CCL5-CCR5 Interactions in Breast Cancer Regulate Cell Metabolism

by

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

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Degree of Doctor of Philosophy, 2017

Graduate Department of Immunology

University of Toronto

Chemokines are chemotactic cytokines that recruit and activate leukocytes. It is now apparent that they also participate actively in the neoplastic process in cancer. This thesis describes the importance of the activation of CCR5 by the inflammatory chemokine CCL5/RANTES in the context of cancer metabolism. Specifically, CCL5-CCR5 interactions promote anabolic metabolism in breast cancer cells and enhance cell proliferation. CCL5 activation of CCR5 invokes the mTOR/AKT signaling pathway and, within minutes, induces phosphorylation of downstream substrates, including GSK-3β and 4E-BP1. Using a panel of CCR5-expressing breast cancer cells, we show that CCL5 also increases surface GLUT-1 expression, glucose uptake, ATP production and the glycolytic capacity of these tumor cells. Using metabolomics, we demonstrate that the metabolic signature of primary mouse mammary tumor cells that express CCR5 is altered by CCL5 treatment and reflects enhanced anabolic metabolism, resulting in the accumulation of biosynthetic precursors. Concomitant with enhanced anabolic metabolism, we show that CCL5 increases the proliferation and invasive capacity of breast cancer cells. Moreover, in an immune-deficient tumor transplant mouse model, we identify a direct correlation between reduced tumor proliferation and decreased metabolic activity, specifically associated with the absence of CCR5 expression by tumor cells. Using Matrix-Assisted Laser Desorption/Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry imaging, we provide evidence that the rapid early
growth of CCR5\(^{+/+}\) triple negative breast cancer cells \textit{in vivo} is attributable to increased levels of glycolytic intermediates required for anabolic processes, in contrast to the slower growth rate of their corresponding CCR5\(^{-/-}\) cells, that exhibit reduced glycolytic metabolism. Collectively, these studies suggest that CCL5-CCR5 interactions in the tumor microenvironment modulate metabolic events during tumor onset to promote tumorigenesis.
ACKNOWLEDGEMENTS

Thank you to all those who have made this possible:

Eleanor for her guidance and my colleagues and friends for their inspiration and support.

My parents for raising me with love and sacrifice and for being my most important teachers

and

Tracy for being the rock of my life.

I am now ready to embark on a new adventure.

I took the one less traveled by,
And that has made all the difference.

Robert Frost
## TABLE OF CONTENTS

- Title Page ........................................................................................................................................... i
- Abstract ................................................................................................................................................ ii-iii
- Acknowledgements ............................................................................................................................... iv
- Table of Contents ..................................................................................................................................... v-vii
- List of Figures ......................................................................................................................................... viii-ix
- List of Tables .......................................................................................................................................... x
- List of Abbreviations ............................................................................................................................. xi-xv

### CHAPTER 1: Introduction .................................................................................................................... 1-57

1.1. Chemokines & Chemokine Receptors ............................................................................................. 2-15
    1.1.1. Classification ............................................................................................................................ 2-5
    1.1.2. Atypical Chemokine Receptors ............................................................................................ 6-7
    1.1.3. Structure .................................................................................................................................... 7-13
    1.1.4. Ligand-Receptor Binding ........................................................................................................ 13-14
    1.1.5. Chemokine-mediated Signal Transduction ........................................................................... 14-15

1.2. Chemokines & Chemokine Receptors Regulate Cell Functions ..................................................... 16-28
    1.2.1. Immune Cell Activation and Survival .................................................................................... 16-17
    1.2.2. Chemotaxis ............................................................................................................................... 17-18
    1.2.3. Signaling through the PI3K / AKT Pathway ......................................................................... 19-21
    1.2.4. mTOR Activation and Metabolic Regulation ......................................................................... 21-28

1.3. Chemokines & Chemokine Receptors in Cancer ............................................................................ 29-41
    1.3.1. Leukocyte Tumor Infiltration ................................................................................................. 29-37
        1.3.1.1. Tumor Infiltrating Lymphocytes .................................................................................... 32-33
        1.3.1.2. Innate Lymphoid Cells ................................................................................................. 33-35
        1.3.1.3. Tumor-associated Macrophages .................................................................................... 35-36
        1.3.1.4. Myeloid-derived Suppressor Cells ............................................................................... 36-37
    1.3.2. Tumorigenesis, Angiogenesis and Metastasis ......................................................................... 37-41
        1.3.2.1. Chemokines and Tumorigenesis .................................................................................. 38-39
        1.3.2.2. Chemokines and Angiogenesis .................................................................................... 39-40
        1.3.2.3. Chemokines and Metastasis .......................................................................................... 40-41

1.4. Cancer Metabolism and Potential Therapy ...................................................................................... 42-56
    1.4.1. Glucose and Glutamine Metabolism ....................................................................................... 42-48
    1.4.2. The Warburg Effect ................................................................................................................. 48-49
    1.4.3. Microenvironment Acidification and Hypoxia ....................................................................... 50-51
    1.4.4. Therapy Targeting Cancer Metabolism .................................................................................. 52-54
    1.4.5. Cytokines and Growth Factors Affecting Metabolism .......................................................... 54-56

1.5. Hypothesis and Objectives ............................................................................................................... 57
CHAPTER 2: Materials & Methods ................................................................. 58-67

2.1. Mice ........................................................................................................59
2.2. Cells and reagents ..................................................................................60
2.3. Immunoblotting & immunoprecipitation .................................................61
2.4. Fluorescence-activated cell sorting analysis ............................................61
2.5. Glucose uptake assay ..............................................................................61
2.6. ATP bioluminescent assay .....................................................................62
2.7. Glucose 6-phosphate, pyruvate & lactate assay .......................................62
2.8. Glycolytic stress test using the Seahorse Extracellular Flux Analyzer .......62-63
2.9. Proliferation assay ...................................................................................63
   2.9.1. Hemocytometer ................................................................................63
   2.9.2. MTT .................................................................................................63
2.10. Chemotaxis assay ................................................................................63-64
2.11. Invasion assay ......................................................................................64
2.12. Metabolomic profiling ..........................................................................64
2.13. ELISA ...................................................................................................65
2.14. Hematoxylin & Eosin staining of thin sections .........................................65
   2.15.1. Materials ........................................................................................65
   2.15.2. Tissue preparation for MALDI-FTICR-MS imaging .......................65-66
   2.15.3. MALDI-FTICR-MS imaging data acquisition ..................................66
   2.15.4. Discriminate metabolite analysis .....................................................66-67
2.16. Statistical analyses ...............................................................................67

CHAPTER 3: CCL5 activation of CCR5 regulates cell metabolism to enhance proliferation of breast cancer cells ...................................................... 68-113

3.1. Abstract ...................................................................................................69
3.2. Introduction .............................................................................................70-71
3.3. Results ...................................................................................................72-108
   3.3.1. CCL5 increases glucose uptake, GLUT-1 expression and glycolysis ........................................................................................................72-92
   3.3.2. Metabolic signature of CCL5-treated cancer cells reflects increased anabolic metabolism .................................................................92-101
   3.3.3. CCL5 increases the proliferation, migration and invasive capacity of breast cancer cells .................................................................102-108
3.4. Discussion ..............................................................................................109-113

CHAPTER 4: CCL5-CCR5 interactions modulate metabolic events during tumor onset to promote tumorigenesis ..................................................... 114-149

4.1. Abstract ..................................................................................................115
4.2. Introduction ............................................................................................116-117
4.3. Results ...................................................................................................118-143
4.4. Discussion ..............................................................................................144-148
4.5. Conclusion .............................................................................................149
CHAPTER 5: Discussion and Future Directions ........................................... 150-163

5.1. CCL5-CCR5 Interactions Regulate Cell Metabolism ............................... 151-155
5.2. CCL5-CCR5 in Diseases ............................................................................. 155-158
5.3. Targeting CCL5-CCR5 Interactions in Cancer ........................................... 158-161
5.4. Cancer Metabolism Therapeutics ............................................................. 162-163

CHAPTER 6: References .................................................................................. 164-188
LIST OF FIGURES

CHAPTER 1

Figure 1.1. Similar structural elements are shared among chemokines................. 8-9

Figure 1.2. Two-dimensional representation of CCR5 and domains responsible for
ligand binding and signal transduction................................................. 11-12

Figure 1.3. CCL5 and the PI3K/AKT/mTOR pathway .................................. 22-23

Figure 1.4. mTOR phosphorylation of 4E-BP1 promotes protein translation ..... 27-28

Figure 1.5. Chemokines modulate tumor microenvironment................................. 30-31

Figure 1.6. The switch towards aerobic glycolysis depends heavily on the glycolytic
pathway................................................................................................. 44-45

CHAPTER 3

Figure 3.1. CCL5 treatment activates the mTOR/AKT pathway in breast cancer cells
................................................................................................................. 73-74

Figure 3.2. CCL5 treatment activates 4E-BP1 downstream of the AKT/mTOR
pathway in breast cancer cells................................................................. 75-76

Figure 3.3. MDA-MB-231, MCF-7 and MMTV-PyMT express CCR5 ............. 77-78

Figure 3.4. CCL5 treatment increases glucose uptake in breast cancer cells mediated
by CCR5 and mTOR .............................................................................. 80-81

Figure 3.5. Time course of glucose uptake in CCL5 treated MDA-MB-231...... 82-83

Figure 3.6. CCL5 treatment increases GLUT-1 expression ......................... 85-86

Figure 3.7. CCL5 increases rate of glycolysis and cellular glycolytic capacity... 87-88

Figure 3.8. CCL5 treatment increases ATP and glycolytic intermediate flux..... 89-91

Figure 3.9. Metabolic signature of CCL5-treated MMTV-PyMT primary mouse
breast cancer cells reflects increased anabolic metabolism............ 93-95

Figure 3.10. Heat maps reveal a metabolic signature in CCL5-treated MMTV-PyMT
primary mouse breast cancer cells......................................................... 96-97

Figure 3.11. CCL5 treatment results in accumulation of metabolites .......... 98-100

Figure 3.12. CCL5 treatment enhances cell proliferation and invasion ........ 103-104

Figure 3.13. CCL5 induces chemotaxis in MDA-MB-231 ......................... 105-106
CHAPTER 4

**Figure 4.1.** MMTV-PyMT.CCR5\(^{+/−}\) mice have delayed tumor onset and increased tumor free survival compared to MMTV-PyMT.CCR5\(^{+/+}\) mice. 119-120

**Figure 4.2.** MMTV-PyMT.CCR5\(^{+/−}\) tumor cells transplanted into NSG mice exhibit delayed tumor growth compared with MMTV-PyMT.CCR5\(^{+/+}\) tumor cells. 122-124

**Figure 4.3.** MMTV-PyMT.CCR5\(^{+/−}\) tumor cells harvested from NSG mice exhibit lower glucose uptake, reduced GLUT-1 expression and lower levels of cellular metabolites than MMTV-PyMT.CCR5\(^{+/+}\) tumor cells. 127-128

**Figure 4.4.** Generation of MDA-MB-231.CCR5\(^{+/−}\) using CRISPR/Cas9. 129-130

**Figure 4.5.** *In vitro*, MDA-MB-231.CCR5\(^{+/−}\) cells are less metabolically active than MDA-MB-231.CCR5\(^{+/+}\) cells. 131-133

**Figure 4.6.** MDA-MB-231.CCR5\(^{+/−}\) cells transplanted in NSG mice exhibit delayed tumor growth compared with MDA-MB-231.CCR5\(^{+/+}\) cells. 135-136

**Figure 4.7.** MDA-MB-231.CCR5\(^{+/−}\) tumor cells harvested from NSG mice exhibit lower glucose uptake, reduced GLUT-1 expression and lower levels of cellular metabolites than MDA-MB-231.CCR5\(^{+/+}\) tumor cells. 137-138

**Figure 4.8.** MALDI-FTICR-MSI analysis confirms that MDA-MB-231.CCR5\(^{+/−}\) cells are less metabolically active than MDA-MB-231.CCR5\(^{+/+}\) cells. 141-143

CHAPTER 5

**Figure 5.1.** Therapies targeting cancer metabolism. 160-161
LIST OF TABLES

CHAPTER 1

Table 1.1. The Classification of Chemokines and Chemokine Receptors.............. 3-4

CHAPTER 3

Table 3.1. CCL5 treatment results in accumulation of metabolites in MMTV-PyMT primary mouse breast cancer cells. ......................................................... 107-108
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>2-DG</td>
<td>2-Deoxy-D-glucose</td>
</tr>
<tr>
<td>2-NBDG</td>
<td>2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose</td>
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<td>3PO</td>
<td>3-(3-Pyridinyl)-1-(4-pyridinyl)-2-propen-1-one</td>
</tr>
<tr>
<td>4E-BP</td>
<td>4E-binding protein</td>
</tr>
<tr>
<td>9-AA</td>
<td>9-Aminoacridine hydrochloride</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ACKR</td>
<td>Atypical chemokine receptor</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AICD</td>
<td>Activation induced cell death</td>
</tr>
<tr>
<td>Aldo</td>
<td>Aldolase</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine 5’-monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>APAF</td>
<td>Apoptotic protease activating factor</td>
</tr>
<tr>
<td>Arp2/3</td>
<td>Actin-related proteins 2/3</td>
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<tr>
<td>ATP</td>
<td>Adenosine 5’-triphosphate</td>
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<td>BCAA</td>
<td>Branched-chain amino acids</td>
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<td>Bcl</td>
<td>B cell lymphoma</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BHBA</td>
<td>3-Hydroxybutyrate</td>
</tr>
<tr>
<td>CAF</td>
<td>Cancer-associated fibroblast</td>
</tr>
<tr>
<td>CCL5</td>
<td>CC chemokine ligand 5</td>
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<td>CCR5</td>
<td>CC chemokine receptor 5</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>Cdc42</td>
<td>Cell division cycle 42</td>
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<tr>
<td>c-Myc</td>
<td>Cellular-myelocytomatosis virus oncogene</td>
</tr>
<tr>
<td>CRISPR/Cas9</td>
<td>Clustered regularly interspaced short palindromic repeats/Cas9</td>
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<tr>
<td>C-terminus</td>
<td>Carboxyl-terminus</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CXCL</td>
<td>CX3C chemokine ligand</td>
</tr>
<tr>
<td>CXCR</td>
<td>CX3C chemokine receptor</td>
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<tr>
<td>CXCL</td>
<td>CXC chemokine ligand</td>
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<td>CXC chemokine receptor</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DCA</td>
<td>Dichloroacetic acid</td>
</tr>
<tr>
<td>DEPTOR</td>
<td>DEP domain containing mTOR interacting protein</td>
</tr>
<tr>
<td>DHAP</td>
<td>Dihydroxyacetone phosphate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
</tr>
<tr>
<td>DNA-PK</td>
<td>DNA-dependent protein kinase</td>
</tr>
<tr>
<td>DSS</td>
<td>Dextran sodium sulphate</td>
</tr>
<tr>
<td>ECAR</td>
<td>Extracellular acidification rate</td>
</tr>
<tr>
<td>ECL</td>
<td>Extra-cellular loop</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetra-acetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis (2-aminoethylether)-N’N’N’N’-tetra-acetic acid</td>
</tr>
<tr>
<td>eIF</td>
<td>Eukaryotic translation initiation factor</td>
</tr>
<tr>
<td>ELR</td>
<td>Glutamate-Leucine-Arginine</td>
</tr>
<tr>
<td>Eno</td>
<td>Enolase</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-related kinase</td>
</tr>
<tr>
<td>F1,6BP</td>
<td>Fructose 1,6-bisphosphate</td>
</tr>
<tr>
<td>F2,6BP</td>
<td>Fructose 2,6-bisphosphate</td>
</tr>
<tr>
<td>F6P</td>
<td>Fructose 6-phosphate</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorter</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FDG-PET</td>
<td>$^{18}$Fluoro-deoxyglucose-position emission tomography</td>
</tr>
<tr>
<td>GFF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FKBP12</td>
<td>FK506-binding protein of 12kDa</td>
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<tr>
<td>G6P</td>
<td>Glucose 6-phosphate</td>
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<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GC/MS</td>
<td>Gas chromatography/mass spectrometry</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanidined nucleotide exchange factor</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GRK</td>
<td>G-protein receptor kinase</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase 3</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
</tr>
<tr>
<td>HER</td>
<td>Human epidermal growth factor receptor</td>
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<td>HIF-1</td>
<td>Hypoxia-inducible factors</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HK</td>
<td>Hexokinase</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ILC</td>
<td>Innate lymphoid cell</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
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<tr>
<td>IP3</td>
<td>Inositol 1,4,5-phosphate</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosomal entry segment</td>
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<td>IRS</td>
<td>Insulin receptor substrate</td>
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<tr>
<td>Jak</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
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</tbody>
</table>
KO  Knock-out
LDH  Lactate dehydrogenase
LPS  Lipopolysaccharide
LTi  Lymphoid tissue inducer
MALDI-FTICR-MS  Matrix-assisted laser desorption/ionization Fourier transform ion cyclotron resonance mass spectrometry
MAPK  Mitogen-activated protein kinase
MCP  Macrophage chemo-attractant protein
M-CSF  Macrophage colony-stimulating factor
MCT  Monocarboxylate transporter
MDSC  Myeloid-derived suppressor cells
Met-CCL5  Methionine-CC chemokine ligand 5
MHC  Major histocompatibility complex
MICA  MHC class I polypeptide-related sequence A
MIP  Macrophage inflammatory protein
MLST8  Mammalian lethal with SEC13 protein 8
MMP  Matrix metalloproteinase
MMTV-PyMT  Mouse mammary tumor virus polyomavirus middle T antigen
mRNA  Messenger RNA
MSC  Mesenchymal stem cells
MSIN1  Mammalian stress-activated protein kinase interacting protein 1
mTOR  Mammalian target of rapamycin
mTORC1  Mammalian target of rapamycin complex 1
MTORC2  Mammalian target of rapamycin complex 2
MTT  3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH  Nicotinamide adenine dinucleotide phosphate
NF-κB  Nuclear factor-kappa B
NK  Natural killer
nM  Nanomolar
NMR  Nuclear magnetic resonance
NP-40  Nonidet-40
NSG  NOD scid gamma
N-terminus  Amino-terminus
OxPhos  Oxidative phosphorylation
p38  38kDa stress-activated kinase
PBS  Phosphate buffered saline
PCR  Polymerase chain reaction
PDH  Pyruvate dehydrogenase
PDK  Phosphoinositide-dependent kinase
PFK  Phosphofructokinase
PGI  Phosphoglucone isomerase
PGK  Phosphoglycerate kinase
PGM  Phosphoglycerate mutase
PH  Pleckstrin homology
PI3K  Phosphatidylinositol 3-kinase
PIKK  Phosphoinositide kinase-related kinase
PIP2  Phosphatidylinositol 4,5-bisphosphate
PIP3  Phosphatidylinositol 3,4,5-phosphate
<table>
<thead>
<tr>
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<th>Full Form</th>
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<tr>
<td>PK</td>
<td>Pyruvate kinase</td>
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<tr>
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<td>Protein kinase C</td>
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<td>PKR</td>
<td>Pyruvate kinase R</td>
</tr>
<tr>
<td>PLCβ</td>
<td>Phospholipase Cβ</td>
</tr>
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<td>PMSF</td>
<td>Phenylmethylsulfonylflouride</td>
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<td>PPP</td>
<td>Pentose phosphate pathway</td>
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<td>PR</td>
<td>Progesterone receptor</td>
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<td>Proline-rich AKT1 substrate 1</td>
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<td>PTEN</td>
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<td>pTx</td>
<td>Pertussis toxin</td>
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<td>S-2-amino-3-[4’-N,N,-bis(2-chloroethyl)amino]phenyl propionic acid N-oxide dihydrochloride</td>
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<td>Rac</td>
<td>Ras-related C3 botulinum toxin substrate</td>
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<td>RANTES</td>
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<td>Raptor</td>
<td>Regulatory associated protein of mTOR</td>
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<td>Ras-homolog enriched in brain</td>
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<td>ROR</td>
<td>RAR-related orphan receptors</td>
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<td>S6 kinase</td>
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<td>Stromal derived factor</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SH2</td>
<td>Src-homology 2</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small (or short) interfering RNA</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TAA</td>
<td>Tumor-associated antigen</td>
</tr>
<tr>
<td>TAK-779</td>
<td>N,N-dimethyl-N-[[2-(4-methylphenyl)-6,7-dihydro-5H-benzocyclohepten-8-yl]amino]benzyl]-tetrahydro-2H-pyran-4-aminium chloride</td>
</tr>
<tr>
<td>TAM</td>
<td>Tumor associated macrophages</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TFF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TIL</td>
<td>Tumor infiltrating T lymphocytes</td>
</tr>
<tr>
<td>TNBC</td>
<td>Triple negative breast cancer</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>TOP</td>
<td>Tract of oligopyrimidines</td>
</tr>
<tr>
<td>TPI</td>
<td>Triose phosphate isomerase</td>
</tr>
<tr>
<td>Tregs</td>
<td>Regulatory T cells</td>
</tr>
<tr>
<td>TSC</td>
<td>Tuberous sclerosis complex</td>
</tr>
<tr>
<td>TXP</td>
<td>Threonine-X-Proline</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>-----------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>UHPLC/MS</td>
<td>Ultrahigh performance liquid chromatography/tandem mass spectrometry</td>
</tr>
<tr>
<td>ULBP</td>
<td>UL16 binding protein 1</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>VDAC</td>
<td>Voltage-dependent anion channel</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WHIM</td>
<td>Warts, hypogammaglobulinemia, immunodeficiency, and myelokathexis</td>
</tr>
<tr>
<td>XCL</td>
<td>XC chemokine ligand</td>
</tr>
<tr>
<td>XCR</td>
<td>XC chemokine receptor</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction
1.1. CHEMOKINES & CHEMOKINE RECEPTORS

