing, densitometry data were normalised to activated control. Graphs shown include data from at least 3 independent experiments. Blots were representative of a minimum of 3 separately conducted experiments. Error bars show SEM. NS=not significant, *=p<0.05
6.2.4 PLB-985 cells lack CD16

The observation that dPLBs responded so poorly to adhesion-dependent stimulation and to immune complexes was intriguing. To understand why the cells responded so weakly, the cell surface integrin and Fcγ receptors on dPLBs and primary neutrophils isolated from peripheral blood of healthy donors were compared. Results from flow cytometry analysis revealed that dPLBs expressed the major neutrophil integrins CD11b (also known as MAC-1, CR3 or integrin αM), CD11a (also known as LFA-1α or integrin αL) and CD18 (also known as integrin β2) to a very similar extent as primary neutrophils. Moreover, dPLBs expressed more surface CD49f (also known as VLA6) than primary neutrophils and both expressed only negligible surface CD49b (also known as integrin α2) (Fig 6.2.4). As for Fcγ receptors, dPLBs expressed the major neutrophil receptor CD32 (FcγRIIA), to a comparable level as primary human neutrophils; in-line with not being activated, neither cell expressed significant CD64 (FcγRI) (Fig 6.2.4). Interestingly, and in stark contrast with primary human neutrophils, no CD16 (FcγRIIIB) was detected on the surface of dPLBs (Fig 6.2.4). It is suspected that the lack of CD16 on differentiated PLBs might be the reason why they were not responsive to stimulation with immune complexes.
Figure 6.2.4 Differentiated PLBs do not express CD16.

For differentiation, PLB-985 cells were differentiated with 1.25% DMSO for 5 days. Human neutrophils isolated from peripheral blood of healthy donors and differentiated PLBs were stained with antibodies against cell surface integrin receptors (CD11b, CD18, CD11a, CD49F and CD49b) and Fcγ receptors (CD64, CD32 and CD16), and were then subjected to flow cytometry analysis. For comparison, histograms of primary human neutrophils (black solid) are copied into dPLBs (black broken). All histograms are representative of 3 independent experiments.
6.2.5 Generation of PLB-985 cells expressing CD16

Following on from the previous observation, it was decided to reconstitute PLB-985 cells with CD16, to test whether dPLBs expressing CD16 would produce a better and more consistent response to stimulation with immune complexes and to integrin ligation. I obtained a retroviral transfer vector encoding (untagged) CD16, generated retroviral particles and transduced PLB-985 cells as detailed in chapter 2.7. Also a population of PLB-985 cells that were antibiotic-resistant was obtained. As shown in Fig 6.2.5, these antibiotic resistant PLB cells expressed cell surface CD16 (although not as much as primary human neutrophils) whilst the parental PLB-985 cells did not. Interestingly, the transduced PLB-985 cells expressed CD16 only once induced to become neutrophil-like (not shown). Expression of CD32 and of the major neutrophil integrins was not affected by CD16 expression (data not shown).
Figure 6.2.5 Transduced PLB-985 cells express CD16.

PLB-985 cells that had (red broken line in histogram) or that had not (black broken line) been reconstituted with CD16 were differentiated with 1.25% DMSO for 5 days; human neutrophils (black line) were isolated from peripheral blood. Cells were stained using an anti-CD16 antibody, followed by analysis by flow cytometry. The shown histogram is representative of 3 independent experiments.
6.2.6 CD16-expressing PLB-985 cells still responded poorly to stimulation with immune complexes and integrin ligands

It was next tested whether the CD16-expressing dPLBs showed an improved response to stimulation with immune complexes and integrin ligands. As shown in Fig 6.2.6 A and B, PKB was not convincingly activated and high Erk background phosphorylation remained upon stimulation of CD16-expressing dPLBs by plating onto immobilised ICs or upon incubation with insoluble ICs (even if the dPLBs had been primed prior to the assay; not shown). Although PKB was activated upon plating of the CD16-expressing dPLBs onto fibrinogen in the presence of TNF, the amplitude of Erk phosphorylation obtained remained unconvincing (Fig 6.2.6 C). It was concluded that, unfortunately, expression of CD16 in PLB985 cells was sufficient to overcome their poor responses to integrin and Fcγ receptor stimulation.
Figure 6.2.6 CD16-expressing dPLBs responded poorly to stimulation with immune complexes and following plating onto integrin ligands.

