Role of the Rho GEF, Lfc, in Macrophage and Neutrophil Function

by

Noah Arie Fine

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Medical Biophysics
University of Toronto

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Doctor of Philosophy
Department of Medical Biophysics
University of Toronto
2012

Abstract

Lfc is a Rho specific guanine nucleotide exchange factor (GEF) that is bound and inhibited by the microtubule (MT) cytoskeleton. In epithelial cells, Lfc promotes actomyosin contractility in response to MT depolymerization; however, its role in leukocytes has not been assessed. Through genetic ablation, we generated an Lfc knockout mouse (Lfc−/−) and tested biochemical and cell biological responses to MT depolymerization in bone marrow derived cells. Lfc was necessary for characteristic actomyosin based contractile behaviours of neutrophils and macrophages, in response to MT depolymerization.

Gout is a painful arthritic inflammatory disease, caused by buildup of monosodium urate (MSU) crystals in the joints. Colchicine, a MT-depolymerizing agent that is used in prophylaxis and treatment of acute gout flare, blocks neutrophil infiltration to sites of MSU crystal-induced inflammation. We found that Lfc was necessary for the ability of colchicine to inhibit MSU-induced neutrophil infiltration in two in vivo models of gout-like inflammation.

Efficient recruitment of leukocytes from the vasculature is a critical step in the immune response to infection. Leukocyte extravasation, which includes rolling, crawling, and diapedesis across the endothelial barrier, is enhanced by fluid shear stress. Through comparison of Lfc+/+ and Lfc−/− mice, we found that Lfc was necessary for in vivo leukocyte crawling and emigration out of the
vasculature. Lfc<sup>−/−</sup> mice also showed defective neutrophil infiltration in response to acute inflammatory insults, and increased mortality in response to polymicrobial infection. *In vitro*, we found that Lfc was necessary for neutrophil responses to shear stress.
Acknowledgments

Foremost, I would like to thank my supervisor, Dr. Rob Rottapel, for his encouragement and support. I am also indebted to past and present members of the Rottapel lab for creating a productive and stimulating work environment. These include: Fiona Guerra, Jane Cullis, Grace Chen, Mauricio Medrano, Korris Lee, Fernando Suarez, Paul Krzyzanowski, Fabrice Sircoulomb, Ioannis Dimitriou, Dedi Meiri, Noam Levaot, and Josée Normand. A special thank you to Jose LaRose for much appreciated guidance and assistance, and for generating the Lfc knockout mouse. My collaborators Jakob Rullo, Eric Gracey, Jack Haitsma, and Björn Petri, have helped me make the science enjoyable and exciting. Finally, I would like to acknowledge my family including: my grandmother Mae, for the chicken soup and brisket; my mom, dad, and two brothers, for their unconditional support and unwavering faith in my abilities; my wonderful wife Rosalie, who has demonstrated her love by enduring all my long nights in the lab; and my daughter Ava, who reminds me to live each moment to its full potential.
# Table of Contents

Acknowledgments .................................................................................................................. iv

Table of Contents ................................................................................................................... v

List of Figures .......................................................................................................................... x

List of Tables ............................................................................................................................ xii

List of Supplemental Videos ................................................................................................... xiii

List of Appendices ..................................................................................................................... xiv

List of Abbreviations ............................................................................................................... xv

Chapter 1 Introduction .............................................................................................................. 1

  1 Lfc and Rho .......................................................................................................................... 2

    1.1 Rho family small GTPases .............................................................................................. 2

    1.2 RhoA ............................................................................................................................... 4

    1.3 Lfc ..................................................................................................................................... 4

    1.4 Regulation of Lfc ............................................................................................................. 6

      1.4.1 Lfc is inhibited on the MT cytoskeleton ..................................................................... 6

      1.4.2 Modes of Lfc Activation ......................................................................................... 6

      1.4.3 Subcellular localization of Lfc regulates its function in distinct cell types .............. 7

    1.5 Lfc in normal and disease states ..................................................................................... 7

    1.6 Lfc expression ............................................................................................................... 8

  2 The innate immune system .................................................................................................... 10

    2.1 Defects of the innate immune response ......................................................................... 12

    2.2 Septic shock ................................................................................................................... 12

  3 Leukocyte migration ............................................................................................................. 13

    3.1 Inflammation at the endothelial surface ......................................................................... 14
3.2 Selectins .......................................................... 15
3.3 Integrins .................................................................. 15
3.4 Diapedesis ............................................................... 16
3.5 Shear stress .............................................................. 17
3.6 Leukocyte migration in three dimensions ...................... 20
3.7 Role of Rho GTPases in leukocyte migration .................... 20
4 Gout ........................................................................... 21
  4.1 Overview and history ................................................. 21
  4.2 The inflammatory response in gout ............................. 22
  4.3 Colchicine and gout therapy .................................... 23
5 Thesis overview .......................................................... 26

Chapter 2 The Microtubule-Associated Guanine Nucleotide Exchange Factor, Lfc, is Necessary for the Anti-Inflammatory Effects of Colchicine in Murine Models of Gout .... 27

6 Abstract ...................................................................... 28
7 Introduction .................................................................. 29
8 Methods ....................................................................... 31
  8.1 Lfc gene-targeted mice .............................................. 31
  8.2 Cell preparation ....................................................... 31
  8.3 Complete blood count (CBC) ................................... 32
  8.4 Live imaging .......................................................... 32
  8.5 RhoA GTPase activity assay ..................................... 34
  8.6 Immunoblotting ...................................................... 34
  8.7 F-actin assay .......................................................... 36
  8.8 Immunofluorescence and confocal imaging .................. 36
  8.9 Uropod assay ......................................................... 37
8.10 Preparation of MSU crystals ................................................................. 37
8.11 Experimental peritonitis .................................................................. 38
8.12 Intra-articular injection and immunohistochemistry .......................... 38
8.13 ROS assay .......................................................................................... 39
8.14 Statistical analysis ............................................................................. 39
9 Results ...................................................................................................... 39
9.1 Lfc+/mice have normal blood cell counts ............................................ 39
9.2 Lfc is required for macrophage tail retraction ........................................ 41
9.3 Lfc is necessary for nocodazole-induced contraction of BMMs .............. 44
9.4 Lfc is necessary for nocodazole-induced RhoA activation and MLC 
phosphorylation in BMMs ....................................................................... 46
9.5 Lfc is necessary for neutrophil contractility and uropod formation in response to 
nocodazole ................................................................................................ 48
9.6 Lfc is necessary for nocodazole-induced neutrophil chemokinesis .......... 52
9.7 Lfc is necessary for nocodazole-induced MLC phosphorylation in neutrophils .... 54
9.8 Lfc is necessary for nocodazole-induced F-actin polymerization in neutrophils .... 54
9.9 Lfc is necessary for inhibition of neutrophil infiltration by colchicine in gout-like 
peritonitis .................................................................................................. 56
9.10 Lfc is necessary for inhibition of neutrophil infiltration by colchicine in an intra-
articular model of gout ............................................................................. 58
9.11 Lfc negatively regulates MSU crystal-induced ROS production in neutrophils..... 60
10 Discussion .............................................................................................. 61

Chapter 3 Lfc Promotes Neutrophil Spreading and Crawling in Response to Shear Stress and 
is Critical for the Innate Immune Response to Infection in Mice .................. 70
11 Abstract .................................................................................................. 71
12 Introduction ............................................................................................ 71
13 Methods ................................................................................................. 73
List of Figures

Figure 1.1: Regulation of RhoGTPases ................................................................. 3
Figure 1.2: Lfc drives RhoA activity in response to microtubule depolymerization ............. 5
Figure 1.3: GeneAtlas microarray RNA expression data for Lfc and GEF-H1 .................... 9
Figure 1.4: Leukocyte recruitment from the circulation ............................................. 14

Figure 2.1: Blood cell counts are normal in Lfc<sup>-/-</sup> mice ................................................. 40
Figure 2.2: Lfc<sup>-/-</sup> BMMs have a tail retraction defect .................................................. 42
Figure 2.3: Lfc is not required for macrophage chemotaxis .......................................... 44
Figure 2.4: Lfc is necessary for BMM contractility upon microtubule depolymerization ........ 46
Figure 2.5: Lfc drives RhoA activation and MLC phosphorylation in BMMs in response to microtubule depolymerization ................................................................. 48
Figure 2.6: Lfc is necessary for the neutrophil contractile response to microtubule depolymerization .............................................................................................................. 51
Figure 2.7: Lfc is necessary for polar localization of pMLC and F-actin in nocodazole stimulated neutrophils .......................................................... 52
Figure 2.8: Lfc is necessary for random migration of neutrophils in response to microtubule depolymerization ............................................................................................................. 53
Figure 2.9: Lfc is necessary for MLC phosphorylation and actin polymerization in neutrophils in response to microtubule depolymerization .................................................. 55
Figure 2.10: Colchicine blocks neutrophil infiltration to the peritoneal cavity in Lfc<sup>+/+</sup> but not Lfc<sup>-/-</sup> mice .................................................................................................................. 58
Figure 2.11: Colchicine blocks neutrophil infiltration to MSU-injected knee joints in Lfc<sup>+/+</sup> but not Lfc<sup>-/-</sup> mice.

Figure 2.12: Colchicine blocks ROS production in response to MSU in Lfc<sup>+/+</sup> and Lfc<sup>-/-</sup> neutrophils.

Figure 2.13: Model for the mechanism of action of colchicine in gout.

Figure 3.1: Neutrophil infiltration to the peritoneal cavity is defective in Lfc<sup>-/-</sup> mice.

Figure 3.2: *In vivo* crawling and emigration of Lfc<sup>-/-</sup> neutrophils is defective.

Figure 3.3: Lfc<sup>-/-</sup> mice have increased mortality in response to acute poly-microbial infection.

Figure 3.4: Lfc<sup>-/-</sup> neutrophils have increased ROS production.

Figure 3.5: Lfc is not necessary for neutrophil crawling in static conditions.

Figure 3.6: Lfc is not necessary for adhesion or transwell migration.

Figure 3.7: Lfc is necessary for shear stress induced neutrophil migration.

Figure 3.8: Lfc is necessary for shear stress induced neutrophil spreading.

Figure 3.9: Model of the Lfc-mediated response to shear stress.

Figure 4.1: Lfc<sup>-/-</sup> neutrophils have reduced F-actin content under shear stress, relative to Lfc<sup>+/+</sup> neutrophils.

Figure 4.2: Lfc<sup>-/-</sup> neutrophils exposed to shear stress have a reduced number of microtubule fibers per cell, compared to Lfc<sup>+/+</sup> neutrophils.
List of Tables

Table 1: Comparison of neutrophil migration under static and shear stress conditions ............... 96
List of Supplemental Videos

Video 1: Chemokinesis of Lfc\(^{+/+}\) BMMs

Video 2: Chemokinesis of Lfc\(^{-/-}\) BMMs

Video 3: Chemotaxis of Lfc\(^{+/+}\) peritoneal macrophages

Video 4: Chemotaxis of Lfc\(^{-/-}\) peritoneal macrophages

Video 5: The contractile response of Lfc\(^{+/+}\) BMMs to microtubule depolymerization

Video 6: The contractile response of Lfc\(^{-/-}\) BMMs to microtubule depolymerization

Video 7: The contractile response of Lfc\(^{+/+}\) neutrophils to microtubule depolymerization

Video 8: The contractile response of Lfc\(^{+/+}\) neutrophils to microtubule depolymerization

Video 9: The contractile response of Lfc\(^{-/-}\) neutrophils to microtubule depolymerization

Video 10: The contractile response of Lfc\(^{-/-}\) neutrophils to microtubule depolymerization
List of Appendices

Appendix A: Supplemental video legends................................................................. 148
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-MEM</td>
<td>Alpha minimum essential medium</td>
</tr>
<tr>
<td>AJC</td>
<td>Apical junction complex</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ArfGAP</td>
<td>ADP-ribosylation factor-directed GTPase activating protein</td>
</tr>
<tr>
<td>ASAP1</td>
<td>ArfGAP with SH3 domain, ankyrin repeat, and PH domain 1</td>
</tr>
<tr>
<td>BMM</td>
<td>Bone marrow derive macrophage</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
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<td>BTK</td>
<td>Burton tyrosine kinase</td>
</tr>
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<td>CARS</td>
<td>Compensatory anti-inflammatory response syndrome</td>
</tr>
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<td>CBC</td>
<td>Complete blood count</td>
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<tr>
<td>CCF</td>
<td>Crystal-derived chemotactic factor</td>
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<td>Cdc42</td>
<td>Cell division cycle 42</td>
</tr>
<tr>
<td>CDGI</td>
<td>CalDAG-GEFI</td>
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<td>CLP</td>
<td>Cecal ligation and puncture</td>
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<td>Cre recombinase</td>
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<td>Colony stimulating factor-1</td>
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<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
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<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
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<td>Dbl</td>
<td>Diffuse B-cell lymphoma</td>
</tr>
<tr>
<td>DH</td>
<td>Dbl-homology</td>
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<td>DIC</td>
<td>Differential interference contrast</td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>Dithiothreitol</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbant assay</td>
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<tr>
<td>EPEC</td>
<td>Enteropathogenic <em>Escherichia coli</em></td>
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<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ERM</td>
<td>Ezrin/radixin/moesin</td>
</tr>
<tr>
<td>ES</td>
<td>Embryonic stem cell</td>
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<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FMF</td>
<td>Familial Mediterranean fever</td>
</tr>
<tr>
<td>fMLP</td>
<td>N-Formyl-L-methionyl-L-leucyl-L-phenylalanine</td>
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<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
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<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GDI</td>
<td>Guanine nucleotide dissociation inhibitors</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
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<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
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<tr>
<td>GFDL</td>
<td>GNU Free Documentation License</td>
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<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
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<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HeLa</td>
<td>Henrietta Lacks cells</td>
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<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cell</td>
</tr>
<tr>
<td>ICAM</td>
<td>Inter cellular adhesion molecule</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>--------------</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol trisphosphate</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>JAM</td>
<td>Junction adhesion molecule</td>
</tr>
<tr>
<td>LAD</td>
<td>Leukocyte adhesion deficiency</td>
</tr>
<tr>
<td>LARG</td>
<td>Leukaemia-associated Rho GEF</td>
</tr>
<tr>
<td>Lbc</td>
<td>Lymphoid blast crisis</td>
</tr>
<tr>
<td>LFA-1</td>
<td>Lymphocyte function-associated antigen-1</td>
</tr>
<tr>
<td>Lfc</td>
<td>Lbc's first cousin</td>
</tr>
<tr>
<td>LIMK</td>
<td>LIM (Lin-1, Isl-1, and Mec-3) domain kinase</td>
</tr>
<tr>
<td>LPA</td>
<td>Lysophosphatidic acid</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucine-rich repeat</td>
</tr>
<tr>
<td>LTB₄</td>
<td>Leukotriene B₄</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MBL</td>
<td>Mannose-binding lectin</td>
</tr>
<tr>
<td>mDia</td>
<td>Mammalian homolog of <em>Drosophila</em> diaphanous</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblast</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential media</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>MLC</td>
<td>Myosin light chain</td>
</tr>
<tr>
<td>MSU</td>
<td>Monosodium urate</td>
</tr>
<tr>
<td>MT</td>
<td>Microtubule</td>
</tr>
<tr>
<td>MTOC</td>
<td>Microtubule organizing centre</td>
</tr>
<tr>
<td>NACHT</td>
<td>NAIP, CIITA, HET-E and TP1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
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</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate-oxidase</td>
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<tr>
<td>NALP3</td>
<td>NACHT, LRR, and pyrin domain-containing protein-3</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NLR</td>
<td>NOD-like receptor</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide oligomerization domain</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
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<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
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<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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<tr>
<td>PAK</td>
<td>p21-activated kinase</td>
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<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular patterns</td>
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<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PECAM-1</td>
<td>Platelet endothelial cell adhesion molecule-1</td>
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<td>PFA</td>
<td>Paraformaldehyde</td>
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<td>PH</td>
<td>Plextrin homology</td>
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<td>P_i</td>
<td>Inorganic phosphate</td>
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<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
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<td>PIP2</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
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<td>PIP3</td>
<td>Phosphatidylinositol 3,4,5-triphosphate</td>
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<tr>
<td>PKA</td>
<td>Protein kinase A</td>
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<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
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<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>PSGL-1</td>
<td>P-selectin glycoprotein ligand-1</td>
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<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
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<td>Rap-1</td>
<td>Ras-related protein 1</td>
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<tr>
<td>Ras</td>
<td>Rat sarcoma</td>
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<td>Rac</td>
<td>Ras-related C3 botulinum toxin substrate</td>
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<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>Rho</td>
<td>Ras-homologous</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative light units</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated protein kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SH3</td>
<td>Src homology 3</td>
</tr>
<tr>
<td>SIRS</td>
<td>Systemic inflammatory response syndrome</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>Src</td>
<td>Sarcoma</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<td>Syk</td>
<td>Spleen tyrosine kinase</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline with Tween</td>
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<td>TCA</td>
<td>Trichloroacetic acid</td>
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<tr>
<td>Tctex1</td>
<td>T-complex testis-expressed 1</td>
</tr>
<tr>
<td>TEM</td>
<td>Transendothelial migration</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
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<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
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<tr>
<td>Term</td>
<td>Definition</td>
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<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
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<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
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<td>V(D)J</td>
<td>Variable, diverse, joining</td>
</tr>
<tr>
<td>VLA-4</td>
<td>Very late antigen-4</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cell count</td>
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</table>
Chapter 1

Introduction
1 Lfc and Rho

1.1 Rho family small GTPases

Rho family small GTPases are biomolecular switches that regulate a wide range of important cellular biological phenomena (Etienne-Manneville and Hall, 2002; Jaffe and Hall, 2005). Their activity is regulated by cycling between an inactive, GDP-bound form, and an active, GTP-bound form. Loading of the small GTPases with GTP induces a conformational change that allows binding and activation of downstream effectors. This is accomplished by guanine nucleotide exchange factors (GEFs), which catalyze the exchange of GDP for GTP, and reversed by the intrinsic enzymatic activity of the GTPases, which cleaves GTP into GDP and inorganic phosphate. GTPase activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs) inactivate small GTPases by accelerating their intrinsic enzymatic activity and by blocking exchange of GDP for GTP, respectively (Fig. 1.1). Downstream effectors of the Rho-GTPases include serine/threonine kinases, such as p21-activated kinase (PAK) (Manser et al., 1994) and Rho-associated protein kinase (ROCK) (Fujisawa et al., 1996), actin nucleating proteins, such as mammalian homologue of Drosophila diaphanous (mDia) (Watanabe et al., 1999), and scaffolding proteins, such as Rhotekin (Reid et al., 1996).

The Rho family of small GTPases contains approximately 20 members. These are activated by a large family of approximately 85 GEFs in mammals (Hall, 2005). The majority of these RhoGEFs are members of the Dbl (diffuse B-cell-lymphoma) family, which contain tandem Dbl homology (DH) and plekstrin homology (PH) domains (Schmidt and Hall, 2002). The core catalytic domain of the GEFs is the DH domain; however the PH domain can enhance nucleotide exchange through direct interaction with the GTPase substrate (Rossman et al., 2005a; Rossman et al., 2005b). Also, interaction of PH domains with membrane phospholipids is necessary for GEF activity in vivo (Rossman et al., 2003).

The prototypical Rho family GTPases are RhoA, Rac1, and Cdc42. Rac1 and Cdc42 are responsible for protrusive F-actin based structures at the leading edge of migrating fibroblasts, known as lamellipodia and filopodia, respectively (Nobes and Hall, 1999). RhoA is required for formation of stress fibers and integrin-based focal adhesions in fibroblasts (Paterson et al., 1990; Riddley and Hall, 1992; Hotchin and Hall, 1995). In addition to cell migration, Rho family small GTPases coordinate a variety of cellular responses (Ridley, 2001) including proliferation and
survival (Olson et al., 1995; Aznar and Lacal, 2001), gene expression (Hill et al., 1995),
activation of the NADPH oxidase complex (Abo et al., 1991; Knaus et al., 1991), and
phagocytosis (Caron and Hall, 1998). Rho family GTPases also contribute to transformation,
metastasis, and invasion in cancer progression (del Peso et al., 1997; Narumiya et al., 2009;
Rathinam et al., 2011).

**Figure 1.1: Regulation of RhoGTPases.** Rho family small GTPases are activated by guanine nucleotide exchange factors (GEFs), and inhibited by GTPase activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs). The active, GTP-bound form of Rho, is generated by GEFs, which exchange GDP for GTP. The catalytic GTPase activity of the Rho proteins, which cleaves bound GTP into GDP and inorganic phosphate (P_i), is enhanced by GAPs. GDIs hinder nucleotide exchange by binding to RhoGDP.
1.2 RhoA

RhoA is an important regulator of the cellular contractile response (Fig. 1.2). In its GTP bound form, RhoA activates its downstream effector ROCK, which in turn activates MLC through direct phosphorylation (Amano et al., 1996), or by inhibiting myosin light chain phosphatase (MLCP) (Kimura et al., 1996). ROCK can also activate LIM kinase (LIMK) through direct phosphorylation. In its active state, LIMK phosphorylates coflin, thus inactivating it and preventing coflin dependent depolymerization of actin (Maekawa et al., 1999). Another RhoA effector, mDia, directly catalyzes F-actin polymerization (Watanabe et al., 1999; Zigmond, 2004). In fibroblasts and epithelial cells, the RhoA-induced contractile response, characterized by cell contraction, and the formation of actin stress fibers and focal adhesions, is induced by thrombin (Ruiz-Loredo et al., 2011), lysophosphatidic acid (LPA) (Amano et al., 1997), and disruption of the MT cytoskeleton (Ren et al., 1999; Danowski, 1989; Enomoto, 1996; Birukova et al., 2004a). Interestingly, cell contraction itself drives stress fiber and focal adhesion formation (Burridge, 1981; Chrzanowska-Wodnicka and Burridge, 1996; Totsukawa et al., 2000). RhoA-dependent focal adhesion strengthening is a biochemical response to intracellular contractile tension, associated with the mechanosensory function of integrins (Riveline et al., 2001).

1.3 Lfc

Lfc is a RhoA specific GEF (Glaven et al., 1996; Ren et al., 1998; Glaven et al., 1999), a truncated form of which was originally identified based on its ability to transform NIH3T3 fibroblasts (Whitehead et al., 1995). For simplicity, we will refer to both the mouse gene and its human homologue, GEF-H1/ARHGEF2, as Lfc. Lfc is a MT-associated protein, and can be activated by MT-depolymerizing agents such as nocodazole and colchicine. In epithelial cells, Lfc is necessary for RhoA-dependent contractility and stress fiber formation in response to MT depolymerization (Krendel et al., 2002; Chang et al., 2008). Hence Lfc serves to promote F-actin and actomyosin based phenomena as a direct consequence of its release from MTs (Fig. 1.2). In distinction to Lfc, p190RhoGEF, another MT associated RhoA specific GEF, does not show increased exchange activity towards RhoA in response to MT depolymerization (van Horck et al., 2001).
Figure 1.2: Lfc drives RhoA activity in response to microtubule depolymerization. Release of MT-associated Lfc as a result of treatment with MT depolymerization agents, such as nocodazole and colchicine, drives RhoA dependent formation of stress fibers and focal adhesions in fibroblasts.
1.4 Regulation of Lfc

1.4.1 Lfc is inhibited on the MT cytoskeleton

The association of Lfc with the MT cytoskeleton depends on its interaction with the dynein light chain, Tctex-1 (Meiri et al., 2009; Conde et al., 2010), although Lfc has been reported to interact directly with tubulin (Glaven et al., 1999; Yoshimura and Miki, 2011). Phosphorylation of serine 885 in the C-terminus of Lfc by PKA (Meiri et al., 2009), PAK1 (Zenke et al., 2004), or PAK4 (Callow et al., 2004), promotes binding to 14-3-3 proteins, which maintains Lfc in an inactive state on the MTs.

1.4.2 Modes of Lfc Activation

In addition to activation by MT-depolymerizing agents, signalling to Lfc can be achieved through stimulation of a number of different kinds of cell surface receptors. Lfc is activated downstream of LPA signalling in fibroblasts (Meiri et al., 2009), thrombin stimulation of endothelial cells (Birukova et al., 2006), TNF-α and epidermal growth factor (EGF) signalling in tubular epithelial cells (Kakiashvili et al., 2009; 2011), Wnt signalling in neuronal cells (Tsuji et al., 2010), NOD-like receptor stimulation in intestinal epithelia and macrophages (Fukazawa et al., 2008; Zhao et al., 2011), and as a result of mechanosensory stimulation of integrins (Guilluy et al., 2011). Lfc is also upregulated downstream of transforming growth factor (TGF)-β in retinal pigment epithelium (Tsapara et al., 2010).

ERK mediated phosphorylation of Lfc on threonine 678 promotes its GEF activity (Fujishiro et al., 2008). Recent evidence indicates that Lfc and another GEF called LARG (leukaemia-associated Rho GEF) are both necessary for RhoA-induced mechanical stiffening in response to force on integrins in fibroblasts (Guilluy et al., 2011). In this system LARG was activated downstream of the Src family tyrosine kinase Fyn, while Lfc was activated by a FAK-Ras-ERK signalling axis. Signalling induced phosphorylation or dephosphorylation of specific serine and threonine residues, release from the MT array, localized MT depolymerization, or some combination of these are all possible mechanisms of Lfc activation that could potentially occur in
different contexts. Elucidation of the relative contribution of these processes may be complicated by the fact that Lfc itself stabilizes MTs (Yoshimura and Miki, 2011). In the case of thrombin-induced Lfc-dependent responses in endothelial cells, partial depolymerization of MTs has been observed (Birukova et al., 2004b). However, in the case of integrin induced mechanotransduction by Lfc, MT depolymerization is not necessary, since the MT stabilizing agent, taxol, did not block Lfc activation (Guilluy et al., 2011).

1.4.3 Subcellular localization of Lfc regulates its function in distinct cell types

Differential association of Lfc with specific binding partners regulates its intracellular localization and activity. In confluent epithelial cells, Lfc is sequestered to the tight junctions through interaction with cingulin (Aijaz et al., 2005), and paracingulin (Guillemot et al., 2008). In neurons, MT-associated Lfc is released in response to membrane depolarization, and binds to neurabin and spinophilin in dendritic spines, where it regulates dendritic spine morphology (Ryan et al., 2005). Lfc binds to ASAP1 (ArfGAP with SH3 domain, ankyrin repeat, and PH domain 1) in fibroblasts, where it negatively regulates podosomes (Shiba and Randazzo, 2011).

1.5 Lfc in normal and disease states

Lfc plays a role in diverse functions in various cell-types, including orientation of the mitotic spindle (Bakal et al., 2005) in fibroblasts; cytokinesis (Birkenfeld et al., 2007), stress fiber formation (Krendel et al., 2002), and cell migration (Nalbant et al., 2009) in human epithelial cervical cancer (HeLa) cells; regulation of tight-junctions, paracellular permeability (Benais-Pont et al., 2003), and G1/S phase transition (Aijaz et al., 2005) in kidney epithelial cells; and neuronal differentiation (Gauthier-Fisher et al., 2009) and morphology (Ryan et al., 2005; Muly et al., 2008).

Lfc plays a role in diverse pathological conditions (Birkenfeld et al., 2008), including cancer (Brecht et al., 2005; Mizuarai et al., 2006; Liao et al., 2011), Huntington's disease (Varma et al., 2010), agonist and ventilator-induced pulmonary dysfunction (Birukova et al., 2006; 2010), and
retinal dysfunction and fibrosis (Tsapara et al., 2010). Epithelial barriers, which are composed of epithelial cells held together by apical junction complexes (AJC), are essential for restricting infiltration of pathogens into the underlying tissue. Crohn's disease and other inflammatory diseases of the colon are characterized by degeneration of these AJCs and infiltration of gut microbes into the tissue of the intestine (Laukoetter et al., 2006). Lfc has been shown to mediate AJC disassembly through RhoA-ROCK mediated actomyosin contractility in response to calcium depletion in human colonic epithelial cells (Samarin et al., 2007). Since calcium depletion induces reorganization of the MT array, and stabilizing the MTs blocks AJC disassembly (Ivanov et al., 2006), the authors speculated that reorganization of apical MTs releases Lfc, which drives AJC disassembly through RhoA. Furthermore, it was recently demonstrated that Lfc expression is upregulated in colon tissue biopsies of patients with Crohn's disease (Zhao et al., 2011). Enteropathogenic Escherichia coli (EPEC), a highly pathogenic strain of E. coli, break through epithelial barriers in the gut by exploiting Lfc-mediated RhoA activation. They do so by injecting toxins that cause MT depolymerization, leading to an Lfc dependent increase in contractility and epithelial breakdown (Matsuzawa et al., 2004). Also, the angiogenesis inhibitor 2-methoxyestradiol, which destabilizes MTs, has been shown to promote ROCK dependent disruption of endothelial barriers (Bogatcheva et al., 2007), suggesting the possibility that, in this context, Lfc activation might contribute to inhibition of blood vessel formation.