1.1.1. Classification

Chemokines are soluble, low molecular weight (8-14kDa) signaling proteins that bind and activate their cognate seven trans-membrane G-protein coupled receptors (GPCRs) to induce cell migration or chemotaxis (Wong & Fish, 2003). Since the discovery of the first chemokine in 1977 (Walz et al., 1977), over 40 human chemokines have since been identified (Table 1.1). They are divided into four sub-families based on the location of their first two cysteine residues found at the N-terminus. The CXC or α family of chemokines has two cysteine molecules separated by one non-conserved amino acid, while the cysteines in the CX3C or δ chemokines are separated by three non-conserved amino acids. The XC or γ family lacks the first consensus cysteine, whereas the CC or β family chemokines have two adjacent cysteines at the N-terminus. In 2000, a new system of nomenclature was introduced that assigns each ligand and receptor an identification number followed by their sub-family name (Murphy et al., 2000). For example, the chemokine growth-regulated protein β (Groβ) has since been known as CXCL2, which indicates CXC chemokine ligand 2, and RANTES (Regulated on Activation Normal T cell Expressed and Secreted) is now classified as CCL5, for CC chemokine ligand 5. Throughout this thesis, chemokine ligands and receptors will be referred to using the newer nomenclature. Their corresponding original names can be found in Table 1.1.

Chemokines can also be classified based on their function. Homeostatic chemokines are constitutively produced and are involved in normal leukocyte development and migration, while inflammatory chemokines are induced by pro-inflammatory stimuli and actively attract immune cells to the site of inflammation (Proudfoot, 2002). Most chemokines are secreted,
Table 1.1. The Classification of Chemokines and Chemokine Receptors
<table>
<thead>
<tr>
<th>CXC Chemokines</th>
<th>Alternate Names</th>
<th>Receptor(s)</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL1</td>
<td>Gro-a</td>
<td>CXCR2, ACKR1</td>
<td>Inducible</td>
</tr>
<tr>
<td>CXCL2</td>
<td>Gro-β</td>
<td>CXCR2, ACKR1</td>
<td>Inducible</td>
</tr>
<tr>
<td>CXCL3</td>
<td>Gro-γ</td>
<td>CXCR2, ACKR1</td>
<td>Inducible</td>
</tr>
<tr>
<td>CXCL4</td>
<td>PF-4</td>
<td>CXCR3β</td>
<td>Inducible</td>
</tr>
<tr>
<td>CXCL5</td>
<td>ENA-78</td>
<td>CXCR2, ACKR1</td>
<td>Inducible</td>
</tr>
<tr>
<td>CXCL6</td>
<td>GCP-2</td>
<td>CXCR1, CXCR2, ACKR1</td>
<td>Inducible</td>
</tr>
<tr>
<td>CXCL7</td>
<td>NAP-2</td>
<td>CXCR2</td>
<td>Inducible</td>
</tr>
<tr>
<td>CXCL8</td>
<td>IL-8</td>
<td>CXCR1, CXCR2, ACKR1</td>
<td>Inducible</td>
</tr>
<tr>
<td>CXCL9</td>
<td>MIG</td>
<td>CXCR3, ACKR1</td>
<td>Dual-function</td>
</tr>
<tr>
<td>CXCL10</td>
<td>IP-10</td>
<td>CXCR3, ACKR1</td>
<td>Dual-function</td>
</tr>
<tr>
<td>CXCL11</td>
<td>I-TAC</td>
<td>CXCR3, ACKR1, ACKR3</td>
<td>Dual-function</td>
</tr>
<tr>
<td>CXCL12</td>
<td>SDF-1</td>
<td>CXCR4, ACKR3</td>
<td>Constitutive</td>
</tr>
<tr>
<td>CXCL13</td>
<td>BCA-1</td>
<td>CXCR5, ACKR1, ACKR4</td>
<td>Constitutive</td>
</tr>
<tr>
<td>CXCL14</td>
<td>BRAK</td>
<td></td>
<td>Constitutive</td>
</tr>
<tr>
<td>CXCL15</td>
<td>WECHE</td>
<td></td>
<td>Constitutive</td>
</tr>
<tr>
<td>CXCL16</td>
<td>SRPSOX</td>
<td>CXCR6</td>
<td>Dual-function</td>
</tr>
<tr>
<td>CXCL17</td>
<td>DMC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CC Chemokines</th>
<th>Alternate Names</th>
<th>Receptor(s)</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL1</td>
<td>I-309</td>
<td>CCR8, ACKR1</td>
<td>Dual-function</td>
</tr>
<tr>
<td>CCL2</td>
<td>MCP-1</td>
<td>CCR2, ACKR1, ACKR2</td>
<td>Inducible</td>
</tr>
<tr>
<td>CCL3</td>
<td>MIP-1α</td>
<td>CCR1, ACKR2</td>
<td>Inducible</td>
</tr>
<tr>
<td>CCL4</td>
<td>MIP-1β</td>
<td>CCR1, CCR5, ACKR2</td>
<td>Inducible</td>
</tr>
<tr>
<td>CCL5</td>
<td>RANTES</td>
<td>CCR5, ACKR1, ACKR2</td>
<td>Inducible</td>
</tr>
<tr>
<td>CCL6 (mice)</td>
<td>MRP-1</td>
<td>CCR1</td>
<td>Inducible</td>
</tr>
<tr>
<td>CCL7</td>
<td>MCP-3</td>
<td>CCR2, ACKR1, ACKR2</td>
<td>Inducible</td>
</tr>
<tr>
<td>CCL8</td>
<td>MCP-2</td>
<td>CCR1, CCR2B, CCR5, ACKR1, ACKR2</td>
<td>Inducible</td>
</tr>
<tr>
<td>CCL9/CCL10 (mice)</td>
<td>MRP-2</td>
<td>CCR1</td>
<td>Constitutive</td>
</tr>
<tr>
<td>CCL11</td>
<td>Eotaxin</td>
<td>CCR2, CCR3, CCR5, ACKR1, ACKR2</td>
<td>Inducible</td>
</tr>
<tr>
<td>CCL12 (mice)</td>
<td>MCP-5</td>
<td>CCR2, CCR3, CCR5, ACKR1, ACKR2</td>
<td>Inducible</td>
</tr>
<tr>
<td>CCL13</td>
<td>MCP-4</td>
<td>CCR2, CCR3, CCR5, ACKR1, ACKR2</td>
<td>Inducible</td>
</tr>
<tr>
<td>CCL14</td>
<td>HCC-1</td>
<td>CCR1, ACKR1, ACKR2</td>
<td>Inducible</td>
</tr>
<tr>
<td>CCL15</td>
<td>HCC-2</td>
<td>CCR1, CCR3</td>
<td>Inducible</td>
</tr>
<tr>
<td>CCL16</td>
<td>HCC-4</td>
<td>CCR1, CCR2, CCR5, CCR8, ACKR1</td>
<td>Dual-function</td>
</tr>
<tr>
<td>CCL17</td>
<td>TARC</td>
<td>CCR4, ACKR1, ACKR2</td>
<td>Dual-function</td>
</tr>
<tr>
<td>CCL18</td>
<td>PARC</td>
<td>ACKR1</td>
<td>Constitutive</td>
</tr>
<tr>
<td>CCL19</td>
<td>ELC</td>
<td>CCR7, ACRK4</td>
<td>Constitutive</td>
</tr>
<tr>
<td>CCL20</td>
<td>LARC</td>
<td>CCR6</td>
<td>Dual-function</td>
</tr>
<tr>
<td>CCL21</td>
<td>SLC</td>
<td>CCR7, ACRK4</td>
<td>Constitutive</td>
</tr>
<tr>
<td>CCL22</td>
<td>MDC</td>
<td>CCR4, ACKR2</td>
<td>Dual-function</td>
</tr>
<tr>
<td>CCL23</td>
<td>MPIF-1</td>
<td>CCR1</td>
<td>Constitutive</td>
</tr>
<tr>
<td>CCL24</td>
<td>Eotaxin-2</td>
<td>CCR3, ACKR2</td>
<td>Inducible</td>
</tr>
<tr>
<td>CCL25</td>
<td>TECK</td>
<td>CCR9, ACRK4</td>
<td>Dual-function</td>
</tr>
<tr>
<td>CCL26</td>
<td>Eotaxin-3</td>
<td>CCR3, ACKR2</td>
<td>Inducible</td>
</tr>
<tr>
<td>CCL27</td>
<td>CTACK</td>
<td>CCR10</td>
<td>Inducible</td>
</tr>
<tr>
<td>CCL28</td>
<td>MEC</td>
<td>CCR3, CCR10</td>
<td>Inducible</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C Chemokines</th>
<th>Alternate Names</th>
<th>Receptor(s)</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>XCL1</td>
<td>SCM-1α</td>
<td>XCR1</td>
<td>Inducible</td>
</tr>
<tr>
<td>XCL2</td>
<td>SCM-1β</td>
<td>XCR1</td>
<td>Inducible</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CX₃C Chemokine</th>
<th>Alternate Names</th>
<th>Receptor(s)</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>CX₃CL1</td>
<td>Fractalkine</td>
<td>CX₃CR1</td>
<td>Inducible</td>
</tr>
</tbody>
</table>
except CX3CL1 and CXCL16, which are normally membrane-bound proteins, but can also be released in soluble form after the proteolytic cleavage of their transmembrane tether (Bazan et al., 1997). Interestingly, only 19 unique GPCRs have been identified for the 47 known chemokines, which suggests that the chemokine system is highly redundant. The high degree of redundancy exists likely to facilitate the fine-tuning of specific biological signaling events and to ensure the robustness of the system in the event of any random mutations. For example, CCR5−/− mice develop a fully sufficient immune system, suggestive that the lack of CCR5 may be compensated by other receptors (Zhou et al., 1998).

There are currently 19 chemokine receptors in addition to 4 atypical chemokine receptors that have been described (Table 1.1). All chemokines induce their physiological functions through binding and activating their cognate GPCR. Similar to chemokines, chemokine receptors are also classified according to the sub-family of the corresponding chemokines (Murphy et al., 2000). For example, CXC chemokines would bind to CXCR or CXC receptors. In particular, CC chemokine receptor 5, or CCR5, has 352 amino acids and a molecular mass of 40.6kDa and can be activated by CCL3, CCL4, CCL5 and CCL8. There is a high degree of similarity between CCR5 and CCR2, as 71% of their amino acids are identical (Appay et al., 2001; Raport et al., 1996). There are several identified non-functional variants of CCR5; the most widely found is the CCR5Δ32 mutation, which has a frequency of 10% amongst Europeans. This mutation results in a truncated CCR5, which prevents it from being expressed on the surface of the cell. Many forms of the human immunodeficiency virus (HIV) use CCR5 as a co-receptor in addition to CD4 for viral entry. Individuals with the CCR5Δ32 mutation are known to be resistant to these strains of HIV (Samson et al., 1996; Wang et al., 1997).
1.1.2. Atypical Chemokine Receptors

Chemokine receptors can be further categorized into the conventional GPCRs and the atypical chemokine receptors (ACKR). There are currently 4 members of ACKRs that are characterized: ACKR1, ACKR2, ACKR3 and ACKR4 (Bachelerie et al., 2014). They were previously referred to as decoy or orphan receptors, but have since been named “atypical” due to their alternative downstream signaling pathways compared to their conventional counterparts. Accumulating evidence suggests that ACKRs do not function to promote chemotaxis but rather modulate the chemokine gradient through binding and sequestering free-flowing chemokine from the microenvironment (Bonecchi et al., 2016). ACKRs lack a functional G-protein-binding domain at the C-terminus, but they have been shown to play a critical regulatory role in both development and diseases by signaling through G-protein-independent pathways (Massara et al., 2016).

ACKRs differ in their function and binding targets. ACKR1 and ACKR2 bind inflammatory chemokines almost exclusively, including CCL2, CCL3 and CCL5 (Bonecchi et al., 2016; Ford et al., 2014), while ACKR4 binds mostly homeostatic chemokines, like CCL19, CCL21 and CCL25 (Bryce et al., 2016; Vacchini et al., 2016). ACKR3 binds an array of both chemokine and nonchemokine ligands, with varying downstream effects (Gustavsson et al., 2017). ACKRs are usually poorly expressed on hematopoietic cells and are mainly found on endothelial and epithelial cells at barrier sites, including the skin, gut and brain (Vacchini et al., 2016).

ACKRs also play an important role in regulating the chemokine system in the context of cancer biology. ACKR1 is constitutively expressed on erythrocytes and endothelial cells.
It functions to decrease angiogenesis and inflammation which inhibit tumorigenesis (Graham et al., 2012). Similar to ACKR1, ACKR2 scavenges and binds to inflammatory chemokines and prevents leukocyte recruitment. ACKR3 expression on tumor cells has been linked to increased proliferation, apoptosis inhibition and accelerated cell cycling (Massara et al., 2016). Interestingly, ACKR3 expressed on endothelial cells has been shown to prevent cancer metastasis by regulating the bioavailability of CXCL12 (Massara et al., 2016). ACKR4 can also negatively regulate tumor cells by blocking pro-proliferative chemokines from binding their cognate receptors (Vacchini et al., 2016). Given their prominent role in cancer regulation, a deeper understanding of the biological consequences between the interactions of ACKRs and GPCRs may be very useful in identifying effective therapies against cancer.

1.1.3. Structure

Despite having a low level of amino acid sequence identity, the structure of chemokines is highly homologous. Using nuclear magnetic resonance (NMR) and X-ray crystallography, it was observed that the three-dimensional structure of CCL5, CCL2, CCL3, CCL4 and CXCL8 have the same “chemokine fold”, which consists of three anti-parallel β-strands overlaid by a C-terminal α-helix and a flexible N-terminal region (Baldwin et al., 1991; Czaplewski et al., 1999). The three-dimensional conformation is stabilized by two disulphide bonds between the first and third, and the second and fourth cysteine residues (Figure 1.1). The N-terminal segment is believed to be important in receptor binding, since modification of the region has been shown to affect chemokine function (Wong & Fish, 2003). Specific modifications of the ligands at the N-terminus can, in fact, neutralize activation completely, effectively creating a potent antagonist to the receptor, which act as
Figure 1.1. Similar structural elements are shared among chemokines.

Despite a low level of amino acid sequence homology, CCL5 (blue), CCL2 (green) and CCL11 (red) exhibit similar structural conformations.
Adapted from Crump et al. (1998)
competitive inhibitors to agonists without inducing signaling. The addition of a methionine residue at the N-terminus of CCL5 (Met-CCL5) and CCL2 (Met-CCL2) has been shown to generate antagonists for CCR5 and CCR2, respectively (Gaertner et al., 2008; Proudfoot et al., 1996).

It is known that chemokines form oligomers in solution. Studies have also shown that chemokine monomers and higher-order multimers have distinct biological functions (Proudfoot et al., 2003). It is now understood that chemokines form dimers through their residues near their N-terminus. They then engage with glycosaminoglycans (GAGs) on the cell surface which facilitates the formation of a chemical gradient for chemotaxis (Appay et al., 2001). GAGs tend to be negatively charged due to the high number of sulphate and carboxylate groups, which help interact and stabilize with the basic, positively charged chemokines. CCL5 has been shown to not only form dimers, but also has a tendency to aggregate into higher-order oligomeric structures (Appay et al., 2000). This aggregation is dependent on the negatively charged glutamine residue Glu-66 and Glu-26 which then form ionic bonds with several positively charged residues on CCL5 surface (Czaplewski et al., 1999). CCL5-aggregation, specifically at minimum, a quaternary oligomer, is required for biological activity in vivo, while CCL5 dimers are sufficient to signal in vitro (El-Asmar et al., 2005; Issafras et al., 2002).