For differentiation, CD16-expressing PLB-985 cells were cultured in complete RPMI medium supplemented with 1.25% DMSO for 5 days. dPLBs were pre-incubated with the pan PI3K inhibitor wortmannin prior to being plated onto dishes that had been coated with (A) BSA or BSAαBSA (immobilised ICs), or (C) fibrinogen in the presence or absence of TNF [Fgn&TNF (20 ng/ml)], or (B) that were kept in suspension and stimulated with insoluble ICs (HSAαHSA, 10 μg/ml) as detailed in chapter 2. Cell lysates were collected after centrifugation and subjected to SDS-PAGE, followed by western blotting of phosphorylated proteins as indicated. β-actin acted as loading control. Blots are representative of 2 independent experiments.
6.3 Discussion

Owing to their similarity to primary human neutrophils, and because they are amenable to culture, transfection and transduction, dPLBs have been widely used as a model cell line in the study of neutrophils [234, 235]. Since PI3K signalling was not conserved between human and mouse neutrophils (see chapter 5), it was hoped to use dPLBs as an alternative model to confirm the findings obtained with primary human neutrophils. Although signalling events appeared to be conserved between dPLBs and human neutrophils, the analysis events was hindered by the poor responses of dPLBs to stimulation with ICs (even when cells have been starved prior to stimulation). Despite the attempt to express CD16 in PLB-985 cells, they still responded poorly to these stimulations. The response window was too small to detect potentially subtle differences caused by knock-down of signalling proteins of interest. Heterologous expression of receptors has often been used to investigate receptor-stimulated signalling [236]. Yet, expressing CD16 in PLB-985 did not permit them to become responsive to immune complexes. In-line with our results, additional experiments performed in the laboratory (not by myself) indicated that PLB-985 responded also very poorly in terms of ROS production to integrin stimulation, and poorer still to immune complex-dependent stimulation. Expression of CD16 did not improve adhesion-dependent of insoluble IC-induced ROS production either, suggesting that the receptor was not functioning in the desired fashion. It could be that PLB-985 are not only insufficiently differentiated to express CD16, but even lack the ability to employ heterologous CD16 to signal.

Expression of CD16 in dPLBs has been explored by different groups, with contradictory results. A very small number of studies are suggestive of CD16
expression in differentiated PLB-985 or HL-60 cells, another pro-myelocytic cell line that is commonly used as a model for neutrophils in response to differentiation with dimethylformamide (DMF) or dimethyl sulphate [237, 238]. Others agree with our findings that CD16 is not expressed in PLB-985 cells that were differentiated with dimethyl sulfoxide (DMSO), dibutyryl-cAMP (dbcAMP) or DMF [233]. We also analysed CD16 expression in induced HL-60 cells, which was negative (data not shown), and attempted induction of HL60 with dimethyl sulphate as described in [237]. Unfortunately, all HL-60 cells died during the course of differentiation with this agent; it is unclear why the results were different to those obtained by the authors of this study.

The differences in CD16 expression observed by different groups may be due to inconsistent PLB-985 cell differentiation. As shown in Fig 6.2.1 B, only some dPLBs exhibited a nuclear morphology that was reminiscent of primary human neutrophils, suggesting that differentiation of PLB-985 cells is somewhat variable. Alternatively, it is possible that the discrepancies in the literature might be due to different substrains of cells being used in different laboratories. CD16 is a marker of mature neutrophils. Its expression initiates only late during granulopoiesis in metamyelocytes and peaks in band and segmented neutrophils. (Fig 1.1.2.2.) [239, 240]. Given that PLB-985 cells were derived from an immature human myeloid leukaemia cell line, at a developmental stage significantly earlier than CD16 induction, it appears possible that they may simply be too immature to express CD16.