1.6 Lfc expression

By northern blot, Lfc expression is highest in mouse thymus, spleen and bone marrow, relative to other tissues (Whitehead et al., 1995). GeneAtlas microarray expression data indicates that Lfc and GEF-H1 mRNA are ubiquitously expressed in many tissue and cell types (Fig. 1.3). For both Lfc and GEF-H1, mRNA expression is highest in hematopoietic tissues, and brain. Of the hematopoietic cell types, expression of Lfc was particularly high in macrophages, natural killer (NK) cells, and mast cells; however Lfc expression was below median in Mac1+GR-1+ granulocytes.
Figure 1.3: GeneAtlas microarray RNA expression data for Lfc and GEF-H1. This data is reproduced from the Genomics Institute of the Novartis Research Foundation in accordance with the GNU Free Documentation License (GFDL). Probe sets are 1421043_s_at for Lfc and 209435_s_at for GEF-H1, and are consistent with other probe sets. Y-axis indicates specific cell and tissue types, and cell lines. X-axis is arbitrary expression units. 3xM is three times the median expression in all samples. Red boxes indicate myeloid lineage cell types.
Tissue microarray data from the human protein atlas (www.proteinatlas.org) indicates ubiquitous expression of GEF-H1 at the protein level, with moderate to strong staining in 77% of cell types tested. Strong immunohistochemical staining of GEF-H1 was reported in bone marrow.

2 The innate immune system

Living organisms are under constant threat of infection. The ability of host organisms to defend themselves against harmful opportunistic microorganisms depends on the coordinated actions of the innate and adaptive immune systems (Janeway et al., 2005). The innate immune system is evolutionarily more primitive than the adaptive immune system, and is an essential feature of the host response to infection and tissue damage. The innate immune response involves a number of white blood cell types and blood-borne molecules that are predisposed to recognize specific danger signals and patterns associated with infectious agents and tissue damage (Janeway and Medzhitov, 2002). Components of the innate immune system include macrophages, neutrophils, dendritic cells, NK cells, mast cells, and the complement system.

Resident tissue macrophages are the first cells to recognize invading pathogenic microorganisms. Binding, through specific cell surface receptors, results in ingestion of pathogens by phagocytosis, and induction of an early induced innate immune response through secretion of inflammatory mediators. Cells of the innate immune system express an array of genome encoded cell surface receptors, which recognize ubiquitous pathogen-associated molecular patterns (PAMPs). The Toll-like receptors (TLRs) are a family of 10 pattern recognition receptors (PRR) that recognize distinct sets of PAMPs. For example, bacterial lipopolysaccharide (LPS), a cell-wall component of gram-negative bacteria, also known as endotoxin, triggers an immune response through stimulation of TLR-4 on macrophages (Poltorak et al., 1998). NOD-like receptors (NLRs) are a family of >20 PRRs (Inohara et al., 2005). In contrast to cell surface expressed TLRs, NLRs are intracellular receptors that detect specific PAMPs within the cytosol. A subset of NLRs, that response to nonmicrobial danger signals, assemble into multi-protein complexes known as inflammasomes, which promote secretion of the proinflammatory cytokines IL-1β and IL-18 (Martinon, 2009). Other PRRs, such as the mannose-binding lectin (MBL) and the complement protein C1q, occur as free protein in the blood plasma. These promote pathogen clearance through activation of the complement system, a group of plasma proteins that produce
a cascading reaction on the surface of pathogens resulting in cell lysis or recruitment of phagocytes. Upon pathogen recognition, macrophages shed inflammatory lipids such as prostaglandins, leukotrienes, and platelet activating factor (PAF), and secrete inflammatory cytokines, such as TNF-α, IL-1β, IL-6, IL-8, IL-12 and IL-18. Within the first 6 hours of an acute inflammatory response, neutrophils arrive at the site of infection in large numbers, followed by monocytes, which subsequently differentiate into mature macrophages.

Neutrophils constitute 60% of white blood cells in the circulation and respond quickly to invading bacterial pathogens. Neutrophils are short lived and are normally absent in healthy tissue. The appearance of pus, a mixture of neutrophils and liquefied tissue, is a characteristic sign of a neutrophil mediated immune response. At the site of infection, neutrophils secrete toxic reactive oxygen species (ROS), ingest bacteria by phagocytosis, and secrete granules laden with anti-microbial agents (Borregaard, 2010).

Responses of innate immune cells, including migration, phagocytosis, and ROS production, rely on members of the Rho family of small GTPases (Bokoch 2005). For example, Rac is a component of the membrane associated NADPH oxidase complex, which is responsible for generating ROS in neutrophils and other cell types. The NADPH oxidase complex transfers electrons from NADPH to molecular oxygen to produce the highly reactive and toxic superoxide free-radical, which subsequently dismutates to hydrogen peroxide and other ROS. The phagocytic NADPH oxidase complex consists of six subunits: NOX2, p22phox, p40phox, p47phox, p67phox, and Rac2. In addition to bacterial killing, ROS production is necessary for Rac mediated neutrophil spreading and chemotaxis, through redox-dependent inhibition of Rho and PTEN (Nimnual et al., 2003; Kuiper et al., 2011).

The induced innate immune response is often insufficient to clear rapidly replicating bacteria in tissues during acute infection. This can occur if a microorganism is able to evade the innate immune defenses or enters the body in sufficient numbers to overwhelm the innate immune system and establish a focus of infection. In this case, mitigation by the adaptive immune system is often sufficient to clear infection. Components of the innate immune response, such as macrophages and dendritic cells, promote an adaptive immune response through antigen presentation and secretion of inflammatory cytokines.
2.1 Defects of the innate immune response

The inflammatory mechanisms of innate immunity are chemically and physically destructive, therefore the ability to respond appropriately to danger signals and to distinguish between self and non-self is critical. Inappropriate neutrophil activity can lead to excessive tissue damage (Weiss, 1989; Nathan, 2006), while compromised neutrophil function, such as in leukocyte adhesion deficiency (LAD) (Anderson and Springer, 1987; Bunting et al., 2002) or chronic granulomatous disease, leads to recurrent bacterial infections (Holland, 2010). LAD is caused by inactivating mutations of the β2-integrin or other adhesion molecules, while chronic granulomatous disease is caused by mutations in the NADPH oxidase complex, and hence defective ROS production.

Inflammatory diseases can be divided into those in which microbial toxicity and inflammation contribute to pathology, such as sepsis, cystic fibrosis, hepatitis C, and leprosy, and those in which inflammation alone is the primary contributor to pathogenesis, which include gout, rheumatoid arthritis, psoriasis, atherosclerosis, multiple sclerosis, and asthma (Nathan, 2002). Although known and unknown infectious causes are possible for some of the later group (Inman et al., 2000; Scher and Abramson, 2011), anti-inflammatory intervention remains the main therapeutic option.

2.2 Septic shock

The same innate immune responses that are necessary for proper clearance of pathogens can, under certain circumstances, lead to the potentially fatal pathogenic condition known as septic shock. Septic shock is characterized by loss of tissue perfusion and oxygen delivery, leading to failure of vital organs such as the kidneys, liver, heart, and lungs, and is mediated primarily by TNF-α (Watanabe et al., 1995). The inflammatory effects of macrophage secreted TNF-α are instrumental in containing localized infection and preventing the spread of bacteria into the blood. However, severe infection or spreading of infection into the bloodstream, known as sepsis or bacteremia, results in systemic secretion of TNF-α by macrophages in the liver, spleen, and other sites. The inflammatory effects associated with systemic TNF-α release, include loss of blood volume due to increased vascular permeability, and severe intravascular blood clotting.
Septic shock is diagnosed when patients exhibit evidence of infection, organ failure, and hypotension combined with systemic inflammatory response syndrome (SIRS); which includes elevated heart rate, respiratory rate, and temperature and greatly reduced or elevated white blood cell (WBC) counts.

In response to inflammatory factors released in sepsis, neutrophil membrane rigidity increases markedly, associated with a corresponding increase in F-actin content at the cortex. Increased membrane rigidity of activated neutrophils in circulation results in sequestration in the capillary beds (Drost et al., 1999; Skoutelis et al., 2000; Saito et al., 2002). These neutrophils fail to transmigrate and cause damage to the endothelial lining and ischemia. As sepsis progresses to septic shock, neutrophil function is greatly inhibited in a phenomenon called compensatory anti-inflammatory response syndrome (CARS) (Alves-Filho et al., 2010). Thus, early neutrophil responses in sepsis and subsequent neutrophil failure due to excessive systemic inflammation contribute directly to exacerbation of vascular damage, and cause further unchecked spread of infection, respectively.

Septic shock illustrates how the innate immune system can act as a double-edged sword. Release of the inflammatory cytokine, TNF-α, protects against localized infection, but excessive TNF-α secretion in response to severe systemic infection can be pathological and fatal. Mutant mice that lack TLR-4, a receptor for bacterial LPS, are resistant to septic shock, but more prone to infection by gram-negative bacteria (Poltorak et al., 1998).

### 3 Leukocyte migration

The induced innate and adaptive immune responses depend on infiltration of circulating leukocytes by a process known as extravasation. This includes cell rolling on the endothelial surface, firm adhesion, crawling, and diapedesis, also known as transendothelial migration (TEM) (Fig. 1.4) (Springer, 1994). Extravasation also encompasses chemotaxis of leukocytes through the three-dimensional tissue matrix towards a gradient of chemokine. What follows is a review of physical and molecular events associated with leukocyte recruitment to inflammatory sites, with particular emphasis on our main cell-type of interest, neutrophils.
Figure 1.4: Leukocyte recruitment from the circulation. Recruitment of leukocytes from the circulation involves a distinct sequence of events at the endothelial surface. Tethering and rolling are mediated by selectin ligand interactions. Spreading, crawling, and diapedesis are determined by dynamic integrin ligand associations and anchoring of integrins to the F-actin cytoskeleton. Integrin activation is facilitated by GPCR signalling in response to endothelial expressed chemoattractants. Selectin and integrin mediated events are potentiated by shear stress.

3.1 Inflammation at the endothelial surface

In order to reach a site of infection, neutrophils and other leukocytes must overcome a number of obstacles. The first step in leukocyte infiltration of the tissue is recruitment from the circulation. Cytokines and lipid mediators, secreted by macrophages, stimulate vascular endothelial cells in the vicinity of the infection, which promotes recruitment of circulating leukocyte in several ways. Cytokines stimulate blood vessels to dilate, increasing the volume and decreasing the velocity of blood flow. Consequently, the endothelium becomes more permeable, allowing leakage of fluid and plasma proteins into the tissue. This results in increased cellularity within the inflamed vasculature, which promotes contact of cells with the endothelial lining, known as margination. Cytokine stimulation also induces expression of adhesion molecules on the
endothelial surface. These include P and E-selectins and integrin ligands such as ICAM-1, ICAM-2, and VCAM-1, which help to capture leukocytes by binding to specific cell surface receptors as detailed below. In addition, endothelial surface expressed cytokines signal through specific G-protein coupled receptors (GPCR), which triggers integrin activation and promotes leukocyte sequestration.

3.2 Selectins

Circulating leukocytes respond quickly to endothelial expressed adhesion molecules and cytokines, which induce them to roll, attach, crawl, and undergo diapedesis across the endothelial wall. In neutrophils, rolling is mediated by tethering of P- and E-selectin on the endothelial surface with neutrophil expressed glycoprotein ligands that contain the tetrasaccharide sialyl Lewis $^x$, including P-selectin glycoprotein ligand-1 (PSGL-1) (Norgard et al., 1993). Rolling is also mediated by tethering of neutrophil expressed L-selectin to its endothelial expressed glycoprotein ligand, CD34 (Baumhueter et al., 1993). Though transient, selectin-ligand interactions are sufficient to allow rolling and promote transition to the attachment regime. L-selectin and PSGL-1 localization to neutrophil microvilli and anchoring to the F-actin cytoskeleton are necessary for their contribution to rolling and adhesion under conditions of shear stress (Buscher et al., 2010). Stable neutrophil rolling also depends on the deformability of microvilli (Pospieszalska and Ley, 2009), which increases upon inhibition of F-actin (Yago et al., 2002).

3.3 Integrins

Arrest of tethered or rolling leukocytes, and subsequent crawling and diapedesis, require dynamic association of integral membrane receptors, known as integrins, with their ligands on the endothelial surface. Of the more than 20 integrin heterodimers that are expressed in various cell types, neutrophils express primarily the $\beta_2$-integrins LFA-1 (\(\alpha_L\beta_2\)) and Mac-1 (\(\alpha_M\beta_2\)), which bind ICAM-1 and ICAM-2, respectively. Integrins take on three conformations: a bent form that is inactive, an extended form with intermediate affinity, and an extended open conformation with high affinity (Evans et al., 2009). Integrin clustering, association with the cortical actin
cytoskeleton, and recruitment of integrin associated protein components can further modulate integrin function and contribute to adhesion strengthening.

In the blood flow, leukocyte integrins are in the inactive, non-ligand-binding conformation, minimizing unsolicited recruitment and inflammation. Integrin unfolding and formation of stable integrin-ligand bonds are induced by inside-out and subsequent outside-in signalling at the inflamed endothelial surface. Chemokine stimulation through GPCRs induces integrin activation through inside-out signalling (Laudanna and Alon, 2006). The small GTPases, RhoA (Laudanna et al., 1996; Pasvolsky et al., 2008), and Rap-1 (Stadtmann et al., 2011) are known to facilitate fast GPCR mediated integrin activation on the endothelial surface. GPCR signalling activates phospholipase C (PLC) mediated hydrolysis of PIP2, generating inositol trisphosphate (IP$_3$) and diacylglycerol (DAG). This leads to activation of Rap-1 by its GEF, CalDAG-GEFI (CDGI). Active Rap-1 promotes integrin activation by triggering the association of talin with the integrin cytoplasmic domain (Kinashi et al., 2005). Talin facilitates unfolding and extension of the extracellular integrin domain and acts as a linker between integrins and the actin cytoskeleton (Tadokoro et al., 2003). The mechanism by which RhoA contributes to GPCR-mediated integrin activation has not been elucidated. One group has speculated that RhoA might function to mediate actin remodelling and linkage formation with ligand-occupied integrins, facilitating the mechanosensory response of integrins (Alon and Ley, 2008). Indeed, ligand-engaged LFA-1 is highly associated with the actin cytoskeleton, while low-affinity LFA-1 is poorly anchored to the cytoskeleton (Shamri et al., 2005). Full activation of integrins requires integrin-ligand stimulated, outside-in activation, which may be facilitated by physical force on the extended ligand-occupied integrin (Astrof et al., 2006; Alon and Ley, 2008). Integrin mediated leukocyte capture on the endothelial surface occurs on the subsecond timescale and involves simultaneous bidirectional activation induced by inside-out and outside-in signalling (Alon, 2010).

3.4 Diapedesis

After adhesion and crawling on the vascular surface, leukocytes must cross the endothelial barrier and the basement membrane through diapedesis. The majority of neutrophils cross the endothelium at intercellular junctions, by paracellular migration; however, transcellular migration directly through individual endothelial cells is also common (Petri et al., 2008). The
predominant mode of transmigration can differ between different white blood cell types (Nieminen et al., 2006) and on different vascular surfaces (Engelhardt and Wolburg, 2004). Differential expression of adhesion receptors on endothelial surfaces helps to determine the mode of leukocyte transmigration (Yang et al., 2005). LFA-1 appears to mediate transcellular migration while Mac-1 mediates paracellular migration, since overexpression of ICAM-1 or ablation of Mac-1 favours transcellular migration (Yang et al., 2005; Phillipson et al., 2006). In addition to integrins, paracellular migration is mediated by platelet endothelial cell adhesion molecule-1 (PECAM-1), CD99, and junction adhesion molecules (JAMs) (Lou et al., 2007; Petri and Bixel, 2006). Furthermore, endothelial cells actively encapsulate neutrophils at the apical-surface by forming dome-like structures, thus minimizing vascular permeability during paracellular and transcellular migration (Phillipson et al., 2008; Petri et al., 2011).

3.5 Shear stress

Diverse cell types exhibit biochemical responses to fluid shear stresses (Chen et al., 2007; Chachisvilis et al., 2006; Alon and Ley, 2008). The environment inside blood vessels is characterized by varying degrees of fluid shear stress due to blood flow. Leukocytes are evolutionarily adapted to respond to mechanical force, to the extent that fluid shear stress, in the range of 1-10 dyn/cm² of pressure, is required in order to achieve optimal leukocyte crawling and diapedesis (Alon and Dustin, 2007; Zarbock and Ley, 2009; Makino et al., 2007). The mechanisms and pathways that contribute to the mechanosensory response of leukocytes to shear stress are beginning to be elucidated, and are likely to be initiated by physical perturbation of load-bearing subcellular structures such as the plasma membrane, integrin based adhesions, and the cytoskeleton (Hoffman et al., 2011), during rolling and crawling of leukocytes on the endothelial surface.

Rolling leukocytes experience shear stress at the endothelial surface as transient selectin-ligand bonds form. Signalling as a result of transient selectin-ligand tethering during rolling, triggers integrin activation in neutrophils but not in lymphocytes (Green et al., 2004; Alon and Ley, 2008). This induces slow rolling and promotes firm adhesion (Smith, 2000). Specifically, ligation of neutrophil expressed PSGL-1 with endothelial expressed E-selectin has been shown to stimulate integrin unfolding (Zarbock et al., 2007). This occurs through a signalling cascade
involving Src family kinases, immunoreceptor tyrosine-based activation motif (ITAM) containing proteins, spleen tyrosine kinase (Syk), and Burton tyrosine kinase (BTK). BTK in turn activates phospholipase Cγ (PLC-γ), phosphoinositide 3-kinase (PI3K), and p38 mitogen-activated protein kinase (MAPK) (Hentzen et al., 2002; Zarbock et al., 2008; Mueller et al., 2010; Yago et al., 2010), resulting in integrin activation.

Leukocytes are also sensitive to shear stress by selectin-independent mechanisms. Unstimulated leukocytes underwent myosin and F-actin dependent cell stiffening within seconds of exposure to shear stress (Coughlin et al., 2008), and showed pseudopod projection and spreading five minutes after exposure to shear stress (Coughlin and Schmid-Schönbein, 2004). Rounded neutrophils had increased volume and intracellular granule velocity 5-10 minutes after exposure to shear stress (Moazam et al., 1997). In contrast, unstimulated cells that were spread on a glass surface showed pseudopod retraction within one minute of exposure to shear stress (Makino et al., 2006). These studies were performed using HL-60 cells, a human promyelocytic leukemia cell line that can be differentiated into neutrophil-like cells. A fluorescence resonance energy transfer (FRET)-based approach showed that GPCR signalling was reduced in HL-60 cells exposed to shear stress. Furthermore, it was demonstrated that HL-60 cells in suspension have a transient increase in RhoA activity between 2 and 5 minutes after exposure to shear stress, while active Rac1 and Rac2 levels are reduced after 10 minutes of shear stress (Makino et al., 2005). The authors suggest a mechanism whereby shear stress directly, or indirectly, blocks GPCRs, resulting in reduced Rac activity and pseudopod retraction in spread neutrophils (Makino et al., 2007). Spread neutrophils that had been centrifuged (Fukuda and Schmid- Schönbein, 2002) or treated with anti-inflammatory steroids (Fukuda et al., 2004) showed pseudopod extension rather than retraction upon exposure to shear stress, indicating that the nature of the neutrophil response to shear stress is highly sensitive to environmental factors.

Although these analyses are informative, it will be important to analyze the morphological response of neutrophils to shear stress when they are spread on physiological ligands, instead of glass. Several studies have begun to probe the neutrophil response to shear stress on activated endothelial surfaces. The rate of neutrophil crawling and diapedesis on TNF-α (100 units/ml, 4 hours) stimulated human umbilical vein endothelial cells (HUVECs) increases with the magnitude of shear stress (Kitayama et al., 2000). Interestingly, increased migration was observed in neutrophils that had been exposed to shear stress even after they had completed
diapedesis, and were therefore no longer exposed to shear. In another study, neutrophil diapedesis was found to be independent of shear stress when HUVEC monolayers were highly activated (200 units/ml TNF-α, 24 hours), but shear stress-dependent when HUVECs were unstimulated, or moderately activated (2 units/ml TNF-α, 4 hours) (Cinamon et al., 2004). In this context, shear stress promoted invagination of neutrophils into the apical endothelial interface and was dependent on β2 integrins and endothelial-presented chemoattractants. The authors propose that neutrophils integrate stimuli from multiple inputs on the endothelial surface, including integrin ligands, endothelial-presented chemoattractants, and shear stress.

In lymphocytes, disruption of paxilin binding to the α4β1 integrin (VLA-4) cytoplasmic tail, blocked integrin association with the actin cytoskeleton, cell capture, and adhesion strengthening in response to shear stress (Alon et al., 2005). In addition, VLA-4 can be activated by shear stress generated by circular stirring (Zwartz et al., 2004), in a manner that depends on GPCR signalling and calcium flux. Shear stress has also been shown to induce adhesiveness of other integrins towards their ligands (Salas et al., 2002; de Chateau et al., 2001) and may do so through a force-induced conformational change in the integrin head domain (Astrof et al., 2006).

Typical protein-protein interactions disassociate under applied force, and are characterized as slip bonds. However, some protein-protein interactions can display a property known as a catch bond, whereby applied force increases the lifetime of the bond. When force loading on integrin-ligand bonds is fast, integrins act as catch bonds, and are more likely to convert to a high affinity state (Thomas et al., 2008; Hoffman et al., 2011). This property of integrins is thought to be essential for the shear stress response of leukocytes, and it predicts a different response on rigid versus compliant surfaces. On rigid surfaces, the force loading on integrins by shear stress is more rapid. Blood vessel dilation during local inflammation causes stretching of endothelial cells, and therefore results in increased rigidity of the endothelial surface. This is likely to result in more efficient capture of circulating leukocytes.

Although shear stress enhances neutrophil migration across cytokine-activated HUVECs, some neutrophil transmigration does occur in the absence of shear stress. In contrast, diapedesis of T-lymphocytes (Cinamon et al., 2001) and eosinophils (Cuvelier and Patel, 2001) absolutely requires shear stress. Thus, the relative contribution of the shear stress response varies for different leukocyte sub-types.
3.6 Leukocyte migration in three dimensions

The chemotactic response of leukocyte in the three-dimensional tissue matrix is distinct from earlier steps of the recruitment cascade. After crossing the endothelial barrier into the underlying tissue matrix, leukocytes migrate towards the inflammatory site by sensing a gradient of chemoattractant. Using a knockout mouse model, Lammermann et al. (2008) demonstrated that integrins are completely dispensable for chemotaxis in a three-dimensional matrix. In this context, leukocytes migrate by integrin-independent flowing and squeezing, which is dependent on RhoA-induced contractility. In contrast, integrins were necessary for attachment and crawling on a two-dimensional surface. Cancer cells transition between two distinct modes of migration in order to migrate across discrete tissue barriers (Sanz-Moreno et al., 2008; Croft and Olson, 2008), mesenchymal migration, which relies on Rac1 activity, and amoeboid migration, which is more dependent on RhoA.

3.7 Role of Rho GTPases in leukocyte migration

Successful extravasation of leukocytes to a site of infection requires highly orchestrated regulation of cell attachment, morphology, and polarity in response to extracellular cues. This is achieved through control of integrin based adhesions, cytoskeletal dynamics, and cell contractility. Rho family small GTPases, such as RhoA, Rac1, and Cdc42, and their activators, the GEFs, are central regulators of these processes and control cell migration (Wittmann and Waterman-Storer, 2001; Raftopoulou and Hall, 2004; Fenteany and Glogauer, 2004; Bokoch, 2005). Soluble factors that promote neutrophil migration include chemokines, formylated peptides of bacterial origin, such as f-Met-Leu-Phe (fMLP), and the complement fragment, C5a. Each of these factors signals through a cognate GPCR. GPCR signalling activates PI3K, which leads to enrichment of phosphatidylinositol 3,4,5-trisphosphate (PIP3) at the leading edge. Enrichment of PIP3 at the leading edge and polarized activation of Rho GTPases are necessary for maintaining polarity in neutrophil chemotaxis (Srinivasan et al., 2003; Van Keymeulen et al., 2006). Rac1 is activated at the leading edge where it promotes F-actin based protrusion, while active RhoA is enriched at the sides and the back of the cell, where it drives myosin based contractile activity (Pestonjamasp et al., 2006). The mutual exclusion of Rac1 to the front of the cell and RhoA to the back is thought to help establish and maintain self-organizing polarity.
during migration (Xu et al., 2003; Wong et al., 2006; Pestojamasp et al., 2006). RhoA dependent cell contraction is necessary for retraction of the trailing cell body in migrating neutrophils (Eddy et al., 2000; Yoshinaga-Ohara et al., 2002; Niggli, 2003a) and monocytes (Worthylake et al., 2001). Although RhoA dependent contractility is necessary for detachment of the tail, it also strengthens integrin based adhesion in neutrophils (Laudanna et al., 1996), lymphocytes (Soede et al., 2001; Giagulli et al., 2004) and fibroblasts (Chrzanowska-Wodnicka and Burridge, 1996).

Maintaining traction for efficient cell migration depends on coordination between adhesion strengthening, protrusion at the leading edge, contraction of the cell body, and de-adhesion in the tail (Lauffenburger and Horwitz, 1996, Schwartz and Horwitz, 2006), and requires cyclical regulation of integrins and their association with the contractile F-actin cytoskeleton.

Observations by Gupton and Waterman-Storer (2006) suggest that an optimal balance between integrin based cell-adhesion and intracellular contractility, is necessary for optimal migration on two-dimensional surfaces.

4 Gout

4.1 Overview and history

Gout was first identified by the ancient Egyptians in 2640 BC. Historically, it was known as "the disease of kings" or "rich man's disease", since dietary risk factors such as meat and alcohol consumption could only be afforded by the affluent (Nuki and Simkin, 2006). Because gout was disproportionately represented among the affluent, it has an interesting and colourful recorded history (Bhattacharjee, 2009).

Gout affects 1-2% of people in the west at some point in their lives, and is caused by deposition of monosodium urate (MSU) crystals in and around joints and tendons, leading to self-resolving bouts of inflammation. Due to evolutionary loss of uricase, the enzyme that converts the purine derivative urate to allantoin, humans and the other hominids eliminate urate by excretion (Wu et al., 1992; Riches et al., 2009). Elevated levels of uric acid in the blood, known as hyperuricemia, can occur due to high intake or de novo synthesis of purines, but is most commonly due to under-
excretion of urate (Terkeltaub et al., 2006; Richette and Bardin, 2010). Inability of the kidneys to adequately excrete urate can be genetic (Unger et al., 2007), or it can occur as a secondary consequence of diuretic drug use or disorders that impair renal function. At saturating concentrations in the serum (>360 µmol/L), urate can crystallize with sodium and form deposits of MSU crystals in the tissue (Mandell, 2008). Although most individuals with hyperuricemia never develop gout, the incidence rate of gout increases exponentially with increasing serum uric acid concentration (Bhole et al., 2010; Campion et al., 1987). In addition to hyperuricemia, major risk factors for gout include genetic factors, old age, male sex, alcohol and meat consumption, obesity, hypertension, diuretic use, and paradoxically, rapid reduction in serum urate levels upon initiation of anti-gout therapy (Roddy and Doherty, 2010).