All chemokine receptors are seven-transmembrane receptors with a cytoplasmic C-terminus that contains multiple serine/threonine and tyrosine phosphorylation residues, seven hydrophobic domains, three intracellular and extracellular loop (ECL) and an extracellular N-terminus involved with ligand binding (Figure 1.2; Oppermann, 2004). The first 25 amino acids of CCR5 are crucial for CCL5 binding, as well as for HIV viral entry.
Figure 1.2. Two-dimensional representation of CCR5 and domains responsible for ligand binding and signal transduction.

Schematic structure of CCR5 with extracellular and intracellular domains indicated. Amino acids critical for CCR5 function are highlighted by filled circles. The grey box marks the location of the membrane bilayer. Disulfide bonds, G-proteins binding domains, tyrosine sulfation sites, tyrosine phosphorylation sites and serine phosphorylation sites are indicated.
Adapted from M. Oppermann (2004)
Post-translational modification also plays an important role in modulating chemokine receptor function. Palmitoylation on three cysteine residues in the C-terminus of CCR5 is required for trafficking the receptor to the cell surface, while glycosylation and tyrosine phosphorylation on its N-terminus is required for ligand binding (Blanpain et al., 2001). The C-terminus of most chemokine receptors is coupled to the heterotrimeric G-protein, which is critical for chemokine signaling (Murooka et al., 2006).

1.1.4. Ligand-Receptor Binding

Mutagenesis studies have shown that the N-terminal domain of CCR2, CCR3, CCR5 and CXCR1 is critical for ligand binding (Duma et al., 2007). Specifically, residues 2-13 are important for CCR5 to respond to CCL4 and CCL5. Site-specific mutagenesis in that region results in weaker signaling (Blanpain et al., 1999). In addition to the N-terminus, the ECL is another region important for binding ligands. Replacement of any of the four cysteine residues on the extracellular domain of CCR5, specifically Cys-20, Cys-101, Cys-178 and Cys-269, will result in significantly lower surface expression (Blanpain et al., 1999). Furthermore, the trans-membrane helices are also important for receptor activation, where a Thr-X-Pro (TXP) motif is essential in binding chemokines and eliciting functional responses (Govaerts et al., 2001).

Post activation, serine residues in the C-terminal region of the receptors are phosphorylated by protein kinase C (PKC) and G-protein receptor kinases (GRKs), which trigger the signaling cascade for receptor internalization. Receptor internalization is a control mechanism that prevents cells from undergoing excessive receptor stimulation or periods of prolonged inactivity. β-arrestin initiates the process by interacting with the phosphorylated
receptor, which uncouples the GPCR from the G-proteins. β-arrestin then binds to clathrin and the clathrin adaptor complex AP-2, which prevents the receptor from leaving the clathrin-coated pit. The clathrin-coated pit begins pinching into a vesicle, while carrying the receptors inside. Once internalized, vesicles accumulate in endosomes as the receptors are dephosphorylated and recycled back to the cell surface (Signoret et al., 2004).

1.1.5. Chemokine-mediated Signal Transduction

Upon binding to their cognate GPCRs, chemokines trigger a rapid conformational change in the receptor, which enables it to function as a guanine nucleotide exchange factor (GEF). GEF can then activate an associated heterotrimeric G-protein by exchanging the GDP bound to the inactive G protein for a GTP. Once bound to GTP, GPCR will undergo dissociation, where the Gαi and the Gβγ subunits separate from each other, leading to the activation of a number of signaling pathways. The dissociated Gβγ subunits can activate phospholipase C (PLC)-β while Gαi can activate adenylyl cyclase. Phospholipase Cβ can further generate two messenger molecules: diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP₃), which activate PKC and induce rapid increases in cytosolic calcium, respectively. Subsequently, activated PKC phosphorylates conserved serine residues on the C-terminus of CCR5, specifically Ser-336, Ser-337, Ser-342 and Ser-349, to induce receptor internalization (Oppermann et al., 1999). Pertussis toxin (pTx), a bacteria-derived toxin, can prevent all G-protein coupled signaling by catalyzing the ADP-ribosylation of the Gαi subunits. Notably, chemokines elicit pTx-insensitive, tyrosine phosphorylation-dependent responses, providing additional complexity and function to the chemokine-chemokine receptor system (Bacon et al., 1995; Mellado, M, 2001).
Amongst others, the Jak-Stat pathway is one that is G-protein-independent. Many cytokines, growth factors and chemokines elicit their biological effects mediated by Jak-Stat signaling. Dimerization of receptors post ligand-binding brings two Janus kinases (Jaks) into close proximity. This event triggers the trans-phosphorylation of Jaks and the phosphorylation and binding of signal transducer and activator of transcription (Stat) molecules. Phosphorylated Stats then dimerize via their SH2-domains, translocate into the nucleus and regulate gene transcription by binding to specific DNA sequences. CCL5-induced rapid phosphorylation of CCR5 leads to the activation of Jak2 and Jak3, which subsequently mediates Stat1:Stat1 and Stat1:Stat3 homo- and hetero-dimerization, first identified in Molt-4 and Jurkat T cells (Wong & Fish, 1998). In HEK-293 cells, CCL5-CCR5 interactions promote Jak1 and Stat5b activation (Rodríguez-Frade et al., 1999). Viewed altogether, the chemokine-chemokine receptor system is complex, induces multiple parallel signaling cascades and is heavily regulated to elicit precise and controlled biological functions.

This thesis will review our general understanding of chemokine-chemokine receptor interactions, signaling and function, with an emphasis on the CC chemokine CCL5 and its receptor, CCR5, in the context of breast cancer metabolism.
1.2. CHEMOKINES & CHEMOKINE RECEPTORS REGULATE CELL FUNCTIONS

1.2.1. Immune Cell Activation and Survival

Accumulating evidence indicates that chemokines affect cell differentiation, activation, apoptosis and survival (Balkwill, 2004; Moser et al., 2004; Proudfoot, 2002; Wong & Fish, 2003). The difference in cell fate is often determined by the type of chemokine, its concentration and the target cell. In addition to their chemotactic properties, chemokines co-stimulate T cells. CXCL12, for example, stimulates the activation of CD4+ T cells to induce the production of interleukin (IL)-2, IL-10 and IL-4 (Nanki et al., 2000). CCL5 functions as a co-stimulatory molecule for T cells. T cells stimulated with anti-CD3 and nM concentrations of CCL5 exhibit increased proliferation. It is also observed that CCL5-deficient T cells have impaired cytokine production (Makino et al., 2002). Furthermore, μM concentrations of CCL5 induce antigen-independent activation of T cells, suggesting that high concentration of CCL5 can bypass the requirement of antigen-specific T cell receptor (TCR) stimulation to activate T cells (Dairaghi et al., 1998). CCL5 with a Glu-26 mutation is unable to activate T cells, likely due to its inability to form higher order oligomers (Czaplewski et al., 1999). The extracellular matrix GAG molecules facilitate the sequestering and aggregation of CCL5 which leads to unusually high local CCL5 concentrations and the formation of higher order CCL5 aggregates (Martin et al., 2001; Murooka et al., 2006; Proudfoot et al., 2003). Chemokine receptors also influence T cell fate and determine T helper cell polarization. Receptors are differentially expressed depending on the effector functions: CXCR3 and CCR5 are more commonly expressed on T helper (Th) 1 cells, while CCR8 and CCR4 are often found on Th2 cells (Luther et al., 2001). Therefore,
through engaging their cognate receptors, chemokines may promote Th1 or Th2 polarization and recruit the appropriate effector T cells to sites of inflammation or insult.

The role of chemokines is also strongly implicated in inducing pro- and/or anti-apoptotic events. CXCL12-CXCR4 interactions activate the ERK1/2 and AKT signaling intermediates for cell survival, while also activating the MAPK p38 pathway for apoptosis (Vlahakis et al., 2002). It has been suggested that AKT signaling is able to “override” the apoptotic signal and promote cell survival (Kennedy et al., 1997). Interestingly, in cells lacking Akt such as neurons, CXCL12 activated signaling downstream of CXCR4 results in cell death (Berndt et al., 1998). CCL5-CCR5 interactions induce cell death (Murooka et al., 2006). At μM concentration, CCL5 can induce apoptosis in Molt-4, PM1 and tumor infiltrating T lymphocytes (TILs) through the release of cytochrome c and activation of caspase-9 and caspase-3. CCL5-mediated induction of apoptosis is independent of GPCR-signaling, but requires the activity of Tyr-339 on the C-terminus of CCR5 (Murooka et al., 2006). The ability for chemokines to induce two distinct outcomes may be important mechanism for the resolution of an immune response. Activation induced cell death (AICD) of T cells may be induced post clearance of pathogens to limit immune activation and prevent further immunopathology (Appay et al., 2000; Ward et al., 1998).

1.2.2. Chemotaxis

Chemotaxis, i.e. directed cell migration, is a highly regulated process and is critical for a number of biological functions, including tissue repair, leukocyte activation and pathogen clearance. Chemotaxis is the result of numerous coordinated signaling events, leading to cytoskeleton rearrangement and the redistribution of receptors, adhesion and signaling molecules on the cell surface, effecting cell polarization (Murooka et al., 2008;
Parent et al., 1998). The process begins when receptors recognize and bind to their cognate chemokine and assemble in a cluster at the side of the cell closer to the stimulus, known as the lamellipodium or the leading edge (Giannone et al., 2007). Molecules like filamentous F-actin further polymerize at the leading edge to form a protrusion, the pseudopod. These pseudopods are extensions of the cell membrane which adhere to the extracellular matrix and act as traction sites (Ridley et al., 2003). To elicit cell migration, the points of adhesion de-assemble at the posterior edge, the uropodium, and re-associate at the leading edge. As such, the leading edge is rich in actin filaments and actin-modifying enzymes, like the Arp2/3 and WAVE/Scar complex, while the uropodium contains high levels of myosin filaments which help stabilize the cell during migration (van Haastert et al., 2004).

Efficient chemotaxis requires tight regulation of molecules involved in cytoskeleton rearrangement. The ubiquitously-expressed Rho family of GTPases is a key regulator of the actin/myosin cytoskeleton, amongst which, Cdc42, Rac1 and Rho have been studied extensively. They function as molecular switches by switching between their GDP-bound inactive forms, to GTP-bound active forms, catalyzed by GEFs. When activated, Cdc42 and Rac accumulate at the leading edge, promoting the polymerization of actin to form pseudopods. Rho, on the other hand, localizes to the uropodium and promotes detachment (Spiering et al., 2011). CCL5 activates Rac and its downstream substrate, PAK2, in macrophages, to induce cell polarization and chemotaxis (Weiss-Haljiti et al., 2004). Other β-chemokines, namely CCL3 and CCL4, have also been shown to induce the formation of lamellipodia in macrophages (Di Marzio et al., 2005).
1.2.3. Signaling through the PI3K / AKT Pathway

Chemokine-mediated cytoskeletal rearrangement is dependent on the activation of the PI3K pathway. The PI3K family of proteins are lipid kinases that phosphorylate the 3’-OH position on the inositol ring of phosphoinositides. PI3K and its lipid product, phosphatidylinositol (3,4,5) triphosphate (PIP₃), are implicated in playing an important role in cell polarity and chemotaxis. There are four identified classes of PI3K, IA, IB, II and III, identified based on their structures and substrate specificities. Class IA and Class IB are heterodimeric enzymes that catalyze the generation of PIP₃ from PIP₂. Class IA consists of one of the four 110kDa catalytic subunit isomers (α, β, γ and δ) and one of five regulatory subunit isomers (p85α, p85β, p55α, p55γ and p50α). Class IB has a p110γ catalytic subunit bound to one of two regulatory subunits, p101 or p84. In contrast to Class I, Class II and Class III PI3Ks differ in their structures and functions. Class II consists of three catalytic isoforms (C2α, C2δ and C2γ) that do not contain regulatory proteins. They phosphorylate PIP₂ inefficiently and their role in immune cell signaling is largely unknown. Class III kinases only generate PIP₃ from phosphatidylinositol and contain both a catalytic and regulatory subunit. Their functions have been implicated in autophagy, endocytosis and vesicle trafficking (Kok et al., 2009).

During chemotaxis, activated PI3K is activated to generate high levels of PIP₃ at the lamellipodium. These phospholipids then function as secondary messengers to recruit proteins that contain that pleckstrin homology (PH) domain to the leading edge and induce a number of signaling cascades. Two of the most important PIP₃-binding proteins are phosphoinositide-dependent protein kinase 1 (PDK1) and the serine/threonine kinase protein kinase B (PKB, or Akt). Co-localization of activated PDK1 with Akt promotes PDK1-
dependent phosphorylation of Akt at Thr-308 (Manning et al., 2007). Full activation of Akt also requires phosphorylation on Ser-473, which can be catalyzed by mechanistic targets of rapamycin complex 2 (mTORC2) or DNA-dependent protein kinase (DNA-PK). Activated Akt further phosphorylates a number of downstream target molecules, many involved in cell survival, metabolism and cell proliferation (Finlay et al., 2010). Constitutive activation of PI3K is associated with hyper-proliferation and tumorigenesis, therefore its activation is tightly regulated by the tumor suppressor protein, Phosphatase and Tensin Homolog Deleted in Chromosome Ten (PTEN) (Stambolic et al., 1998). PTEN commonly localizes at the uropod of the cell, where it dephosphorylates PIP₃ to PIP₂, resulting in a transient increase in PIP₃ levels at the leading edge, which ensures the continual polarization of the cell during chemotaxis. Notably, PTEN-deficient cells generate pseudopodia in multiple directions, which significantly impairs directional movement towards chemoattractants (Iijima et al., 2002).

Chemokines, including CCL5, CCL19 and CCL21 rapidly induce phosphorylation and activation of PI3K. Using inhibitors such as wortmannin and Ly294002, the importance of PI3K during chemokine-mediated cell migration has been well defined (Turner et al., 1995; Wymann et al., 2005). It is now clear that localization of activated PI3K at the lamellipodium is critical for the establishment of a chemotactic signaling gradients. Using knock-out (KO) mice, evidence was provided that PI3Kγ (p110γ) is a critical regulator of chemotaxis. Neutrophils and macrophages from PI3Kγ-KO mice are unable to generate PIP₃ at the lamellipodium and fail to activate Akt in response to chemoattractants (Hannigan et al., 2002). Interestingly, B cells deficient in p110γ but not p110δ are able to migrate to Peyer’s
patches towards CXCL13, suggesting other PI3K isoforms and other PI3K-independent pathways may also be important for efficient cell migration (Reif et al., 2004).

Downstream of PI3K activation, the serine/threonine kinase Akt has a central role in regulating cellular processes, including metabolism, apoptosis, proliferation and migration. Three members of the AKT subfamily, Akt1, Akt2 and Akt3 have been identified. Akt1 is the predominant member of the subfamily and has been shown to be involved in survival pathways, by inhibiting apoptosis. Akt1-KO mice exhibit deficiencies in tissue growth and increased apoptosis in the thymus (Chen et al., 2001). Akt2 has an important role in the insulin signaling pathway, by inducing glucose transport and is often overexpressed in breast cancer cells. Akt2-KO mice display diabetic phenotypes, suggesting a role for Akt2 in regulating insulin receptor signaling (Hill et al., 2002). Akt3 has a play a role in brain development, but its mechanism of action remains largely unclear (Yang et al., 2004). Upon phosphorylation-activation, Akt directly upregulates glycolysis, increases glucose uptake through regulation of glucose transporters, promotes lipid synthesis, as well as indirectly activates its downstream substrate, mTOR (Figure 1.3).

1.2.4. mTOR Activation and Metabolic Regulation

mTOR is an evolutionarily conserved sensor of cellular metabolism, proliferation and survival. It belongs to a family of kinases called the phosphatidylinositol kinase-related kinase (PIKK), to which PI3K also belongs (Peter et al., 2010; Sinclair et al., 2008). Rapamycin, an anti-fungal macrolide, is a potent immune-suppressive agent that binds directly to a small protein receptor called FKBP12 (FK506-binding protein 12kDa) and interacts specifically with mTOR to inhibit its function (Edinger et al., 2003). mTOR exists in two complexes: mTORC1, which is sensitive to rapamycin inhibition and mTORC2,
Figure 1.3. CCL5 and the PI3K/AKT/mTOR pathway.

CCL5 binding to CCR5 activates the PI3K/AKT/mTOR pathway. TAK-779 and maraviroc are CCR5 antagonists and prevent CCL5-CCR5 interaction. Akt is a central regulator of cellular metabolism. Akt directly upregulates glycolysis, increases glucose uptake through regulation of glucose transporters, and promotes lipid synthesis. mTOR activation promotes cap-dependent mRNA translation, leading to enhanced chemotaxis and proliferation. Rapamycin binds directly to mTOR and prevents its activation. The glycolysis pathway generates glucose 6-phosphate which can be shuttled into the pentose phosphate pathway (PPP), leading to nucleotide and amino acid synthesis.
which is rapamycin resistant and phosphorylates Akt. mTORC1 contains mTOR, Regulatory Associated Protein of mTOR (Raptor), Proline-rich AKT1 substrate 1 (PRAS), DEP domain-containing mTOR-interacting protein (DEPTOR) and mammalian lethal with SEC13 protein 8 (mLST8), while mTORC2 consists of mTOR, Rapamycin-Insensitive Companion of mTOR (Rictor), mLST8 and mammalian stress-activated protein kinase interacting protein 1 (mSin1) (Frias et al., 2006; Kim et al., 2002). mTOR activation is important for integrating signals from growth factors, nutrients and influences energy status to modulate cell fate and metabolism, and therefore its induction is highly regulated. Following activation of Akt, it phosphorylates and inactivates the tuberous sclerosis complex (TSC) 1/2, which indirectly inhibits mTOR (Inoki et al., 2003). Mutations in TSC1/2 lead to a hyper-active mTOR, leading to tumorigenesis. TSC2 functions as a GTP-ase Activating Protein (GAP) which stimulates the GTPase activity of Ras-homolog enriched in brain (Rheb), while TSC1 acts as a stabilizer to protect TSC2 from degradation. TSC1/2 promotes the hydrolysis of GTP and inactivates Rheb. In its active form, the GTP-bound Rheb interacts with mTOR and stimulates its activity (Long et al., 2005). Activated mTOR can regulate cell proliferation and survival through signaling through the p70 ribosomal S6 kinase 1 (S6K1) and the inhibition of eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) (Inoki et al., 2003).