In conclusion, although dPLBs are widely used as a model of human neutrophils, this work has identified that they are unfortunately not a useful model for the study of
signalling induced by Fcγ receptor ligation. Therefore, to confirm the findings obtained with human neutrophils, an alternative model will need to be found.
7 Final discussions and future directions

The aim of this thesis was to elucidate the mechanism by which PI3K activates Erk in insoluble IC-stimulated neutrophils and to identify the neutrophil function that this signalling pathway regulates. It was demonstrated using pharmacological inhibitors, that instead of using the canonical Ras-Raf-Mek-Erk pathway, a novel, pro-apoptotic PI3K-Cdc42-Pak-Mek-Erk pathway operates downstream of Fcγ receptors in the human neutrophil.

A PI3K-regulated Cdc42 GEF

The findings with human neutrophils demonstrated that Cdc42 activation, at least following stimulation with insoluble ICs, is PI3K-dependent. This was a novel observation: in contrast to the situation with Rac, where several PI3K-regulated Rac GEFs have been described (e.g., P-Rex and DOCK2 [152, 153, 156]), no PI3K-regulated Cdc42 GEF has yet been described. There are only few other observations that also suggest PI3K-dependent activation of Cdc42 in other contexts. For example, Cdc42 activation in MTLn3 carcinoma cells was suggested to be dependent on PI3K activity [241]. In contrast, PI3K has been demonstrated to regulate Cdc42 inactivation, acting via Cdc42-GAP, which is expressed in neutrophils, where it was identified to be a PIP3 binding protein. Interestingly, Cdc42-GAP-deficient neutrophils have been shown to be characterised by chemotaxis and recruitment defects owing to a directionality defect [107, 150]. The results reported in this thesis suggest that a PI3K-regulated Cdc42 GEF acts downstream of Fcγ receptors in human neutrophils (please see chapter 3). Activation of this GEF by PI3K could be direct, or indirect. Given that
the experiments suggest that neutrophils also use non-canonical PI3K-Erk signalling when stimulated by plating onto immobilised ICs or integrin ligands (Figure 3.2.1 and Figure 3.2.3), and that integrins and Fcγ receptors have been shown to share downstream signalling pathways in other contexts [242, 243], it is conceivable that this PI3K-activated Cdc42 GEF may also function in adhesion-dependent contexts. However, due to time restriction of my PhD studentship, this possibility has not yet been formally explored. It would clearly be interesting to gain a better understanding of this PI3K-regulated Cdc42 GEF with a view to identify it. Candidates for this PI3K-regulated Cdc42 GEF can be unearthed bioinformatically by first aligning sequences of all GEFs [GEFs of Rho family GTPases typically contain a DH domain (conventional) or an unrelated domain identified in DOCK-family proteins (atypical) [244, 245]], and those that are expressed in neutrophils can be identified by examining their expression level using mRNA analysis. Potential candidates can be further analysed for their ability to bind to PIP (e.g. with regards to their PH domains) using bioinformatics tools. Interesting candidates might be found amongst the relatively poorly understood Cdc42 GEFs, for example zizimin, which form part of the DOCK-family GEFs [246]. With most cell types, it would be feasible to perform a knock-down based screen to identify this GEF. However, human neutrophils are unfortunately not very tractable cells. As laid out in Chapter 6 (and please also see below), identifying a cell line that replicates Fcγ receptor signalling in human neutrophils is not a simple, straightforward process. For this reason, at least at present, in order to identify and examine this GEF, biochemical purification of this GEF form primary neutrophil cytosol is required, and an in vitro activity assay would need to be designed and therefore used as a read out.
A model system for the study of Fcγ receptor signalling in human neutrophils