4.2 The inflammatory response in gout

Leukocytes are activated by MSU crystals through stimulation of the NALP3 inflammasome (Martinon et al., 2006; Martinon et al., 2010b). The inflammasomes are a family of intracellular multiprotein complexes that are organized around NOD-like receptor (NLR) family PRRs, and activate inflammatory caspases in response to PAMPs in the cytosol (Kufer et al., 2005). Stimulation of the NALP3 inflammasome results in activation of caspase-1. In turn, active caspase-1 generates mature IL-1β from its precursor, pro-IL-1β. The NALP3 inflammasome appears to specialize in recognition of large particulate insults, including alum, silica, asbestos, and calcium pyrophosphate crystals, in addition to MSU crystals (Dostert et al., 2008; Martinon et al., 2006). Signalling to the NALP3 inflammasome is thought to be triggered by crystal phagocytosis, and may involve lysis of the lysosomal membrane by phagocytosed crystals (Martinon, 2010a). The precise mechanism of activation of the NALP3 inflammasome is not known, however ROS production and reduced intracellular potassium concentration are necessary (Pétrilli et al., 2007; Martinon, 2010b). Also, the response to MSU crystals by the NALP3 inflammasome is enhanced by co-stimulation through TLR-4 (Giamarellos-Bourboulis et al., 2009).

Recent evidence indicates that uric acid released from dying cells can crystallize and act as an endogenous danger signal, triggering an innate immune response to infection or tissue injury (Shi et al., 2003). It is therefore not surprising that pathological accumulation of MSU crystal in the
joints also elicits an inflammatory response. The inflammatory response to MSU crystals in gout involves recognition and phagocytosis of crystals first by resident macrophages and then by neutrophils that have been recruited in the early inflammatory response (Martin et al., 2009). Exposure of resident macrophages to MSU crystals activates the NALP3 inflammasome, which catalyzes the release of inflammatory cytokines such as IL-1β, IL-18 and TNF-α (Martinon et al., 2006). This in turn leads to local activation of synoviocytes and endothelial cells, and recruitment of neutrophils from the circulation. Neutrophils that arrive at the site of inflammation also respond to MSU crystals by secreting inflammatory cytokines. Acute episodes of gout are generally self-limiting. Within a week after initiation, maturing monocytes begin to secrete the anti-inflammatory factor TGF-β, leading to resolution of inflammation. If untreated, chronic tophaceous gout can develop. Chronic gout is characterized by multiple deposits of MSU crystals in the form of tophi, associated low-level inflammation of the joints, and significant bone destruction (Richette and Bardin, 2010).

4.3 Colchicine and gout therapy

Colchicine, an alkaloid extract of the meadow saffron *colchicum,* has been used to treat gout for more than 1500 years (Nuki, 2008), and is effective for prophylaxis and treatment of acute gout. More recently, colchicine has also been used to treat familial Mediterranean fever (FMF), Behçet's disease, and a number of other inflammatory disorders (Ben-Chetrit and Levy, 1998a; Niel and Scherrmann, 2006; McCarty, 2008). One regular 0.6 mg daily dose or 2-4 daily doses of 0.5 mg are recommended for prophylaxis and treatment of acute gout flare, respectively. High doses of colchicine cause significant toxic side effects and can be lethal (Jayaprakash et al., 2007). A recent clinical trial indicated that, if administered early in the onset of acute gout flare, low-dose oral colchicine is just as effective as high dose colchicine, while circumventing its harmful side effects (Terkeltaub et al., 2010). In this study, the low dose of colchicine was 1.2 mg followed by 0.6 mg 1 hour later, and the high dose was 4.8 mg over a 7 hour period. Prophylactic effects of low dose colchicine have been substantiated in two randomized controlled clinical trials (Paulus et al., 1974; Borstad et al., 2004). Most of the colchicine that is absorbed in the intestine is either metabolized in the liver or cleared by biliary or renal excretion (Niel and Scherrmann, 2006). After a single 1 mg oral dose of colchicine, the average peak
plasma concentrations in healthy male volunteers was 13.8 µM, after 62 minutes, and dropped to less than 1 µM in the first 8 hours, as the colchicine was either cleared or absorbed by the tissue (Rochdi et al., 1994). With repeated daily doses of 1 mg/ml, steady state colchicine concentrations in the blood can range from 7.5 nM to 6 µM (Niel and Scherrmann, 2006). Tissue absorption of colchicine is highly variable, and depends on binding to tubulin and cellular efflux by the membrane pump, P-glycoprotein. Colchicine has been shown to accumulate at higher concentrations in neutrophils than other leukocyte subtypes (Chappey et al., 1993), possibly because they do not express the P-glycoprotein efflux pump (Ben-Chetrit and Levy, 1998b).

Colchicine inhibits MT polymer formation by binding to the tubulin heterodimer (Borisy and Taylor, 1967), and preventing it from adopting a straightened conformation that is necessary for incorporation into microtubules (Ravelli et al., 2004). Colchicine effectively depolymerizes the neutrophil MT cytoskeleton (Malawista and Bensch, 1967). Its therapeutic efficacy is assumed to stem from this ability to inhibit MTs, since several of its effects on leukocytes are blocked by the MT stabilizing agent, taxol (Molad, 2002).

Colchicine exhibits pleiotropic effects on macrophages, neutrophils, and endothelial cells at varying concentrations (Nuki, 2008); however the precise effects that are responsible for its therapeutic efficacy in gout are not known. It has been demonstrated that colchicine blocks neutrophil recruitment from the blood without affecting early MSU-induced TNF-α and IL-8 production in rabbit synovial joint fluid (Matsukawa et al., 1998), suggesting that neutrophils are the relevant therapeutic target of colchicine. Through depletion of neutrophils, it was demonstrated that an early phase of MSU-induced IL-1β production (2 hours), which was inhibited by colchicine, depended on neutrophils (Matsukawa et al., 1998). In vitro, colchicine blocks MSU crystal-induced surface expression of selectins (Cronstein et al., 1995) and cytokine secretion (McCarty, 2008) by neutrophils at nanomolar concentrations, while inhibition of cytokine secretion by macrophages occurs in the micromolar range (Martinon et al., 2006), supporting the model that low prophylactic dosages of colchicine act through inhibition of neutrophil responses. Colchicine blocks a number of relevant neutrophil responses in MSU crystal-induced inflammation, including upregulated surface expression of adhesion molecules (Cronstein et al., 1995), attachment (MacGregor, 1976; Fordham et al., 1981; Cronstein et al., 1995), motility (Phelps, 1969; Ehrenfeld et al., 1980), tyrosine phosphorylation (Roberge et al., 1993), ROS production (Roberge et al., 1996; Chia et al., 2008), and secretion of chemoattractant
factors such as crystal-derived chemotactic factor (CCF) (Phelps, 1970; Spilberg et al., 1979; McCarty, 2008), IL-1β (Roberge et al., 1994), and leukotriene B4 (LTB4) (Ouyang et al., 1989). An in vivo study demonstrated that doses of colchicine that block MSU-crystal induced ROS production by neutrophils (0.05 µmol/kg), are much lower than those required to block neutrophil infiltration into the peritoneal cavity (5 µmol/kg) (Chia et al., 2008). This suggests that multiple distinct pharmacological effects of low-dose colchicine might contribute to its therapeutic efficacy. In addition, colchicine inhibits neutrophil attachment to endothelial cells by modifying the surface distribution of adhesion molecules on endothelial cells (Cronstein et al., 1995). Microarray analysis indicated that high dose colchicine profoundly affects gene expression in HUVECs (Ben-Chetrit et al., 2006). After 12-24 hours, 0.1-1 µM colchicine suppressed expression of a cohort of genes involved in neutrophil recruitment and inflammation, however low dose colchicine (10 nM) had no effect. The authors suggest that colchicine might function through two distinct anti-inflammatory mechanisms; a slow (12-24 hours) effect at high concentrations, that functions through gene expression changes, and a fast (30-120 min) low dose effect, that occurs through direct physical effects on neutrophils and endothelial cells.

In modern times, new, highly effective treatments for gout have been developed (Dalbeth 2010; Terkeltaub, 2010). Non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids can be used to treat acute gout, while drugs that lower serum urate levels are commonly used to prevent repeat attacks. Allopurinol and febuxostat effectively reduce blood urate levels by inhibiting xanthine oxidase, the enzyme responsible for generating urate from its metabolic precursor, xanthine. Probenecid reduces blood urate levels by increasing uric acid excretion in the urine (Neogi, 2011). Recent clinical trials have shown efficacy of drugs that interfere with IL-1 signalling, such as Rilonacept, and the IL-1-receptor antagonist, anakinra, for gout therapy (Terkeltaub et al., 2009; Mcgonagle et al., 2007). These might be particularly useful when other approaches are contraindicated or too toxic. Although colchicine has been used to treat gout since antiquity, it has a relatively narrow therapeutic index, and high mortality associated with overdose. NSAIDs and corticosteroids are as effective as colchicine, and each has its own set of potentially adverse side effects and contraindications (Eggebeen, 2007; Terkeltaub, 2010). Due to the relatively narrow therapeutic index of colchicine and its toxic side effects, including gastrointestinal, hepatic, renal, neuromuscular, and hematopoietic complications, it is currently employed as a second-line therapy for gout.
5 Thesis overview

Lfc is an important regulator of cell morphology and migration, but its role has not been assessed in fast moving cells of the immune system. Through genetic ablation, we generated an Lfc knockout (Lfc\textsuperscript{-/-}) mouse model and performed \textit{in vivo} and \textit{in vitro} analysis of leukocytes. We found that Lfc is necessary for actomyosin based contractility of macrophages and neutrophils in response to MT depolymerization. Lfc was also found to be necessary for the anti-inflammatory effects of the MT-depolymerizing agent, colchicine, in two models of gout-like inflammation. We propose a model in which colchicine potentiates Lfc-mediated contractility in leukocytes or other cell types, which interferes with the inflammatory response to MSU crystals. In addition to its role downstream of MT depolymerization, we observed a defect in the innate immune response of Lfc\textsuperscript{-/-} mice compared to Lfc\textsuperscript{+/+} mice, using \textit{in vivo} models of inflammation. Lfc\textsuperscript{-/-} mice had decreased leukocyte crawling, and diapedesis \textit{in vivo}, decreased neutrophil infiltration in response to acute inflammation, and increased mortality in response to experimentally induced sepsis. \textit{In vitro}, a spreading and crawling defect of Lfc\textsuperscript{-/-} neutrophils was observed only under conditions of shear stress. These results are the first evidence of a role for Lfc in leukocyte migration, and specifically in the mechanosensory response to shear stress. Also, our discovery of the role of Lfc in the pharmacological effect of the anti-inflammatory drug, colchicine, has important implications with respect to its therapeutic mechanism of action.
Chapter 2

The Microtubule-Associated Guanine Nucleotide Exchange Factor, Lfc, is Necessary for the Anti-Inflammatory Effects of Colchicine in Murine Models of Gout

- The Lfc⁻/⁻ and Lfc⁺/+ mice were generated by Jose LaRose
- The experiment in Figure 2.11 was performed in collaboration with Eric Gracey
6 Abstract

In gout, resident macrophages respond to a buildup of monosodium urate (MSU) crystals in the synovial fluid, resulting in painful accumulation of neutrophils. The microtubule (MT)-depolymerizing agent colchicine has been used to treat gout since antiquity, yet the mechanism underlying its anti-inflammatory effects is still unknown. Lfc/GEF-H1 is a MT-associated Rho-specific, guanine nucleotide exchange factor (GEF) that mediates cross-talk between the MT and actin cytoskeletons in fibroblasts. Here we showed that Lfc orchestrates dramatic actomyosin based mechanocellular responses to MT depolymerization in macrophages and neutrophils, and was necessary for the anti-inflammatory efficacy of colchicine in two mouse models of gout-like inflammation.

We performed live imaging of primary bone marrow macrophages (BMMs) and neutrophils derived from an Lfc knockout (Lfc−/−) mouse. MT depolymerization induced robust contractile behaviour in wild type (Lfc+/+) leukocytes, which was absent in Lfc−/− leukocytes. The contractile responses of Lfc−/− leukocytes were accompanied by increased RhoA-GTP, myosin light chain (MLC) phosphorylation, and F-actin content, which were also absent in Lfc−/− leukocytes. We demonstrated that responses of Lfc−/+ leukocytes to MT depolymerization depended on Rho-associated kinase, myosin, and actin, by the use of specific inhibitors.

To test the possible role of Lfc in the therapeutic mechanism of action of colchicine in gout, we performed intraperitoneal and intra-articular injection of monosodium urate (MSU) crystals in Lfc+/+ and Lfc−/− mice. When colchicine was co-administered, MSU crystal-induced neutrophil infiltration to the peritoneal cavity and to the synovium was impaired in Lfc+/+ mice, but not in Lfc−/− mice. We suggest that, upon MT depolymerization, Lfc-dependent contractile activity, or some other Rho-dependent function, interferes with leukocyte responses to MSU crystals, which may account for the therapeutic effects of colchicine in gout.
7 Introduction

Gout is characterized by acute inflammatory episodes caused by build-up of monosodium urate (MSU) crystals in the joints and surrounding tissue. Recognition of MSU crystals by resident macrophages results in activation of the NALP3 inflammasome, and secretion of inflammatory cytokines such as IL-1β and TNF-α (Martinon et al., 2006; Martin et al., 2009). Subsequent local inflammation of the vasculature promotes recruitment of circulating neutrophils. Upon exposure to MSU crystals, neutrophils amplify the inflammatory response through secretion of a number of inflammatory mediators, including IL-1β, IL-8, leukotriene B4 (LTB4), and crystal-derived chemotactic factor (CCF) (Popa-Nita and Naccache, 2010).

Colchicine, an extract of the meadow saffron *colchicum*, is an anti-inflammatory agent that has been used to treat gout for centuries (Nuki, 2008). Colchicine blocks neutrophil infiltration to inflamed joints when administered prior to or during an acute episode of gout, and also shows therapeutic efficacy in familial Mediterranean fever (FMF), Behçet's disease, and a select group of other inflammatory conditions (Ben-Chetrit and Levy, 1998a; McCarty, 2008). The therapeutic potential of colchicine is thought to stem from its ability to inhibit microtubule (MT) polymerization (Molad, 2002; Nuki, 2008). Colchicine has pleiotropic effects on different cell types; however it is unclear which of these are responsible for its therapeutic potential in gout. Colchicine inhibits MSU-induced secretion of inflammatory cytokines by THP1 monocytes (Martinon et al., 2006), and blocks cytokine induced adhesiveness of endothelial cells by modifying surface expression of adhesion molecules (Cronstein et al., 1995). Colchicine also blocks a number of neutrophil functions, including attachment (MacGregor, 1976; Fordham et al., 1981; Cronstein et al., 1995), motility (Phelps, 1969; Ehrenfeld et al., 1980), phagocytosis (Dallaverde et al., 1982), secretion of chemoattractants (McCarty, 2008), superoxide production (Roberge et al., 1996; Chia et al., 2008), and tyrosine phosphorylation (Roberge et al., 1993).

Activation of the small GTPase RhoA and stimulation of actin stress fibers and cellular contractility have long been known to occur as a result of MT depolymerization in fibroblasts (Danowski, 1989; Enomoto, 1996). The tensegrity model has proposed that the MT-cytoskeleton acts to buttress the cell membrane, and in this way opposes pre-existing intracellular tension (Ingber, 1993; Wang et al., 2001). However, more recent results demonstrate that MT depolymerization drives actomyosin based cell contraction and stress fiber formation in
epithelial cells (Krendel et al., 2002; Chang et al., 2008), through release of the MT associated guanine nucleotide exchange factor (GEF), GEF-H1. Upon release from the MT array, GEF-H1, which we will refer to by its mouse homologue Lfc, activates RhoA by catalyzing its transition from the inactive GDP-bound form to the active GTP-bound form. In its GTP-bound form, RhoA activates a number of downstream effectors, including ROCK and mDia, which leads to phosphorylation of myosin light chain (MLC) and actin polymerization. In neutrophils, MT depolymerization promotes cell polarization and migration through potentiation of actomyosin based contractility (Dziezanowki et al., 1980; Keller et al., 1984; Keller and Niggli, 1993; Niggli, 2003b; Rossy et al., 2009). Functional polarization of neutrophils by MT-depolymerizing agents or chemokines is characterized by formation of an F-actin rich leading edge and a uropod at the posterior of the cell (Niggli, 2003b; Wong et al., 2006). Most of the tractionsal stresses that drive neutrophil migration are concentrated in the uropods (Smith et al., 2007), which are highly contractile and enriched for active RhoA (Pestonjamasp et al., 2006). Uropods are functional repositories of adhesion molecules (Alonso-Lebrero et al., 2000), and a number of protein components that localize to neutrophil uropods have been identified, including ezrin/radixin/moesin (ERM), P-selectin glycoprotein ligand1 (PSGL-1), and Flotilin1 and 2 (Rossy et al., 2009). Lfc has been shown to localize to uropods of polarized lymphocytes (Heasman et al., 2010).

The role of Lfc in the contractile response of fibroblasts to MT depolymerization prompted us to determine whether Lfc plays a similar role in other cell types. Using primary bone marrow macrophages (BMM) and neutrophils derived from Lfc knockout (Lfc^−/−) and wild type (Lfc^{+/+}) mice we tested cellular and biochemical responses to MT depolymerization. We observed that Lfc potentiates the contractile behaviour of leukocytes in response to MT depolymerization. Based on this observation and the fact that the MT-depolymerizing agent, colchicine, blocks neutrophil and macrophage functions in gout, we queried whether Lfc was required for the therapeutic actions of colchicine. Using peritoneal and intra-articular models of gout-like inflammation, we found that colchicine blocked MSU-induced neutrophil infiltration in Lfc^{+/+} mice, but not in Lfc^−/− mice. This provides genetic evidence that Lfc is necessary for the ability of colchicine to block MSU crystal-induced inflammation.
8 Methods

8.1 Lfc gene-targeted mice

Lfc<sup>+/+</sup> and Lfc<sup>-/-</sup> mice were generated by targeted gene replacement. A targeting construct was designed to insert a loxP site upstream of exon 2, and a loxP-flanked neomycin resistance cassette (in reverse orientation) downstream of exon 2 of the Lfc gene. Removal of exon 2 produces a frame-shift, ensuring that no functional Lfc transcript or protein will be produced. The construct was electroporated into the E14K embryonic stem cell (ES) line. Correctly targeted ES cells were injected into recipient blastocysts, which were implanted in the uterus of recipient foster mothers. The resulting chimeric mice were bred to C57BL/6 females to establish the colony. The Lfc flox mice were then bred with CMV-Cre mice. The resulting mice lacking both exon 2 and the floxed neo cassette were selectively bred to remove the CMV-Cre transgene. Heterozygous mice were backcrossed to C57BL/6 mice for at least 4 generations and then bred together to generate homozygous mice. Lfc flox mice that were not crossed with CMV-Cre mice were also backcrossed and used as wild type controls. Mice were maintained under specific pathogen-free conditions according to University Health Network animal care committee guidelines.

8.2 Cell preparation

To generate primary bone marrow macrophages (BMMs), whole bone marrow was flushed from femurs and tibias of 8-16 week old mice with Hanks balanced salt solution lacking Ca<sup>2+</sup>/Mg<sup>2+</sup> (Hanks<sup>-/-</sup>) + 0.1% BSA, and red blood cells (RBCs) were lysed with 0.155 M NH<sub>4</sub>Cl, 10 mM KHCO<sub>3</sub>, 0.1 mM EDTA. The remaining leukocytes were plated overnight on tissue culture treated plastic dishes in macrophage growth media. Macrophage growth media was α-MEM, supplemented with 10% (v/v) foetal bovine serum (FBS), and 10% (v/v) CMG-14-12 conditioned media. Cells were maintained at 37°C in a humidified incubator with 5% CO<sub>2</sub>. The following day, non-adherent cells were replated (1 x 10<sup>6</sup> cells/ml) and fresh media was added four days later. Sub-confluent macrophages were imaged or used in experiments the following day. To lift cells off the plate, cells were incubated with 10 mM EDTA for 15 minutes followed by vigorous pipetting.
Naive peritoneal macrophages were harvested from peritoneal cavities by 2 washes with 4 ml of Hanks-+. Cells were spun down at 300g for 10 minutes, washed once, and resuspended at 6 x 10^6 cells/ml in RPMI 1640 media containing 10% FBS and used immediately in experiments.

Neutrophils were isolated as described by Lowell et al. (1996). Leukocytes were isolated from bone marrow as above, washed twice with Hanks-+ and resuspended in 3 ml of a 45% Percoll (GE healthcare, Buckinghamshire, UK) solution in Hanks-+. The cell suspension was loaded on top of a Percoll density gradient prepared in a 15-ml polystyrene tube by gently layering 2 ml each of 62%, 55%, and 50% Percoll solutions on top of 3 ml of an 81% Percoll solution. Cells were then centrifuged at 2500 rpm for 30 minutes at room temperature. Cells at the interface between the 81% and 62% layer were harvested using a Pasteur pipette, and washed twice with Hanks-+ + 0.1% BSA. The isolated mature neutrophils were resuspended in Hanks balanced salt solution containing Ca^{2+}/Mg^{2+} (Hanks+/-) before each of the experiments unless otherwise indicated. Preparations were ~80-90% neutrophils based on analysis of the murine granulocyte marker Gr-1 (BD biosciences, NJ, USA) by flow cytometry.

### 8.3 Complete blood count (CBC)

For blood collection, mice were warmed next to a lamp for 5 minutes and restrained in a 50 ml conical tube. Mouse legs were shaved and the saphenous vein was punctured with a 25 gauge needle. One hundred µl of blood was collected in EDTA microvette tubes (Sarstedt, Nümbrecht, Germany) by capillary action, and maintained on ice. A Hemavet 950FS (Drew Scientific, CT, USA) was used for automated analysis of fresh blood samples.

### 8.4 Live imaging

Time lapse live-cell imaging of macrophages and neutrophils was performed on an inverted microscope (Axiovert 200M; Zeiss, Stuttgart, Germany) equipped with a video camera (Coolsnap HQ; Roper Scientific, Ottobrunn, Germany) and a large environmental chamber maintained at 37°C and 5% CO₂. Metamorph software (Molecular Devices, CA, USA) was used for automated image acquisition.
BMMs (3 x 10^4) were plated in 4-well Labtek II chambered coverslips (Nunc, Roskilde, Denmark). The following day, cells were starved for 5 hours in macrophage starvation media (α-MEM, 0.2% BSA, 20mM Hepes) and imaged by phase contrast microscopy with a 10x objective lens (0.5 NA; Fluar, Nikon, Tokyo, Japan) or a 32x objective lens (0.4 NA; A-Plan, Zeiss) for 1 hour upon stimulation with macrophage growth media. Alternatively, BMMs were imaged every 5 seconds for 5 minutes in starvation media, and for an additional 10 minutes upon addition of nocodazole (Calbiochem, CA, USA) in pre-warmed starvation media to a final concentration of 10 µM. In some experiments, cells were monitored for up to 2 hours. The spread area of macrophages before and after treatment with nocodazole was measured using ImageJ (National Institutes of Health, MD, USA), and used to determine the percentage contraction from at least 10 cells in 3 independent experiments.

Macrophage chemotaxis was assessed using the μ-slide chemotaxis assay (Ibidi, Martinsried, Germany) as per the manufacturer's instructions. Peritoneal macrophages (3.6 x 10^4 cells) were plated in the viewing channel of μ-slides for 3 hours at 37°C. The channel was washed once with 15 µl of RPMI media containing 10% FBS, and the upper and lower reservoirs were filled with media. Fifteen µl of C5a (30 ng/ml) was added to the upper chamber and slides were imaged every hour for 10 hours in the environmental chamber. Macrophage tracks were generated using ImageJ with the manual tracking plug-in. Twenty to 50 randomly selected cells were tracked per experiment and three independent repeats were performed. At least 100 cells were tracked per condition. Cell paths were plotted and migration parameters were determined using the Chemotaxis and Migration tool (Ibidi). Moving cells were defined as those that migrated a minimum distance of 30 µm. Forward migrational index in the y-axis (yFMI) was defined as y-directional distance/accumulated distance. Directionality was defined as displacement/accumulated distance, and is a measure of straightness. A directionality of 1 represents straight motion. Linear chemotactic gradients in the μ-slide are stable for over 48 hours.

Freshly prepared mouse neutrophils were maintained on ice at 12.5 million/ml and diluted to a final concentration of 2.5 million/ml in warm Hanks+/+ containing 20 mM Hepes, immediately prior to use. Neutrophils were plated in Labtek II chambered coverslips, allowed to settle for 5 minutes, and imaged for 5 minutes by phase contrast microscopy with a 63x oil immersion objective lens (1.4 NA; Plan-ApoChromat, Nikon, Tokyo, Japan). Nocodazole in pre-warmed
media was added, to a final concentration of 10 µM, and cells were imaged for an additional 30 minutes.

For crawling assays, neutrophils were treated with DMSO or nocodazole (10 µM) for 30 minutes, or fMLP (10 µM) for 5 minutes, prior to time lapse imaging. Cells were sandwiched between a slide and a glass cover-slip and imaged at 1 minute intervals for 10 minutes with a 10x objective. Neutrophil tracks were generated using ImageJ with the manual tracking plug-in. Scatter-plots were generated and cell velocities were determined using the Chemotaxis and Migration tool (Ibidi). Moving cells were defined as those that migrated a minimum distance of 10 µm. The experiment was repeated three times and at least 100 cells were tracked per experiment.

8.5 RhoA GTPase activity assay

RhoA GTPase levels were assessed using the RhoA GLisa kit (Cytoskeleton, CO, USA) as per the manufacturer's instructions. BMMs (2 x 10^6) were starved in 6-well plates and treated with DMSO or 10 µM nocodazole for 2 minutes or 5 minutes. Cells were washed, lysed in 70µl of lysis buffer, cleared, snap frozen in liquid nitrogen, and stored at -70ºC. Total GTP bound RhoA was determined from cell lysates in duplicate. Mean values from three independent experiments are shown with SEM. Lysates were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and total protein and RhoA content were assessed by protein immunoblot analysis with GAPDH and RhoA antibodies, respectively. Equal protein loading was also confirmed with the Precision Red Advanced Protein Assay Reagent (Cytoskeleton)

8.6 Immunoblotting

BMMs (1 x 10^6) were plated in 6-cm-diameter dishes overnight. Cells were starved as above and pre-incubated with or without the Rho-associated kinase (ROCK) inhibitor Y-27632 (10 µM, Calbiochem) for 30 minutes, prior to treatment with DMSO or nocodazole (10 µM) for 2 minutes or 5 minutes at 37ºC. After treatment, cells were rinsed with ice-cold phosphate buffered saline (PBS) and lysed in 200 µl of SDS lysis buffer (50 mM Tris pH 6.8, 1% SDS, 1 mM
EDTA, 1mM Na$_3$VO$_4$, 1mM PMSF, and 10 mM DTT). Lysis buffer contained 1 Mini, EDTA-free protease inhibitor cocktail tablet (Roche, IN, USA) per 10 mls. Lysates were sonicated briefly, boiled, and supplemented with 6X Laemmli sample buffer (Laemmli, 1970). Lysates (50 µl) were resolved by SDS-PAGE, transferred to Immobilon-FL polyvinylidene fluoride (PVDF) membranes (Millipore, MA, USA), and membranes were blocked with TBST (50 mM Tris, 150 mM NaCl, 0.1% Tween) containing 5% milk powder, before probing with primary antibodies and fluorescently labeled secondary antibodies. For detection of pMLC, membranes were blocked in TBST containing 5% BSA, and incubated with primary antibody overnight at 4°C. Secondary antibodies were diluted in TBS containing 5% milk powder, 0.05% triton, and 0.02% SDS and incubated for 30 minutes in the dark at room temperature. After extensive washing in TBST, membranes were dipped in methanol and dried before imaging. Membranes were scanned and quantified using the Odyssey infrared imaging system and Odyssey software (Li-cor, NE, USA). Primary antibodies were as follows: sheep anti-Lfc, 1:500 (Exalpha Biologicals, MA, USA); rabbit anti-pMLC, 1:500 (Cat #3674, Cell Signaling, MA, USA); mouse anti-MLC, 1:4000 (Sigma-Aldrich, MO, USA); mouse anti-GAPDH, 1:26000 (Sigma-Aldrich). Secondary antibodies were diluted 1 in 15000 as follows: IRDye 800CW goat anti-mouse (Li-cor); IRDye 680LT goat anti-rabbit (Li-cor); Alexa Fluor 680 donkey anti-sheep (Molecular Probes, OR, USA).