Following mTOR-mediated activation, S6K1 phosphorylates the 40S ribosomal protein S6 (rpS6), which promotes the translation of a subset of mRNA with the 5’ tract of oligopyrimidine (TOP) sequence. These mRNAs encode ribosomal proteins and elongation factors, and translation of these mRNAs upregulates the global cellular capacity for protein translation. mTOR also promotes 4E-BP1-dependent protein translation (Figure 1.4; Wullschleger et al., 2006). Following transcription of mRNAs, ribosomes are recruited to the
5’ end of an mRNA where the start codon AUG is located. In cap-dependent translation, eukaryotic translation initiation factors (eIFs) recognize and bind to the 7-methyl guanosine residue (m7GpppX, where ‘m’ is a methyl group and ‘X’ is any nucleotide) that forms the 5’ cap of all mRNAs (Hay et al., 2004). eIF4E forms the eIF4F complex with other initiation factors, including eIF4G, eIF4A and eIF4B around the 5’ cap. The eIF4F complex is responsible for unwinding the mRNA and allowing ribosomes to bind efficiently (Richter et al., 2005). eIF4E-binding proteins are a family of translation inhibitors, consisting of 4E-BP1, 4E-BP2 and 4E-BP3. These repressor proteins compete with eIF4G for the overlapping binding sites on eIF4E. The removal of eIF4E prevents the formation of the eIF4F initiation complex and mRNA translation is suppressed. Upon activation, mTOR phosphorylates 4E-BP1 on two sites, Thr37/46, followed by subsequent phosphorylation of Thr70 and Ser65. This releases eIF4E from 4E-BP1 and promotes cap-dependent translation (Hay et al., 2004).

mTOR can sense cellular nutrients, especially amino acids. It has been shown that in the absence of leucine, downstream mTOR effectors rapidly dephosphorylate (Wullschleger et al., 2006). It has been suggested that activation of mTOR by amino acids is independent of TSC1/2, given that S6K1 remained sensitive to amino acid levels in TSC1/2 KO cells. It is now understood that the presence of amino acids can activate Rag GTPase heterodimers, which can localize mTOR to the surface of lysosome where they interact directly with Rheb-GTP (Sancak et al., 2008). Interestingly, studies have suggested that the class III PI3K, vps34, may play a role in amino acid sensing. vps34 is important in TSC1/2-independent activation of mTORC1 in response to amino acids, as siRNA knockdown and sequestration of vps34 both resulted in the inhibition of mTORC1 activity, as measured by S6K1.
phosphorylation (Backer, 2008). However, the exact mechanism by which vps34 induces mTOR activation remains unclear.
Figure 1.4. mTOR phosphorylation of 4E-BP1 promotes protein translation.

Upon mTOR-mediated phosphorylation, 4E-BP1 dissociates from eIF4E, allowing eIF4E to be incorporated into the eIF4F complex, which also includes eIF4G and eIF4A. Subsequently, eIF4B and the 43S pre-initiation complex bind around the 5’ cap to initiate cap-dependent protein translation.
1.3. CHEMOKINES & CHEMOKINE RECEPTORS IN CANCER

Leukocytes are present in both the tumor milieu and the tumor-supporting stroma (Seyfried et al., 2013). It is now clear that the tumor microenvironment is characterized by inflammatory cells and chemokines that regulate tumor development. Tumorigenesis often involves abnormal expression of chemokine and chemokine receptors. Specifically, CXCL12, CCL21, CCL2, CCL5 and CCR5 have all been implicated in promoting the malignant phenotype in breast cancer (Biswas et al., 2014; Luboshits et al., 1999; Müller et al., 2001; See et al., 2014). By binding and activating chemokine receptors on the target cell surface, cancer-associated chemokines can promote tumorigenesis directly, by activating signals to proliferate, and indirectly, by mediating the influx of leukocytes into the microenvironment (Wong & Fish, 2003). Accumulating evidence suggests that chemokine and chemokine receptors play a critical role in the control of immune infiltration into solid tumors and the regulation of tumorigenesis, angiogenesis and metastasis (Figure 1.5; Murooka et al., 2005).

1.3.1. Leukocyte Tumor Infiltration

Infiltrating leukocytes found in most solid tumors can account for up to 50% of the tumor mass, including tumor-infiltrating lymphocytes (TILs), innate lymphoid cells (ILCs), tumor-associated macrophages (TAMs), and myeloid-derived suppressor cells (MDSCs) (Leek & Harris, 2002; Salgado et al., 2015; Schlecker et al., 2012). The influx of immune cells into the tumor microenvironment was initially believed to be an anti-tumor immune response. However, it is now understood that the immune system plays a dual role in cancer development. Various anti-tumor mechanisms, including antibody-dependent cellular cytotoxicity and complement activation, can directly target and kill tumor cells.
Figure 1.5. Chemokines modulate tumor microenvironment.

Chemokines modulate the tumor microenvironment in multiple ways. They support the infiltration of leukocytes into solid tumors, provide signals for tumor growth, induce local angiogenesis and ultimately metastasis.
1 – Neoplastic Transformation
Abnormal chemokine and chemokine receptor expression in transformed cells up-regulate growth and survival factor

2 – Leukocyte Infiltration
Tumor-derived chemokines attract circulating leukocytes, infiltrating the tumor mass

3 – Tumor Cell Growth
Tumor-associated chemokines function as growth and survival factors for tumor cells through a autocrine and/or paracrine loop

4 – Angiogenesis
Tumor-produced chemokines stimulate angiogenesis, causing neighbouring blood vessels to grow into the tumor

5 – Metastasis
Aberrant chemokine receptor expression causes active migration of tumor cells out into the vasculature

6 – Organ Homing
Expression of chemokine receptors on tumor cells allows for specific organ homing

Legend
- Monocyte
- Red Blood Cell
- Dividing Tumor Cell
- Chemokine Receptor
- Macrophage
- Tumor Cell
- Endothelial Cell
- Chemokine

Murooka et al. (2005)
On the other hand, immune responses may be deleterious, through the generation of an immunosuppressive microenvironment that supports tumorigenesis and metastasis. It has been shown that tumor-derived chemokines can also attract specific leukocytes to the tumor milieu in order to promote tumor proliferation and angiogenesis (Schlecker et al., 2012). Therefore an understanding of the different subsets of tumor infiltrating lymphocytes will provide a better understanding of the underlying mechanisms that support anti- and pro-tumor processes.

### 1.3.1.1. Tumor Infiltrating Lymphocytes

TILs are mononuclear leukocytes that infiltrate solid tumors. They are found in most types of cancer, including breast cancer, cervical cancer, lung cancer and melanoma. Often, the presence and the number of TILs correlates with positive prognosis, specifically, clinical data suggest that a high level of TILs is a predictor of a favorable clinical outcome. Earlier studies showed that TILs mainly comprise cytotoxic CD8+ T lymphocytes (CTLs), and *ex vivo* studies confirmed the cytolytic activity of TILs isolated from solid tumors (Salgado et al., 2015). Interestingly, tumors with reduced human leukocyte antigen (HLA) class I expression also had reduced CTL infiltration. CTLs recognize tumor-associated antigen (TAAs) and can directly attack tumor cells. Several chemokines and their receptors have been shown to regulate CTL migration, including CXCR3 and CXCL16. In a murine cancer model, increased expression of CXCR3 ligands – CXCL9 and CXCL10 – enhanced tumor infiltration of CD4+ and CD8+ lymphocytes (Li et al., 2015). Furthermore, CXCL16 expression levels correlate with TIL infiltration as well as improved disease prognosis (Mukaida et al., 2014).
Prior to activation, CTLs require co-stimulation from professional antigen-presenting cells such as dendritic cells (DCs) that present the TAAs on their surface using major histocompatibility complex (MHC) I and II. DCs express an array of chemokine receptors, including CCR1, CCR2, CCR4, CCR5, CCR6, CCR8 and CXCR4. Upon capturing antigens and activation by inflammatory stimuli, mature DCs begin to only express a limited subset of receptors, including CCR7 and CXCR4, and migrate to T-cell areas in the local lymph nodes to induce primary immune responses (Knutson et al., 2005). Studies have further shown that a CTL response against apoptotic tumor cells requires CCL3-CCR5/CCR1-mediated migration of DCs to the draining lymph nodes.

1.3.1.2. Innate Lymphoid Cells

Innate lymphoid cells (ILCs) were first characterized in 2008, when several laboratories independently identified a group of innate leukocytes derived from the common lymphoid progenitor that lacked rearranged antigen-specific receptors (like the TCR and BCR) (Walker et al., 2013). In 2013, a nomenclature was proposed to classify ILCs into three groups (Spits et al., 2013). Group 1 ILCs are characterized by their constitutive expression of the transcription factor T-bet and the production of Th1 cytokines, such as TNFα and IFNγ. This group comprises natural killer (NK) cells and ILC1s which are analogous to CTLs and Th1s, respectively (Dadi et al., 2016). Group 2 ILCs, or ILC2s, produce type 2 cytokines, including IL-4, IL-5, IL-9 and IL-13. Their maturation is dependent on the expression of the transcription factors GATA-3 and RORα. ILC2s are often regarded as the innate counterparts of Th2 cells (Hoyler et al., 2012; Moro et al., 2010; Wong et al., 2012). Group 3 ILCs are analogous to Th17 cells and share the common transcription factor RORγt. Group 3 ILCs include ILC3s and lymphoid tissue inducer (LTi) cells. ILC3s
express IL-22 and the NK cell receptor NKp46, but lack cytotoxic effectors, such as granzymes and perforin. LTi cells are found in the fetus and are required for the development of lymphoid tissues, e.g. lymph nodes and the Peyer’s patches (Cupedo et al., 2009; Klose et al., 2013). In the context of cancer surveillance, ILCs can also be categorized into cytotoxic ILCs, such as NK cells, and the helper-like ILCs, which include ILC1s, ILC2s and ILC3s.

Unlike T and B cells, NK cells do not recognize target cells using antigen-specific receptors, instead lysis of tumor cells by NK cells is mediated through alternative receptors, such as NKG2D, NKp44 and NKp46. NKG2D can recognize a number of ligands commonly expressed on tumor cells, such as UL16 binding protein 1 (ULBP) and MHC class I polypeptide-related sequence A (MICA) (Krneta et al., 2015; Waldhauer et al., 2008). NK cells also express inhibitory receptors that recognize MHC class I molecules, which normally present antigen to CTLs. A common hallmark of tumor cells is the downregulation of surface MHC class I molecules, in order to evade CTL detection. It has been suggested that NK cells evolved to respond to reduced MHC I and to complement the adaptive immune response, a mechanism called “missing-self recognition” (Kärre et al., 1986). In vitro, NK cells kill a wide range of tumor cells via direct cellular cytotoxicity. They have also been implicated in eliminating many spontaneous and transplanted tumors in vivo (Vallentin et al., 2015). NK cells express a number of chemokine receptors to enable their migration to lymph nodes as well as tumor sites, including CCR1, CCR2, CCR5, CXCR3 and CX3CR1 (Mukaida et al., 2014). Accordingly, ligands for these receptors will regulate NK cell trafficking and functions. Helper-like ILCs also contribute to tumor surveillance. ILC1s often polarize other immune cells into an inflammatory phenotype by secreting IFNγ and TNFα, while ILC2s can recruit eosinophils to suppress tumor growth, through IL-5 production. ILC3s have been
shown to contribute to tumor development and progression by producing IL-17, IL-22 and IL-23. The production of IL-22 is associated with tumorigenesis, the inhibition of tumor apoptosis and enhanced metastasis (Carrega et al., 2016; Grivennikov et al., 2010). However, despite the multifaceted functions ILCs have in the tumor microenvironment, the exact role of ILCs in cancer remains unclear.

1.3.1.3. Tumor-associated Macrophages

The predominant subset of TILs is TAMs. They are derived from circulating monocytes that are attracted to the tumor area by tumor- and stroma-secreted chemokines, including CCL2, CCL5, CCL7, and macrophage colony stimulating factor (M-CSF) (Solinas et al., 2009). TAMs elicit pro-tumor effects by secreting various growth factors, such as epidermal growth factor (EGF), fibroblast growth factor (FGF), and the angiogenic mediator, vascular endothelial growth factor (VEGF) (Mantovani et al., 1992). TAMs exhibit the properties of M2-macrophages and are an abundant source of IL-10, transforming growth factor (TGF)-β and arginase, which inhibit immune activation and generate an immunosuppressive environment that promotes tumor proliferation and metastasis (Riabov et al., 2014).

It was first observed in 1983 that CCL2 is highly expressed by most tumor cell lines (Bottazzi et al., 1983). Since then, it has been shown that local production of CCL2 is directly linked to the recruitment of TAMs in breast, ovarian and lung cancer (Allavena et al., 2008; Baay et al., 2011; Lewis et al., 2006). In a dextran sodium sulphate (DSS)-induced colon cancer mouse model, CCL2 blockade significantly reduces the infiltration of CCR2+ TAMs, both of which are important in maintaining tumorigenesis (Popivanova et al., 2009). Studies have also identified a correlation between CCL2 levels and several pro-tumor factors,
including VEGF and CXCL8 (Lazennec et al., 2010). Other studies have characterized the role of tumor-secreted CCL5 in monocyte recruitment. Breast cancer cell lines that express CCL5 in culture will induce monocyte migration in vitro (Adler et al., 2003). Additionally, the administration of the CCR5 antagonist, Met-CCL5, reduced macrophage infiltration in a murine breast cancer model, which led to reduced tumor load (Robinson et al., 2003). Similarly, it was reported that mammary carcinoma cells expressing low levels of CCL5 have decreased tumorigenesis in vivo (Adler et al., 2003). In addition to promoting recruitment, CCL2 and CCL5 stimulate the production and secretion of pro-tumor factors by TAMs in the tumor microenvironment, including matrix metalloproteinase (MMP)-9 and TNFα (Ding et al., 2016; Riabov et al., 2014).

1.3.1.4. Myeloid-derived Suppressor Cells

MDSCs are heterogeneous myeloid immune cells. They are characterized by the co-expression of the myeloid markers GR-1 and CD11b in mice, while they are identified as CD14- CD11b+CD33+ in humans (Gabrilovich, 2017). GR-1 in mice is made up of two molecules, Ly6C and Ly6G. As such, murine MDSCs can be further classified into monocytic (Ly6C$^{hi}$ and Ly6G$^{lo}$) and granulocytic (Ly6C$^+$ and Ly6G$^{hi}$). A lack of a human equivalent to the murine GR-1 marker makes direct comparison between MDSCs from the two species difficult (Talmadge et al., 2013). Although their functionality is comparable, there is no consensus on how the human subsets of MDSCs should be classified.

Under normal circumstances, myeloid progenitors differentiate into macrophages, dendritic cells and neutrophils. However, under inflammatory conditions, myeloid differentiation tends to shift towards MDSC expansion in the presence of G-CSF and GM-CSF (Gabrilovich et al., 2009). These MDSCs enter the inflammatory sites and inhibit...
immune responses. They express and secrete immunosuppressive factors, namely arginase, inducible nitric oxide synthetase (iNOS), TGFβ and IL-10, to suppress the proliferation and activation of T and NK cells (Marvel et al., 2015). CCL2 has been implicated in the recruitment of MDSCs in several murine cancer models, including lung carcinoma, melanoma and lymphoma (Gabrilovich, 2017). Interestingly, a CCR2 deficiency results in a shift from MDSCs of a macrophage lineage to those of a neutrophil lineage, without influencing tumorigenesis (Sawanobori et al., 2008). Granulocytic MDSCs found in colonic tumors express high levels of CXCR2 and its ligands, namely CXCL1, CXCL2 and CXCL5, are also abundant in the tumor microenvironment. A loss of CXCR2 results in a significant decrease in tumor growth, through the inhibition of MDSC infiltration (Katoh et al., 2013).

Viewed altogether, chemokines and chemokine receptors are certainly important for the recruitment of inflammatory cells into the tumor microenvironment.

1.3.2. Tumorigenesis, Angiogenesis and Metastasis

In addition to recruiting leukocytes to the tumor milieu, chemokines can act directly as growth and survival factors for many types of tumors. Signaling events mediated by chemokines may be autocrine, where tumor cells secrete and respond to chemokines, or paracrine, where the tumor-supporting stroma, such as cancer-associated fibroblasts (CAFs) and mesenchymal stem cells (MSCs), secrete soluble factors to support the tumor microenvironment (Lazennec et al., 2010; Nicolson, 1993; Roy et al., 2016). Chemokine signaling has the potential of promoting tumor growth, enhancing the development of new vasculature and ultimately leading to tumor metastasis.
1.3.2.1. Chemokines and Tumorigenesis

The CXCL12/CXCR4 signaling pathway has been well-documented to directly support tumorigenesis. CXCR4 upregulation has been reported in a number of cancers, including melanomas, lymphomas, breast cancers and prostate cancers (Chatterjee et al., 2014; Sarvaiya et al., 2013). CXCL12 increases DNA synthesis in an Erk1/2-dependent manner, as well as contributing to Akt activation (Xueqing Sun et al., 2010). CXCL12 expression is also associated with a lower rate of apoptosis in human myelodysplastic syndrome. Interestingly, CXCR4 with a C-terminal deletion led to a gain-of-function phenotype, resulting in enhanced cell proliferation and motility in breast carcinoma cells (Ueda et al., 2006). This deletion is also the underlying cause of the Warts, Hypogammaglobulinemia, Immunodeficiency, and Myelokathexis (WHIM) syndrome (Kawai et al., 2009).

The role of CCL5-CCR5 in promoting tumorigenesis has been widely studied. Specifically, CCL5 levels are elevated in more aggressive forms of breast cancer and are predictive of rapid disease progression in stage II breast cancer patients (Azenshtein et al., 2002; Luboshits et al., 1999; Niwa et al., 2001). Furthermore, serum CCL5 levels are elevated in patients with high-grade tumors compared to those with low-grade tumors (Niwa et al., 2001). Several breast cancer cell lines respond to CCL5 with enhanced proliferation and increased metastasis (Robinson et al., 2003; Velasco-Velázquez et al., 2012), suggesting that locally produced CCL5 may directly signal and modulate cancer cells in the tumor microenvironment. In addition, previous work from our lab has shown that CCL5, at physiological levels, actively promotes the translation of survival and proliferative proteins, such as cyclin D1 and c-Myc in a CCR5- and mTOR-dependent manner, resulting in
significant increase in proliferation in vitro (Murooka et al., 2009). Other chemokines have also been implicated in the activation of the mTOR pathway to enhance tumorigenesis. CXCR4 expression has been reported to correlate with increased proliferative and migratory potential in gastric carcinoma cells, while CXCL8 upregulates the translation of cyclin D1 in prostate cancer (Chatterjee et al., 2014; Liu et al., 2016).