As laid out in chapters 5 and 6 of this thesis, I was unable to confirm genetically that Pak and Cdc42 are indeed involved in the pro-apoptotic signalling downstream of PI3K in insoluble IC-stimulated neutrophils. Conditionally immortalised (mouse) neutrophil progenitors have been shown to be amenable to genetic modification (using retrovirus) whilst being compatible with the in vitro differentiation of neutrophils [247-249]. This system relies on the estrogen-inducible expression of ER-HoxB8 which drives the reversible immortalisation of myeloid progenitor cells until ER-HoxB8 inactivation (by tamoxifen withdrawal). Coupled with the administration of suitable cytokines (e.g. SCF for neutrophils; GM-CSF for macrophages), the progenitors are committed to differentiate to a myeloid lineage of choice. This model system appears to be very promising for in vitro work with mouse neutrophils. Unfortunately, the regulation of haematopoiesis in mouse and human is somewhat divergent and there is no direct counterpart of the HOXB8 transcription factor [250]. Given that the signalling cascade identified is not conserved between human and mouse (see chapter 5), only a human model system would be useful for this particular project. Nevertheless, it would still be interesting to test whether HoxB8 neutrophils are more mature than DMSO-induced promyeloid cancer cell lines, such that Fcγ receptor and integrin signalling are intact. Whilst not being useful for this particular research project, such cells might be invaluable for other projects, where signalling is conserved between human and mouse neutrophils.
**FcγR signalling in mouse neutrophils**

Although mouse neutrophils did not prove to be useful for this particular project, in that the signalling cascade of interest was not conserved between the two species, they did make some observations that are interesting in their own right. As shown in chapter 5, contrasting to human neutrophils, Erk activation was PI3K- and Raf-dependent in mouse neutrophils that had been stimulated with insoluble ICs. No further analysis of signalling events in mouse neutrophils was pursued, and the mechanism by which PI3K activated Erk in insoluble IC-stimulated mouse neutrophils remains unknown. However, it would be interesting to explore whether this might be a situation in which PI3K activates Erk by acting upstream of Ras (which has been suggested to occur [163]). It would be straightforward to test this by analysing Ras activation in insoluble IC-stimulated mouse neutrophils in the presence and absence of wortmannin (or other PI3K inhibitors). Due to time limitation, I chose to focus on elucidating signalling in human neutrophils instead of mouse neutrophils, this experiment was therefore not carried out.

**Importance of priming**

All experiments presented in this thesis were carried out under defined situations *in vitro*, with neutrophils that had been freshly prepared from peripheral blood of healthy individuals (or derived from bone marrow in the case of mouse neutrophils). *In vivo*, a complex mixture of inflammatory cytokines and chemokines is present at inflammatory sites to drive neutrophil recruitment and priming. It is possible that neutrophil priming might result in differential intracellular signalling. One way to test
this possibility would be by carrying out experiments with neutrophils that have been primed, so they are ‘inflammatory like’. This might be an interesting avenue to follow in the future, to more closely analyse signalling events in a particular physiological disease (e.g. rheumatoid arthritis, RA). Soluble immune complexes are also known to induce neutrophil signalling (and functional responses) but only stimulate primed cells [160] (see also below). By working with unprimed cells, any confusion due to events inadvertently induced by stimulation with soluble immune complexes could be excluded. Furthermore, differential priming conditions have been shown to drive different neutrophil functions [251]. This suggests any priming condition would needs to be carefully chosen to reflect an inflammatory situation of choice; yet, at least to my knowledge, it is unclear exactly how neutrophils in any given conditions, such as RA, are primed, nor indeed whether this is uniform. However, it will still be very interesting to analyse whether this novel pathway does indeed operate under conditions that human neutrophils encounter at inflammatory sites in vivo.