Neutrophil immunoblots were performed essentially as described by V. Niggli (2003b). Neutrophils (2 x 10$^6$) were resuspended in 450 µl of Hanks$^{+/-}$ media and maintained in a 37°C water bath with periodic mixing. Some samples were pre-incubated with Y-27632 (10 µM) for 30 minutes, prior to treatment with DMSO, nocodazole (10 µM), fMLP (10 µM), or C5a (1 µg/ml), as described in the results section. An equal volume of a solution containing 20% (w/v) trichloroacetic acid (TCA), 40 mM NaF and 10 mM Na$_2$HPO$_4$ was added to stop reactions. Samples were incubated on ice for 20 minutes, centrifuged, and precipitates were washed once with 0.5 ml of 5% TCA and once with 0.5% TCA. Pellets were dissolved in 100 µl of Laemmli sample buffer and analyzed as above.
8.7  F-actin assay

Neutrophils were resuspended in Hanks\textsuperscript{+/+} and maintained in a 37°C water bath. Cells were either untreated or pre-treated with Y-27632 (10 µM) for 30 minutes, prior to stimulation with 10 µM nocodazole or vehicle control for different time intervals. Cells were fixed with 3.7% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained with Texas Red phalloidin (4 µl) (Molecular Probes). The geometric mean fluorescence intensity (MFI) of F-actin was determined by flow cytometry. All measurements were performed in triplicate and repeated in at least three independent experiments. The percentage increase in F-actin content was expressed relative to DMSO treated control cells.

8.8  Immunofluorescence and confocal imaging

BMMs (2 x 10\textsuperscript{5}) were plated in 6-well plates on glass cover slips. Cells were starved as above and treatment with DMSO or nocodazole (10 µM) for 30 minutes at 37°C. Cells were fixed for 20 minutes at room temperature in 3.7% paraformaldehyde, permeabilized for 5 minutes with 0.1% Triton X-100, blocked with 5% BSA and stained with Texas Red-X phalloidin (Molecular Probes) for 30 minutes at room temperature. Cover-slips were mounted in GelTol mounting media (Thermo Electron Corporation, OH, USA), and cells were imaged on an Olympus IX81 inverted confocal microscope with a 60x oil immersion objective lens (1.4 NA; PlanApo, Nikon), using FluoView software (Olympus, Tokyo, Japan). All images were acquired with constant microscope sensitivity settings.

Neutrophils were resuspended in Hanks\textsuperscript{+/+} and maintained in a 37°C water bath with periodic mixing. Cells were stimulated in suspension for 30 minutes with DMSO or 10 µM nocodazole, and fixed for 20 minutes with 3.7% paraformaldehyde. Cells were adhered to slides by cytopinning, permeabilized with 0.1% Triton X-100, blocked with 5% BSA, and incubated with a mouse anti-pMLC antibody (Cat #3675, Cell Signaling) at 4°C overnight. The next morning cells were incubated with an Oregon Green 488 conjugated anti-mouse IgG (Molecular probes) and stained with Texas Red-X phalloidin for 30 minutes at room temperature. After mounting, 10 random cells were imaged on an Olympus IX81 inverted confocal microscope with a 60x oil immersion objective lens (1.4 NA; PlanApo, Nikon) at 5x zoom, using FluoView software.
(Olympus, Tokyo, Japan). All images were acquired with constant microscope sensitivity settings. The percentage of cells that had F-actin caps and clear polarization of F-actin and pMLC signals was determined from three independent experiments.

8.9 Uropod assay

Neutrophils were resuspended in Hanks+/+ at 1 x 10⁶ cells/ml and incubated at 37°C in the presence or absence of inhibitors for 30 minutes as follows; ROCK inhibitor (Y-27632, 10 µM), myosin inhibitor (blebbistatin, 100 µM, Sigma-Aldrich), or F-actin inhibitor (latrunculin A, 10 µM, Alexis Corporation, CA, USA). Cells were then stimulated with nocodazole (10 µM) or colchicine (10 nM - 10 µM) for 30 minutes and fixed in 1% gluteraldehyde for 20 minutes. After washing in PBS, cells were mounted on a slide and 20 random fields of view were imaged at 40x magnification. Cells were scored for the presence or absence of uropods in three independent experiments.

8.10 Preparation of MSU crystals

MSU crystals were prepared essentially as described (Roberge et al., 1993). Briefly, 250 mg of uric acid was boiled in 45 mls of a 0.03 M solution of NaOH (pH 7.5). The solution was filtered and 1 ml of 5 M NaCl was added to accelerate crystal formation. The solution was incubated at RT with gentle stirring for 24 h or until a milky-white precipitate formed. Crystals were kept sterile, washed with ethanol and acetone, and dried at room temperature. MSU crystals were re-suspended in sterile PBS at 24 mg/ml, sonicated, and examined under bright field and polarized light microscopy. MSU crystals were between 5 and 20 µm in length, and displayed negative birefringence under polarized light microscopy. MSU crystal preparations were free of endotoxin as assessed by the E-toxate (Sigma-Aldrich) limulus assay (<0.052 EU/mg).
8.11 Experimental peritonitis

To test the role of Lfc in the anti-inflammatory response to colchicine in gout, we used a peritoneal model of MSU induced inflammation. Lfc\(^{+/+}\) and Lfc\(^{-/-}\) mice received an i.p. injection of PBS or colchicine (5 nmol/g) immediately prior to injection of a 3 mg/ml slurry of MSU (0.5 ml) or vehicle. Mice were sacrificed 5 hours after injection and peritoneal cavities were washed with 3 mls of cold Hanks\(^{+}\). Peritoneal cells were washed and absolute numbers of cells per ml of lavage fluid were determined by manual counting with a hemocytometer. Cells were stained with Gr-1-PE and analyzed by flow cytometry. Side scatter and Gr-1 positive fluorescence were used to gate neutrophils and monocytes as per Lagasse and Weissman (1996). Cell counts and percentages were used to calculate absolute recovery of neutrophils and monocytes per ml of lavage fluid.

8.12 Intra-articular injection and immunohistochemistry

Mice were anaesthetized with isoflurane (2\%) and administered a subcutaneous injection of meloxicam (2 mg/kg) for analgesia. Mice were given a subcutaneous injection of colchicine (5 nmol/g) or PBS and a 10 µl volume of MSU crystals (240 µg) was microinjected directly into the knee joint cavity with a microinjection pump (CMA/100 Microinjection Pump; Carnegie Medicin, Stockholm, Sweden). Contralateral knees were injected with endotoxin free PBS. After 6 hours, mice were sacrificed and knees were removed and fixed in 10% buffered formalin. Knees were decalcified, embedded in parafin wax, sectioned, and mounted, prior to immunohistological staining with the neutrophil specific antibody Ly-6G (BD Pharmingen, cat no. 551459) at a 1:600 dilution, for 1 hour at room temperature. Slides were imaged at 10x magnification and neutrophil infiltration into the synovium was determined using ImageJ software. RGB (Red-green-blue) images were split into their basic channels, and equal saturation thresholding was applied to the blue images to identify the area, in pixels, occupied by neutrophils in each histological section. This was performed because differences between background staining and neutrophil specific signal were clearly evident in this channel. The area occupied by neutrophils was divided by the approximate number of pixels per neutrophil to obtain an estimate of the number of neutrophils per histological section. Three to 4 mouse knees were examined per condition.
8.13 ROS assay

Neutrophil ROS production was assessed in the presence of luminol, a chemiluminescent substrate that releases energy in the form of light when it is oxidized by ROS. Neutrophils were resuspended in Hanks\textsuperscript{+/+} and 5 x 10\textsuperscript{5} cells were plated per well in a 96-well plate. Freshly prepared luminol (Sigma-Aldrich) was added to a final concentration of 100 µM and plates were incubated at 37°C for 30 minutes in the presence or absence of 10 µM colchicine. MSU (3 mg/ml) was added to each well in triplicate and luminescence was measured every 2 minutes using a DTX 880 multimode detector (Beckman Coulter, CA, USA). Samples were mixed by pipetting up and down with a multi-channel pipetter, prior to each measurement.

8.14 Statistical analysis

Averages from at least three independent experiments are presented as mean ± SEM unless otherwise noted. Student’s \( t \)-Test was used to determine the statistical significance of differences between groups. Statistical significance was defined as *\( P < 0.05 \).

9 Results

9.1 Lfc\textsuperscript{-/-} mice have normal blood cell counts

In order to confirm that Lfc protein was not expressed in Lfc\textsuperscript{-/-} mice, we performed immunoblot analysis of cells derived from the Lfc\textsuperscript{+/+} and Lfc\textsuperscript{-/-} mice. Probing BMM lysates with an anti-Lfc antibody confirmed the presence of an ~115 kDa band in Lfc\textsuperscript{+/+} lysates, which was absent in lysates of cells derived from the Lfc\textsuperscript{-/-} mouse (Fig. 2.1A). Although Lfc\textsuperscript{-/-} mice produced viable offspring, litter sizes were much smaller than those of Lfc\textsuperscript{+/+} mice. The Lfc\textsuperscript{-/-} mice appeared normal, however they were jumpier than Lfc\textsuperscript{+/+} control mice. A behavioural phenotype would be consistent with the role of Lfc in neurogenesis during embryonic mouse brain development (Gauthier-Fisher et al., 2009). We performed a complete blood count (CBC) test on Lfc\textsuperscript{+/+} and Lfc\textsuperscript{-/-} mice, to determine the relative abundance of the different blood cell types. We did not
Figure 2.1: Blood cell counts are normal in Lfc−/− mice. A) Lysates from Lfc+/+ and Lfc−/− BMMs were resolved by SDS-PAGE and probed with an anti-Lfc antibody. Total actin is used as a loading control. Numbers indicate molecular weights in kilodaltons. A complete blood count (CBC) test was performed on Lfc+/+ and Lfc−/− mice (B-F). Red blood cell (RBC) counts (B), hemoglobin (C), hematocrit (D), and WBC counts (E) are indicated. A differential analysis (F) indicates the percentages of several WBC subtypes, including neutrophils (neut), lymphocytes (lymph), monocytes (mono), eosinophils (eos), and basophils (baso). All values are expressed as mean ± SEM, from 8 Lfc+/+ and 8 Lfc−/− mice.
observe any difference in the mean hemoglobin concentration, hematocrit, or red and white blood cell counts from \( \text{Lfc}^{+/+} \) and \( \text{Lfc}^{-/-} \) mice (Fig. 2.1B-E). Similarly, there was no difference between \( \text{Lfc}^{+/+} \) and \( \text{Lfc}^{-/-} \) mice in the relative proportions of the major white blood cell subtypes, including neutrophils, lymphocytes, monocytes, eosinophils, and basophils (Fig. 2.1F). These results indicate that leukopoiesis is normal in \( \text{Lfc}^{-/-} \) mice.

### 9.2 Lfc is required for macrophage tail retraction

Since RhoA is necessary for tail retraction in leukocytes, we analyzed the morphology and migrational capacity of macrophages derived from \( \text{Lfc}^{+/+} \) and \( \text{Lfc}^{-/-} \) mice. To generate macrophages, bone marrow derived cells were maintained in tissue culture media containing colony stimulating factor-1 (CSF-1) for 6 days. \( \text{Lfc}^{-/-} \) BMMs displayed highly elongated cell bodies when compared to \( \text{Lfc}^{+/+} \) BMMs (Fig. 2.2A). Live cell imaging indicated that elongation of the cell body was due to a defect in retraction of the posterior end of \( \text{Lfc}^{-/-} \) macrophages as they crawled forward (Video 1 and 2). The mean length of \( \text{Lfc}^{-/-} \) BMMs was \( \approx 130 \, \mu\text{m} \), compared to \( \approx 80 \, \mu\text{m} \) for \( \text{Lfc}^{+/+} \) BMMs (\( P = 1 \times 10^{-4} \), Fig. 2.2B).

Due to the role of Lfc in macrophage tail retraction we tested migration of naive \( \text{Lfc}^{+/+} \) and \( \text{Lfc}^{-/-} \) peritoneal macrophages towards a gradient of the chemotactic complement fragment, C5a (Fig. 2.3). Macrophage migration was monitored over a 10 hour period. \( \text{Lfc}^{+/+} \) and \( \text{Lfc}^{-/-} \) macrophages were observed to migrate towards the chemotactic gradient (Video 3 and 4). Many of the migrating \( \text{Lfc}^{-/-} \) macrophages displayed long tail-like projections (Video 4), however this did not impede their migration during the period of analysis. There was no difference between \( \text{Lfc}^{+/+} \) and \( \text{Lfc}^{-/-} \) macrophages with respect to the mean percentage of migrating cells (Fig. 2.3B), displacement (Fig. 2.3C), migrational index in the direction of the chemotactic gradient (Fig. 2.3D), or directionality of motion (Fig. 2.3E).
Figure 2.2: Lfc<sup>−/−</sup> BMMs have a tail retraction defect. A) Lfc<sup>+/+</sup> and Lfc<sup>−/−</sup> BMMs were imaged by phase contrast microscopy at 10x magnification. Bar is 50 µm B) Cell lengths were measured manually from phase contrast images. The experiment was performed three times and over 700 cells were measured in each condition. Mean cell length and 95% confidence intervals are shown. * indicates statistical significance. * indicates statistical significance.
Figure 2.3: Lfc is not required for macrophage chemotaxis. Peritoneal macrophages from Lfc\(^{+/+}\) and Lfc\(^{-/-}\) mice were plated on \(\mu\)-slides and imaged for 10 hours in the presence of a shallow gradient of C5a. A) Individual cell tracks from a representative experiment are shown. Tracks of cells that moved towards and away from the chemotactic gradient are shown in black and red, respectively. The average final position of all cells is indicated by \(\oplus\). Distance from the origin is indicated on the X- and Y-axes in \(\mu\)m. The direction of the chemotactic gradient is indicated. The mean percentage of moving cells (B), displacement (C), forward migrational index (D), and directionality (E) are shown \(\pm\) SEM from three independent experiments. More than 100 cells were tracked per condition.

9.3 Lfc is necessary for nocodazole-induced contraction of BMMs

Since Lfc is activated by MT depolymerization in fibroblasts, we assessed the cellular response of Lfc\(^{+/+}\) and Lfc\(^{-/-}\) BMMs to the MT-depolymerizing agent nocodazole. The response of BMMs to treatment with nocodazole was monitored by time-lapse video-microscopy. Immediately upon stimulation with 10 \(\mu\)M nocodazole, Lfc\(^{+/+}\) BMMs underwent a robust contractile response (Fig. 2.4A; Video 5). Within 2 minutes, the cells had begun to pull away from the substrate, and by 5 minutes had rounded up and were refractile to light by phase contrast microscopy. After 10 minutes, all of the Lfc\(^{+/+}\) BMMs had rounded up, displaying an average of less than 40\% of their original spread area (Fig. 2.4C). Lfc\(^{-/-}\) BMMs failed to contract in response to nocodazole (Fig. 2.4A-C; Video 6). On average, only 1.4\% of Lfc\(^{-/-}\) BMMs had rounded up after 30 minutes, compared to 99.9\% of Lfc\(^{+/+}\) BMMs \((P < 1 \times 10^{-4};\) Fig. 2.4B). In one experiment only two out of 72 Lfc\(^{-/-}\) BMMs had rounded up 2 hours after exposure to the MT-depolymerizing agent.

Next we analyzed the effect of nocodazole on the F-actin cytoskeleton of BMMs. Lfc\(^{+/+}\) and Lfc\(^{-/-}\) BMMs were fixed 30 minutes after treatment with nocodazole or DMSO, cells were fluorescently stained with phalloidin, and imaged by confocal microscopy (Fig. 2.4D). Lfc\(^{+/+}\) BMMs that had been treated with nocodazole had a highly contracted morphology, with F-actin staining concentrated at the cell cortex in the rounded up cell bodies, while Lfc\(^{-/-}\) BMMs looked similar to untreated controls with respect to cell morphology and F-actin staining. Both Lfc\(^{+/+}\) and Lfc\(^{-/-}\) BMMs showed intact filopodia formation, suggesting normal Cdc42 activity.
Figure 2.4: Lfc is necessary for BMM contractility upon microtubule depolymerization. A) Lfc$^{+/+}$ and Lfc$^{-/-}$ BMMs were treated with nocodazole and imaged by phase contrast time-lapse video-microscopy, at 32x magnification. Time after addition of nocodazole is indicated. Bar is 50 µm. B) Rounded and spread Lfc$^{+/+}$ and Lfc$^{-/-}$ BMMs were scored 30 minutes after treatment with nocodazole. The mean percentage of rounded cells was determined in three independent experiments. More than 200 cells were scored in each condition. Mean percentage of rounded cells ± SEM are shown for Lfc$^{+/+}$ and Lfc$^{-/-}$ BMMs. A p-value was determined using Student’s t-Test. * indicates statistical significance. C) The spread area of Lfc$^{+/+}$ and Lfc$^{-/-}$ BMMs was determined at time zero and 10 minutes after treatment with nocodazole. The mean percentage contraction was determined in three independent experiments. A total of 30 cells were measured in each condition. Mean percentage contraction ± SEM are shown for Lfc$^{+/+}$ and Lfc$^{-/-}$ BMMs. A p-value was determined using Student’s t-Test. * indicates statistical significance. D) Lfc$^{+/+}$ and Lfc$^{-/-}$ BMMs were treated with DMSO or nocodazole for 30 minutes, fixed and stained for F-actin. BMMs were imaged by confocal fluorescence microscopy at 60x magnification. Bar is 50 µm.

9.4 Lfc is necessary for nocodazole-induced RhoA activation and MLC phosphorylation in BMMs

Lfc$^{+/+}$ and Lfc$^{-/-}$ BMMs were treated with nocodazole for 2 or 5 minutes, or DMSO for 2 minutes, and total RhoA-GTP levels were assessed (Fig. 2.5A). At both time points nocodazole treatment stimulated a significant increase in total RhoA-GTP levels, relative to DMSO treated controls, in Lfc$^{+/+}$ but not in Lfc$^{-/-}$ BMMs (Fig. 2.5A). We next examined the levels of MLC phosphorylation in Lfc$^{+/+}$ and Lfc$^{-/-}$ BMMs following nocodazole treatment by western blot (Fig. 2.5B). pMLC levels, normalized to total MLC protein, were significantly higher in Lfc$^{+/+}$ BMMs at time points 2 and 5 minutes after treatment with nocodazole, compared to DMSO treated controls. In distinction, nocodazole-induced MLC phosphorylation was absent in Lfc$^{-/-}$ BMMs. The defects in RhoA-GTP and pMLC in Lfc$^{-/-}$ BMMs, were statistically significant after 2 ($P = 0.017$) and 5 minutes ($P = 0.004$) of stimulation with nocodazole. In order to determine whether MLC phosphorylation was dependent on ROCK activation Lfc$^{+/+}$ BMMs were pre-treated with the ROCK inhibitor Y-27632 prior to exposure to nocodazole. Y-27632 effectively blocked nocodazole-induced MLC phosphorylation in Lfc$^{+/+}$ BMMs (Fig. 2.5B).
Figure 2.5: Lfc drives RhoA activation and MLC phosphorylation in BMMs in response to microtubule depolymerization. A) Lfc<sup>+/+</sup> and Lfc<sup>-/-</sup> macrophages were treated with DMSO or nocodazole for the indicated times and RhoA activity was measured in duplicate from lysates using a colorimetric ELISA-based assay. Constitutively active RhoA was used as a positive control (c). Lysates were resolved by SDS-PAGE and equal protein loading and total RhoA levels were confirmed by immunoblotting with an anti-GAPDH antibody, and an anti-RhoA antibody, respectively. Mean absorbance at 490 nm from three independent experiments is shown with SEM. p-values were determined using Student’s t-Test. B) Lfc<sup>+/+</sup> and Lfc<sup>-/-</sup> BMMs were either untreated or pre-treated with Y-27632 (10 µM) for 30 minutes, prior to stimulation with DMSO or nocodazole for the indicated times. Cell lysates were resolved by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies. The mean ratio of pMLC to total MLC from three independent experiments is shown with SEM. p-values were determined using Student’s t-Test. * indicates statistical significance.

9.5 Lfc is necessary for neutrophil contractility and uropod formation in response to nocodazole

To determine the effect of nocodazole on neutrophil morphology, we performed time-lapse video-microscopy on primary bone marrow derived neutrophils. A dramatic contractile response was readily evident in Lfc<sup>+/+</sup> neutrophils in suspension, but completely absent in Lfc<sup>-/-</sup> neutrophils, upon addition of nocodazole. By 2 minutes of treatment with nocodazole, Lfc<sup>+/+</sup> neutrophils had undergone a subtle morphological transformation characterized by the appearance of small deformations of the membrane cortex (Fig. 2.6A; Video 7). After 3 minutes of treatment with nocodazole, Lfc<sup>+/+</sup> neutrophils exhibited membrane blebbing and increased morphological activity (Fig. 2.6A; Video 7), which culminated in polarized neutrophils with highly dynamic uropods (Fig. 2.6B; Video 8). In distinction, nocodazole-treated Lfc<sup>-/-</sup> neutrophils lacked signs of the morphological or contractile phenomena observed in Lfc<sup>+/+</sup> neutrophils (Video 9 and 10). After 30 minutes of stimulation with nocodazole approximately 30% of Lfc<sup>+/+</sup> neutrophils had uropods, compared to ~3% of DMSO treated controls. The percentage of Lfc<sup>-/-</sup> neutrophils that had uropods after nocodazole treatment was similar to DMSO treated controls (Fig. 2.6C). Nocodazole-induced uropod formation was completely blocked by inhibition of ROCK (Y-27632), myosin (blebbistatin), or F-actin (latrunculin A) (Fig. 2.6C), suggesting that MT depolymerization-induced uropod formation depends on Lfc-induced ROCK activity and actomyosin contractility. Treatment with the MT-depolymerizing agent, colchicine, also induced uropod formation in ~30-38% of Lfc<sup>+/+</sup> neutrophils, but not in Lfc<sup>-/-</sup>
neutrophils (Fig. 2.6D). Concentrations of colchicine ranging from 100 nM to 10 µM effectively stimulated uropod formation in Lfc+/+ neutrophils.

To assess the role of pMLC and F-actin in nocodazole-induced neutrophil polarization, we analyzed fixed and stained neutrophils by confocal microscopy. Naive Lfc+/+ and Lfc−/− neutrophils displayed a rounded morphology with F-actin and pMLC preferentially localized at the cortex. After stimulation with nocodazole, approximately 35% of Lfc+/+ neutrophils had formed uropods, and had strong cortical staining of pMLC and F-actin (Fig. 2.7A and B). F-actin caps and pMLC staining were polarized to opposite ends of the cell. Lfc−/− neutrophils did not form uropods or display polarization of pMLC and F-actin at the cortex after treatment with nocodazole. Since myosin and actin inhibitors blocked neutrophil polarization in response to nocodazole, we tested their effects on F-actin and pMLC levels by immunofluorescence. Total F-actin levels were significantly reduced in latrunculin A treated Lfc+/+ and Lfc−/− neutrophils (Fig. 2.7C), confirming that the inhibitor was effective. Twenty cells were imaged for each condition and no uropods were observed. Pre-treatment of neutrophils with myosin and actin inhibitors blocked polarization and F-actin cap formation, but failed to block accumulation of pMLC in Lfc+/+ neutrophils after nocodazole treatment (Fig. 2.7C). Thus F-actin and myosin are necessary to support neutrophil polarization in response to MT depolymerization.
Figure 2.6: Lfc is necessary for the neutrophil contractile response to microtubule depolymerization. A) Lfc^+/+ and Lfc^-/- neutrophils were treated with 10 µM nocodazole and imaged by phase contrast microscopy for 5 minutes in LabtekII chambered coverslips at 63x magnification. Membrane blebbing is indicated by arrowheads. Bar is 5 µm. B) Lfc^+/+ and Lfc^-/- neutrophils were imaged prior to, and 30 minutes after stimulation with 10 µM nocodazole by phase contrast microscopy at 63x magnification. Arrowheads indicate uropods. Bar is 5 µm. C) Lfc^+/+ and Lfc^-/- neutrophils were untreated or pre-treated for 30 minutes with Y-27632 (10 µM), blebbistatin (100 µM), or latrunculin A (10 µM), followed by an additional 30 minute incubation with DMSO, or nocodazole (10 µM). Cells were fixed and scored for presence or absence of uropods. D) Lfc^+/+ and Lfc^-/- neutrophils were treated with colchicine for 30 minutes at the indicated concentrations (µM), fixed, and scored for presence or absence of uropods. Percentages of cells with uropods were determined from at least 10 fields of view and the mean ± SEM from three independent experiments is shown. p-values were determined using Student’s t-Test. * indicates statistical significance.
Figure 2.7: Lfc is necessary for polar localization of pMLC and F-actin in nocodazole stimulated neutrophils. A) Lfc<sup>+</sup>/− and Lfc<sup>−/−</sup> neutrophils were treated with DMSO or nocodazole for 30 minutes, fixed, and stained for F-actin (red) and pMLC (green). Cells were imaged by confocal fluorescence microscopy at 60x magnification. Bar is 5 µm. B) The percentage of neutrophils with polarized F-actin and pMLC was determined from 10 randomly selected cells, and the mean ± SEM from three independent experiments is shown. A p-value was determined using Student’s t-Test. * indicates statistical significance. C) Lfc<sup>+</sup>/− and Lfc<sup>−/−</sup> neutrophils were pre-treated for 30 minutes with blebbistatin or latrunculin A prior to treatment with DMSO or nocodazole for 30 minutes, fixed, and stained for F-actin (red) and pMLC (green). Twenty cells were imaged for each condition tested, and no uropods were observed. Cells were imaged by confocal fluorescence microscopy at 60x magnification. Bar is 5 µm.

9.6 Lfc is necessary for nocodazole-induced neutrophil chemokinesis

We next determined the requirement for Lfc in MT depolymerization and fMLP-induced chemokinesis. Neutrophils were treated with DMSO, nocodazole, or fMLP, and random migration was monitored by time-lapse video microscopy (Fig. 2.8). Lfc<sup>+</sup>/− neutrophils stimulated with nocodazole showed an increase in the percentage of migrating cells, from 8.6% to 19.0% and a doubling of the mean velocity of moving cells from ~3 µm/min to ~6 µm/min (Fig. 2.8B and C). In contrast, Lfc<sup>−/−</sup> neutrophils failed to show an increase in the percentage of migrating cells or the mean velocity of moving cells compared to DMSO treated controls. Differences between the mean percentage movers and the mean velocity of nocodazole stimulated Lfc<sup>+</sup>/− and Lfc<sup>−/−</sup> neutrophils were significant, as determined by Student's t-Test. Lfc<sup>+</sup>/− and Lfc<sup>−/−</sup> neutrophils stimulated with fMLP showed an increase in the fraction of migrating cells, compared to DMSO treated controls, from 8.6% to 47.6%, and from 8.7% to 43.1%, respectively (Fig. 2.8B). The mean velocity of migrating fMLP stimulated Lfc<sup>+</sup>/− and Lfc<sup>−/−</sup> neutrophils increased from ~3 µm/min to ~7 µm/min (Fig. 2.8C). This suggests that Lfc is not required for fMLP-induced chemokinesis.
Figure 2.8: Lfc is necessary for random migration of neutrophils in response to microtubule depolymerization. A) Lfc\textsuperscript{+/-} and Lfc\textsuperscript{-/-} neutrophils were treated with DMSO or nocodazole for 30 minutes, or fMLP for 5 minutes, and random kinetic migration was monitored for 10 minutes by time-lapse video-microscopy. Motion tracks generated from a representative experiment are shown. Distance from the origin is indicated on X- and Y-axes in µm. B) The mean percentages of moving cells, defined as those that traveled a minimum accumulated distance of 10 µm, are indicated, with SEM from three independent experiments. At least 100 cells were tracked per experiment. A p-value was determined using Student’s t-Test. C) The mean velocities of moving cells are shown, with SEM from three independent experiments. At least 100 cells were tracked per experiment. A p-value was determined using Student’s t-Test.
9.7 Lfc is necessary for nocodazole-induced MLC phosphorylation in neutrophils

To determine if the downstream targets of Rho are activated by nocodazole in neutrophils and require Lfc, we examined the phosphorylation status of MLC, a substrate of the Rho-activated kinase, ROCK. Lfc<sup>+/+</sup> and Lfc<sup>-/-</sup> neutrophils were treated with nocodazole or DMSO, and MLC phosphorylation was assessed by western blot (Fig. 2.9A). After 1 minute of nocodazole stimulation, Lfc<sup>+/+</sup> neutrophils displayed a pronounced increase in pMLC levels, which peaked at 2.5 minutes and then decreased gradually over 30 minutes. The ROCK inhibitor, Y-27632, blocked nocodazole-induced MLC phosphorylation, confirming that MLC phosphorylation was ROCK dependent. In distinction to wild type neutrophils, nocodazole-induced MLC phosphorylation was absent in Lfc<sup>-/-</sup> neutrophils. Neutrophil stimulation with fMLP or C5a caused robust MLC phosphorylation in Lfc<sup>+/+</sup> and Lfc<sup>-/-</sup> neutrophils (Fig. 2.9B), suggesting that chemoattractant stimulated MLC phosphorylation occurs through a distinct pathway from nocodazole, that does not require Lfc.