1.3.2.2. Chemokines and Angiogenesis

Angiogenesis is the physiological process of forming new blood vessels from existing vasculature. This process is tightly regulated by a balance of pro- and anti-angiogenic factors. A tumor consists of a population of rapidly dividing cells that require a steady source of nutrients and oxygen in order to fuel their proliferation. However, the rate of tumorigenesis often outpaces that of angiogenesis and hypoxia occurs in the tissue furthest away from the vasculature. Nonetheless, tumor cells have the ability to secrete angiogenic factors directly, namely VEGF, and promote the growth of capillaries into the tumor. Furthermore, accumulating evidence suggests that CXC chemokines can directly regulate angiogenesis and play a role in tumor formation. The chemokines that contain the glutamic acid-leucine-arginine (ELR+) motif at their N-terminus are critical for neovascularization, mainly through activating signaling mediated by CXCR2 (Liu et al., 2016). ELR+ chemokines include CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, and CXCL8 (Mukaida et al., 2014). In contrast, ELR- CXC chemokines are angiostatic factors, which inhibit the formation of new vessels (Strieter et al., 2005). ELR- chemokines include CXCL4, CXCL9 and CXCL10. Interestingly, CXCL12 is ELR- but is a potent angiogenic factor (Teicher et al., 2010). The balance between pro- and anti-angiogenesis is determined by the relative abundance of angiogenic and angiostatic factors.
In addition to secreting immunosuppressive factors, TAMs and MDSCs also contribute to tumor angiogenesis by producing various angiogenic factors, including VEGF, TGFβ, MMP-2 and MMP-9. Several chemokines, including CCL2 and CCL5, can induce angiogenesis by recruiting TAMs and MDSCs (Soria et al., 2008). CXCL6 exerts its angiogenic functions by recruiting neutrophils (Gijsbers et al., 2005). Additionally, CCL5 has been shown to directly upregulate MMP-9 in breast cancer cells (Azenshtein et al., 2002). Given the importance of increased vasculature in support of tumor growth, the angiogenic activity of chemokines may have profound implications in tumorigenesis.

1.3.2.3. Chemokines and Metastasis

Metastasis is the spread of cancer from the primary tumor to secondary sites in the host. Differential chemokine and chemokine receptor expression correlate with patterns in breast cancer metastasis (Müller et al., 2001). In breast cancer, tumors typically metastasize to local lymph nodes, the bone marrow, the lungs and the liver. CXCR4 and CCR7 are highly expressed in malignant breast cancer tumors that metastasize to those sites. CXCL12 – the ligand for CXCR4, is also found in the typical sites for metastasis (Sun et al., 2014). Interestingly, low levels of CXCL12 are found in areas not associated with breast cancer metastasis, including the skin, the kidneys and the brain. Elevated levels of CCL19 and CCL21, ligands for CCR7, are found in the lymph nodes of breast cancer patients (Müller et al., 2001). CCL21 is expressed at higher levels, suggesting that it may play a more significant role than CCL19 in interacting with CCR7. Other chemokines and receptors have also been implicated in enhancing metastasis. CXCR1 and CXCR2 are expressed at high levels in metastatic human melanoma cells (Liu et al., 2016). Notably, chemokine receptor antagonists inhibit proliferation and invasiveness of melanoma cells (Sharma et al., 2010).
CCL5 activation of CCR5 promotes breast cancer metastasis. Insulin-like growth factor (IGF)-1-mediated migration of breast cancer cells was shown to be dependent on CCL5-CCR5 interactions (Mira et al., 2001). In a murine model of melanoma, CCR5-deficient mice develop significantly fewer metastases to the lung than their wildtype counterparts (van Deventer et al., 2008). In addition, CAFs have been documented to play an important role in promoting tumor metastasis. Bone marrow-derived MSCs, which contribute up to 25% of CAFs, can actively migrate towards cancer cells to increase their metastatic potential (Guo et al., 2008). MSCs can be stimulated to secrete CCL5 which enhances the motility, invasion and metastasis of cancer cells in a CCR5-dependent manner (Karnoub et al., 2007). Furthermore, CCR5-expressing pulmonary MSCs are responsible for lung metastases, mediated through MMP-9 expression (van Deventer et al., 2008). Data from our lab provided additional support that CCL5-CCR5 signaling promotes mTOR-dependent translation of motility-related proteins, which further potentiates the migratory phenotype (Murooka et al., 2009).

Clearly, in addition to functioning as chemoattractants, chemokines and chemokine receptors are also involved in modulating the tumor microenvironment, specifically promoting tumorigenesis, angiogenesis and metastasis.
1.4. CANCER METABOLISM AND POTENTIAL THERAPY

The onset of tumorigenesis is often accompanied by altered cell metabolism, either as a cause or as a result. Cancer cells are more reliant on glucose, specifically glycolysis, to generate sufficient energy and nutrients (Gatenby et al., 2004). They are also more resilient to apoptosis, including responses associated with mitochondrial permeabilization (Kennedy et al., 1997). Tumors secrete and also respond to soluble factors that enhance the metabolism of activated leukocytes, which in turn promote the proliferation of malignant cells. A deeper understanding of the classical hallmarks of cancer metabolism could therefore be useful in identifying targets for potential treatments.

1.4.1. Glucose and Glutamine Metabolism

It is increasingly clear that cancer cells consume greater levels of glucose compared with normal cells. To enable this, cancer cells enhance their rate of extracellular glucose uptake (Vander Heiden et al., 2010). The dependency of tumor proliferation on glucose is a common hallmark for most cancer types; as such the utilization of $^{18}$fluoro-deoxyglucose-position emission tomography (FDG-PET) has become a clinical standard to diagnose cancers. A more recent examination of cancer metabolism suggested that glutamine is also an important nutrient for tumor proliferation, providing a carbon source when glucose levels fluctuate. Furthermore, hyperactivation of glutaminase, that catalyzes the conversion between glutamine to glutamate, can further accelerate cancer cell metabolism (Dilshara et al., 2017). In this section, both glucose and glutamine metabolism will be examined.

The glycolysis pathway is critical to tumorigenesis (see 1.4.3. Microenvironment Acidification and Hypoxia). It can generate energy 100 times faster than oxidative
phosphorylation (OXPHOS), but yields only 1/18th the ATP (Liberti et al., 2016), i.e. cancer cells trade efficiency for the ability to quickly generate and consume energy. The glycolysis pathway is made up of ten enzyme-catalyzed reactions, three of which are virtually irreversible and are tightly regulated (Figure 1.6). These reactions are catalyzed by hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK), to generate glucose 6-phosphate (G6P), fructose 1,6-bisphosphate (F1,6BP) and pyruvate, respectively (Li et al., 2015).

The first step is catalyzed by hexokinase, which phosphorylates glucose into G6P. This reaction functions to maintain a low concentration of glucose, which promotes the transport of extracellular glucose via surface glucose transporters (GLUT) (Harrington et al., 2003). In addition, this step prevents glucose from leaving the cell. G6P cannot freely diffuse out of the cell due to its charged nature, and the cell lacks an active transporter for G6P. Additionally, G6P can be shuttled into adjacent anabolic pathways, including the pentose phosphate pathway (PPP) (Zhang et al., 2014). There are four isoforms of hexokinase, HK I, II, III and IV. HK II has the highest affinity for glucose and the one most often overexpressed in cancers (Patra et al., 2013). Accumulating data have implicated a role for HK II in cancer promotion, including its role in generating precursors for the biosynthesis of nucleotides and its interactions with mitochondrial proteins to suppress apoptosis (Pastorino et al., 2008) (see 1.4.3. Microenvironment Acidification and Hypoxia). Interestingly, in most normal mammalian tissues, HK II is only expressed at low levels (Mathupala et al., 2006). The PI3K/AKT pathway has been implicated in regulating glucose metabolism. Akt directly stimulates the expression of GLUT1 and induces surface translocation of GLUT4. It can also
Figure 1.6. The switch towards aerobic glycolysis depends heavily on the glycolytic pathway.

Cancer cells upregulate glycolysis to sustain their rapid proliferation. Key enzymes (in bold) regulating rate-limiting reactions are often overexpressed in tumors. Glycolytic intermediates act as precursors for biosynthetic pathways, including the pentose phosphate pathway and lipid synthesis. Lactate secretion into the microenvironment induces acidification and promotes tumorigenesis. Aldo, aldolase; Eno, enolase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GLUT1, glucose transporter 1; HK, hexokinase; LDH, lactate dehydrogenase; PFK-I, phosphofructokinase 1; PGI, phosphoglucone isomerase; PGK, phosphoglycerate kinase; PGM, phosphoglycerate mutase; PK, pyruvate kinase; TPI, triose phosphate isomerase.
Extracellular glucose
  ↓
GLUT1
  ↓
Glucose
  ↓
HK
  ↓
Glucose 6-phosphate
  ↓
PGI
  ↓
Fructose 6-phosphate
  ↓
PFK-1
  ↓
Fructose 1,6-bisphosphate
  ↓
Aldo
  ↓
Dihydroxyacetone phosphate
  ↓
TPI
  ↓
Glyceraldehyde 3-phosphate
  ↓
GAPDH
  ↓
1,3-Bisphosphoglycerate
  ↓
PGK
  ↓
3-Phosphoglycerate
  ↓
PGM
  ↓
2-Phosphoglycerate
  ↓
Eno
  ↓
Phosphoenolpyruvate
  ↓
PKM2
  ↓
Pyruvate
  ↓
LDH
  ↓
Lactate
  ↓
Secreted lactate
  ↓
Acidification of the tumor environment
  ↑
  ↓
Nucleotide Synthesis
  ↓
Pentose Phosphate Pathway
  ↓
Lipid synthesis
  ↓
Acetyl-CoA

Adapted from Annibaldi & Widmann (2010)
activate PFK2 to accelerate glycolysis (Yang et al., 2004). Additionally, Akt can stimulate the association of HK II with residual ATP from mitochondria, thus improving the catalysis in the first rate-limiting step of glycolysis (Rathmell et al., 2003).

The second rate-limiting step in glycolysis is the phosphorylation of fructose 6-phosphate (F6P) into F1,6BP by phosphofructokinase-1 (PFK-1). PFK-1 can control the rate of glycolysis through allosteric regulation. PFK-1 is activated to accelerate glycolysis and generate additional ATP when the ADP/ATP ratio is high (Carlet et al., 2010). Interestingly, PFK-2, another member of the PFK family, phosphorylates fructose 6-phosphate at the 2’ position to generate fructose 2,6-bisphosphate (F2,6BP), the most potent activator of PFK-1 (Rider et al., 2004). Therefore, a high concentration of F6P as a result of increased upstream glycolytic flux will further accelerate downstream reactions. Consequently, cancer cells with high glucose uptake will trigger a feed-forward reaction, furthering amplifying the rate of glycolysis. Another regulator of glycolysis is AMP-activated protein kinase (AMPK). AMPK is a highly conserved heterotrimeric protein that detects the AMP/ATP or ADP/ATP ratio and will inhibit anabolic activities under low energy conditions. AMPK promotes glycolysis directly by activating PFK2 and promotes glucose uptake via translocation of GLUT4 to the cell surface by phosphorylating the Rab-GTPase-activating protein TBC1D1 (Ros et al., 2013). Interestingly, many cancer cells have mutations in the upstream AMPK kinase, LKB1, which is required for the activation of AMPK (Shackelford et al., 2009). These mutations often lead to inactivation of LKB1 and subsequently AMPK, resulting in uncontrolled proliferation.

In the third rate-limiting step and the final reaction in glycolysis, PK catalyzes the dephosphorylation of phosphoenolpyruvate to pyruvate. There are four isoforms of PK, PKM1,
PKM2, PKR and PKL. PKM2 is the embryonic isoform, but is also expressed in normal proliferating cells, such as lymphocytes (Christofk et al., 2008). PKM2 is commonly found in its inactive dimeric form, while PKM1 is constitutively in its active form. PK activity can be allosterically regulated by the upstream F1,6BP, which activates PKM2 by promoting the conversion from the inactive dimeric form into the active tetrameric form and increases its enzymatic activity 25-fold (Anastasiou et al., 2012). The regulation of PKM2 is thought to enable the accumulation of phosphorylated glycolytic intermediates, which can serve as precursors for biosynthetic pathways. Forced PKM1 expression over PKM2 in mouse embryonic fibroblasts resulted in arrested proliferation and an inability to produce deoxynucleotides (Muralidhar et al., 2016). Evidently, regulation of PK activity in cancer cells may be important in coordinating glycolysis with the synthesis of DNA and cell replication.

In addition to glucose, glutamine can also be metabolized to produce ATP and function as a precursor for the synthesis of lipids, proteins and nucleotides. Once taken up by the cell, most of the glutamine is metabolized to glutamate, catalyzed by glutaminase, which is often overexpressed and upregulated in cancers (Carr et al., 2010). Both glutamine and glutamate contribute to anabolic processes in the cell. Glutamine provides nitrogen for nucleotide synthesis, while glutamate donates nitrogen groups to generate other amino acids. Furthermore, glutamate can be converted into pyruvate and α-ketoglutarate and be metabolized in the tricarboxylic acid (TCA) cycle (Lu et al., 2010). The oncogene c-myc has been implicated in driving cellular dependence on glutamine. Inducible c-myc enhances the expression of glutamine transporters and glutaminase (Lukey et al., 2013). Given that metabolites upstream of pyruvate are fed into adjacent anabolic pathways, the amount of
pyruvate entering the TCA cycle is reduced. However, intermediates of the TCA cycle are required for the synthesis of amino acids and lipids. Therefore, cancer cells may depend on glutamate to replenish TCA intermediates via its conversion to α–ketoglutarate by glutamate dehydrogenase. Citrate and malate in the TCA cycle can further be used for biosynthetic pathways (Altman et al., 2016).

1.4.2. The Warburg Effect

Unlike differentiated tissue, transformed cells are more dependent on glycolysis rather than oxidative phosphorylation, even in the presence of oxygen. This phenomenon is called the Warburg effect, named after the German physicist Otto Heinrich Warburg, who first made the observation (Hsu et al., 2008; Warburg, 1956). This dependency on glycolysis may seem counterintuitive, considering cancer cells require high energy levels to divide and proliferate, yet they are more reliant on a less efficient form of ATP production. A number of hypotheses have been proposed to explain this dependency on glycolysis.

Unlike oxidative phosphorylation, which is completely catabolic, breaking down glucose into CO₂, the glycolysis pathway generates a number of key metabolites, which can be channeled into other anabolic pathways in the cell (Cairns et al., 2011). As discussed previously, cancer cells often use the less active isoform of pyruvate kinase, PKM2, to further accumulate those metabolites and allow them to be more efficiently shuttle to other pathways, namely the PPP for nucleotide and amino acid synthesis (Diers et al., 2012). Furthermore, PPP generates nicotinamide adenine dinucleotide phosphate (NADPH), which provides antioxidant defense against reactive oxygen species (ROS) and chemotherapeutic agents. In proliferating cells, pyruvate can enter a truncated TCA cycle, where it is catalyzed into acetyl-CoA by pyruvate dehydrogenase (PDH). Instead of completing the TCA cycle,
the resultant acetyl-CoA is exported from the mitochondrial matrix and becomes available for the synthesis of fatty acids and cholesterol.

In addition to generating metabolic intermediates for the biosynthesis of proteins, lipids and fatty acids, an altered metabolism may also provide tumor cells with another proliferative advantage: avoidance of apoptosis (Kroemer et al., 2008). Oxidative phosphorylation generates ROS, which have the potential to damage DNA, RNA and protein, resulting in cellular apoptosis. Furthermore, increased flux through the electron transport chain in mitochondria promotes the release of cytochrome c into the cytoplasm, where it would bind to apoptotic protease activating factor-1 (Apaf-1), leading to apoptosis. The use of dichloroacetate (DCA), an inhibitor of pyruvate dehydrogenase kinase and an FDA-approved treatment for congenital lactose acidosis, activates oxidative phosphorylation and promotes apoptosis (Locasale et al., 2010). By uncoupling glycolysis from oxidative respiration, tumor cells could potentially reduce the production and release of ROS and cytochrome c, thus prevent cell death.

A reliance on glycolysis also provides tumor cells with an advantage for invasion and metastasis. As tumor cells hyperproliferate, they become hypoxic as they reach the oxygen diffusion limit, which forces the cells to adapt to the glycolytic phenotype (Vander Heiden, 2011). The anaerobic tumor microenvironment would be lethal to cells that depend on oxygen for survival. A persistent increase in glycolysis results in local environment acidosis, due to lactate production. Increased intra-tumoral lactate concentrations are associated with increased incidence of metastasis. Notably, acidosis itself may be mutagenic, possibly through inhibition of DNA repair.
1.4.3. Microenvironment Acidification and Hypoxia

The proliferation of differentiated cells is controlled by the availability of growth factors. Accessibility to nutrients and oxygen, which are critical for metabolism and cell proliferation, depends on a blood supply or angiogenesis. As aggressive replication effectively moves cancer cells further away from capillaries and therefore nutrients and oxygen, the tumor microenvironment becomes acidic, due to lactate secretion, and hypoxic. A critical transcription factor involved in the adaptation to hypoxia is the hypoxia-induced factor 1α (HIF-1α). HIF-1α is a heterodimer consisting of a stable β subunit and an unstable α subunit, that is degraded under normoxia (Semenza, 2010). Under low oxygen conditions, HIF-1α stimulates the glycolysis pathway by upregulating the glucose transporter GLUT1 and HK II, while promoting the production and secretion of lactate through activating lactate dehydrogenase A (LDHA) and monocarboxylate transporter 4 (MCT4) (Annibaldi et al., 2010; Kroemer et al., 2008). In addition, HIF-1α promotes glycolysis and suppresses OXPHOS by activating PDH kinase 1 (PDK1) which inhibits PDH and the conversion of pyruvate to lactate. HIF-1α can also induce VEGF and promotes angiogenesis (Semenza, 2010).

It has also been suggested that HK II can interact with the voltage-dependent anion channel (VDAC) on the outer membrane of mitochondria in cancer cells (Krasnov et al., 2013). VDAC normally acts as a gate for small molecules, such as ATP, and also as the metabolic interface between mitochondria and the cytosol. Furthermore, VDAC participates in the release of apoptotic factors, such as cytochrome c, which activates Apaf-1 and the degradation cascade. The binding of HK II to VDAC promotes the closure of the channel and directly prevents the release of pro-apoptotic factors. B-cell lymphoma-extra large (Bcl-xL),
an anti-apoptotic protein in the Bcl-2 family, can bind VDAC and inhibit the pro-apoptotic protein Bax/Bak, which forms pores in the mitochondrial wall (Cory et al., 2002). HK II-VDAC interactions prevent Bcl-xL from binding to VDAC and promotes Bcl-xL-Bax/Bak interactions, thus indirectly preventing apoptosis (Pastorino et al., 2008). The HK II-VDAC association is further facilitated by Akt, which directly phosphorylates HK II and inhibits GSK-3β phosphorylation of VDAC. Under homeostatic conditions, GSK3-β can phosphorylate VDAC on threonine 51, which results in the detachment of HK II and the sensitization of cancer cells to pro-apoptotic factors, including chemotherapeutic agents (Pastorino et al., 2008).