**Potential significance of IC-induced apoptosis in autoimmune disease**

The pathway that has been described in this thesis regulates immune complex-induced neutrophil apoptosis. At present it remains unclear what the physiological significance of this pathway might be, and many questions remain open. This will be an interesting area to explore further in the future. PI3K signalling plays a key role in controlling inflammation, including in autoimmune diseases, such as RA, where immune complex-driven neutrophilic inflammation is a major contributor of the disease. Under normal conditions, insoluble ICs are rapidly cleared [252], whilst in certain disease
states immune complexes deposit on biological surfaces (in the kidney – glomerulonephritis; in the vessel – ANCA vasculitis) [68]. Interestingly, the synovial fluid of RA patients contains abundant insoluble and soluble ICs, and in Felty’s Syndrome patients (a subsection of RA), they are abundant even in the circulation [253]. Clearance of immune complexes is known to be poor in certain autoimmune diseases (e.g. SLE and Felty’s Syndrome) [254, 255]. The results described in this thesis demonstrated that insoluble ICs stimulate neutrophils for their own uptake and induce neutrophil apoptosis, suggesting that neutrophils may be involved in the clearance of immune complexes at inflammatory sites. In doing so, they may contribute to the resolution of inflammation. It is therefore of interest to identify whether this process might be dysregulated in neutrophils of RA patients, and whether this results in their inefficiency to remove immune complexes. Moreover, whether insoluble ICs induce apoptosis in neutrophils of RA patients remains unknown.

**Can soluble and immobilised ICs also induce neutrophil apoptosis?**

The human work presented here was carried out by stimulating neutrophils with insoluble ICs. In contrast, I did not explore signalling and functional responses induced by soluble ICs.

Since the synovial fluid of RA patients is also a source abundant of soluble ICs, it would be interesting to also analyse neutrophil signalling and functions induced by these soluble ICs. Insoluble and soluble ICs have been shown to induce neutrophil functions via distinct mechanisms. In contrast to insoluble ICs, stimulation of neutrophils with soluble ICs depends on neutrophil priming. The requirement of Fcγ
receptors (FcγRIIA and FcγRIIIB) through which they induce neutrophil functions is also different [160, 166]. Interestingly, soluble ICs have been shown to be internalised by neutrophils via an endocytic mechanism that depends on FcγRIIA. This internalisation has been shown to induce NET formation, offering an obvious pro-inflammatory mechanism [256]. Given that it is not certain whether NET release always induces neutrophil death (‘NETosis’), it remains unclear whether soluble ICs also promote neutrophil apoptosis. It will be interesting to test whether soluble ICs also induce neutrophil apoptosis, and, if so, which signalling cascade regulates this process.

Similarly, it would also be interesting to test whether immobilised ICs, which are also important contributors to many autoimmune diseases (including RA and ANCA vasculitis) are capable of inducing neutrophil apoptosis. I commenced, but did not follow up the analysis of signalling induced by plating neutrophils onto immobilised ICs due to the degree of protein degradation observed (Fig 3.2.2.1). Judging from the limited work with immobilised ICs (not all of which is shown in chapter 3), it appears possible that they also induce pro-apoptotic PI3K-Cdc42-Pak-Mek-Erk signalling. Whilst it will not be practical to analyse apoptosis in adherent cells by flow cytometry and cytocentrifuge preparation as described in chapter 4, it would be possible to analyse induction of apoptosis by using immunofluorescence, for example by performing a TUNEL stain on neutrophils that are fixed at different time points after plating onto the immobilised immune complexes.
IC-induced neutrophil apoptosis as an in-built protection from excessive inflammation

In conclusion, the novel, pro-apoptotic signalling pathway identified in this thesis suggests insoluble IC-induced apoptosis could be a form of intrinsic apoptosis that acts as a protective measure to avoid excessive neutrophilic inflammation under conditions where insoluble ICs are present. The fact that none of the neutrophil functions tested, other than apoptosis, were regulated by PI3K-Cdc42-Pak-Mek-Erk signalling suggests that pro-and anti-inflammatory effects induced by insoluble ICs might be uncoupled. If so, it might be possible to pharmacologically affect pro-inflammatory but not this pro-apoptotic pro-resolution pathway. This could have important implications for treatment design and modulation of disease.
References


δ regulate neutrophil oxidase activation in response to Aspergillus fumigatus hyphae. The Journal of Immunology 186, 2978-2989.


MEK1 complex formation and activation during cellular adhesion. Molecular and cellular biology 24, 2308-2317.


238. Selmeczy, Z., Szelényi, J., Német, K., and Vizi, E.S. (2003). The inducibility of TNF-α production is different in the granulocytic and monocytic differentiated forms of wild type and CGD-mutant PLB-985 cells. Immunology and cell biology 81, 472-479.