9.8 Lfc is necessary for nocodazole-induced F-actin polymerization in neutrophils

Rho activity promotes actin cytoskeletal polymerization. We next determined whether the formation of total cellular filamentous actin (F-actin) was dependent on Lfc. Lfc<sup>+/+</sup> or Lfc<sup>-/-</sup> neutrophils were treated with DMSO or nocodazole, stained with Texas Red-conjugated phalloidin, and analyzed by flow cytometry (Fig. 2.9C). The F-actin content of Lfc<sup>+/+</sup> neutrophils increased by 33% at its peak, 2.5 minutes following nocodazole treatment, relative to DMSO controls. Increased F-actin content was significant by Student's t-Test 1, 2.5, 5, and 30 minutes after stimulation of Lfc<sup>+/+</sup> neutrophils with nocodazole. In contrast, there was little change in the F-actin content in Lfc<sup>-/-</sup> neutrophils after treatment with nocodazole. Pretreatment of Lfc<sup>+/+</sup> neutrophils with Y-27632 significantly reduced the nocodazole stimulated increase in F-actin content ($P = 2 \times 10^{-4}$), demonstrating a role for ROCK activation in F-actin formation.
Figure 2.9: Lfc is necessary for MLC phosphorylation and actin polymerization in neutrophils in response to microtubule depolymerization. A) Lfc<sup>+/+</sup> and Lfc<sup>−/−</sup> neutrophils were either untreated or pre-treated with Y-27632 (10 µM) for 30 minutes, prior to stimulation with DMSO or nocodazole for the indicated times. Cell lysates were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. B) Lfc<sup>+/+</sup> and Lfc<sup>−/−</sup> neutrophils were treated with...
fMLP (10 µM) for 1 minute, C5a (1 µg/ml) for 2 minutes, or DMSO as a control. Cell lysates were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. C) Lfc+/+ and Lfc−/− neutrophils were either untreated or pre-treated with Y-27632 (10 µM) for 30 minutes, prior to stimulation with DMSO or nocodazole for the indicated times. Cells were fixed and stained with Texas Red-phalloidin and total F-actin content was determined by flow cytometry. The geometric mean fluorescence of F-actin was determined in triplicate in at least four independent experiments and expressed as the mean percentage increase in F-actin relative to DMSO treated cells ± SEM. p-values were determined using Student’s t-Test. *, P < 0.02; **, P < 1 x 10⁻⁴.

9.9 Lfc is necessary for inhibition of neutrophil infiltration by colchicine in gout-like peritonitis

Neutrophils are the cellular agents responsible for pathogenesis in gout, a disease that is frequently treated with the microtubule inhibitor colchicine. We next addressed the question of whether a component of the pharmacologic activity of colchicine in treating gout is related to Lfc-dependent effects on leukocyte activity or function. Specifically, we sought to determine whether Lfc is responsible for the therapeutic efficacy of colchicine in gout. We used a peritoneal mouse model (Martin et al., 2009) to test the inflammatory response to monosodium urate (MSU) crystals, and the anti-inflammatory effects of colchicine in Lfc+/+ and Lfc−/− mice. A colloidal suspension of MSU crystals in PBS, or PBS alone, was injected into the peritoneal cavity of Lfc+/+ and Lfc−/− mice. Additionally, mice received a 50 µl injection of colchicine (5 µmol/kg) or PBS. Lavage fluid was harvested 5 hours later, the number of cells per volume of lavage fluid was determined, and the percentage of Gr-1 positive neutrophils and mononuclear cells was determined by flow cytometry (Fig. 2.10A). On average, 0.5 million neutrophils were recovered from Lfc+/+ mice per ml of peritoneal lavage fluid (Fig. 2.10B). This yield was comparable to published results (Martin et al., 2009). A ~51% reduction in the mean peritoneal yield of neutrophils was observed in Lfc−/− mice in response to MSU crystals, compared to Lfc+/+ mice (P = 3 x 10⁻⁴). These data demonstrate that Lfc is required for optimal recruitment in response to urate mediated peritonitis. These differences could not be ascribed to defects in leukopoiesis as the proportion of blood neutrophil was similar in wild type and Lfc deficient mice (Fig. 2.1F). Treatment with colchicine effectively blocked wild type neutrophil infiltration into the peritoneal cavity (P = 1 x 10⁻⁷), while the migratory behavior of Lfc deficient neutrophils was unaffected by colchicine treatment (P = 0.94, Fig. 2.10B).
Figure 2.10: Colchicine blocks neutrophil infiltration to the peritoneal cavity in Lfc<sup>+/+</sup> but not Lfc<sup>-/-</sup> mice. A) Lfc<sup>+/+</sup> and Lfc<sup>-/-</sup> mice received a peritoneal injection of PBS or colchicine, followed immediately by an injection of MSU crystals. A representative flow cytometric analysis of peritoneal cell recovery 5 hours later is shown. Side scatter (SSC) and Gr-1 staining were used to identify neutrophil (large rectangle) and mononuclear cell (small rectangle) populations. Numbers indicate the percentages of each population. Absolute numbers of neutrophils (B) and mononuclear cells (C) recovered per ml of peritoneal lavage fluid are shown for individual mice. Yields are indicated as mean ± SEM. * indicates statistical significance.

Recovery of mononuclear cells was comparable for wild type and Lfc deficient mice 5 hours after intraperitoneal injection of MSU crystals (Fig. 2.10C). Colchicine inhibition of mononuclear cell infiltration was significant for Lfc<sup>+/+</sup> (P = 4 x 10<sup>-4</sup>) and Lfc<sup>-/-</sup> (P = 0.011) mice. These results demonstrate that, while Lfc is required for optimal neutrophil migration into the peritoneum, it is also required for the inhibitory effect of colchicine on neutrophil recruitment in urate induced peritonitis.

9.10 Lfc is necessary for inhibition of neutrophil infiltration by colchicine in an intra-articular model of gout

To more closely mirror gout in the mouse, we performed intra-articular injection of MSU crystals into the synovial tissues of Lfc<sup>+/+</sup> and Lfc<sup>-/-</sup> mice in the presence or absence of colchicine. Immunohistochemical staining with the marker Ly-6G was used to identify neutrophils that had infiltrated the synovium 6 hours after MSU or PBS injection into the knee joints (Fig. 2.11A). Neutrophil infiltration was assessed by automated scoring of 3-4 knees for each condition (Fig. 2.11B). MSU-induced synovial accumulation of neutrophils was greater in the Lfc<sup>+/+</sup> mice than in Lfc<sup>-/-</sup> mice, consistent with an impaired migratory behaviour of Lfc deficient neutrophils in response to MSU-mediated inflammatory stimuli. Nevertheless, while colchicine blocked wild type neutrophil accumulation in the synovium, on average it did not block neutrophil accumulation in Lfc<sup>-/-</sup> mice. These results confirm that Lfc is necessary for inhibition of neutrophil infiltration by colchicine in models of MSU-induced gout-like inflammation.
**Figure 2.11: Colchicine blocks neutrophil infiltration to MSU-injected knee joints in Lfc<sup>+/−</sup> but not Lfc<sup>−/−</sup> mice.** A) Colchicine and vehicle treated mice underwent intra-articular injection with 240 µg of MSU crystals or PBS. Histological sections of PBS- and MSU-injected femorotibial joints from Lfc<sup>+/−</sup> and Lfc<sup>−/−</sup> mice were immunostained with Ly-6G antibody. The synovium was imaged by bright field microscopy at 10x or 20x magnification. Representative 20x magnification images are shown. Bar is 200 µm. B) Neutrophil infiltration into the synovium was determined using ImageJ software. Three to 4 immunostained histological sections were scored for each condition tested. Neutrophil counts for individual knees are shown. Mean neutrophil counts ± SEM are indicated for each condition tested. *p*-values were determined using Student’s *t*-Test. * indicates statistical significance.

### 9.11 Lfc negatively regulates MSU crystal-induced ROS production in neutrophils

Upon phagocytosis of MSU-crystals, neutrophils secrete harmful ROS that contributes to disease severity in gout. This MSU-induced response is dampened by colchicine (Chia et al., 2008). Since Lfc was necessary for inhibition of MSU-induced neutrophil infiltration by colchicine, we tested whether Lfc was also necessary for the effect of colchicine on MSU-induced ROS. Wild type or Lfc deficient neutrophils were treated with a colloidal suspension of MSU and monitored for ROS production using the chemiluminescent substrate, luminol. Peak ROS production in Lfc deficient neutrophils (~750 RLU) was 1.5 fold greater than in wild type neutrophils (~500 RLU) following exposure to MSU crystals (Fig. 2.12). These results provide genetic evidence that Lfc limits ROS production in neutrophils.

Pre-treatment of neutrophils with colchicine effectively suppressed MSU-induced ROS production in Lfc<sup>+/−</sup> and Lfc<sup>−/−</sup> neutrophils. However, colchicine suppressed ROS production by Lfc<sup>−/−</sup> neutrophils to levels that were comparable to those achieved by MSU stimulated Lfc<sup>+/−</sup> neutrophils in the absence of colchicine.
Figure 2.12: Colchicine blocks ROS production in response to MSU in Lfc<sup>+/+</sup> and Lfc<sup>−/−</sup> neutrophils. Lfc<sup>+/+</sup> and Lfc<sup>−/−</sup> neutrophils were mixed with luminol and plated in triplicate in 96-well plates. Neutrophils were pre-incubated with colchicine or vehicle for 30 minutes and stimulated with MSU crystals, and the luminescent reaction was measured over time. Mean relative light unit (RLU) measurements from a representative experiment are shown. Error bars indicate standard deviation of the mean.

10 Discussion

By comparing leukocytes derived from Lfc<sup>+/+</sup> and Lfc<sup>−/−</sup> mice, we have demonstrated that the robust contractile response of BMMs and neutrophils to MT depolymerization is Lfc-dependent. In vivo observations indicated that Lfc is also essential for the inhibition of MSU-induced neutrophil recruitment by the MT-depolymerizing agent colchicine. While it has previously been assumed that colchicine is effective because the intact MT-cytoskeleton is indispensable for the leukocyte response to MSU crystals, our results suggest that Lfc activation, rather than dissolution of the MT-cytoskeleton per se, accounts for some of colchicine's anti-inflammatory effects. The role of Lfc in the anti-inflammatory effects of colchicine is likely to be related to the induction of cellular contractility by Lfc upon MT-depolymerization. Further studies will be necessary to determine which cell types and cellular functions are critically inhibited by Lfc-dependent effects of colchicine in gout therapy.
To address the role of Lfc in the contractile response of leukocytes to MT depolymerization, we cultivated BMMs from Lfc<sup>+/+</sup> and Lfc<sup>-/-</sup> mice. A tail retraction defect, characterized by long string-like projections that extended from the posterior end of crawling Lfc<sup>-/-</sup> BMMs, was readily apparent by phase contrast imaging and live-cell video-microscopy (Fig. 2.2A and B; Video 1 and 2). The role of Lfc in tail retraction in cultured BMMs suggests that Lfc promotes intracellular tension and connectivity, and is consistent with the known role of RhoA and ROCK in leukocyte tail retraction (Worthylake et al., 2001; Yoshinaga-Ohara et al., 2002; Pestonjamasp et al., 2006). Despite the tail retraction defect of Lfc<sup>-/-</sup> macrophages, migration of Lfc<sup>-/-</sup> macrophages towards a chemotactic gradient was not defective over a 10 hour period, compared to Lfc<sup>+/+</sup> macrophages (Fig. 2.3; Video 3 and 4). Therefore, Lfc is not necessary for directional migration of macrophages.

Based on the role of Lfc in fibroblast contractility in response to MT depolymerization (Krendel et al., 2002), we tested the effects of nocodazole on our BMMs. We found that BMMs demonstrate Lfc-dependent contraction in response to MT depolymerization (Fig. 2.4A-C; Video 5 and 6). Robust contraction and rounding-up of Lfc<sup>+/+</sup> BMMs occurred within minutes of treatment with nocodazole, while Lfc<sup>-/-</sup> BMMs had no signs of a contractile response upon MT depolymerization and maintained their spread morphology. We also observed acute increases in RhoA-GTP and pMLC levels in nocodazole stimulated Lfc<sup>+/+</sup> BMMs, on a time-scale that coincided with contractile events (Fig. 2.5A and B). These biochemical changes were absent in Lfc<sup>-/-</sup> BMMs. Furthermore, nocodazole-induced pMLC could be blocked in Lfc<sup>+/+</sup> BMMs with the ROCK inhibitor, Y-27632. Through fluorescent staining with phalloidin, we observed a dramatic restructuring of the F-actin cytoskeleton in Lfc<sup>+/+</sup> BMMs that had been treated with nocodazole (Fig. 2.4D). F-actin had become highly concentrated at the cortex in the rounded up cell bodies of these cells, while F-actin staining in Lfc<sup>-/-</sup> BMMs was unaffected by MT depolymerization. This indicates that changes in the actin cytoskeleton are characteristic of Lfc-dependent BMM contractility in response to MT depolymerization. To our knowledge, the effects of MT depolymerization on macrophages have not been tested before; however, our results are consistent with previous studies showing that RhoA promotes contraction and rounding up of monocytes and macrophages (Aepfelbacher et al., 1996; Allen et al., 1997). In contrast to fibroblasts, where RhoA-induced contractility results in actin stress-fiber formation and focal adhesion strengthening, the contractile response of macrophages and other cell types,
such as neurons (Kozma et al., 1997), causes cell rounding. This is likely due to the fact that macrophages form weak focal contacts that facilitate fast migration, while fibroblasts form strong focal adhesions (Hall, 1998).

In contrast to the contractile response in macrophages, MT depolymerization causes neutrophils to polarize and undergo random migration (Niggli, 2003b). Our results show that Lfc is necessary for the neutrophil contractile response to MT depolymerization, culminating in neutrophils with functionally polarized uropods. Two minutes after stimulation with nocodazole, Lfc<sup>+/+</sup> neutrophils began to show obvious morphological alteration at the cortex in the form of small uniform bumps, giving the cortex a rough appearance (Fig. 2.6A). A similar morphological feature has been described in fMLP and phorbol 12-myristate 13-acetate (PMA) stimulated neutrophils and correlated with accumulation of F-actin at the cortex (Roos et al., 1987; Watts et al., 1991; Fernández-Segura et al., 1995). By 3 minutes of stimulation with nocodazole, a subset of Lfc<sup>+/+</sup> neutrophils began to show membrane blebbing and displayed a heaving and shoving motion in suspension due to their contractile activity (Fig. 2.6A; Video 7). At later time points, neutrophil polarization, characterized by uropod formation, was apparent in Lfc<sup>+/+</sup> neutrophils. Thirty minutes after stimulation with nocodazole, ~30% of Lfc<sup>+/+</sup> neutrophils had uropods (Fig. 2.6B and C), and live imaging showed that uropods were highly contractile and dynamic, forming and dissipating continuously (Video 8). Lfc<sup>−/−</sup> neutrophils failed to display nocodazole-induced morphological and contractile phenomena, or uropod formation (Fig. 2.6A-C; Video 9 and 10). Nocodazole-induced uropod formation could be blocked with ROCK, myosin, and actin inhibitors (Fig. 2.6C). Consistent with the role of MT depolymerization in Lfc-dependent neutrophil contractility, colchicine also induced uropod formation in Lfc<sup>+/+</sup> neutrophils, but not in Lfc<sup>−/−</sup> neutrophils (Fig. 2.6D). Colchicine effectively stimulated uropod formation at concentrations as low as 100 nM. From confocal imaging of fluorescently labeled cells, Lfc<sup>+/+</sup> neutrophils, that had undergone nocodazole-induced polarization, also had polar localization of F-actin and pMLC to opposite ends of the cell (Fig. 2.7A and B), a phenomenon that has been previously described (Keller and Niggli, 1993). These results indicate that the neutrophil contractile response to MT depolymerization, which culminates in cell polarization, occurs through an Lfc-Rho-ROCK signalling axis, and requires actin and myosin.

Since neutrophils undergo random chemokinetic migration in response to MT depolymerization and fMLP stimulation, we tested this in Lfc<sup>+/+</sup> and Lfc<sup>−/−</sup> neutrophils. We observed a significant
increase in random migration of Lfc<sup>+/+</sup> neutrophils that were treated with nocodazole, relative to DMSO treated controls (Fig. 2.8A-C). This chemokinetic response to MT depolymerization was not observed in Lfc<sup>-/-</sup> neutrophils. Nocodazole-induced migration of Lfc<sup>+/+</sup>, but not Lfc<sup>-/-</sup> neutrophils, is consistent with our observation that Lfc<sup>-/-</sup> neutrophils lack the contractile behaviour and polarization that was typical of Lfc<sup>+/+</sup> neutrophils in response to the MT-depolymerizing agent. Neutrophil polarization and migration in response to MT depolymerization is similar to the response to fMLP in many respects, including activation of RhoA and its downstream targets: ROCK and myosin light chain (MLC). However, MT depolymerization-induced polarization and migration are not inhibited by pertussis toxin or wortmannin and are therefore independent of heterotrimeric G<sub>i</sub> proteins, and PI3K signalling, respectively (Niggli, 2003b). We found that Lfc<sup>+/+</sup> and Lfc<sup>-/-</sup> neutrophils had similar chemokinetic migration upon stimulation with fMLP, indicating that Lfc is not necessary for fMLP induced migration. This is consistent with the observation that the neutrophil migratory responses to MT depolymerization and fMLP rely on different signalling pathways (Niggli, 2003b). Furthermore, another RhoA specific GEF, PDZRhoGEF, has been shown to drive RhoA dependent contractility in uropods of fMLP stimulated neutrophils (Wong et al., 2007), indicating that a role for Lfc in this pathway might be redundant.

Consistent with the requirement for myosin and actin in the neutrophil contractile response, we observed nocodazole-induced increases in pMLC and F-actin in Lfc<sup>+/+</sup> neutrophils, which coincided with the morphological changes that we observed by live imaging (Fig. 2.9A and C). Both of these biochemical changes were absent in Lfc<sup>-/-</sup> neutrophils, and were sensitive to the Rock inhibitor Y-27632. A previous study by Keller et al. (1993) failed to detect an increase in polymerized F-actin in response to MT depolymerization in human neutrophils, although they did observe increased cytoskeleton-associated actin by analysis of Triton X-100 insoluble fractions. This discrepancy could be due to differences in experimental technique or differences between human and mouse neutrophils. Although MLC phosphorylation and F-actin polymerization peak 1 to 5 minutes after nocodazole stimulation, cell-polarization and chemokinesis occur after 20 to 30 minutes of membrane blebbing and cellular contortions. Chemokine stimulated neutrophil polarization is characterized by actomyosin dependent formation of detergent-resistant membrane raft domains that eventually fuse to form uropods (Seveau et al., 2001), with characteristic segregation of membrane-associated protein.
components. It is likely that nocodazole-induced Lfc-dependent contractility bypasses proximal signalling events downstream of chemokines and leads to fusion of membrane-raft domains, polarization, and uropod formation, through an analogous mechanism.

Normal MLC phosphorylation was observed in Lfc<sup>−/−</sup> neutrophils upon stimulation with fMLP or C5α (Fig. 2.9B). Although Lfc is known to be activated downstream of certain GPCRs in fibroblasts (Meiri et al., 2009; Birukova et al., 2006), our results suggest that in neutrophils, at least for fMLP and C5α, this is not the case. These results confirm that Lfc plays a unique role in the neutrophil contractile response downstream of MT depolymerization, but not as a result of chemokine receptor signalling.

Since Lfc plays an important role in leukocyte responses to MT depolymerization, we hypothesized a possible role for Lfc in the therapeutic application of the MT-depolymerizing agent colchicine, in gout. To test this model, we performed peritoneal injection of monosodium urate (MSU) crystals into Lfc<sup>+/+</sup> and Lfc<sup>−/−</sup> mice, with and without co-injection of colchicine. We observed a significant defect in neutrophil infiltration in Lfc<sup>−/−</sup> mice in response to MSU crystals, compared to Lfc<sup>+/+</sup> mice. This defect is discussed in more detail in chapter 3. When Lfc<sup>+/+</sup> mice received an injection of colchicine coincident with MSU crystals, neutrophil infiltration to the peritoneal cavity was effectively blocked (Fig. 2.10A and B). Although fewer neutrophils infiltrated the peritoneal cavity of Lfc<sup>−/−</sup> mice compared to Lfc<sup>+/+</sup> mice, colchicine did not cause a further reduction of MSU crystal-induced neutrophil infiltration in Lfc<sup>−/−</sup> mice. In contrast, colchicine blocked MSU crystal-induced peritoneal infiltration of monocytes in Lfc<sup>+/+</sup> and Lfc<sup>−/−</sup> mice (Fig. 2.10C).

Next we performed intra-articular injection of MSU crystals into knee joints of Lfc<sup>+/+</sup> and Lfc<sup>−/−</sup> mice. Lfc<sup>+/+</sup> mice that received a subcutaneous injection of colchicine showed greatly reduced infiltration of neutrophils to the synovium in response to MSU crystals (Fig. 2.11). Similarly to peritoneal injection, there was significantly less neutrophil infiltration in the synovium of Lfc<sup>−/−</sup> mice; however there was no further reduction in neutrophil infiltration when these mice were treated with colchicine. These results strongly support our hypothesis that Lfc is required for the anti-inflammatory activity of colchicine.

Production of ROS is an important component of neutrophil bactericidal activity (Borregaard, 2010). Treatment with colchicine dampens MSU-induced ROS production by neutrophils
Roberge et al., 1996; Chia et al., 2008), suggesting that one of colchicine's *in vivo* therapeutic actions is to suppress tissue damaging ROS production in neutrophils following crystal phagocytosis. We found that Lfc<sup>−/−</sup> neutrophils produced greater amounts of ROS in response to MSU than Lfc<sup>+/−</sup> neutrophils (Fig. 2.12). Colchicine caused similar decreases in MSU stimulated ROS production in Lfc<sup>+/−</sup> and Lfc<sup>−/−</sup> neutrophils, relative to ROS production in the absence of colchicine, indicating that the inhibitory effect of colchicine on ROS production does not depend on Lfc. However, since ROS production has been shown to be necessary for activation of the NALP3 inflammasome by MSU crystals (Dostert et al., 2008; Pétrilli et al., 2007; Martinon, 2010), it is possible that the elevated ROS production in Lfc<sup>−/−</sup> neutrophils, relative to Lfc<sup>+/+</sup> neutrophils, compensates for reduction of ROS by colchicine, facilitating an inflammatory response.

Although we did not explore the mechanism of elevated ROS production by Lfc<sup>−/−</sup> neutrophils, our result suggests that Lfc-dependent Rho activity might act to limit ROS production. One possible mechanism by which Rho activity might limit ROS production is through repression of Rac2. The Rho family small GTPase Rac2 is an essential component of NADPH oxidase (Bokoch 2005), the enzyme complex that is responsible for ROS production in phagocytes. Active Rho and Rac segregate to the uropod and leading edge of polarized neutrophils, respectively, in a mutually exclusive manner (Xu et al., 2003; Wong et al., 2006; Pestonjamasp et al., 2006), and Rac-mediated ROS production has been shown to inhibit Rho activity (Nimmual et al., 2003). Our results suggest a reciprocal pathway, whereby Lfc-dependent Rho activity might limit Rac-mediated ROS production.

We have shown that Lfc is necessary for inhibition of MSU-induced neutrophil recruitment by colchicine, however the precise mechanism by which Lfc contributes to this effect is not known (Fig. 2.13). The role of Lfc in the anti-inflammatory mechanism of action of colchicine is likely to be related to the induction of actomyosin-based contractility by Lfc in response to MT depolymerization. MT depolymerization promotes Lfc-dependent contractility of neutrophils and macrophages, and this might interfere with the normal physiological response of leukocytes to MSU crystals. There are a number of MSU crystal-induced responses that MT depolymerization-dependent Lfc-activity could potentially block. Colchicine-induced Lfc-dependent intracellular tension might interfere with neutrophil rolling and attachment on the inflamed vasculature, since cells with higher cortical tension, and hence decreased deformability, have a greater rolling
velocity and a decreased probability of attachment and transition to the spreading regime (Lei et al., 1999; Bose, 2010). Neutrophil responses to MT depolymerization, which are Lfc-dependent, mimic the effects of chemotactic peptide, and could constitute a form of pre-stimulation, that subsequently interferes with the normal response of neutrophils to MSU-crystal induced inflammation. Indeed, chemotactic activation of neutrophils prior to contact with endothelial cells greatly reduced selectin-dependent tethering under flow (Smith et al., 1991), and pre-activation of neutrophils with LPS inhibited firm-adhesion to activated endothelial monolayers in the presence of shear stress, despite increased surface expression of integrin (Ploppa et al., 2010). Alternatively, Lfc-dependent contractility might interfere with phagocytosis of MSU crystals, activation of the NALP3 inflammasome, or cytokine secretion by neutrophils (McCarty, 2008). Colchicine has also been shown to reduce surface expression of L-selectin on neutrophils and to modify surface expression of E-selectin on endothelial cells (Cronstein et al., 1995), phenomena that could occur as a result of Lfc-dependent contractile events. Finally, colchicine might interfere with crystal phagocytosis or crystal-induced cytokine secretion by resident synovial macrophages, by eliciting Lfc-dependent contraction in these cells. Future work will allow us to determine which of these functions is Lfc-dependent, and to help us identify the effects of colchicine that are responsible for its anti-inflammatory efficacy.

Although colchicine has a long history as a therapeutic agent for gout, and is thought to function through inhibition of the MT-cytoskeleton, its precise mechanism of action remains unknown. We have shown that the MT associated guanine nucleotide exchange factor (GEF), Lfc, is necessary for suppression of MSU-induced neutrophil recruitment by colchicine, and for leukocyte actomyosin-based contractile responses to MT depolymerization. These observations suggest novel implications for the anti-inflammatory mechanism of colchicine in gout.
Figure 2.13: Model for the mechanism of action of colchicine in gout. Colchicine blocks neutrophil recruitment in gout through an Lfc-dependent mechanism. Colchicine has pleiotropic effects on different cell types; however, which of these effects is through Lfc remains to be determined. A) Colchicine could block neutrophil recruitment in response to MSU crystals through Lfc-dependent effects on resident macrophages or endothelial cells. Lfc-dependent effects might interfere with crystal recognition, crystal phagocytosis, or secretion of cytokines such as IL-1β, by resident macrophages; or upregulation of adhesion molecules by endothelial cells. B) Lfc-dependent effects of colchicine could also interfere with neutrophil extravasation or...
neutrophil responses upon exposure to MSU-crystals at the site of inflammation. Lfc is not necessary for inhibition of MSU-induced ROS production by colchicine. C) In neutrophils, Lfc-induced contractility could potentially interfere with selectin, integrin, or GPCR mediated events at the endothelial surface, which include tethering, rolling, spreading, crawling, and diapedesis.
Chapter 3

Lfc Promotes Neutrophil Spreading and Crawling in Response to Shear Stress and is Critical for the Innate Immune Response to Infection in Mice

- The experiments in Figure 3.2 were performed by Dr. Björn Petri
- The experiment in Figure 3.3A was performed in collaboration with Dr. Jack Haitsma
- The experiments in Figure 3.3B-F were performed by Dr. Jack Haitsma
- The experiments in Figures 3.7 and 3.8 were performed in collaboration with Jacob Rullo
11 Abstract

Leukocyte emigration from the vasculature is a critical early step of the immune response to invading pathogens. In response to inflammatory signals, leukocytes roll, attach, and crawl on the surface of the vascular endothelium, prior to undergoing diapedesis. Through a mechanism that has not been fully elucidated, shear stress at the endothelial surface potentiates leukocyte emigration. RhoA is a member of a family of small GTPases that integrate spatial and temporal cues to regulate cell adhesion, polarity, and migration. RhoA controls cytoskeletal dynamics and actomyosin based contractility, and is known to participate in the response of leukocytes to shear stress. Guanine nucleotide exchange factors (GEFs), which activate the small GTPases, also play important roles in cell migration. We sought to determine the role of the RhoA specific GEF, Lfc, in neutrophil migration and extravasation in mice.