An upregulation of LDHA and MCT4 results in the acidification of the tumor microenvironment. Low pH can impair anti-tumor immune responses, while promoting invasion and metastasis. The acidic environment inhibits dendritic cell maturation during antigen-specific T cell stimulation and suppresses NK cell activity (Calcino et al., 2012). Furthermore, high acid concentration impairs CTL functions, as demonstrated by reduced IL-2, granzyme and perforin production and an elevated rate of apoptosis. Interestingly, providing stronger T cell signaling via CD28 stimulation was able to rescue this acidity-induced anergy in vitro, suggesting that the activation threshold for T cells may be pH-dependent (Huber et al., 2017). An acid environment enhances the recruitment of TAMs and MDSCs, while directly increasing invasion and metastasis of cancer cells. The metastatic phenotype is likely driven by the pH-dependent activation of metalloproteinases and cathepsins, that break down basement membranes and the extracellular matrix (Seyfried et al., 2013). Melanoma cells pre-treated with acidic medium, similar to the tumor microenvironment, are more likely to metastasize to the lung (Kato et al., 2013).
1.4.4. Therapy Targeting Cancer Metabolism

Therapies that directly target glycolysis have shown some promise in cancer treatments. Silibinin is a GLUT inhibitor that can induce G1 cell cycle arrest and suppress angiogenesis (Singh et al., 2005). However, due to its poor tumor penetrance and short half-life, it had only modest results in a Phase I trial for hepatocellular carcinoma. WZB117, a GLUT-1-specific inhibitor, inhibits cancer cell grow both in vitro and in vivo preclinical studies as well as inducing cell-cycle arrest, leading to apoptosis (Liu et al., 2012). 2-deoxyglucose (2-DG), a non-metabolized analogue of glucose, is currently in clinical trials for treatment of advanced breast, prostate, lung and gastric cancers (Sborov et al., 2015). 2-DG competes with glucose for interactions with GLUT and HK II. However, 2-DG lacks a 2’ oxygen group, which prevents it from being metabolized further. As a result, it is able to suppress glycolysis and cell proliferation. Lonidamine is a HK II inhibitor that promotes mitochondrial permeability and cellular apoptosis (Nath et al., 2016). However, after 2 negative Phase III trials, further development has been terminated. Another drug that inhibits HK II is 3-bromopyruvate, that depletes cellular ATP, and has shown significant anti-cancer effects in preclinical studies (Pedersen, 2012). 3-(3-Pyridinyl)-1-(4-pyridinyl)-2-propen-1-one) (3PO) is a novel molecule that can inhibit PFK activity, decrease glucose uptake and inhibit cancer growth in vitro (Xintaropoulou et al., 2015). In addition, TEPP-46, an activator of PKM2, has shown efficacy in inhibiting tumorigenesis in preclinical studies by decreasing the availability of glycolytic intermediates (Galluzzi et al., 2013).

In addition to targeting glycolysis, a number of inhibitors have been developed to suppress anabolic pathways. Pictilisib, or GDC-0941, is a potent inhibitor of pan-class I PI3K. In a Phase II clinical trial for estrogen receptor (ER)+ advanced breast cancer,
pictilisib and fulvestrant (a drug that targets and causes the degradation of the ER) treatment led to a progression-free survival rate of 6.6 months, an increase of 1.5 month over fulvestrant alone (Sarker et al., 2015). BKM120, or buparlisib, is another promising PI3K inhibitor which is currently in a Phase III clinical trial (Massacesi et al., 2016). MK-2206 is a small molecule that can allosterically inhibit Akt. It has strong affinity for all three isoforms of Akt and is currently in Phase II clinical trials (Lee et al., 2015). One of the most widely used mTOR inhibitors is rapamycin or sirolimus. It was initially developed as an immunosuppressant for transplant patients. Since then, several derivatives of rapamycin, or rapalogues, have been developed for cancer treatment. Temsirolimus has been approved for renal cell carcinoma, while everolimus is approved for the treatment of breast cancer, neuroendocrine tumors of the pancreas and subependymal giant cell astrocytomas (Meng et al., 2015). Metformin is another drug that showed promise in its latest clinical trials for patients with endometrial hyperplasia. It is the most commonly prescribed medication for type 2 diabetes, but can also suppress mTOR activity indirectly by activating AMPK (Buzzai et al., 2007; Vakana et al., 2012). It is currently being examined for treatment for colorectal cancer, endometrial cancer, non-small cell lung cancer and prostate cancer.

Small molecules have been developed to also target the unique tumor microenvironment. Given the importance of HIF-1α in promoting tumorigenesis, small molecules have been designed to target its function at multiple stages (Mooring et al., 2011; Onnis et al., 2009; Xia et al., 2012). EZN-2968 is an RNA antagonist against HIF-1α mRNA and showed potent dose-dependent downregulation of HIF-1α and VEGF expression in a phase I trial. PX-478 (S-2-amino-3-[4′-N,N-bis(2-chloroethyl)amino]phenyl propionic acid N-oxide dihydrochloride) reduces HIF-1α deubiquitination and leads to degradation of HIF-
1α proteins. In a phase I trial, 35% of the patients with advanced cancer who were treated with PX-478 had stable disease. Acriflavine, a topical antiseptic, was shown to inhibit the dimerization of HIF-1α by binding to the PAS-B subdomain in a xenotransplant mouse model. However, there is currently no clinical trial testing its effectiveness as a potential cancer therapeutic. A number of inhibitors have also been developed to block HIF-1α binding to target DNA, including polyamides and echinomycin, and to reduce HIF-α transcriptional activity, such as chetomin and bortezomib. However, no HIF-1α inhibitor is currently approved for treatment of cancer. Monocarboxylate transporter inhibitors have been developed to prevent lactate efflux and therefore microenvironment acidification. One such example, α-cyano-4-hydroxy-cinnamic acid, showed efficacy in decreasing tumor size and sensitizing hypoxic tumors to radiotherapy (Ganapathy-Kanniappan et al., 2013). LDHA inhibitors, oxamate and FX11 have also been shown to reduce cellular ATP levels, promote AMPK activation and induce apoptosis through generating reactive oxygen species in preclinical studies (Martinez-Outschoorn et al., 2016; Porporato et al., 2011). In summary, the search for effective metabolic inhibitors is an area of intense activity. The development of highly specific, isoform-selective inhibitors will add a new dimension to therapeutic interventions against cancers.

1.4.5. Cytokines and Growth Factors Affecting Metabolism

Cytokines and growth factors have long been associated with modulating normal and malignant cell metabolism. Type I IFNs are central to antiviral and anti-cancer immunity. IFN-α induces rapid tyrosine phosphorylation of the insulin receptor substrate-1 (IRS-1), which subsequently interacts with the p85 subunit of PI3K via SH2 domains (Uddin et al., 1995). This finding suggests that IFN-α elicits its responses in part through the IRS-1
pathway, which is downstream of the insulin signaling cascade. IRS-1 has been implicated in its activation of the PI3K/AKT pathway, which leads to the translocation of GLUT4 to the surface and enhances glucose uptake. More recently, it was shown that IFN-β also engage directly in metabolic changes to better support the induction of an effective immune response in mouse peritoneal macrophages (Jiang et al., 2016). IFN-β preferentially activates PFK2 in macrophages to promote glycolytic activity. This IFN-induced accelerated cellular metabolism enhanced antiviral activity, including phagocytosis and the removal of virally infected cells (Jiang et al., 2016).

IL-4, similar to type I IFNs, can activate the insulin growth factor (IGF) pathway via IRS signaling, in breast cancer cells (Jackson et al., 1998). Not surprisingly, IGF stimulates the insulin pathway with greater potency. Nonetheless, breast cancer cells may leverage soluble IL-4 to enhance cell proliferation. Leptin is another cytokine that has been implicated in cancer metabolism. It plays an important role in fatty acid synthesis, regulating energy balance and inhibiting hunger. Leptin, produced by adipose tissue, is regulated by insulin. Leptin binds to its receptor LEP-R or OB-R and activates Jak2 leading to Stat3 phosphorylation, resulting in the proliferation and angiogenesis of breast, pancreas, lung, prostate and ovarian cancers (Mullen et al., 2016). Indeed, Stat3 signaling is well documented for inducing the proliferation and migration of malignant cells (Yu et al., 2014). Specifically, Stat3 upregulates the transcription of cyclin D1 and VEGF. Notably, leptin has been shown to induce IRS phosphorylation, likely in a Jak2 and Stat5b-dependent manner.

CCL5-CCR5 interactions invoke both Jak/Stat signaling (Wong et al., 2001) and the PI3K/AKT/mTOR pathway (Murooka et al., 2009), both implicated in modulating cellular metabolism, as described above. Additionally, studies from our research group provided
evidence that CCL5-CCR5 interactions upregulate glycolysis and glucose uptake in activated T cells in an mTOR-dependent manner (Chan et al., 2012). It is understood that the neoplastic process is heavily dependent on cell metabolism and given that mTOR is a nutrient sensor that regulates cell proliferation and that CCL5 is a predictor of disease progression in breast cancer, studies described herein are focused on the role of CCL5 in modulating anabolic pathways in support of enhanced cancer cell proliferation. This thesis will investigate the role of CCL5-CCR5 interactions in the context of breast cancer metabolism.
1.5. Hypothesis and Objectives

Hypothesis

CCL5-CCR5 interactions modulate metabolic events in breast cancer cells to promote proliferation.

Objectives:

1. Examine the role of CCL5-CCR5 interactions in regulating cell metabolism to enhance proliferation of breast cancer cell \textit{in vitro}.

2. Investigate the effects of CCL5-CCR5-mediated metabolic events on tumorigenesis \textit{in vivo}.
Chapter 2

Materials & Methods
2.1. Mice

All mice were housed in a pathogen-free environment and all experiments were approved by the Animal Care Committee of the Toronto General Hospital Research Institute. C57BL/6 MMTV-PyMT mice were provided by P. Ohashi (University Health Network, Toronto). The MMTV-PyMT transgene (MMTV-PyMT) has the mouse mammary tumor virus (MMTV) long terminal repeat upstream of a cDNA sequence encoding the polyomavirus middle T antigen (PyMT). Female mice develop palpable tumors by five weeks of age. C57BL/6 CCR5−/− mice were purchased from the Jackson Laboratory. C57BL/6 MMTV-PyMT.CCR5−/− were generated by breeding MMTV-PyMT.CCR5+/+ mice with C57Bl/6 CCR5−/− mice, selecting for PyMT.CCR5+/− F1 progeny, and then crossing PyMT.CCR5+/− with C57BL/6 CCR5−/− mice and selecting for PyMT.CCR5−/− F2 progeny. Multifocal tumors (100–700 mm³) were harvested from MMTV-PyMT and MMTV-PyMT.CCR5−/− mice, tissue minced, then digested with digestion buffer (F-12, 100 units ml⁻¹ penicillin, 100 mg ml⁻¹ streptomycin, 1 mg ml⁻¹ DNase, 100 U ml⁻¹ Collagenase type I). The resultant cell suspension was passed through a 40 µm filter to obtain single cell suspension. The single cell suspensions were maintained in F12 medium supplemented with 10% fetal calf serum (Sigma), 100 units ml⁻¹ penicillin and 100 mg ml⁻¹ streptomycin (Invitrogen). NOD scid gamma (NSG) mice were purchased from the Cancer Stemcell Colony at the University Health Network, Toronto. Eight week old female NSG mice were injected with 2.5×10⁶ cells in 200 µL PBS into their lower mammary fat pads. Tumor growth was monitored externally using calipers.
2.2. Cells and reagents

Human breast cancer cell lines MDA-MB-231 and MCF-7 were a gift from L. Penn (University Health Network, Toronto). Cell lines were maintained in DMEM/F12 medium supplemented with 10% fetal calf serum (Sigma), 100 units ml\(^{-1}\) penicillin and 100 mg ml\(^{-1}\) streptomycin (Invitrogen). CCR5 expression was confirmed by flow cytometry using an anti-human (BD BioSciences) and anti-mouse (BioLegend) CCR5 (CD195) antibody. Antibodies for phospho-mTOR (Ser-2448; #2971), mTOR (#2972), phospho-AKT (Ser-473; #9271), AKT (#9272), phospho-GSK-3β (Ser-9; #9336), GSK-3β (#9315) and phospho-4E-BP1 (#2855) were purchased from Cell Signaling Technology. MDA-MB-231.CCR5\(^{-/-}\) cells were generated using a commercial clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9) CCR5 gene knockout kit (KN216008) from Origene according to the manufacturer’s protocol. GLUT-1, -2, -3 and -4 antibodies were obtained from Santa Cruz Biotechnology Inc. and R & D Systems. CCL5 was a generous gift from A. Proudfoot (Geneva Research Centre, Merck Serono International, Switzerland). Maraviroc and TAK-779 (N,N-dimethyl-N-[[2-(4-methylphenyl)-6,7-dihydro-5H-benzocyclohepten-8-yl]amino]benzyl)-tetrahydro-2H-pyran-4-aminium chloride) were provided by D. Branch (University of Toronto). Rapamycin was obtained from Calbiochem. 2-DG, the ATP bioluminescent assay, pyruvate assay and glucose-6-phosphate assay kits were purchased from Sigma. Oligomycin was obtained from Cell Signaling Technology. MTT and puromycin were obtained from ThermoFisher Scientific.
2.3. Immunoblotting & immunoprecipitation

Cells were serum starved for 16 h, then incubated with 10 nM CCL5 for the indicated times, pelleted by centrifugation, then lysed in 100 µl of lysis buffer (1% Triton X-100, 0.5% NP-40, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF). In total, 40 µg of protein lysate was denatured and resolved by SDS-PAGE. The separated proteins were transferred to a nitrocellulose membrane and membranes were probed with the specified antibodies. Proteins were visualized using the ECL detection system.

2.4. Fluorescence-activated cell sorting analysis

In total, 10^6 cells were incubated with the specified primary antibodies or isotype control antibodies, for 30 min at room temperature, followed by 20 min with FITC/PE-conjugated secondary antibodies, then stained and fixed immediately in 2% PFA. Cells were analysed using the FACSCalibur and FLOWJO software (BD Biosciences).

2.5. Glucose uptake assay

In total, 3 × 10^5 cells in 2% FCS DMEM/F12 medium were plated in individual wells of 12-well plates overnight. Cells were either left untreated, pre-treated with inhibitors for 1 h prior to CCL5 treatment or treated with CCL5 alone for the indicated times, then pulsed with 50 µM 2-deoxy2-[(7-nitro-2, 1,3-benzoxadiazol-4-yl) amino]-d-glucose (2-NBDG) for 15 min or as described. Cells were washed three times with PBS, detached using 1 mM EDTA and analysed using the FACSCalibur and FLOWJO software (BD Biosciences).
2.6. ATP bioluminescent assay

Intracellular ATP levels were examined using the ATP bioluminescent assay kit, according to the manufacturer's protocol (Sigma-Aldrich). Briefly, $2 \times 10^4$ cells were either left untreated, pre-treated with inhibitors for 1 h prior to treatment with CCL5 or treated with CCL5 alone. Bioluminescence was measured using a VICTOR™ X3 Multilabel Plate Reader (PerkinElmer).

2.7. Glucose 6-phosphate, pyruvate & lactate assay

Intracellular G6P, pyruvate and lactate levels were examined using the respective assay kits, according to the manufacturer's protocol (Sigma-Aldrich). In total, $2.5 \times 10^5$ cells were either left untreated, treated with inhibitors for 1 h or treated with CCL5. 0.1g of tissue was used for all ex vivo lactate assays. Bioluminescence was measured using a VICTOR™ X3 Multilabel Plate Reader (PerkinElmer).

2.8. Glycolytic stress test using the Seahorse Extracellular Flux Analyzer

Real-time measurements of ECAR were used as indicators of the cellular rate of glycolysis. In total, $5 \times 10^4$ cells were seeded into individual wells of a 96-well plate overnight. For experiments involving inhibitors, cells were pre-treated for 1 h with the indicated inhibitor prior to experimentation. The culture medium was replaced with DMEM/F12 (without bicarbonate), to allow for a swift and rapid change in pH. The basal reading was normalized. Subsequently, compounds were injected into each individual well as indicated, to measure effects on the rate of glycolysis. Rate of change in pH was measured every 5 min. To examine glycolytic capacity, 2 µM oligomycin was injected into each well.
and the rapid surge and the subsequent plateau in ECAR was interpreted as the cellular transition from mitochondrial respiration to glycolysis for the production of ATP. The difference between the resulting maximum and the normalized basal ECAR was interpreted as the glycolytic capacity.

2.9. Proliferation assay

2.9.1. Hemocytometer

In total, $2 \times 10^4$ cells were seeded into individual wells of 24-well plates in 2% FCS DMEM/F12. Cells were either left untreated or incubated with 10 nM CCL5 for the indicated times, then collected and counted with a hemocytometer. In CCR5 blocking studies, cells were pre-treated with inhibitors for 1 h prior to CCL5 stimulation. The medium containing CCL5 and/or inhibitors was replaced every other day.

2.9.2. MTT

$1 \times 10^4$ cells were seeded into individual wells of 96-well plates in 2% FCS DMEM/F12. Cells were either left untreated, treated with inhibitor or CCL5 for 4 days. On day 4, 10μL of 12mM MTT stock solution was added to each well and incubated for 4 hours. 50μL of DMSO was added to each well before measuring absorbance.

2.10. Chemotaxis assay

Cell chemotaxis was assayed using 24-well Transwell chambers with 8 μm pores (Corning). In total, $1 \times 10^5$ cells in 100 μl chemotaxis buffer (DMEM/F12/0.5% BSA) were placed in the upper chambers. CCL5, diluted in 600 μl chemotaxis buffer, was placed in the lower wells and the chambers were incubated for 18 h at 37°C. Cells that migrated to the
bottom wells were collected and counted with a hemocytometer. For experiments involving inhibitors, cells were pre-treated for 1 h with the indicated inhibitor and then placed in the upper chambers.

2.11. Invasion assay

The invasive capacity of cells was assayed using a QCM ECMatrix Cell Invasion kit with 8 µm pore size, according to the manufacturer's protocol (Millipore). In total, $10^5$ cells in 100 µl buffer containing CCL5 were placed in the upper chambers. Cells were allowed 24 h to invade through the ECMatrix into the lower chamber. Cells were subsequently quantified using CyQuant GR DyeCells. Fluorimetric readings were measured using a VICTOR™ X3 Multilabel Plate Reader (PerkinElmer).

2.12. Metabolomic profiling

Unbiased, metabolomic profiling analysis was conducted using the Metabolon platform, as described (Gatenby et al., 2004). Packed pellets of $3 \times 10^7$ frozen MMTV-PyMT cells, treated as indicated, were transferred to Metabolon Inc. (Durham, NC) for metabolomics analysis. Using cold methanol extraction, supernatants from the samples were purified for mass spectrometry. To identify metabolites, the platform used ultrahigh performance liquid chromatography/tandem mass spectrometry (UHPLC/MS-MS) and gas chromatography/mass spectrometry (GC/MS) to identify features in the experimental samples against a reference library of chemical standards that include molecular weight, retention time and MS spectra. The information was then curated into standard formats for presentation purposes, including heat maps, pathways and box plot analyses, described in Figures 3.9 – 3.11.
2.13. ELISA

CCL5 levels in culture supernatants were measured using anti-mouse CCL5 (DY478) and anti-human CCL5 (DRN00B) ELISA kits from R&D Systems, according to the manufacturer’s protocol.

2.14. Hematoxylin & Eosin staining of thin sections

Mice were euthanized, their tumors harvested, then fixed in 10% formalin. Thin sections (5µm) were prepared and stained with hematoxylin and eosin (H&E).

2.15. Matrix-Assisted Laser Desorption/Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (MALDI-FTICR-MS) imaging

2.15.1. Materials

9-Aminoacridine hydrochloride (9-AA) was purchased from Sigma–Aldrich. Water and Methanol was purchased from Fischer Scientific at the highest purity grade available and used without further purification.

2.15.2. Tissue preparation for MALDI-FTICR-MS imaging

Frozen tumor tissue sections (10 microns) were prepared with a Microm HM550 (Thermo Scientific) cryostat and were placed on histology slides for MALDI-FTICR-MS imaging. Serial sections (5µm) were H&E stained for morphological analysis. Imaging slides were stored at -20°C and were then slowly brought to room temperature in a desiccator and prepared for MALDI-MSI analysis. A uniform coating of 9-AA was added using an HTX TM Sprayer (HTX Technologies Carrboro, NC, Germany) and a solution of 10 mg/mL 9-AA
in 80:20 MeOH/H₂O. Slides were then stored in a desiccator for 1 h before analysis in the mass spectrometer.

2.15.3. MALDI-FTICR-MS imaging data acquisition

All experiments were performed using a 7T solariX XR FTICR mass spectrometer (Bruker Daltonics, Billerica, MA, USA) using a 50-µm pixel size and 200 laser shots per pixel. The FTICR mass spectrometer was calibrated prior to the MALDI-MSI experiments using the peaks of NaTFA. Each pixel’s mass spectrum was then recalibrated using the matrix peak of 9-AA as an internal lock mass during data collection. Mass spectra were acquired in negative ion mode within a mass range of 150–1100 m/z and 1M data points collected. Molecular images were visualized using the FlexImaging software (version 4.1, Bruker) using total ion current normalization for visualization of images.

2.15.4. Discriminate metabolite analysis

Data from FlexImaging was exported to SCiLS Lab (ver.2015b-3.02.7804: Bremen, Germany) was used for processing the datasets as follows: raw data was imported and normalized to total ion current (TIC). To determine peaks with differential abundance in WT and KO tumor tissue, 200 random spectra from each region were selected for discriminate analysis in SCiLS. Hypothesis testing was then performed using the Student’s t-test on the mean spectra of each tissue region for each m/z region identified. To obtain the most accurate m/z for potential metabolite peaks, data from FlexImaging were exported into Data Analysis (version 4.4 Bruker) and internal calibration was performed using 9AA, ATP and ADP (endogenous tissue metabolite peaks, verified using an ATP standard). Compounds were identified by accurate mass match of obtained high resolution FTICR m/z values to the
Metlin database of metabolites. High mass accuracy, using a mass tolerance of 1 ppm, was then used to filter the discriminate peaks and assign provisional identifications.

2.16. Statistical analyses

Statistical significance was analysed by Student's $t$-test unless specified otherwise. Data generated using the Metabolon platform were analysed using the Welch's $t$-test. A level of $p < 0.05$ identified significance. Data are expressed as mean ± S.E.
Chapter 3

CCL5 activation of CCR5 regulates cell metabolism to enhance proliferation of breast cancer cells

Chapter 3 was published as:


All experiments were performed by D.Gao unless stated.
3.1. Abstract

In earlier studies, we showed that CCL5 enhances proliferation and survival of MCF-7 breast cancer cells in an mTOR-dependent manner and we provided evidence that, for T cells, CCL5 activation of CCR5 results in increased glycolysis and enhanced ATP production. Increases in metabolic activity of cancer cells, specifically increased glycolytic activity and increased expression of glucose transporters, are associated with tumor progression. In this report, we provide evidence that CCL5 enhances the proliferation of human breast cancer cell lines (MDA-MB-231, MCF-7) and mouse mammary tumor cells (MMTV-PyMT), mediated by CCR5 activation. Concomitant with enhanced proliferation we show that CCL5 increases cell surface expression of the glucose transporter GLUT-1, and increases glucose uptake and ATP production by these cells. Blocking CCL5-inducible glucose uptake abrogates the enhanced proliferation induced by CCL5. We provide evidence that increased glucose uptake is associated with enhanced glycolysis, as measured by extracellular acidification. Moreover, CCL5 enhances the invasive capacity of these breast cancer cells. Using metabolomics, we demonstrate that the metabolic signature of CCL5-treated primary mouse mammary tumor cells reflects increased anabolic metabolism. The implications are that CCL5–CCR5 interactions in the tumor microenvironment regulate metabolic events, specifically glycolysis, to promote tumor proliferation and invasion.
3.2. Introduction

Inflammation is critical in tumor progression (Jiang & Shapiro, 2014; Mantovani et al., 2008; Vendramini-Costa & Carvalho, 2012), creating a tumor microenvironment largely defined by soluble secreted factors and an influx of inflammatory cells. Together, these participate in the neoplastic process to regulate the proliferation, survival and migration of tumor cells (de la Cruz-Merino et al., 2013; Ubaldo Martinez-Outschoorn et al., 2014). Notably, tumor cells have acquired some of the effectors of the innate immune system, such as chemokines and their receptors, to facilitate metastasis and invasion.

Chemokines are chemotactic cytokines responsible for orchestrating leucocyte migration. Chemokine binding to G-protein-coupled receptors initiates signaling cascades that promote directional migration through cytoskeletal rearrangement, cell polarization and integrin activation. Chemokines also regulate numerous migration-unrelated responses, including cell survival, apoptosis, protein translation, embryogenesis, angiogenesis and tumor growth (Griffith et al., 2014). In the context of breast cancer, chemokines have been implicated in promoting the malignant phenotype: CXCL3, CXCL12, CXCL13, CCL21 and CCL5 are associated with tumor progression and metastasis in breast cancer (Biswas et al., 2014; Luboshits et al., 1999; Müller et al., 2001; See et al., 2014; Zhang et al., 2013).

CCL5 (RANTES) is a chemokine that exerts an important role in inflammation, by orchestrating the migration of monocytes and T cells to injured, infected and tumor sites (Marques et al., 2013). While CCL5 may promote efficient anti-tumor immune responses, it has also been associated with cancer progression and metastasis (Luboshits et al., 1999; Zhang et al., 2013). Notably, CCL5 engagement with its cognate receptor, CCR5, results in
the rapid upregulation of mRNA translation of pro-survival factors in MCF-7 breast cancer cells (Murooka et al., 2009). Furthermore, CCL5 is a predictor of disease progression in stage II breast cancer patients. Circulating CCL5 levels are elevated in patients with high-grade tumors compared with low-grade tumors (Yaal-Hahoshen et al., 2006).

Unlike differentiated tissue, tumor cells are more reliant on anaerobic glycolysis, even in the presence of oxygen (Warburg, 1956). The glycolysis pathway can generate a number of key metabolites that provide a proliferative advantage to tumor cells. Increases in metabolic activity of cancer cells, specifically increased glycolytic activity and increased expression of glucose transporters, are associated with tumor progression (Boroughs et al., 2015; Szablewski, 2013). In earlier studies, we provided evidence that CCL5–CCR5 signaling can stimulate growth of MCF-7 cells in an mTOR-dependent manner (Murooka et al., 2009). In addition, for T cells, CCL5 activation of CCR5 results in increased glucose uptake and enhanced intracellular ATP, all associated with enabling CCL5-mediated chemotaxis (Chan et al., 2012).

In this chapter, we provide the first evidence that CCL5–CCR5 interactions regulate breast cancer cell metabolism to enhance proliferation, migration and invasion. The novelty of these data suggests targeting CCL5–CCR5 interactions that increase tumor metabolism may be an effective therapeutic strategy to limit tumor proliferation and tissue invasion.
3.3. Results

3.3.1. CCL5 increases glucose uptake, GLUT-1 expression and glycolysis

In an earlier publication, we provided evidence that CCL5 treatment promotes the proliferation of MCF-7 human breast cancer cells, through mTOR-dependent mRNA translation of a subset of proteins associated with cell cycle progression and survival (Murooka et al., 2009). mRNA translation is an energy dependent process. Moreover, the TORC1 complex in the mTOR pathway functions to integrate many different signals beyond mRNA translation, including those associated with nutrient sensing. In a first series of experiments, we examined the effects of CCL5 on activation of the AKT/mTOR pathway in MDA-MB-231 human breast cancer cells. In western immunoblots of cell lysates, we identified the rapid and transient phosphorylation of AKT and mTOR following CCL5 treatment (Figure 3.1), with evidence of phosphorylation of the downstream target of mTOR, 4E-BP1, being phosphorylated (Figure 3.2). In addition, we provide evidence that CCL5 phosphorylates glycogen synthase kinase 3β (GSK-3β), thereby de-repressing/releasing signaling downstream of CCR5 associated with the mTOR pathway (Figure 3.1). Using the CCR5 inhibitor TAK-779, we show that these CCL5-mediated signaling events are mediated by CCR5 (Figure 3.1). CCL5 activation of mTOR signaling is consistent with our earlier findings (Murooka et al., 2009).

In all subsequent experiments, we employed MCF-7 and MDA-MB-231 breast cancer cells and primary cells from mammary tumors harvested from MMTV-PyMT mice. Cell surface expression of CCR5 was confirmed by fluorescence-activated cell sorting (FACS) analysis in all three breast cancer cell types (Figure 3.3).
Figure 3.1. CCL5 treatment activates the mTOR/AKT pathway in breast cancer cells.

A Cells were either left untreated, treated with 10nM CCL5 for the indicated times, or pretreated with 2uM TAK-779 for 1hr prior to CCL5 treatment. Cell lysates were prepared and resolved by SDS-PAGE then immunoblotted with the indicated antibodies. Data shown are representative of 3 independent experiments. B Bands were quantified by densitometry using Image J software and normalized relative to the corresponding unphosphorylated proteins. Bar graphs represent means +/- S.E. of the combined data from the 3 experiments. Statistical analysis was performed comparing different treatments: *p<0.05, **p<0.01 and ***p<0.001.
A

CCL5 CCL5 + Tak-779

mins 0 15 30 60 0 15 30 60

p-mTOR (ser2448)
mTOR

p-AKT (ser473)
AKT

p-GSK3β (ser9)
GSK3β

B

■ CCL5 □ CCL5 + TAK-779

<table>
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<th>mins post-CCL5 treatment</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>60</th>
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<td>p-GSK3β (Normalized Index)</td>
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MDA-MB-231
Figure 3.2. CCL5 treatment activates 4E-BP1 downstream of the AKT/mTOR pathway in breast cancer cells.

A MDA-MB-231 cells were either left untreated, or treated with 10nM CCL5 for the indicated times. Cell lysates were prepared, resolved by SDS-PAGE, then immunoblotted with the indicated Thr37/46 antibodies. Data shown are representative of 2 independent experiments. B Bands were quantified by densitometry using Image J software and normalized relative to the tubulin loading control. Bar graphs represent means +/- S.E. of the combined data from the 2 experiments. Statistical analysis was performed comparing different treatments: * $p<0.01$ and ** $p<0.01$. 
A

CCL5

mins 0 5 15 30

p-4E-BP1 (T37/46)

β-tubulin

B

mins post-CCL5 treatment

p-4E-BP1 (Normalized Index)

lower band

both bands
Figure 3.3. MDA-MB-231, MCF-7 and MMTV-PyMT express CCR5.

Cells were fixed with BD Perm/Wash Buffer™ prior to staining for CCR5 and FACS analysis. MDA-MB-231 and MCF-7 were stained using FITC mouse anti-human CD195 antibody from BD Biosciences (cat: 555992), MMTV-PyMT and PyMT.CCR5<sup>−/−</sup> were stained using PE anti-mouse CD195 antibody from Biolegend (cat: 107005). Grey histogram corresponds to isotype controls; open histograms indicate anti-CD195 stained cells.
In T cells, we have shown that CCL5 regulates glucose uptake and ATP generation, required to enable their migration (Chan et al., 2012). Notably, CCL5 regulation of glucose uptake was mTOR-dependent. Having shown that T cell chemotaxis is facilitated by CCL5 regulating glucose uptake (Chan et al., 2012), we speculated that CCL5 may enhance tumor cell proliferation, invasion and metastasis by a similar mechanism, because cancer cells exhibit significant dependence on glucose for their growth and survival (Zhao, 2014). At the outset, we examined the effects of CCL5 treatment of the different breast cancer cell types on glucose uptake. For these experiments, we employed the fluorescent glucose analogue 2-NBDG. The results in Figure 3.4A-C show that at a physiologic dose of 10 nM, CCL5 increases glucose uptake for all three breast cancer cell lines and that this uptake is CCR5- and mTOR-dependent, as both maraviroc (CCR5 inhibitor) and rapamycin (mTOR inhibitor) inhibit uptake. Our time course studies revealed that glucose uptake is elevated 1 h after CCL5 treatment, reaching a maximum by 3 h and remaining elevated for 18 h (Figure 3.5). These findings were similar whether 10 or 50 nM of CCL5 was administered. Notably, when cells derived from tumors harvested from MMTV-PyMT.CCR5−/− mice (described in Material and methods) are treated with CCL5, we observe no enhancement in glucose uptake, in support that CCR5 is the receptor mediating this effect (Figure 3.4D). Glucose transporter (GLUT) proteins facilitate glucose uptake across the plasma membrane. In human cells, 14 different GLUT isoforms have been identified, of which GLUT-1, GLUT-3 and GLUT-12 have been reported in clinical breast cancer cells (Kocdor et al., 2013). Given that GLUT-1 expression is upregulated by Akt activation (Fang et al., 2014) and we have shown CCL5 activation of Akt, we examined the effects of CCL5 treatment on GLUT-1 cell surface
Figure 3.4. CCL5 treatment increases glucose uptake in breast cancer cells mediated by CCR5 and mTOR.

Cells were either left untreated, treated with 10nM CCL5 for 3 h, or pre-treated with 2µM maraviroc or 50nM rapamycin for 1 h prior to CCL5 treatment. Glucose uptake was measured at 3 h, using 2-NDBG, as described in Materials and Methods. Glucose uptake is expressed as percent-change relative to untreated cells. Values are means +/- S.E. of triplicate assays and each data point combines the data from 3 independent experiments. Statistical analysis was performed comparing different treatments, with p values as indicated, or comparing CCL5 treatment with untreated cells: * p<0.05 and ** p<0.01.
Figure 3.5. Time course of glucose uptake in CCL5 treated MDA-MB-231.

Cells were either left untreated, or treated with 10nM CCL5 for 1, 2, 3, 4, 6, 8 and 18 h. Glucose uptake was measured using 2-NDBG, as described in Materials and Methods. Glucose uptake is expressed as percent change relative to untreated cells. Values are means +/- S.E. of triplicate assays and each data point combines the data from 4 independent experiments. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$. 
Percent change in intracellular glucose

MDA-MB-231

hrs post-CCL5 treatment
expression for the different breast cancer cells. Oligomycin (inhibitor of mitochondrial oxidative respiration) treatment, that diverts glucose preferentially for glycolysis, served as the positive control. The results in Figure 3.6 show that CCL5 treatment increases cell surface GLUT-1 expression over 3 h for all three breast cancer cell types. CCL5 treatment had no effect on GLUT-2, GLUT-3 or GLUT-4 cell surface expression.

Glucose uptake drives anaerobic glycolysis and ATP production. Most cancers rely on glycolysis for ATP production despite an available oxygen source. Glycolytic production of lactate serves as a measure of metabolic activity. Accordingly, we undertook a series of time course experiments using the Seahorse Metabolic Flux analyzer to evaluate the effects of CCL5 on glycolytic flux, using extracellular acidification rate (ECAR) as the readout. The data in Figure 3.7A-C represent the time course of ECAR measurements when cells are treated with CCL5. Although CCL5 treatment increased ECAR, pre-treatment with rapamycin or maraviroc ablated this increase in all three breast cancer cell types. Oligomycin treatment served again as the positive control for glycolysis. To investigate the effect of CCL5 on cellular capacity to conduct glycolysis, cells were pre-treated with CCL5 for 3 or 23 h, at which time oligomycin was introduced to preferentially force glycolysis. The difference between the basal level of ECAR and the maximal ECAR reached in the presence of oligomycin is the glycolytic reserve/capacity of the cell. Cells that were treated with CCL5 exhibit an elevated glycolytic reserve compared with untreated cells (Figure 3.7D-F). 2-Deoxy-D-glucose (2-DG), an inhibitor of glycolysis, was subsequently added, validating that the observed changes in ECAR were due to changes in the glycolytic rate. In support of the effects of CCL5 treatment on enhancing glycolysis, we observed a concomitant increase
Figure 3.6. CCL5 treatment increases GLUT-1 expression.

A-C Cells were treated with 10nM CCL5 for 1, 2 and 3 h, or 2µM oligomycin for 3 h, then fixed with 2% formalin to prevent receptor internalization prior to staining for cell surface GLUT-1 expression and FACS analysis. GLUT-1 expression is expressed as percent-change relative to untreated cells. Values are means +/- S.E. of triplicate assays and each data point combines the data from 3 independent experiments. Statistical analysis was performed comparing untreated and CCL5/oligomycin treated samples: * p<0.05, ** p<0.01 and *** p<0.001.
Figure 3.7. CCL5 increases rate of glycolysis and cellular glycolytic capacity.