Utilizing an Lfc knockout (Lfc<sup>-/-</sup>) mouse model we observed deficient intravascular crawling and emigration of neutrophils compared to wild type (Lfc<sup>+/+</sup>) controls, by intravital-microscopy. We also observed defective neutrophil infiltration in response to thioglycolate and cecal ligation and puncture (CLP) induced peritonitis. Furthermore, Lfc<sup>-/-</sup> mice that underwent CLP had a higher mortality rate than Lfc<sup>+/+</sup> mice, indicating a defective innate immune response to infection. Primary neutrophils derived from the Lfc<sup>-/-</sup> mouse showed defective spreading and crawling in response to shear stress, suggesting a mechanosensory role of Lfc. The defective shear stress response of Lfc<sup>-/-</sup> neutrophils is likely to account for the defective in vivo leukocyte migration, reduced neutrophil infiltration in response to inflammatory insults, and increased mortality of Lfc<sup>-/-</sup> mice in response to polymicrobial infection.

12 Introduction

Neutrophils are a critical component of the innate immune system and the first responders to infection. Recruitment of neutrophils from the circulation depends initially on recruitment to the activated endothelial surface and the ability of neutrophils to crawl and undergo diapedesis, also known as transendothelial migration (TEM). These events depend on integrin engagement with ligands expressed on the endothelial surface and chemokine stimulation of G-protein coupled receptors (GPCRs). Furthermore, neutrophils and other leukocytes, being adapted to conditions
of blood flow, crawl and transmigrate optimally in the presence of shear stress (Alon and Dustin, 2007; Alon and Ley, 2008). Transmigration of neutrophils across a layer of activated human umbilical vein endothelial cells (HUVECs) is accelerated in a shear dependent manner, and correlates with the magnitude of shear (Kitayama et al., 2000). Shear stress also promotes neutrophil invagination into the apical endothelial interface (Cinamon et al., 2004). The mechanosensory mechanism by which shear stress promotes neutrophil migration is not well understood.

Cell crawling depends on propulsive, adhesive, and contractile forces, all of which are regulated by Rho family small GTPases such as RhoA, Rac1, and Cdc42 (Nobes and Hall, 1999; Zhelev et al., 2002). Rho GTPases are molecular switches that cycle between active GTP-bound and inactive GDP-bound states. They are activated by the guanine nucleotide exchange factors (GEFs), which catalyze the exchange of GDP for GTP. In fibroblasts, actin-based protrusion at the leading edge is driven by Rac1 and Cdc42 in the form of lamellipodia and filopodia, respectively. RhoA drives tail retraction through control of cellular contractility (Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996; Wojciak-Stothard et al., 2003), however RhoA dependent actomyosin contractility also contributes to adhesion strengthening through the mechanosensory function of integrins (Ridley and Hall, 1992; Nobes and Hall, 1995; Chrzanowska-Wodnicka and Burridge, 1996; Amano et al., 1997; Totsukawa et al., 2000; Matthews et al., 2006). Active RhoA localizes in the tail and at the leading edge of polarized fibroblasts (Kurokawa and Matsuda, 2005; Pertz et al., 2006) and lymphocytes (Heasman et al., 2010). Active RhoA concentrates at the sides and back of polarized HL-60 neutrophil-like cells, but also occurs transiently along the entire periphery in spread cells immediately prior to polarization (Wong et al., 2006). In neutrophils, RhoA is necessary for adhesion (Laudanna et al., 1996), polarization (Pestonjamasp et al., 2006), de-adhesion (Liu et al., 2002; Yoshinaga-Ohara et al., 2002; Cavnar et al., 2011), and migration (Alblas et al., 2001). GPCR signalling through RhoA induces integrin-dependent adhesion and actin assembly in neutrophils (Laudanna et al., 1996; Laudanna et al., 1998) and lymphocytes (Laudanna et al., 1996; Giagulli et al., 2004; Pasvolsky et al., 2008). Two downstream effectors of Rho, Rho-associated kinase (ROCK) and myosin II, are implicated in de-adhesion in the tail and adhesion at the leading edge, respectively, during neutrophil migration (Shin et al., 2010). Furthermore, RhoA is implicated in the mechanosensory response of neutrophils to shear stress (Makino et al., 2005).
A number of RhoA specific GEFs have been implicated in neutrophil migration, including LARG (Shi et al., 2009), PDZRhoGEF (Wong et al., 2007), and Lsc (Francis et al., 2006). GEF-H1, which we will refer to by its mouse homologue, Lfc, is a microtubule (MT) associated RhoA specific GEF. It has diverse roles in a variety of cellular phenomena, including regulation of tight junctions and paracellular permeability (Benais-Pont et al., 2003; Kakiashvili et al., 2009) and migration of fibroblast (Nalbant et al., 2009), epithelial cells (Tsapara et al., 2010), and T-cells (Heasman et al., 2010). Recently it has been demonstrated that Lfc regulates the mechanical response to force on integrins (Guilluy et al., 2011) and relocates to integrin based adhesion complexes in response to intracellular tension (Kuo et al., 2011) in fibroblasts.

Based on the role of Lfc in cell migration, we were interested in determining its function in the innate immune response. We performed in vivo analysis of neutrophil migration in wild type (Lfc<sup>+/+</sup>) and Lfc deficient (Lfc<sup>−/−</sup>) mice. We used intravital microscopy to observe in vivo neutrophil rolling, adhesion, and emigration across the endothelial lining of postcapillary venules in mouse cremaster preparations. We also tested infiltration of neutrophils to a local inflammatory site by experimentally induced acute peritonitis, and mortality in a mouse model of sepsis. Finally, we tested the neutrophil chemokinetic response in the presence or absence of shear stress.

13 Methods

13.1 Experimental peritonitis

To test neutrophil infiltration in response to a local inflammatory insult, 3% thioglycolate was administered by intraperitoneal injection (0.5 ml in Hanks<sup>−/−</sup>) into Lfc<sup>+/+</sup> and Lfc<sup>−/−</sup> mice (n ≥ 5). Mice were sacrificed 5 hours later and their peritoneal cavities were washed with 3 mls of cold Hanks<sup>−/−</sup>. Peritoneal cells were washed and absolute numbers of cells per ml of lavage fluid was determined by manual counting with a hemocytometer. Cells were stained with Gr-1-PE and analyzed by flow cytometry. Side scatter and Gr-1 positive fluorescence were used to gate neutrophils and monocytes as per Lagasse and Weissman (1996). Cell counts and percentages were used to calculate absolute recovery of neutrophils and monocytes per ml of lavage fluid.
13.2 Intravital microscopy

To monitor neutrophil behaviour \textit{in vivo} on the inflamed mouse vasculature, male Lfc\textsuperscript{+/+} and Lfc\textsuperscript{-/-} mice were anesthetized with a mixture of 200 mg/kg ketamine hydrochloride (Bimeda-MTC, Cambridge, ON, Canada) and 10 mg/kg xylazine (Bayer Inc., Toronto, ON, Canada), injected intraperitoneally. The right jugular vein was cannulated for administration of additional anesthetic. The cremaster muscle was used to study leukocyte responses upon stimulation as previously described (Liu et al., 2005; Phillipson et al., 2009). Briefly, an incision was made in the scrotal skin to expose the left cremaster muscle, which was exteriorized following dissection from associated tissues, and placed on a clear viewing pedestal. A longitudinal cut was made in the cremaster muscle, which was then held flat against the pedestal by securing silk sutures to the perimeter of the muscle. The muscle was initially superfused with bicarbonate-buffered saline (pH 7.0, 37°C), followed by superfusion with a 1 \(\mu\)M concentration of the synthetic formylated peptide fMLP (Sigma-Aldrich, MO, USA).

The preparation was visualized with an intravital microscope (Axiolskip; Zeiss, Stuttgart, Germany) connected to a video camera (5100 HS; Panasonic, Osaka, Japan) using 25x (0.35 NA; Fluotar, E. Leitz, Munich, Germany) and 40x (0.80 NA; Achronplan, Zeiss) objective lenses. The same five sections of single unbranched cremasteric venules (20-40 \(\mu\)m in diameter) were observed throughout the experiment. The images were recorded on a videocassette and rolling flux, rolling velocity, adhesion, emigration, and crawling of leukocytes were determined during video playback. Rolling leukocytes were defined as those cells moving at a velocity less than that of erythrocytes within a chosen vessel. The rolling flux was measured as the number of rolling leukocytes that pass through a 100 \(\mu\)m section of vessel per minute. The rolling velocity was calculated from the time required for a cell to roll along a 100 \(\mu\)m length of vessel and is expressed as \(\mu\)m per second. A cell was deemed adherent if it remained stationary for at least 30 seconds and total adhesion was quantified as the number of adherent cells within a 100 \(\mu\)m length of venule in 5 minutes. Leukocyte emigration constitutes the total number of cells observed in the extravascular space adjacent to the observed venule within the microscopic field of view. Previous studies have shown that more than 90% of emigrated cells are neutrophils (Cara et al., 2001; Thompson et al., 2001). The percentage of crawling cells was determined from the number of adherent cells that showed a clear crawling behaviour.
13.3 Microvascular permeability measurement

Microvascular permeability was quantified based on the degree of vascular albumin leakage from cremasteric venules of Lfc<sup>+/+</sup> and Lfc<sup>-/-</sup> mice, as described previously (Petri et al., 2011). Briefly, 25 mg/kg FITC-labeled BSA (Sigma-Aldrich) was administered to the mice intravenously 10 minutes before each experiment. The exposed cremaster muscle was superfused with 1 μM fMLP in warm (37°C) bicarbonate-buffered saline and FITC-derived fluorescence (excitation wavelength, 450-490 nm; emission wavelength, 520 nm) was detected using a spinning disk confocal fluorescent microscope (BX51WI, Olympus, Center Valey, PA) with a 20x/0.95 water XLUMPlan FL objective (Olympus). The microscope was equipped with a confocal light path (WaveFx, Quorum, Guelph, ON) based on a modified Yokogawa CSU-10 head (Yokogawa Electric Corporation, Tokyo, Japan). Image analysis software (ImageJ, 1.44; National Institutes of Health, MD, USA) was used to determine fluorescence intensity in the venule lumen and in the adjacent perivascular tissue. The index of vascular albumin leakage (permeability index) at different time points after fMLP superfusion was determined according to the following ratio expressed as a percentage: (mean interstitial intensity - background)/(venular intensity - background).

To determine the influence of neutrophil/endothelial interactions on microvascular permeability changes, mice were injected intraperitoneally with 150 μg of anti-Gr-1 antibody 24h before fMLP superfusion. This treatment has been shown previously to deplete more than 95% of mouse neutrophils (Bonder et al., 2004).

13.4 Cecal ligation and puncture

To test the neutrophil response to polymicrobial infection, Lfc<sup>+/+</sup> and Lfc<sup>-/-</sup> mice were anesthetized with 200 mg/kg ketamine and 10 mg/kg xylazine by intraperitoneal injection. Using aseptic technique, a ventral midline incision (~1 cm) was made and the cecum was exteriorized. The cecum was ligated 1 cm from the apex with 3–0 silk suture and penetrated through-and-through with a 22-gauge needle. The cecum was then returned to the stomach cavity, and the abdominal incision was closed in two layers with 4–0 nylon suture. Mice were monitored every 12 hours for 1 week. For sham surgeries, the cecum was exteriorized and manipulated but not
ligated or punctured. For peritoneal recovery, mice were sacrificed 12 hours after CLP or sham surgeries and the peritoneal cavities were washed twice with 3 mls of Hanks\(^{-}^{+}\). Peritoneal cells were washed and counted using a hemocytometer. Cells were stained with Gr-1-PE, CD11b-FITC, and annexin V-APC and analyzed by flow cytometry.

Determination of inflammatory cytokines in serum and bronchoalveolar lavage (BAL) fluid, cellularity in the lung, and bacterial load were determined 24 hours after CLP or sham surgery as previously described (Mei et al., 2010). Whole blood was collected by cardiac puncture and spun down, and plasma was stored at -80\(^{\circ}\)C for further analysis. BAL fluid was obtained by 3 washes with 1 ml of cold PBS. BAL was centrifuged at 4\(^{\circ}\)C for 10 minutes at 400 g and the supernatant was snap-frozen in liquid nitrogen and stored at -80\(^{\circ}\)C for further analysis. Cells were resuspended in 0.5 ml of PBS, counted on a hemocytometer, and normalized to volume of recovered BAL fluid. Cytokine levels in serum and BAL fluid were measured using a LiquiChip multiplex cytokine bead array kit (Qiagen, CA, USA), according to the manufacturer's instructions. To determine bacterial load, spleens were homogenized in 1 ml of sterile PBS and colony forming units (CFU) were determined by plating 10-fold serial dilutions on blood agar plates, which were incubated at 37\(^{\circ}\)C overnight.

### 13.5 Isolation of bone marrow neutrophils

Neutrophils were isolated from the femurs and tibias of 8-16 week old mice as described by Lowell et al. (1996). Bone marrow was flushed using Hanks\(^{-}^{+}\) + 0.1% BSA and red blood cells (RBCs) were lysed with 0.155 M NH\(_4\)Cl, 10 mM KHCO\(_3\), 0.1 mM EDTA. The remaining leukocytes were washed twice with Hanks\(^{-}^{+}\) and resuspended in 3 ml of a 45% Percoll (GE healthcare, Buckinghamshire, UK) solution in Hanks\(^{-}^{+}\). The cell suspension was loaded on top of a Percoll density gradient prepared in a 15-ml polystyrene tube by gently layering 2 ml each of 62, 55, and 50% Percoll solutions on top of 3 ml of an 81% Percoll solution. Cells were then centrifuged at 2500 rpm for 30 minutes at room temperature. Cells at the interface between the 81% and 62% layer were harvested using a Pasteur pipette, and washed twice with Hanks\(^{-}^{+}\) + 0.1% BSA. The isolated mature neutrophils were resuspended in Hanks balanced salt solution containing Ca\(^{2+}\)/Mg\(^{2+}\) (Hanks\(^{-}^{+/+}\)) before each of the experiments unless otherwise indicated.
Preps were ~80-90% neutrophils based on analysis of the murine granulocyte marker Gr-1 by flow cytometry.

13.6 ROS assay

To test production of ROS, neutrophils were resuspended in Hanks\(^{+/-}\) and 5 x 10\(^5\) cells were plated in triplicate in a 96-well plate. Freshly prepared luminol (Sigma-Aldrich) was added to a final concentration of 100 µM and plates were incubated at 37°C for 10 minutes in the presence or absence of 250 units of superoxide dismutase (SOD, Sigma-Aldrich). Phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) was added to each well at a final concentration of 1 µM and luminescence was measured every 30 seconds on a DTX 880 multimode detector (Beckman Coulter, CA, USA).

13.7 Transwell migration assays

HUVECs were maintained in EBM-2 media supplemented with 10% FBS (Lonza, MD, USA). For transwell migration assays, neutrophils were labeled with CellTracker Red (Invitrogen, CA, USA) as per the manufactures instructions, resuspended in Hanks\(^{+/-}\) with 0.1% BSA, and 5 x 10\(^5\) cells were plated in triplicate on top of confluent HUVECs in transwell inserts with a 3 µm pore-size (Corning). Inserts were placed in 24-well plates and cells were allowed to transmigrate for 1 hour towards 1 µM MLP or DMSO in the wells below each filter. Inserts were removed and plates were spun for 1 min at 500g to collect transmigrated cells onto BSA coated (0.1%) coverslips at the bottom of each well, and fixed with PFA. Coverslips were mounted on microscope slides in mounting media and imaged by confocal microscopy at 10x magnification. Automated cell counting was performed on 5 random fields of view using CellProfiler software (Broad Institute, MA, USA).
13.8 Adhesion assays

For adhesion assays neutrophils were labeled with CellTracker Red, resuspended in Hanks\(^{+/+}\) with 0.1% BSA, and 5 x 10^5 cells were plated in triplicate on confluent HUVECs in 96-well plates. Cells were allowed to settle for 10 minutes at 37°C, 5% CO\(_2\), and supplemented with DMSO or fMLP (1 µM final) for an additional 20 minutes. Plates were spun upside down for 1 minute at 100g, and 5 x 10^5 labeled neutrophils were plated in triplicate into empty wells to control for fluorescence. Mean fluorescence intensity (MFI) was determined by spectrofluorometry.

13.9 Chemokinesis

To test random migration of Lfc\(^{+/+}\) and Lfc\(^{-/-}\) neutrophils, Labtek II chambered coverslips (Nunc, Roskilde, Denmark) were coated with fibronectin (10 µg/ml, Sigma-Aldrich) or ICAM-1. For ICAM-1 coating, a small area of each well was coated with a 20 µl puddle of goat anti-human IgG (100 µg/ml, Jackson ImmunoResearch, PA, USA) in PBS\(^{+/-}\), blocked with 5% FBS, and then coated with Fc-ICAM-1 chimera (20 µg/ml, Sigma-Aldrich), each for 1 hour at room temperature and then stored in block at 4°C until use. Freshly prepared primary mouse neutrophils were maintained on ice at 12.5 million/ml and diluted to a final concentration of 2.5 million/ml in warm Hanks\(^{+/+}\) containing 20 mM Hepes and 1 µM fMLP, immediately prior to use. Neutrophils were plated and allowed to settle for 5 minutes, gently washed twice, and media containing 1 µM fMLP was replaced. Neutrophil crawling was imaged for 10 minutes with an inverted microscope (Axiovert 200M, Zeiss) equipped with a large environmental chamber maintained at 37°C and 5% CO\(_2\). Images were captured at 1 minute intervals with a 10x objective (0.5 NA; Fluar, Nikon, Tokyo, Japan) and a Roper CoolSnap HQ camera (Ottobrunn, Germany) using Metamorph software (Molecular Devices, CA, USA). Neutrophil tracks were generated using ImageJ with the manual tracking plug-in. Migration parameters were determined and scatter-plots were generated using the Chemotaxis and Migration tool (Ibidi). Moving cells were defined as those that migrated a minimum distance of 15 µm. The experiment was repeated three times and at least 100 cells were tracked per experiment.
13.10 Migration under shear stress

HUVECs were grown to confluence on 35 mm tissue culture plates (Corning, NY, USA) and stimulated with TNF-α (100 ng/ml, Sigma-Aldrich) for 4 hours. ICAM-1 coating on and neutrophil preparation and maintenance were performed as above. A parallel plate flow chamber was assembled by vacuum sealing a gasket between HUVECs or ICAM-1 coated surfaces and a glass plate. The flow chamber was mounted on an inverted microscope (Axiovert, Zeiss) with a heated stage. Neutrophils were infused into the flow chamber and allowed to settle on top of activated HUVEC monolayers or ICAM-1 coated surfaces for 5 minutes. One random field of view was selected and cell motion was imaged for 10 minutes at 20x magnification upon introduction of shear stress. Four dynes/cm² of shear stress is equivalent to 40 μneutons/cm² of pressure, and is similar to physiological levels of shear stress that occur in the postcapillary venules, where extravasation occurs in vivo. Laminar shear flow was introduced with an automated syringe vacuum pump attached to the outlet of the flow chamber. The experiment was repeated three times and at least 60 cells were tracked per experiment. Any cells that detached or underwent visible sliding during the course of the experiment were eliminated from the analysis. Moving cells were defined as those that migrated a minimum distance of 15 μm. Cell migration was analyzed as above.

13.11 Immunofluorescence and confocal imaging

Neutrophils were allowed to settle on top of ICAM-1 coated glass coverslips as above followed by treatment in the presence or absence of 4 dynes/cm² of shear stress for 5 minutes, and then fixed with 3.7% PFA for 10 minutes at room temperature. Glass coverslips were gently separated from the flow chamber using an acupuncture needle, and cells were stained for 1 hour at room temperature with Texas Red-X phalloidin (Molecular probes, OR, USA) and 5 min with 4’,6-diamidino-2-phenylindole (DAPI).

Confocal imaging was performed with an Olympus IX81 inverted microscope using a 60x (1.4 NA; PlanApo, Nikon) objective, and FluoView software (Olympus, Tokyo, Japan). For a given experiment, all images were acquired with constant microscope sensitivity settings. For analysis of neutrophil spread area, at least 10 random confocal images for each condition were analyzed.
using CellProfiler software. DAPI images were used to identify objects and phalloidin images were used, subsequently, to determine cell peripheries and the area in pixels\(^2\) of each cell. Spread versus rounded cells were distinguishable in phase contrast images based on the presence or absence of a broad lamellipodium, and were determined empirically from at least 10 random fields of view in four independent experiments.

13.12 Statistical analysis

Averages from at least three independent experiments are presented as mean \(\pm\) SEM unless otherwise noted. Student’s \(t\)-Test was used to determine the statistical significance of differences between groups with the following exceptions. Statistical significance was defined as \(*P < 0.05\).

Data from intravital microscopy experiments was analyzed using GraphPad Instat 3 software by ANOVA with Bonferroni’s correction. A \(p\)-value of \(< 0.05\) (\(*P < 0.05\); ** \(P < 0.001\)) was considered statistically significant.

Survival curves were analyzed with GraphPad Prism 5 software. Significance was assessed by the log-rank (Mantel-Cox) test.

14 Results

14.1 \(\text{Lfc}^{-/-}\) mice have reduced neutrophil infiltration in experimental acute peritonitis

A mouse model of peritoneal inflammation was used to test infiltration of neutrophils. Lavage fluid was recovered 5 hours after intraperitoneal injection of 3% thioglycolate, total cell counts per volume of lavage fluid were ascertained, and the proportion of neutrophils was determined by flow cytometry (Fig. 3.1A). Relative to \(\text{Lfc}^{+/+}\) mice, \(\text{Lfc}^{-/-}\) mice had an average of 36% fewer neutrophils recruited into the intraperitoneal space following injection of thioglycolate (Fig. 3.1B, \(P = 0.02\)). There was no difference in the percentage of mononuclear cells recovered from \(\text{Lfc}^{+/+}\) mice and \(\text{Lfc}^{-/-}\) mice after thioglycolate injection.
Figure 3.1: Neutrophil infiltration to the peritoneal cavity is defective in Lfc<sup>−/−</sup> mice. A) A representative flow cytometric analysis of peritoneal cell recovery from Lfc<sup>+<//+</sup> and Lfc<sup>−/−</sup> mice five hours after injection of 3% thioglycolate. Side scatter (SSC) and Gr-1 staining were used to identify neutrophil (large rectangle) and mononuclear cell (small rectangle) populations. Numbers indicate the percentages of each population. B) Yields of neutrophils and mononuclear cells per ml of peritoneal lavage fluid, five hours after intraperitoneal injection of PBS (n=2) or thioglycolate (Lfc<sup>+<//+</sup>, n=6; Lfc<sup>−/−</sup>, n=5), are shown for individual Lfc<sup>+<//+</sup> and Lfc<sup>−/−</sup> mice. Mean yields of neutrophils and mononuclear cells are indicated ± SEM. p-values were determined using Student’s t-Test. * indicates statistical significance.
14.2 *In vivo* intravascular crawling and emigration of Lfc<sup>−/−</sup> leukocytes is defective

To determine why neutrophil infiltration is reduced in Lfc<sup>−/−</sup> mice, intravital microscopy was used to assess *in vivo* rolling, adhesion, crawling and emigration of Lfc<sup>+/+</sup> and Lfc<sup>−/−</sup> leukocytes in the inflamed microvasculature. The cremaster muscle was surgically exposed and time-lapse videomicroscopy was used to monitor cell behaviour within cremasteric venules upon superfusion of the tissue with fMLP. Emigration from the vasculature was scored based on accumulation of leukocytes in the extravascular space. Ninety minutes after superfusion, an average of 41% fewer leukocytes were observed in the extravascular space per field of view in Lfc<sup>−/−</sup> mice, relative to Lfc<sup>+/+</sup> mice ($P < 0.05$) (Fig. 3.2A). At earlier time points a similar defect in emigration was observed in Lfc<sup>−/−</sup> mice; however these differences did not reach statistical significance. Upon superfusion, accumulation of Lfc<sup>+/+</sup> and Lfc<sup>−/−</sup> leukocytes was observed on the vascular surface over time, however at all time points there was a greater accumulation of Lfc<sup>−/−</sup> leukocytes compared to Lfc<sup>+/+</sup> leukocytes (Fig. 3.2B). The increased accumulation of leukocytes on the Lfc<sup>−/−</sup> vasculature relative to the Lfc<sup>+/+</sup> vasculature was statistically significant 30 ($P < 0.05$), 60 ($P < 0.001$), and 90 minutes ($P < 0.001$) after superfusion. When emigration was expressed in proportion to the number of leukocyte that had accumulated on the endothelial surface, severely defective diapedesis of Lfc<sup>−/−</sup> leukocytes was apparent at all time points, relative to Lfc<sup>+/+</sup> leukocytes (Fig. 3.2C). We also determined the proportion of attached cells that exhibited a clear crawling behavior. On average, 70% fewer attached Lfc<sup>−/−</sup> cells crawled compared to Lfc<sup>+/+</sup> cells (Fig. 3.2D, $P < 0.001$).

Prior to stimulation there was an average of ~20% fewer rolling leukocytes observed in the postcapillary venules of Lfc<sup>−/−</sup> mice relative to Lfc<sup>+/+</sup> mice (Fig. 3.2E). This difference was statistically significant ($P < 0.05$), but is not expected to contribute significantly to adhesion upon stimulation, since the number of cells that are rolling is not limiting with respect to those that adhere. It has been previously demonstrated that, in order to see any reduction in adhesion, >90% inhibition of leukocyte rolling is necessary (Kubes et al., 1995; Petri et al., 2011). The mean number and velocity of rolling leukocytes in the postcapillary venules dropped significantly thirty, sixty, and ninety minutes after superfusion of the exposed cremaster with fMLP, consistent with a robust local inflammatory response (Fig. 3.2E-F). These changes were similar for Lfc<sup>+/+</sup> and Lfc<sup>−/−</sup> mice.
Figure 3.2: In vivo crawling and emigration of Lfc/^−^ neutrophils is defective. Leukocyte recruitment was induced by superfusion of the exposed cremaster with 1 μM fMLP and emigration (A), cell accumulation (B), crawling behaviour of adherent cells (D), rolling flux (E), and rolling velocity (F) were determined in the Lfc/^+/^ (n=3) and Lfc/^−^ (n=3) vasculatures. (C) Emigration was expressed relative to accumulated cells. *, P < 0.05; **, P < 0.001; ns, not significant. G) Vascular leakage of FITC-labeled BSA was measured upon superfusion of cremasteric muscle preparations with fMLP, with or without prior depletion of neutrophils (depl). Representative images show fluorescence in the vasculature, and in the extravascular space, of neutrophil depleted Lfc/^+/^ and Lfc/^−^ mice at various time points after superfusion. Bar is 50 μm. H) Graph showing the time course of changes in the vascular permeability index of cremasteric venules in Lfc/^+/^ and Lfc/^−^ mice, with or without prior depletion of neutrophils. Values are expressed as mean ± SEM from two mice.
14.3 Vascular permeability is elevated in Lfc<sup>−/−</sup> mice

Studies have shown that Lfc localizes to tight junctions and that overexpression of Lfc potentiates increased paracellular permeability of epithelial monolayers (Benais-Pont et al., 2003), while knockdown of Lfc abrogates signalling-induced permeability (Kakiashvili et al., 2009; Waheed et al., 2010). Furthermore, knockdown of Lfc blocked vascular endothelial leakage and leukocyte recruitment in response to ventilator induced lung injury (Birukova et al., 2010). These studies suggest that the vasculature of Lfc<sup>−/−</sup> mice might be more resistant to diapedesis. To verify that the neutrophil emigration defect in Lfc<sup>−/−</sup> mice was not due to a tight junction defect, we tested permeability of the microvasculature in Lfc<sup>+/+</sup> and Lfc<sup>−/−</sup> mice by intravital microscopy. We monitored leakage of a fluorescently labeled albumin tracer into the extravascular space in normal and neutrophil depleted Lfc<sup>+/+</sup> and Lfc<sup>−/−</sup> mice, upon superfusion with fMLP. Upon stimulation, in non-depleted Lfc<sup>+/+</sup> and Lfc<sup>−/−</sup> mice, similar increases in vascular permeability index were observed (Fig. 3.2G and H). However, when neutrophils had been depleted, the vasculature of Lfc<sup>+/+</sup> mice was not permeable to the fluorescently labeled tracer, while Lfc<sup>−/−</sup> mice still had significant permeability, as determined by the increase in vascular permeability index over time. Permeability of the Lfc<sup>−/−</sup> vasculature was only marginally higher when neutrophils were not depleted, relative to after neutrophil depletion. These data demonstrate that, rather than potentiating paracellular permeability, Lfc is required to maintain endothelial integrity.

14.4 Lfc<sup>−/−</sup> mice are susceptible to poly-microbial infection

Since we observed defective neutrophil migration in Lfc<sup>−/−</sup> mice, we tested whether Lfc deficient mice could mount an effective immune response to poly-microbial infection. Cecal ligation and puncture (CLP) surgery was carried out on Lfc<sup>+/+</sup> and Lfc<sup>−/−</sup> mice and peritoneal lavage fluid was harvested 12 hours later in order to assess the cellular immune response relatively early after initiation of a poly-microbial infection (Fig. 3.3A). Total cells per ml of lavage fluid were counted and percentages of neutrophils were determined by flow cytometry. Relative to Lfc<sup>+/+</sup> mice, Lfc<sup>−/−</sup> mice had on average 60% fewer neutrophils recruited to the peritoneal cavity following CLP (Fig. 3.3A, \( P = 0.01 \)). Mortality was monitored in Lfc<sup>+/+</sup> and Lfc<sup>−/−</sup> mice over a seven day period following CLP. Seventy-five percent of Lfc<sup>−/−</sup> mice died within 48 hours of
CLP, while only 25% of the Lfc<sup>+/+</sup> mice succumbed to infection (Fig. 3.3B). The difference in survival of Lfc<sup>+/+</sup> and Lfc<sup>-/-</sup> mice in response to CLP was significant by the log-rank (Mantel-Cox) test ($P = 0.033$).

Next we tested the profile of inflammatory cytokines in the serum and lung, bacterial load in the spleen, and total cell counts in the lung after CLP (Fig. 3.3C-F). These parameters were determined in Lfc<sup>+/+</sup> and Lfc<sup>-/-</sup> mice 24 hours after CLP surgery. Of the 11 Lfc<sup>-/-</sup> mice, two (18%) died prior to the 24 hour time point, precluding their inclusion in the analysis. Of the surviving Lfc<sup>-/-</sup> mice, three (27% of the total) had significantly elevated levels of various inflammatory cytokines in the serum (Fig. 3.3C) and lung (Fig 3.3F), including IL-1β, TNF-α, IL-6, IL-10, RANTES, MCP-1, and KC. These same three mice also had high colony forming units (CFU) in the spleen (Fig. 3.3D) and low cellularity in the lung (Fig. 3.3E). These three mice may represent a failure to control bacterial replication and a consequent hypercytokinemia. Furthermore, three of the surviving Lfc<sup>-/-</sup> mice (27%) had increased cellularity in the lung relative to the other mice. Two of these mice had relatively high levels of TNF-α and IL-1β in the BAL fluid. These mice might be at an early stage in the septic response. Consistent with the survival rate of Lfc<sup>-/-</sup> mice in response to CLP, only 28% of Lfc<sup>-/-</sup> mice showed no signs of a septic phenotype.
Figure 3.3: Lfc\(^{-/-}\) mice have increased mortality in response to acute poly-microbial infection. A) Lfc\(^{+/+}\) and Lfc\(^{-/-}\) mice underwent sham (n=3), or CLP (Lfc\(^{+/+}\), n=7; Lfc\(^{-/-}\), n=9) surgeries and neutrophil yield was assessed in peritoneal lavage fluid twelve hours later by flow cytometry. Neutrophil yields per ml of peritoneal lavage fluid are shown for individual Lfc\(^{+/+}\) and Lfc\(^{-/-}\) mice. Mean yields of neutrophils are indicated ± SEM. \(p\)-values were determined using Student's \(t\)-Test. * indicates statistical significance. B) Lfc\(^{+/+}\) (n=8) and Lfc\(^{-/-}\) (n=12) mice underwent CLP surgery, and survival was monitored every 12 hours for 1 week. A \(p\)-value was obtained using the Log-rank (Mantel-Cox) test for statistical significance. * indicates statistical significance. Lfc\(^{+/+}\) (n=9) and Lfc\(^{-/-}\) (n=9) mice were sacrificed 24 hours after CLP surgery and serum cytokine levels (C), colony forming units (CFU) per spleen (D), total cells per ml of bronchoalveolar lavage (BAL) fluid (E), and cytokine levels in BAL fluid (F) were assessed. Three Lfc\(^{-/-}\) mice that had elevated inflammatory cytokines and CFU/spleen, compared to the other Lfc\(^{-/-}\) mice, are separated here, and designated -/-2. * indicates statistically significant differences.
14.5 Lfc suppresses phorbol ester-induced ROS production in neutrophils

Reactive oxygen species (ROS) production by neutrophils contributes to bacterial killing. ROS is produced by the NADPH oxidase complex, which is in turn activated by the small GTPase Rac. We tested the efficiency of ROS production in Lfc−/− neutrophils in response to stimulation with the phorbol ester, phorbol 12-myristate 13-acetate (PMA), a known inducer of ROS. We observed that ROS was maximally produced in Lfc+/+ and Lfc−/− neutrophils 10 minutes after PMA stimulation. This signal was quenched by addition of superoxide dismutase (SOD), demonstrating the specificity of the signal. ROS production was ~20% higher in Lfc−/− neutrophils (Fig. 3.4). These data provide genetic evidence that Lfc suppresses maximal ROS production in response to PMA.

![Graph showing ROS production in Lfc+/- and Lfc-/- neutrophils](image)

**Figure 3.4: Lfc−/− neutrophils have increased ROS production.** Lfc+/+ and Lfc−/− neutrophils were mixed with luminol and plated in triplicate in 96-well plates. Neutrophils were either untreated or stimulated with PMA and the luminescent reaction was measured over time. As a negative control, cells were pre-incubated with SOD prior to PMA stimulation. This result was repeated in three independent experiments. Mean relative light unit (RLU) measurements from a representative experiment are shown. Error bars indicate standard deviation of the mean.
14.6  *In vitro* chemokinesis, chemotaxis, and adhesion to HUVECs is normal in Lfc$^{-/-}$ neutrophils

Based on the *in vivo* observations of decreased intravascular crawling and emigration of Lfc$^{-/-}$ neutrophils (Fig. 3.2), we tested the *in vitro* random chemokinetic migration of primary bone marrow derived neutrophils from Lfc$^{+/+}$ and Lfc$^{-/-}$ mice. Lfc$^{+/+}$ and Lfc$^{-/-}$ neutrophils were plated on fibronectin (Fig. 3.5A-C) or ICAM-1 (Fig. 3.5D-F) coated surfaces in the presence of 1 µM fMLP, and cell migration was monitored for 10 minutes. Under these conditions approximately 20-25% of neutrophils exhibited random chemokinetic movement, with a mean displacement during this time frame of ~20 µm on fibronectin and ~30 µm on ICAM-1. The percentage Lfc$^{+/+}$ and Lfc$^{-/-}$ neutrophils that migrated and the mean displacement per neutrophil under these conditions was comparable.

We next tested the ability of Lfc$^{+/+}$ and Lfc$^{-/-}$ neutrophils to migrate across a confluent monolayer of HUVECs in a transwell system. Optimal chemotaxis of neutrophils was stimulated by a 1 µM solution of fMLP below the transwell inserts. There was no difference in the mean number of Lfc$^{+/+}$ and Lfc$^{-/-}$ neutrophils that transmigrated across confluent HUVEC monolayers (Fig. 3.6A).

We also tested neutrophil adhesion to HUVECs. Fluorescently labeled neutrophils were added to a confluent monolayer of HUVECs and allowed to attach in the presence or absence of 1 µM fMLP. Based on recovery of fluorescence intensity, fMLP induced a 2-fold increase in neutrophil adhesion. There was no difference in the mean number of Lfc$^{+/+}$ and Lfc$^{-/-}$ neutrophils that attached under basal (DMSO) or stimulated conditions (Fig. 3.6B).
Figure 3.5: Lfc is not necessary for neutrophil crawling in static conditions. Lfc+/- and Lfc-/- neutrophils were plated on fibronectin (A-C) or ICAM-1 (D-F) coated surfaces and stimulated with 1 µM fMLP. Images were acquired every minute for 10 minutes in 5-10 fields of view and individual cell tracks were determined and used to calculate the mean percentage of cells that migrate and the mean displacement of migrating cells. Migrating cells were defined as those that moved at least 15 µm. Scatter-plots from a representative experiment, indicating final positions of neutrophils relative to the origin, are shown. Means ± SEM from three independent experiments are shown.
**Figure 3.6: Lfc is not necessary for adhesion or transwell migration.**

A) Fluorescently labeled neutrophils were plated in triplicate on confluent HUVECs in transwell filters. Cells were allowed to transmigrate for 1 hour towards 1 µM fMLP in the wells below each filter. Transmigrated cells were imaged by confocal microscopy and automated cell counting was performed using CellProfiler software. Three independent experiments were performed. Mean cell counts and standard deviations from a representative experiment are shown.

B) Fluorescently labeled Lfc^{+/+} and Lfc^{-/-} neutrophils were plated in triplicate on confluent HUVECs for 10 minutes, and treated with fMLP or DMSO for an additional 20 minutes. After removing non-attached cells mean fluorescence intensity (MFI) per well was determined. Labeled neutrophils were plated in empty wells as a positive control. Shown are means of triplicate measurements with standard deviations.
14.7 Lfc<sup>-/-</sup> neutrophil crawling behaviour is defective under shear stress

To address the apparent discrepancy between the in vivo and in vitro migratory behaviour of Lfc deficient neutrophils we analyzed neutrophil migration under conditions of shear stress, thus simulating the in vivo effects of blood flow. In a parallel-plate flow chamber, neutrophils were adhered to a monolayer of TNF-α-activated HUVECs or an ICAM-1-coated surface in the presence of 1 µM fMLP. After 5 minutes, cells were exposed to 4 dynes/cm<sup>2</sup> constant shear flow and cell migration was imaged for 10 minutes. Under these conditions the majority of motile cells migrated in the direction of flow. On ICAM-1 coated plastic 57 ± 2.2% of Lfc<sup>+/+</sup> neutrophils migrated, compared to 27 ± 7.7% of Lfc<sup>-/-</sup> neutrophils (P = 0.01, Fig. 3.7A and B). There was no significant difference in the mean displacement of migratory Lfc<sup>+/+</sup> (57.9 ± 8.8 µm) and Lfc<sup>-/-</sup> (47.3 ± 6.9 µm) neutrophils under shear stress (P = 0.38, Fig. 3.7C). Shear stress induced an almost 3-fold increase in the percentage of migratory Lfc<sup>+/+</sup> neutrophils compared to static conditions, which was statistically significant (P = 1 x 10<sup>-4</sup>). In contrast, the percentage of migratory Lfc<sup>-/-</sup> neutrophils under conditions of shear stress was marginally higher than in static conditions, and this difference was not statistically significant (P = 0.59). Observed increases in the mean displacement of migratory Lfc<sup>+/+</sup> and Lfc<sup>-/-</sup> neutrophils under shear stress relative to static conditions were not statistically significant. A similar shear dependent, statistically significant, defect in the fraction of migratory Lfc<sup>-/-</sup> neutrophils, relative to Lfc<sup>+/+</sup> neutrophils, was observed on a monolayer of TNF-α activated HUVECs (P = 0.02, Fig. 3.7D and E).

Neutrophil migration parameters in the presence and absence of shear stress are summarized in table 1.
Figure 3.7: Lfc is necessary for shear stress induced neutrophil migration. Lfc<sup>+/+</sup> and Lfc<sup>−/−</sup> neutrophils were plated on ICAM-1 coated surfaces (A-C) or TNF-α activated HUVECs (D-F), and stimulated with 1 µM fMLP in the presence of 4 dynes/cm<sup>2</sup> constant shear stress. Cell motion was imaged for 10 minutes and individual cell tracks were generated and used to calculate the mean percentage of migrating cells and the mean displacement of migrating cells. Migrating cells were defined as those that moved at least 15 µm. Cells that detached or rolled were eliminated from the analysis. Scatter-plots from a representative experiment, indicating final positions of neutrophils relative to the origin, are shown. Arrows indicate the direction of flow. Means ± SEM from three independent experiments are shown. p-values were determined using Student’s t-Test. * indicates statistical significance.
Table 1: Comparison of neutrophil migration under static and shear stress conditions. The mean percentage of moving (>15 µm) Lfc<sup>+/+</sup> and Lfc<sup>-/-</sup> neutrophils, and the mean displacement of moving neutrophils over a 10 minute period on various substrates, and in the presence or absence of shear stress, are summarized. Motion tracking was performed on fibronectin and ICAM-1 coated surfaces, and on TNF-α stimulated HUVECs. Mean values are indicated ± SEM from three independent experiments. nd, not done.

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<td>Lfc&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>24.3 ± 5.0</td>
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<td>20.6 ± 1.6</td>
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<td>Lfc&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>23.4 ± 6.1</td>
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<td>Lfc&lt;sup&gt;+/+&lt;/sup&gt;</td>
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<td>31.2 ± 3.5</td>
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<tr>
<td>Lfc&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>18.2 ± 0.3</td>
<td>nd</td>
<td>32.3 ± 2.9</td>
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14.8 Shear stress-induced spreading of Lfc<sup>-/-</sup> neutrophils is defective

Lfc<sup>+/+</sup> and Lfc<sup>-/-</sup> neutrophils were adhered to ICAM-1-coated coverslips for 5 minutes in the presence of 1 µM fMLP. Cells were washed and maintained under static conditions or exposed to 4 dynes/cm² of shear stress for an additional 5 minutes. Cells were fixed with PFA and fluorescently stained with phalloidin and DAPI prior to imaging by confocal microscopy.

Confocal images were used to determine the spread area of Lfc<sup>+/+</sup> and Lfc<sup>-/-</sup> neutrophils under static and shear conditions (Fig. 3.8A and B). When individual cell areas are represented in a rank order distribution it is clear that shear stress induced cell spreading of Lfc<sup>+/+</sup> neutrophils, but failed to induce spreading of Lfc<sup>-/-</sup> neutrophils. Spread versus rounded up cells were easily distinguishable in phase contrast images based on the presence or absence of lamellipodia, and the mean percentage of spread cells for each condition was determined empirically (Fig. 3.8C and D). Under static conditions the percentage of Lfc<sup>+/+</sup> (36%) and Lfc<sup>-/-</sup> (39%) neutrophils that displayed a spread morphology was comparable. However, under shear stress there was a significant increase in the percentage of Lfc<sup>+/+</sup> neutrophils that had a spread morphology (56%). In contrast, only 36% of Lfc<sup>-/-</sup> neutrophils were spread after exposure to shear stress. The difference between the percentage of spread Lfc<sup>+/+</sup> and Lfc<sup>-/-</sup> neutrophils after exposure to shear stress was significant, as determined by Student's t-Test (Fig. 3.8D).
Figure 3.8: Lfc is necessary for shear stress induced neutrophil spreading. Lfc<sup>+/+</sup> and Lfc<sup>−/−</sup> neutrophils were plated on ICAM-1 coated coverslips in the presence of 1 µM fMLP. Cells were exposed to 4 dynes/cm<sup>2</sup> constant shear stress for 5 minutes, fixed and stained with Texas Red-X phalloidin and DAPI. At least 10 random fields of view were imaged per condition, at 60x magnification. Automated determination of mean spread area from confocal images was performed using CellProfiler software. A) Representative images of phalloidin staining with cell outlines overlaid in white. The direction of flow is indicated by an arrow. Bar is 20 µm. B) Rank/order distribution of cell spread area from a representative experiment. C) Representative phase contrast images. Arrow heads indicate spread cells with a broad lamellipodium. The direction of flow is indicated by an arrow. Bar is 20 µm. D) Mean percentages of spread cells were determined from 10 random fields of view in four independent experiments. At least 200 cells were scored per condition. Mean percentages of spread cells ± SEM are shown. <i>p</i>-values were determined using Student’s <i>t</i>-Test. * indicates statistical significance.

15 Discussion

Shear stress due to blood flow is essential for efficient recruitment of neutrophils from the vasculature in response to inflammatory stimuli, but the genes and pathways that mediate this process have not been elucidated. Here we show that the Rho GEF, Lfc, plays a unique role in neutrophil spreading and migration in response to shear stress, implicating Rho mediated effector functions in this process. We show that Lfc is necessary for shear-induced spreading and crawling <i>in vitro</i>, efficient <i>in vivo</i> leukocyte crawling and emigration out of the inflamed vasculature, infiltration of neutrophils to a local site of inflammation, and mouse survival in a model of polymicrobial sepsis.

Severe sepsis culminating in septic shock is associated with high hospital mortality rates (Levinson et al., 2011). The hyper-inflammatory response to infection in sepsis causes
widespread endothelial activation, increased vascular permeability, systemic leukocyte infiltration in the periphery, and activation of the coagulation response, leading to hypoperfusion and death due to multiple organ dysfunction syndrome (Cohen, 2002). Although alteration of neutrophil function in the chronic phase of sepsis, in particular neutrophil paralysis (Alves-Filho et al., 2010), contributes in a fundamental manner to disease progression and severity (Drost et al., 1999; Skoutelis et al., 2000; Watanabe et al., 1995; Ploppa et al., 2010; Reddy and Standiford, 2010), timely therapeutic intervention and the ability to quickly limit or contain the infection is a decisive moment for ultimate disease outcome (Raghavan and Marik, 2006). Early neutrophil infiltration in sepsis improves bacterial clearance and is predictive for survival (Craciun et al., 2010), while interventions at later stages of sepsis are ineffective (Lukaszewicz and Payen, 2010).

Neutrophils are the first cells to arrive during an acute inflammatory response. Peak neutrophil infiltration into the peritoneal cavity occurs within 6 hours after injection of 3% thioglycolate (Lagasse and Weissman, 1996). In the current study, we observed a significant reduction in infiltrating neutrophils in Lfc⁻/⁻ mice, relative to Lfc⁺/⁺ mice, 5 hours after peritoneal injection of 3% thioglycolate (Fig. 3.1), and 12 hours after initiation of a polymicrobial infection through CLP (Fig. 3.3A). On average, 36% and 60% fewer neutrophils were recovered from the peritoneal cavity of Lfc⁻/⁻ mice, relative to Lfc⁺/⁺ mice, in response to thioglycolate and CLP, respectively. A similar defect in peritoneal recruitment of neutrophils was observed in response to MSU crystals, as demonstrated in chapter 2 (Fig. 2.10A and B). Furthermore, Lfc⁻/⁻ mice had increased susceptibility to severe sepsis as evidenced by increased mortality after CLP surgery, compared to Lfc⁺/⁺ mice (Fig. 3.3B). Three out of 9 surviving Lfc⁻/⁻ mice had highly elevated cytokine levels in the blood and lung, 24 hours after CLP surgery, and showed a large bacterial burden in the spleen (Fig. 3.3 C-E). Despite this state of hyper-inflammation, leukocyte counts in the lungs of these mice were low (Fig. 3.3 F), indicating defective leukocyte responses. This phenomenon of high cytokine levels and bacterial burden combined with low leukocyte infiltration could be due to neutrophil paralysis, and suggests that these mice are progressing towards lethal sepsis. Early defective neutrophil infiltration in Lfc⁻/⁻ mice is likely to exacerbate sepsis, leading to increased mortality at later time points. However, the possible contribution of other factors to this outcome cannot be ruled out. For example, increased vascular permeability
in Lfc<sup>−/−</sup> mice (Fig. 3.2G and H) could contribute to sepsis through increased vascular leakage in the periphery.

Using *in vivo* intravital microscopy, we analyzed leukocyte behavior in the postcapillary venules in response to acute stimulation with the bacterial chemotactic peptide, fMLP. We observed a profound defect in crawling and diapedesis of Lfc<sup>−/−</sup> leukocytes. Relative to Lfc<sup>+/+</sup> leukocytes, we observed a 71% reduction in the mean number of adherent Lfc<sup>−/−</sup> leukocytes that crawled (Fig. 3.2D), and a 41% reduction in the number of Lfc<sup>−/−</sup> leukocytes that successfully emigrated into the extravascular space, 90 minutes after superfusion (Fig. 3.2A). The discrepancy between these two values could be due to a subset of leukocytes undergoing transcellular migration, or docking directly at intercellular junctions, and thus circumventing the need to crawl on the endothelial surface. Upon stimulation, increased accumulation of leukocytes on the vascular surface was observed in Lfc<sup>−/−</sup> mice, relative to Lfc<sup>+/+</sup> mice (Fig. 3.2B). This was proportional to the relative decrease in cells that had completed diapedesis, suggesting that defective crawling of Lfc<sup>−/−</sup> leukocytes resulted in reduced emigration and a concomitant accumulation of leukocytes on the inflamed vasculature. Although difficult to test, we propose that reduced leukocyte emigration across the vasculature and reduced recruitment to the inflamed peritoneum in Lfc<sup>−/−</sup> mice, are two manifestations of the same defect. The 41% decrease in leukocyte emigration in the Lfc<sup>−/−</sup> mice is comparable to the ~36% and 60% defect in neutrophil infiltration into the peritoneal cavity that was observed in response to thioglycolate and CLP, respectively (Fig. 3.1 and 3.3A).

To address the well documented role of Lfc in tight junction permeability (Benais-Pont et al., 2003; Waheed et al., 2010), we measured microvascular permeability in Lfc<sup>+/+</sup> and Lfc<sup>−/−</sup> mice (Fig. 3.2G and H). Our results show that upon superfusion, the vasculatures of Lfc<sup>+/+</sup> and Lfc<sup>−/−</sup> mice exhibit similar increases in permeability index. When neutrophils were depleted prior to superfusion, there was no increase in the permeability index in Lfc<sup>+/+</sup> mice, while the permeability index of the Lfc<sup>−/−</sup> mouse vasculature increased significantly with time. High vascular permeability was observed in the Lfc<sup>−/−</sup> mouse, whether neutrophils were depleted or not, with marginally increased permeability observed when neutrophils were not depleted. This suggests that the vascular endothelium of Lfc<sup>−/−</sup> mice is inherently leaky compared to the Lfc<sup>+/+</sup> endothelium. The observation that the Lfc<sup>−/−</sup> vasculature is inherently leaky, goes contrary to the current model, whereby Lfc promotes permeability of tight junctions, and could reflect
differences between transient knock down and germ-line ablation of Lfc, suggesting a possible developmental role for Lfc. Leakiness of the Lfc<sup>-/-</sup> vasculature supports the interpretation that defective crawling of Lfc<sup>-/-</sup> leukocytes likely accounts for the defect in emigration from the vasculature, rather than reduced tight junction permeability, which might have been predicted based on previously published studies. Also in support of our model, one study showed that endothelial permeability is not rate-limiting with respect to diapedesis of lymphocytes (Cinamon et al., 2001). Although the observed high permeability of the microvasculature in Lfc<sup>-/-</sup> mice allays the possibility that the previously acknowledged role of Lfc in tight junction permeability contributes to the neutrophil emigration defect, the possibility remains that some other defect in endothelial cell function could play a role. In order to differentiate between leukocyte and vessel wall effects, reciprocal bone marrow transplantation or use of a leukocyte specific Cre-mouse will be necessary.

Production of ROS is an important component of neutrophil bactericidal activity (Borregaard, 2010). We observed elevated secretion of ROS in response to PMA stimulation in Lfc<sup>-/-</sup> neutrophils, relative to Lfc<sup>+/+</sup> neutrophils (Fig. 3.4). This was similar to the elevated ROS production by Lfc<sup>-/-</sup> neutrophils in response to MSU crystals, discussed in chapter 2. The fact that ROS production is not defective in Lfc<sup>-/-</sup> neutrophils supports the model that defective neutrophil recruitment, rather than anti-microbial activity, is responsible for the increased mortality that we observed in Lfc<sup>-/-</sup> mice in response to CLP. In future studies it will be necessary to test other neutrophil effector functions, such as phagocytosis, degranulation, and formation of extracellular traps.

Despite profound in vivo neutrophil defects, our in vitro analysis initially failed to uncover a defect in migration on ICAM-1 or fibronectin coated surfaces (Fig. 3.5), adhesion to HUVECs (Fig. 3.6A), or transwell migration across a monolayer of HUVECs (Fig. 3.6B). In light of the in vivo defect in neutrophil crawling we tested cell locomotion in the presence of shear stress, a condition that more closely recapitulates the environment of the inflamed vasculature. Under these conditions there was a significant defect in the percentage of Lfc<sup>-/-</sup> neutrophils that migrated (~27%), compared to Lfc<sup>+/+</sup> neutrophils (57%), but no difference in the mean displacement of migrating cells (Fig. 3.7A-C; Table 1), indicating that Lfc is necessary for neutrophil migration in response to shear stress, but does not control migratory velocity. On TNF-α activated HUVECs, a similar shear-dependent defect in migration of Lfc<sup>-/-</sup> neutrophils
was observed (Fig. 3.7D-F). These *in vitro* results recapitulate the *in vivo* leukocyte crawling defect on the mouse vasculature, where 22% of Lfc<sup>−/−</sup> neutrophils crawled, compared to 78% of Lfc<sup>+/+</sup> neutrophils (Fig. 3.2D).

The percentage of Lfc<sup>+/+</sup> neutrophils that migrated on ICAM-1 coated surfaces under shear stress (~57%, Fig. 3.7B; Table 1) was significantly higher than the fraction that migrated in static conditions (~20%, Fig. 3.5E). In contrast, there was no significant increase in the percentage of migratory Lfc<sup>−/−</sup> neutrophils under shear stress (~27%, Fig. 3.7B) relative to static conditions (22%, Fig. 3.5E). Comparison between static and shear conditions is problematic, since, in static conditions, non-adherent neutrophils are washed away immediately prior to motion tracking, while exposure of neutrophils to shear stress is essentially analogous to a continuous wash. Loss of cells under conditions of shear stress could theoretically enrich for migrating cells, however, the degree to which this occurs is not known since both migratory and non-migratory cells were lost during exposure to shear. Also, most of the cells that were lost during the shear stress regime, as opposed to at the onset of shear, were lost within the first few minutes, and it is therefore not possible to determine how many of these might have migrated.

To address the role of Lfc in neutrophil crawling in response to shear stress, we measured the spread area of Lfc<sup>+/+</sup> and Lfc<sup>−/−</sup> neutrophils after exposure to static or shear stress conditions. Automated analysis indicated that Lfc<sup>+/+</sup> neutrophils, but not Lfc<sup>−/−</sup> neutrophils, were more spread after exposure to shear stress compared to static conditions (Fig. 3.8A and B). Visually counting cells with spread versus round morphologies indicated that shear stress induced an increase in the percentage of spread Lfc<sup>+/+</sup> cells, and had no effect on the percentage of spread Lfc<sup>−/−</sup> cells (Fig. 3.8C and D). The spreading defect of Lfc<sup>−/−</sup> neutrophils in response to shear stress closely mirrors the crawling response to shear, suggesting that these two responses are functionally related.

Small GTPases and their activators, the GEFs, integrate spatial and temporal cues to regulate cell migration, and are likely to be involved in the response to shear stress. In fibroblasts, Lfc (Guilluy et al., 2011) and its target, RhoA (Matthews et al., 2006), promote adhesion strengthening and cell stiffening in response to mechanical force. RhoA controls neutrophil adhesion and crawling through F-actin dependent regulation of integrin function (Anderson et al., 2000; Laudanna el al., 1996; Laudanna et al., 1998). One study showed that RhoA is activated in neutrophils in response to shear stress (Makino et al., 2005), however these cells
were in suspension rather than associated with a physiological ligand, and the GEF responsible for this activity was not identified. We propose a model whereby Lfc promotes RhoA dependent adhesion strengthening and dynamics in response to shear stress, through effects on integrins and the F-actin cytoskeleton (Fig. 3.9). This is consistent with the known function of RhoA downstream of GPCR signalling in neutrophils (Laudanna et al., 1996). Our results are also consistent with a study showing that inhibition of F-actin causes increased accumulation, and decreased crawling and diapedesis of lymphocytes on activated HUVECs under shear stress (Cinamon et al., 2001). Defective crawling of Lfc−/− neutrophils in response to shear stress could be a direct result of defective spreading or due to some other RhoA dependent function, such as cell polarization, de-adhesion, or propulsion.

Further analysis will be necessary in order to identify the shear stress responsive pathways that lie upstream of Lfc. Some evidence implicates GPCRs in the response to shear stress in neutrophils (Makino et al., 2006), and Lfc is known to be activated downstream of GPCR signalling in fibroblasts and endothelial cells (Meiri et al., 2009; Birukova et al., 2006). Lfc is phosphorylated and activated by the MAP kinase, ERK (Fujishiro et al., 2008), and a signalling pathway involving FAK, Ras, and ultimately ERK mediated phosphorylation and activation of Lfc has been implicated in the response to mechanical force on integrins in fibroblasts (Guilluy et al., 2011). Consistent with this model of shear induced Lfc activation, MAPK inhibitors block neutrophil emigration but not rolling or adhesion on stimulated mouse cremaster preparations (Cara et al., 2001). Calcium flux is also implicated in the neutrophil response to shear stress, and is triggered immediately following arrest on the vasculature and prior to polarization and migration (Schaff et al., 2008), potentiating RhoA and actin dependent adhesion strengthening. Since Lfc is bound and inhibited by microtubules (Ren et al., 1998), it will be of interest to determine the effect of shear stress on the MT cytoskeleton in neutrophils.

The influence of shear flow on leukocyte recruitment from the vasculature is well established, and some aspects of the mechanosensory mechanism have been identified (Alon and Ley, 2008). In humans, leukocyte adhesion disorder type 1 (LAD-1) is caused by defective leukocyte integrin activation under shear stress, and results in recurrent infections, leukocytosis, and bleeding (Alon et al., 2003), emphasizing the importance of the shear stress response in immune function. We have shown that shear stress-induced spreading and crawling of neutrophils is dependent on Lfc, indicating that Lfc is part of the mechanosensory mechanism that drives this response.
Furthermore, *in vivo* leukocyte crawling on the inflamed vasculature was defective, resulting in reduced diapedesis and accumulation of cells on the endothelial surface in Lfc<sup>-/-</sup> mice. Finally, we found that Lfc was necessary for an efficient innate immune response to infection, since Lfc<sup>-/-</sup> mice had greatly diminished neutrophil infiltration to a local inflammatory site and increased mortality in a mouse model of sepsis, compared to Lfc<sup>+/+</sup> mice.

Neutrophils are adapted to the environment on the endothelial surface of blood vessels to the extent that their capacity to undergo diapedesis is enhanced under conditions of fluid shear stress. Since leukocyte recruitment at the vascular interface is a crucial aspect of the inflammatory response, a more complete knowledge of how cells detect shear stress, and the signalling pathways that translate this information into meaningful biomechanical behavior, will give us a better understanding of normal and pathological inflammatory responses. The RhoGEF, Lfc, is a novel component of the neutrophil mechanosensory response to shear stress on the endothelial surface, which is essential for efficient neutrophil infiltration to an acute site of infection. Further studies will be necessary to elucidate the mechanism by which Lfc potentiates neutrophil responses to shear stress.
Figure 3.9: Model of the Lfc-mediated response to shear stress. A) Lfc is necessary for neutrophil spreading and crawling in response to shear stress. B) Unknown mechanosensory mechanisms, which could include GPCR mediated events, promote Lfc activity. During shear stress-induced spreading, Lfc is likely to promote adhesion strengthening through Rho-mediated effects on F-actin and integrins. Lfc might also contribute to Rho-mediated de-adhesion in order to facilitate subsequent crawling.
Chapter 4

Future Directions
16 Abstract

Lfc is an important regulator of the mechanosensory response in fibroblasts (Guilluy et al., 2011). We have shown that Lfc is necessary for the migratory response of neutrophils to shear stress. Here we show that Lfc potentiates the shear stress response in neutrophils through direct or indirect effects on the F-actin and MT cytoskeletons. Preliminary studies indicate a profound defect in F-actin and MT content in Lfc⁻/⁻ neutrophils under conditions of shear stress. Further studies are suggested in order to confirm and further elucidate the mechanism of Lfc-dependent neutrophil responses to shear stress.

17 Introduction

The F-actin cytoskeleton, composed of polymerized monomers of actin, and the MT cytoskeleton, composed of polymerized α and β-tubulin dimers, are highly dynamic scaffolding networks that permeate the cytoplasm and are central to almost every aspect of cell biology. They orchestrate cell morphology, polarity, migration, intracellular transport, and separation of the chromosomes and cytokinesis during cell-division. Furthermore, the two cytoskeletons undergo cross-talk and are significantly correlated in migrating cells (Small et al., 1999; Rodriguez et al., 2003; Wehrle-Haller and Imhof, 2003). The MT cytoskeleton regulates polarity of migrating cells by regulating actomyosin contractility and adhesion dynamics (Elbaum et al., 1999; Small and Kaverina, 2003), possibly through Lfc (Krendel et al., 2002). Furthermore, MT polymerization is stimulated downstream of RhoA through a compensatory feedback mechanism (Gundersen et al., 2005).

Polarization and orientation of the MT cytoskeleton, with the MT organizing centre (MTOC) in front of the nucleus and stable detyrosinated α-tubulin at the leading edge, is an important aspect of cell polarity in migration of many cell types, and is regulated by the Rho family GTPases (Palazzo et al., 2001a). Uniquely, neutrophils organize polarity with the MTOC “behind” the nucleus and MTs extending radially into the posterior end of the cell (Eddy et al., 2002). The purpose of MTs in the rear of migrating neutrophils is not known, however, it could function to help localize Lfc in the posterior end of the cell, where Rho-mediated contractility must be concentrated in order to maintain polarity, facilitate de-adhesion, and propel the cell forward. In
neutrophils, the MT cytoskeleton is even more dynamic than in other cell types, with a half life of less than 1 minute (Ding et al., 1995). Gross changes in MT and F-actin configuration must occur during a very short time frame when leukocytes undergo dramatic changes in cell morphology, such as during transition from a round to a spread morphology. Here we showed defective F-actin and MT polymerization in the absence of Lfc, in spread neutrophils that were exposed to shear stress. These data show that Lfc is required for actin and MT polymerization or stabilization in response to shear stress, and provides some mechanistic insight into the role of Lfc in the neutrophil shear stress response.

18 Methods

18.1 Immunofluorescence and confocal imaging

Neutrophils were allowed to settle on top of ICAM-1 coated glass coverslips for 5 minutes, followed by treatment in the presence or absence of 4 dynes/cm² of shear stress for an additional 5 minutes. For F-actin staining, cells were fixed with 3.7% PFA for 10 minutes at room temperature. For immunofluorescent staining of MTs, cells were fixed for 5 minutes with 0.7% gluteraldehyde, followed by a 15 minute extraction with 0.5% SDS, as previously described (Ding et al., 1995). After fixation, glass coverslips were gently separated from the flow chamber using an acupuncture needle, and cells were stained for 1 hour at room temperature with Texas Red-X phalloidin (Molecular probes, OR, USA), or mouse α-tubulin antibody (DM1A - Amersham, IL, USA).

Confocal stacks were acquired with an Olympus IX81 inverted microscope using a 60x (1.4 NA; PlanApo, Nikon) objective, and FluoView software (Olympus, Tokyo, Japan). For a given experiment, all images were acquired with constant microscope sensitivity settings. For determination of F-actin staining, 8-10 randomly chosen spread cells were imaged for each condition analyzed. CellProfiler software was used to determine the perimeter of each cell and the average fluorescent intensity per pixel from F-actin footprint slices. For tubulin stained samples, 10 z-stack slices were acquired per cell at increments of ~0.4 µm. By scanning through 3D z-stacks, Individual MT fibers in each cell were counted by eye. Only MT fibers that
projected into the posterior end of cells were counted. The mean number of MT fibers per cell was determined from at least 10 randomly selected spread, polarized neutrophils.

19 Results

19.1 F-actin content is reduced in Lfc−/− neutrophils under shear stress

Lfc+/+ and Lfc−/− neutrophils were adhered to ICAM-1 coated surfaces, exposed to static or shear stress conditions, fixed, and stained with phalloidin. For analysis of F-actin content, 10 randomly selected spread cells were imaged at high magnification. Although stacks were generated, these cells were extremely flat and most of the F-actin content occurred in the footprint slice, where the cells were in contact with the substrate. We analyzed the average pixel intensity per cell from footprint slices using CellProfiler. Under conditions of shear stress, but not in static conditions, there was a significant defect in the mean fluorescence pixel intensity in Lfc−/− neutrophils (Fig. 4.1A and B).

19.2 Microtubule content is reduced in Lfc−/− neutrophils under shear stress

Lfc+/+ and Lfc−/− neutrophils were adhered to ICAM-1 coated surfaces, exposed to static or shear stress conditions, fixed, and stained with an anti-tubulin antibody. MTs extended into the posterior end of the cell in spread adherent neutrophils, as previously demonstrated (Eddy et al., 2002). Individual MT fibers in the posterior of spread, polarized Lfc+/+ and Lfc−/− neutrophils were determined from confocal z-stacks. Under conditions of shear stress, but not in static conditions, there was a significant defect in the mean number of MT fibers extending into the posterior of Lfc−/− neutrophils (Fig. 4.2A and B).
Figure 4.1: *Lfc*<sup>−/−</sup> neutrophils have reduced F-actin content under shear stress, relative to *Lfc*<sup>+/+</sup> neutrophils. *Lfc*<sup>+/+</sup> and *Lfc*<sup>−/−</sup> neutrophils were adhered to ICAM-1 coated coverslips and exposed to static or shear stress conditions, prior to fixation and staining with phalloidin. Eight to 10 randomly selected, spread, polarized neutrophils were imaged per condition. A) Representative confocal images of phalloidin staining at the interface of the cell with the substrate. The direction of flow is indicated by an arrow. Bar is 5 µm. B) Cell profiler software was used to determine cell perimeters and the mean fluorescence intensity per pixel within the footprint of each cell. Individual measurements and mean values of MFI/pixel from three independent experiments are indicated ± SEM. *p*-values were determined using Student’s *t*-Test. * indicates statistical significance.
Figure 4.2: Lfc<sup>−/−</sup> neutrophils exposed to shear stress have a reduced number of microtubule fibers per cell, compared to Lfc<sup>+/+</sup> neutrophils. Lfc<sup>+/+</sup> and Lfc<sup>−/−</sup> neutrophils were adhered to ICAM-1 coated coverslips and exposed to static or shear stress conditions, prior to fixation and staining with α-tubulin antibody. At least ten randomly selected, spread, polarized neutrophils were imaged per condition. A) Representative phase contrast images and projections of 3D z-stack confocal images of tubulin immunofluorescence are shown. The direction of flow is indicated by an arrow. Bar is 10 µm. B) The number of MTs in individual cells and the mean number of MTs per cell are indicated ± SEM. Data were compiled from three independent experiments. p-values were determined using Student’s t-Test. * indicates statistical significance.
20 Discussion

Lfc is an important mediator between the MT and F-actin cytoskeletons, and is necessary for the spreading and crawling response of neutrophils to shear stress. We analyzed the structure and abundance of polymerized actin and MT cytoskeletal structures in crawling neutrophils after exposure to shear stress, which have never been previously assessed. We exposed Lfc<sup>+/+</sup> and Lfc<sup>-/-</sup> neutrophils to shear stress for 5 minutes, fixed them, and stained total F-actin and tubulin in these cells. The average total F-actin content per cell in spread, polarized Lfc<sup>-/-</sup> neutrophils was reduced compared to Lfc<sup>+/+</sup> neutrophils, only under conditions of shear stress. Similarly, there was a profound defect in MT organization in Lfc<sup>-/-</sup> neutrophils exposed to shear stress.

In chapter 3, we demonstrated that Lfc<sup>-/-</sup> neutrophils had defective spreading and crawling relative to Lfc<sup>+/+</sup> neutrophils, specifically under conditions of shear stress. Here we showed that spread Lfc<sup>-/-</sup> neutrophils had reduced footprint-associated F-actin content when exposed to shear stress. This result is consistent with the role of RhoA in mediating F-actin polymerization through its effector, mDia. Reduced footprint-associated F-actin in spread Lfc<sup>-/-</sup> neutrophils did not appear to inhibit their ability to migrate, since the Lfc<sup>-/-</sup> neutrophils that did migrate under shear stress had a similar velocity as migratory Lfc<sup>+/+</sup> neutrophils (Fig. 3.7), however it is possible that cells that are not fully spread, as those which we have observed, here are the relevant and more highly motile cells. Cell spreading and lamellipodium formation are prerequisites to crawling, and we have shown that Lfc promotes the transition of neutrophils to a spread morphology in response to shear stress. Our results suggest that this might occur through Lfc-dependent effects on F-actin polymerization, which can promote integrin clustering and cell adhesion.

The reason for defective MT fiber formation in Lfc<sup>-/-</sup> neutrophils after exposure to shear stress is not known. However, there are at least two possible explanations. Since Rho activity can stimulate MT capping and stabilization through mDia (Ishizaki et al., 2001; Palazzo et al., 2001b; Eng et al., 2006) and Lfc overexpression stabilizes MTs (Yoshimura and Miki, 2011), the absence of Lfc could directly cause MT destabilization. It has been demonstrated that actin and myosin are necessary for polar localization of MTs in neutrophil uropods (Eddy et al., 2002) and the actin and MT cytoskeletons are known to be closely coupled in a number of systems.
Reduced F-actin might indirectly lead to the MT defect. In turn, mislocalization of Lfc, as a result of defective MT formation, could potentiate the F-actin defect.

The question remains why the F-actin and MT defects of Lfc−/− neutrophils are uncovered by shear stress. Lfc has recently been shown to be part of the mechanosensory response of integrins (Guilluy et al., 2011) and relocalizes to integrins in response to intracellular contractility (Kuo et al., 2011). Shear stress-induced integrin adhesion could induce translocation of Lfc from the MTs and facilitate an Lfc-dependent response in leukocytes. Shear stress promotes neutrophil adhesion and invagination into endothelial cells through unknown mechanisms (Cinamon et al., 2004). Shear stress-induced Lfc-dependent contractility could be critical to limit this effect, by facilitating de-adhesion and propulsion. Consistent with this model, direct targeting of fibroblast focal adhesions (FA) by MTs promotes FA disassembly and remodeling of the actin cytoskeleton (Kaverina et al., 1999). Several possible mechanisms of action for MT-mediated FA dissolution have been proposed, including the sequestration of FA-associated Lfc (Small and Kaverina, 2003).

A series of future experiments are proposed in order to develop a more thorough understanding of cytoskeletal dynamics during the shear stress response in neutrophils, and the role of Lfc in this process. Since harvesting cells from the parallel-plate flow chamber would not provide us with enough cells to analyze by western blot, other approaches will be necessary to probe the biochemical responses to shear stress in Lfc+/− and Lfc−/− neutrophils. Neutrophils in suspension can be exposed to shear stress using a cone-and-plate device (model DV II, Brookfield engineering laboratories, MA, USA). This device consists of a rotating 0.8º cone placed over a stationary plate. Cell suspensions are placed in the gap between the cone and the plate and a uniform fluid shear field is generated by turning the cone at a constant speed. Using this approach, Makino et al. (2005), showed that RhoA-GTP levels are elevated 5 minutes after exposure to shear stress. We can use this approach to test whether Lfc is the GEF responsible for RhoA stimulation in response to shear stress, or activation of downstream targets such as MLC. Since integrin engagement with physiological ligands is likely to be important for the shear stress response, cells can be incubated with ICAM-1 coated beads prior to spinning. Alternatively, adherent neutrophils can be incubated with ligand coated magnetic beads and exposed to a magnetic field (Zhao et al., 2007; Guilluy et al., 2011). This approach generates mechanical force at integrins that would mimic shear stress. In the above contexts, cells can be pre-treated...
with the MT stabilizing agent, taxol, to determine the contribution of MT depolymerization to the Lfc-dependent shear stress response.

Very little is known about the mechanics of the actin and MT cytoskeletons during neutrophil spreading and crawling, or in response to shear stress. Most of what is known has been gleaned from IF analysis of fixed cells, and the use of inhibitors of polymerization or depolymerization. However, powerful imaging technologies are now available that allow high-resolution live-imaging of cytoskeletal components as cells undergo fundamental biological processes. Transfectable reagents exist for fluorescent tagging of actin and tubulin in live cells. Since primary neutrophils are short-lived and difficult to transfect, these experiments will need to be carried out in neutrophil-like cell lines, such as HL-60s, after RNA interference (RNAi)-mediated silencing of Lfc. Real-time, high resolution fluorescent imaging of actin and MTs can be carried out in adherent neutrophils upon exposure to shear stress. This has never been done, and has the potential to address many unanswered questions.

Further studies will be necessary to determine how Lfc is activated in response to shear stress. One possibility is that Lfc is activated by MAPK, which has been shown to activate Lfc in response to mechanical stress in fibroblasts (Guilluy et al., 2011). In order to test this, HL-60 cells can be treated with the MAPK inhibitor U1026 prior to exposure to shear stress. Cells can be transfected with an activated form of Lfc to rescue the effects of the MAPK inhibitor on shear stress induced migration.

The shear stress response is an important adaptation that allows leukocytes to quickly respond to inflammatory signals at the vascular surface by enhancing adhesion, crawling, and infiltration into the tissue. Very little is known about the role of RhoGTPase-mediated cytoskeletal changes in this process. Future elucidation of how the MT-associated RhoGEF, Lfc, contributes to the shear stress response in neutrophils will give us greater insight into this fundamental process.
Concluding Remarks

In chapter two, we showed that Lfc is necessary for the capacity of colchicine to block neutrophil recruitment in MSU-induced inflammation. This indicates that Lfc may be partially responsible for the curative effects of colchicine, a potent therapeutic agent used to treat gout and a number of other inflammatory diseases. Given the complex series of events that facilitate leukocyte recruitment in an inflammatory immune response, a number of cell types and responses could potentially be targeted by colchicine in an Lfc-dependent manner. Here, I will suggest future experimental approaches towards elucidating the target cell functions and mechanism of action of colchicine, using the Lfc−/− mouse model.

The therapeutic capacity of colchicine as an anti-inflammatory agent is highly specific to a select group of inflammatory diseases, which include gout, familial Mediterranean fever (FMF), and Bechet's disease. Although colchicine inhibits neutrophil, macrophage, and endothelial functions, its therapeutic specificity implies that the aspect or aspects of the inflammatory response that it targets, which are responsible for its therapeutic effects, are unique to this subset of inflammatory diseases. It seems unlikely that the Achilles' heel in gout is some aspect of neutrophil function that is common to inflammatory diseases where colchicine is not effective.

Roberge et al. (1995) have shown that colchicine and other MT-depolymerizing agents inhibit a characteristic pattern of MSU crystal-induced tyrosine-phosphorylation in neutrophils, but have no effect on fMLP or C5a induced tyrosine phosphorylation. Other unique aspects of the neutrophil response to MSU crystals that are inhibited by colchicine include crystal phagocytosis and secretion of crystal-induced chemotactic factor (CCF). Neutrophil extravasation is common to many inflammatory diseases, including rheumatoid arthritis, where colchicine is not effective. Therefore, it seems unlikely that colchicine exerts its anti-inflammatory effects by blocking neutrophil rolling, crawling, or diapedesis, per se. However, this can not be ruled out since neutrophils play a more prominent role in diseases that are treatable with colchicine. Also, it is possible that neutrophil extravasation in response to unique MSU-induced inflammatory signatures is targeted.

Martinon et al. (2006) have shown that MSU crystals activate the NALP3 inflammasome in macrophages, resulting in secretion of IL-1β. They also show that processing and secretion of IL-1β by macrophages is inhibited by colchicine. Interestingly, FMF is associated with mutations in
the NALP3 inflammasome that result in increased production of IL-1β (Papin et al., 2007). This suggests that high IL-1β production may be a common characteristic of diseases that are treatable by colchicine, and that interference with the NALP3 inflammasome could be a therapeutically relevant action of colchicine in gout. One study has shown that colchicine blocks neutrophil infiltration without affecting TNF-α and IL-8 levels in the synovial fluid (Matsukawa, 1998), suggesting that resident macrophages may not be the relevant target of colchicine. The authors suggest that colchicine blocks the amplification of MSU-induced inflammation by infiltrating neutrophils, since colchicine blocked an early peak of IL-1β production (2 hours) that was dependent on infiltrating neutrophils. Also, inhibition of neutrophil and endothelial cell functions by colchicine occurs at concentrations (nM range) that are more likely to be achieved during therapy, than the concentrations that inhibit IL-1β production by macrophages (µM). This suggests that neutrophils and endothelial cells are more likely to be the therapeutic targets of colchicine than macrophages (Nuki, 2008). Since Lfc is necessary for the therapeutic efficacy of colchicine, testing the effects of colchicine on MSU crystal-induced responses in Lfc+/+ and Lfc−/− leukocytes should allow us to identify the leukocyte function or functions that are meaningfully targeted by colchicine in gout therapy.

To test the effects of colchicine on resident macrophage responses, the levels of inflammatory cytokines in the peritoneal lavage fluid can be assessed soon after injection of MSU crystals into Lfc+/+ and Lfc−/− mice. Levels of IL-1β, TNF-α, and IL-6 in peritoneal lavage fluid peak 4 hours after injection of MSU crystals (Martin et al., 2009). However, as noted above, infiltrating neutrophils are already contributing to IL-1β production as early as 2 hours after MSU injection. MSU crystal-induced cytokine secretion can also be assessed using cultured Lfc+/+ and Lfc−/− macrophages, in the presence and absence of colchicine. If colchicine has a comparable effect on cytokine secretion from Lfc+/+ and Lfc−/− macrophages, then its therapeutic efficacy is likely to be through another cell type.

Using Lfc+/+ and Lfc−/− cells, we can determine whether colchicine exerts its anti-inflammatory effects through interference with neutrophil function. A number of neutrophil responses can be tested, including cytokine secretion, phagocytosis, adhesion, migration, and tyrosine phosphorylation. Similarly, the effects of colchicine on endothelial cells can be tested. To accomplish this, it will be necessary to knock down Lfc expression in an endothelial cell line, such as HUVECs, since it is difficult to purify mouse endothelial cells. Surface expression of
adhesion molecules and adhesive capacity for neutrophils can also be tested in these cells, as per Cronstein et al. (1995).

Since different dosages of colchicine are used for prophylaxis and treatment of acute gout flare, different mechanisms of action might be responsible for these two effects (Cronstein et al., 1995; Nuki, 2008). In our in vivo studies, we have been looking at the prophylactic effects of colchicine, since colchicine is administered in tandem with MSU crystals. In future studies, it will be of interest to test whether Lfc is necessary for the curative effects of colchicine in gout flare, i.e. after the effects of MSU crystal-induced inflammation have set in. To test this, colchicine will need to be administered to Lfc$^{+/+}$ and Lfc$^{-/-}$ mice after MSU crystal-induced inflammation has peaked, and the curative effects monitored at later time points.

Colchicine has been used to treat gout for over 1500 years, and is one of the first known pharmaceutical agents used by mankind to treat a specific medical condition. We have uncovered novel and unexpected results, which establish a new working model for the mechanism of action of this drug, and lay the groundwork for future studies.
References


Appendix

Appendix A: Supplemental video legends

Supplemental video’s can be found online at T-Space (https://tspace.library.utoronto.ca/).

Video 1: Chemokinesis of Lfc\(^{+/+}\) BMMs. Lfc\(^{+/+}\) BMMs were starved for 5 hours and treated with media containing 10% CMG-14-12 immediately prior to time-lapse imaging. Cells were imaged for 1 hour using phase contrast microscopy at 10x magnification.

Video 2: Chemokinesis of Lfc\(^{-/-}\) BMMs. Lfc\(^{-/-}\) BMMs were starved for 5 hours and treated with media containing 10% CMG-14-12 immediately prior to time-lapse imaging. Cells were imaged for 1 hour using phase contrast microscopy at 10x magnification.

Video 3: Chemotaxis of Lfc\(^{+/+}\) peritoneal macrophages. Lfc\(^{+/+}\) peritoneal macrophages were plated in \(\mu\)-chemotaxis slides and exposed to a shallow gradient of C5a. Cell motion was imaged for 10 hours using differential interference contrast (DIC) time-lapse video-microscopy at 5x magnification. The direction of the chemotactic gradient is indicated.

Video 4: Chemotaxis of Lfc\(^{-/-}\) peritoneal macrophages. Lfc\(^{-/-}\) peritoneal macrophages were plated in \(\mu\)-chemotaxis slides and exposed to a shallow gradient of C5a. Cell motion was imaged for 10 hours using differential interference contrast (DIC) time-lapse video-microscopy at 5x magnification. The direction of the chemotactic gradient is indicated.

Video 5: The contractile response of Lfc\(^{+/+}\) BMMs to microtubule depolymerization. Time-lapse imaging of Lfc\(^{+/+}\) BMMs was performed for 5 minutes in the absence of stimulus, followed by an additional 10 minutes upon stimulation with 10 \(\mu\)M nocodazole. Cells were imaged by phase contrast microscopy at 32x magnification.

Video 6: The contractile response of Lfc\(^{-/-}\) BMMs to microtubule depolymerization. Time-lapse imaging of Lfc\(^{-/-}\) BMMs was performed for 5 minutes in the absence of stimulus, followed by an additional 10 minutes upon stimulation with 10 \(\mu\)M nocodazole. Cells were imaged by phase contrast microscopy at 32x magnification.
**Video 7: The contractile response of Lfc<sup>+/+</sup> neutrophils to microtubule depolymerization.**
Lfc<sup>+/+</sup> neutrophils were imaged for 1 minute in the absence of stimulus, followed by an additional 5 minutes upon stimulation with 10 µM nocodazole. Cells were imaged by phase contrast microscopy at 63x magnification.

**Video 8: The contractile response of Lfc<sup>+/+</sup> neutrophils to microtubule depolymerization.**
Lfc<sup>+/+</sup> neutrophils were imaged for 5 minutes, 25 minutes after stimulation with 10 µM nocodazole. Cells were imaged by phase contrast microscopy at 63x magnification.

**Video 9: The contractile response of Lfc<sup>−/−</sup> neutrophils to microtubule depolymerization.**
Lfc<sup>−/−</sup> neutrophils were imaged for 1 minute in the absence of stimulus, followed by an additional 5 minutes upon stimulation with 10 µM nocodazole. Cells were imaged by phase contrast microscopy at 63x magnification.

**Video 10: The contractile response of Lfc<sup>−/−</sup> neutrophils to microtubule depolymerization.**
Lfc<sup>−/−</sup> neutrophils were imaged for 5 minutes, 25 minutes after stimulation with 10 µM nocodazole. Cells were imaged by phase contrast microscopy at 63x magnification.