A Seahorse Extracellular Flux Analyzer was used to detect real-time changes in extracellular acidification rate (ECAR) as measurements of the rate of glycolysis. A-C: The indicated compounds were introduced at the indicated time. Percent change in ECAR were measured. □-medium; ■-CCL5; ○-CCL5 + maraviroc; ●-CCL5 + rapamycin; Δ- Oligomycin. D-F: Glycolytic stress test. Cells were untreated (medium alone), or treated with 10nM CCL5 for 3 or 23 hr. As indicated, 2µM oligomycin was introduced followed by the glycolysis inhibitor 2-DG, at the indicated times. Percent change in ECAR was measured. □-medium; ■-10 nMCCL, 3hr; ●-10nM CCL5, 23hr. Values are the means +/- S.E. of 3 combined independent experiments, with all treatments being performed as 12 replicates. Statistical analysis was performed comparing no treatment with CCL5 treatment: * p<0.05 and ** p<0.01.
Figure 3.8. CCL5 treatment increases ATP and glycolytic intermediate flux.

A-D Cells were either left untreated, treated with 10nM CCL5 for 3 h, or pre-treated with 2µM maraviroc or 50nM rapamycin for 1 h prior to CCL5 treatment for 3 h. Oligomycin is included as a control. The percent change in intracellular ATP levels relative to untreated (medium alone) cells is shown. Values are means +/- S.E. of triplicate assays and each data point combines the data from 2 independent experiments. Statistical analysis was performed comparing different treatments, with p values as indicated, or comparing untreated cells with CCL5 treatment: * p<0.05, ** p<0.01, *** p<0.001. MDA-MB-231 cells were either left untreated, treated with 10nM CCL5 for 3 h, or pre-treated with 2µM maraviroc or 10nM rapamycin for 1 h prior to CCL5 treatment for 3 h. The percent change in intracellular pyruvate (E) and glucose 6-phosphate (G6P) (F) relative to untreated (medium alone) cells is shown. Values are means +/- S.E. of triplicate assays and each data point combines the data from 2 independent experiments. Statistical analysis was performed comparing different treatments, with p values as indicated, or comparing untreated cells with CCL5 treatment: * p<0.05.
in intracellular ATP levels following CCL5 treatment, sensitive to maraviroc and rapamycin treatments (Figure 3.8A-C). As for glucose uptake, treatment of tumor cells from MMTV-PyMT.CCR5+/− mice with CCL5 did not cause an increase in ATP production. Moreover, CCL5 treatment increased levels of glucose 6-phosphate (G6P) and pyruvate, sensitive to treatment with maraviroc and rapamycin (Figure 3.8 D-E). For G6P, the first intermediate in glycolysis, levels were significantly higher than for pyruvate, the final intermediate, suggesting that the majority of the glycolytic intermediates are shuttled into other, potentially anabolic, pathways.

### 3.3.2. Metabolic signature of CCL5-treated cancer cells reflects increased anabolic metabolism

Global unbiased metabolomic analysis was conducted to identify any changes in levels of metabolites that occurred following CCL5 treatment of the primary MMTV-PyMT tumor cells for 3 and 6 h (Figure 3.9A). This approach identified a total of 491 metabolites. The distribution of metabolites affected by CCL5 treatment according to specific metabolic pathways is shown, with a significant proportion involved in lipid, amino acid and carbohydrate metabolism (Figure 3.9B). Closer analysis revealed that CCL5 treatment for 3 h altered the levels of 93 metabolites, increasing 90 and decreasing 3, whereas CCL5 treatment for 6 h increased the levels of 230 and decreased the levels of 17 (Figure 3.9C). Comparing the metabolome of cells treated with CCL5 for 6 h to untreated, nearly half of the metabolites detected in peptide, carbohydrate, lipid, amino acid and nucleotide pathways have increased, providing evidence for a global increase in cellular metabolism (Figure 3.9D). Heat maps of the 491 metabolites arranged by their metabolic pathways are shown in Figure 3.10A, and by glycolytic metabolites, in Figure 3.10B (Table 3.1 for quantitation). Consistent with our
Figure 3.9. Metabolic signature of CCL5-treated MMTV-PyMT primary mouse breast cancer cells reflects increased anabolic metabolism.

MMTV-PyMT cells were treated with medium alone or 10nM CCL5 for 3 or 6 h (n=4 for each treatment). Cells were pelleted by centrifugation and stored frozen for transfer to Metabolon. A Schematic diagram illustrating the fold-change of metabolites in different metabolic pathways in MMTV-PyMT mouse breast cancer cells after 3 and 6 h of CCL5 treatment. The size of the circle indicates the degree of change. The colour indicates the direction of the change in flux, as indicated. B 491 metabolites were identified, grouped according to their classification and as a percentage of the total number identified. C Welch 2-sample t-tests were used to identify metabolites whose levels changed significantly upon CCL5 treatment at the indicated times, relative to their levels in untreated cells (p<0.05). Red indicates an increase in expression and green, a decrease. D Pie chart distinguishing changes in levels of metabolites following 6 h of CCL5 treatment.
Figure 3.10. Heat maps reveal a metabolic signature in CCL5-treated MMTV-PyMT primary mouse breast cancer cells.

A Heat map showing hierarchical clustering of metabolic flux in MMTV-PyMT cells treated with CCL5 for 3 and 6 h. (4 replicates per each treatment group for a total of 12 samples). The Y-axis is clustered according to metabolic pathways. B Plotted in the heat map is a hierarchical clustering of glycolytic metabolites affected by CCL5 treatment.
Figure 3.11. CCL5 treatment results in accumulation of metabolites.

Box plots of metabolites affected by CCL5 treatment involved in A glycolysis, B pentose phosphate pathway, C fatty acid β-oxidation, D amino acid catabolism and E glutamine metabolism, are identified. Box legend:+ inside box represents mean value, bar inside box represents median value, upper bar represents maximum of distribution, lower bar represents minimum of distribution. Scaled intensity is the normalized concentration of the metabolite relative to the Bradford protein concentration. Statistical analysis was performed comparing untreated and CCL5-treated cells: * $p<0.05$, ** $p<0.01$. Schematics of the distinct pathways are indicated.
in vitro findings, closer scrutiny of this metabolomic analysis revealed an increase in metabolites involved in glucose metabolism (Figure 3.11A). Following cellular uptake, glucose is phosphorylated to G6P, which can be metabolized either by glycolysis or by the pentose phosphate pathway. By 6 h after CCL5 treatment, the glycolytic intermediates pyruvate and lactate were elevated (Figure 3.11A). Similarly, specific metabolites of the pentose phosphate pathways, namely G6P and ribose 5-phosphate levels, were elevated after CCL5 treatment (Figure 3.11B).

Fatty acids are a critical source of energy for mitochondrial oxidation and cellular ATP generation. Fatty acids are broken down into acetyl-CoA, which can then be directed into the tricarboxylic acid (TCA) cycle for ATP production. When in excess, acetyl-CoA can be synthesized into ketone bodies, namely 3-hydroxybutyrate (BHBA) during ketogenesis. CCL5 treatment resulted in a transient increase in β-oxidation at 3 h, as reflected in the modest decrease in the levels of fatty acids, including stearoylcarnitine, stearamide, as well as a significant increase in the level of acetyl-CoA and its downstream product BHBA (Figure 3.11C). By 6 h after CCL5 treatment, these changes were reversed.

CCL5 treatment increased the accumulation of branched-chain amino acids (BCAAs), including leucine (Figure 3.11D). As shown, CCL5 induces a steady and consistent increase in the concentrations of BCAAs, while the level of β-hydroxyisovaleryl carnitine, an intermediate for BCAA catabolism, is decreased over the course of the treatment (Figure 3.11D). In addition, consistent with other anabolic pathways examined, the levels of glutamine as well as its downstream intermediates, including glutamate and α-ketoglutarate, increased post-CCL5 treatment (Figure 3.11E; Table 3.1).
3.3.3. CCL5 increases the proliferation, migration and invasive capacity of breast cancer cells

Consistent with our earlier data, in a 5 day time course study, we show that CCL5 treatment increases the proliferation of the three breast cancer cell lines (Figure 3.12 A-C). As anticipated, MMTV-PyMT.CCR5\textsuperscript{−/−} cells show no increase in proliferation/normal growth when treated with CCL5. Although maraviroc treatment alone has no effect on the proliferation of each of the cell lines (within S.E. of proliferation index), as anticipated, rapamycin treatment reduced their proliferation over the 5-day time period. When cells are pre-treated with maraviroc or rapamycin, then CCL5 added, the proliferative response to CCL5 is significantly reduced over the 5-day time course. Cells grown in glutamine-free medium exhibit a significantly blunted proliferation compared with their growth in medium with glutamine. When treated with CCL5, however, cell proliferation is enhanced. In a final series of treatments in these proliferation studies, we show that CCL5 treatment does not enhance cell proliferation when glucose uptake is blocked using 2-DG. Next, we examined the effects of CCL5 treatment on the migration/chemotaxis and invasive capacity of the different breast cancer cell types. We confirmed our earlier findings in MCF-7 cells that CCL5 promotes cell migration in a CCR5- and mTOR-dependent manner, using MDA-MB-231 cells (Figure 3.13). We provide evidence that CCL5-mediated cell invasion is sensitive to the nutrient supply in the extracellular environment: although CCL5 treatment induced an approximately twofold increase in invasion under standard cell culture glucose concentrations (18 mM), at lower glucose concentrations (12 mM, 6 mM or 0 mM), CCL5 treatment did not increase invasion (Figure 3.12D).
**Figure 3.12. CCL5 treatment enhances cell proliferation and invasion.**

A-C Cells were either left untreated (medium alone), treated with 2µM maraviroc (control), 50nM rapamycin (control), or 10nM CCL5, or pre-treated with the aforementioned inhibitors for one hour prior to CCL5 treatment. In addition cells were pre-treated with 2mM 2-DG for one hour prior to CCL5 treatment. In contrast to all the previous treatments in medium containing 5mM glutamine, an additional time course was conducted with 10nM CCL5 treatment, in medium that contained no glutamine (CCL5-glutamine). For all, medium was changed every other day and the treatment(s) reapplied. At the indicated time points, cells were trypsinized and counted with a hemocytometer. The proliferation index is normalized against untreated input cell number (i.e. cells at time zero). Values are means +/- S.E. of triplicate assays and each data point combines the data from 3 independent experiments. Statistical analysis was performed comparing untreated cells with CCL5-treated cells, and inhibitor-treated cells with CCL5 + inhibitor treated cells, or comparing CCL5-treated with CCL5 + inhibitor-treated cells: *p<0.05, **p<0.01. D 10^5 cells in 100µl buffer containing CCL5 at the indicated dose were placed in the upper compartment of the invasion chambers. Cells were allowed 24 h to invade into the bottom compartment through the ECMatrix. Cells were subsequently quantified using CyQuant GR DyeCells. Values are means +/- S.E. of triplicate assays and each data point combines the data from 2 independent experiments. Statistical analysis was performed comparing different treatments, with p values as indicated: *p<0.05, **p<0.01.
Figure 3.13. CCL5 induces chemotaxis in MDA-MB-231.

10^5 cells in 100µl chemotaxis buffer (DMEM/F12/0.5% BSA) were introduced in the upper chamber of 24-well Transwells. CCL5, diluted in 600µl chemotaxis buffer, was introduced into the lower chamber, and the whole incubated for 18 h at 37°C. Cells that migrated to the bottom chamber were collected and counted with a hemocytometer. For experiments involving inhibitors, cells were pre-treated for 1 h with the indicated inhibitor, then placed in the upper chamber. Bar graphs represent means +/- S.E. of the combined data from 3 independent experiments, each treatment performed as a triplicate assay in each experiment. The migration index is normalized against medium control. Statistical analysis was performed comparing different treatments, with p values as indicated, or comparing CCL5 (10nM)-treated and CCL5 + inhibitor-treated cells. *** p<0.001.
\[ p = 0.0003 \]

\[ p = 0.0011 \]
Table 3.1. CCL5 treatment results in accumulation of metabolites in MMTV-PyMT primary mouse breast cancer cells.

Metabolite levels of intermediates involved in major metabolic pathways are presented. Fold changes as well as p and q values are shown. Grey box highlights $p<0.05$. 
## Glycolysis, Gluconeogenesis, and Pyruvate Metabolism

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<th>Pathway</th>
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<th>CCL5 6h</th>
<th>Field Changes</th>
<th>p-value</th>
<th>q-value</th>
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3.4. Discussion

Breast cancer is the most common cancer and the most common cause of cancer-related death among women. CCL5 and CCR5 are overexpressed in basal and HER-2+ breast cancer subtypes, but not in normal breast epithelial cells (D’Esposito et al., 2012; Picon-Ruiz et al., 2016). Notably, the CCL5 gene is located in close proximity to Her2 on chromosome 17, probably contributing to their associated expression patterns. Accumulating data indicate that the CCL5–CCR5 axis is associated with promoting breast cancer proliferation, invasion and metastasis. Herein, we provide the first evidence of CCL5-inducible signaling events in breast cancer cells, mediated by CCR5, that regulate metabolic activity to support enhanced cell proliferation, migration and invasion facilitating tumor progression.

We demonstrate CCL5-inducible rapid activation of the mTOR/AKT pathway as well as phosphorylation of a downstream substrate, GSK-3β, in breast cancer cells. mTOR and AKT are central sensors of nutrients, regulating the biosynthesis of proteins, nucleotides and lipids and inhibiting catabolic processes such as autophagy (Shimobayashi et al., 2014). The mTOR/AKT pathway is associated with the transcriptional induction of genes that encode critical enzymes for the pentose phosphate pathway and for de novo pyrimidine synthesis and genes associated with lipogenesis. Moreover, mTOR-dependent regulation of glucose uptake and GLUT-1 expression has been reported (Buller et al., 2008). In agreement, we demonstrate that CCL5 treatment increases glucose uptake by breast cancer cells, mediated by CCR5 and mTOR-dependent. We observe a concomitant increase in GLUT-1 cell surface expression. Akt regulation of glycolysis includes localization of GLUT-1 to the cell surface and regulation of hexokinase activity, required for phosphorylation of G6P (Rathmell et al.,
We provide evidence that CCL5 treatment increases G6P and pyruvate levels, also mediated by CCR5 and sensitive to rapamycin. We provide evidence that elevated levels of G6P were evident at 6 h after CCL5 treatment, but not after 3 h, yet the levels of subsequent downstream intermediates were elevated at both the 3 and 6 h time points. This is probably due to the increased demand for G6P at 3 h following CCL5 treatment, as a substrate for both glycolysis and the pentose phosphate pathway, while hexokinase activity, which is associated with replenishing the G6P supply by catalysing the phosphorylation of glucose, may not be sufficiently elevated to support this increase in G6P demand. By 6 h after CCL5 treatment, the rate of G6P production may be sufficient to exceed that of G6P consumption, resulting in a significant increase in G6P flux. Furthermore, hexokinase activity is self-limiting and therefore depends on the concentration of G6P. Cells may temporarily limit the level of G6P in order to maximize the catalytic activity of hexokinase, allowing for sustained and elevated glycolytic flux.

Our metabolomic analysis revealed CCL5-induced increases in both pyruvate and lactate levels. The relative levels of pyruvate and lactate are maintained at steady state equilibrium. Lactate is produced from pyruvate in a reaction catalysed by lactate dehydrogenase. The reaction is so rapid that pyruvate and lactate can be considered to always be in equilibrium. Our data support this (Table 3.1). We found that CCL5 treatment increased extracellular acidification, a readout for glycolytic flux, and also increased the glycolytic capacity of breast cancer cells. Our experiments demonstrated that CCL5-inducible increases in glucose uptake and elevated glycolytic flux led to ATP production.

Using the Metabolon platform, we further interrogated the CCL5-inducible changes in metabolism and identified increases in the levels of intermediates in multiple anabolic
pathways, including glycolysis, the pentose phosphate pathway, amino acid synthesis and lipid metabolism. This accumulation of metabolites suggests the potential for diversion of intermediates into alternate pathways for macromolecule synthesis, a metabolic signature consistent with requirements for cell proliferation and invasion. Indeed, we provide evidence that CCL5 treatment enhances the proliferation of breast cancer cells and promotes their migration and invasion. Moreover, the accumulation of the BCAAs valine, isoleucine and leucine and their metabolites allows for their integration into proteins and/or processing to meet energy demands. Likewise, the accumulation of glutamine, glutamate and α-ketoglutarate suggests that CCL5 may also influence glutamine catabolism as an alternative source of carbon for biosynthetic pathways. Glucose and glutamine are the primary carbon sources for ATP production and biosynthesis in proliferating cells. This requirement for glutamine is particularly relevant in tumor cells, when glutamine donates its nitrogen and carbon for different growth-promoting pathways and may often become conditionally essential. The data in Figure 3.12. show that the growth of the three breast cancer cell lines is severely limited in glutamine-deficient medium, yet addition of CCL5 enhances their growth. The metabolomics data provided evidence for CCL5 increasing levels of glutamine, from which we infer that CCL5-induced production of glutamine facilitates the enhanced proliferation observed in glutamine-deficient medium. In the tumor microenvironment, where breast cancer cells interact with adipocytes, the fibrous stroma and the immune cells of the inflammatory infiltrate, CCL5 production increases and is sustained (Picon-Ruiz et al., 2016), thereby ensuring that CCL5 activation of CCR5 will provide a steady supply of glutamine to support the aggressive proliferation of breast cancer cells. Notably, there is evidence that adipocytes secrete higher amounts of CCL5 in the presence of glucose and
fatty acids (D’Esposito et al., 2012), suggesting autocrine regulation of CCL5 in the tumor microenvironment.

Acetyl-CoA is produced during glycolysis and from fatty acid degradation (β-oxidation) and shuttles into the TCA cycle to generate energy. CCL5 treatment increases glycolysis and at 3 h we found a corresponding spike in acetyl-CoA levels that coincided with a reduction in stearoylcarnitine and several fatty acid derivatives and an increase in β-hydroxybutyrate, which is synthesized from acetyl-CoA. By 6 h, acetyl-CoA and β-hydroxybutyrate levels are reduced, probably reflecting the increased demand for acetyl CoA for lipogenesis.

Viewed altogether, the data indicate that the effects of CCL5 treatment on the different breast cancer cell types are associated with enhanced metabolic activity that would support the energy and biosynthetic demands of tumor cell proliferation, migration and invasion. We have evidence of CCL5 invoking increases in cell proliferation and migration, mediated by CCR5, and that CCL5 promotes invasion, dependent on glucose. Certainly, published reports of a role for CCR5 in increased breast tumor metastasis and invasion (Velasco-Velázquez et al., 2012; Zhang et al., 2013) align with these findings of CCL5 activating CCR5 to invoke a cascade of signaling pathways increasing the metabolic capacity of cells to enable these events. Further consideration of the role of the alternate CCR5 ligands, CCL3 and CCL4, on metabolic events is warranted. It is intriguing to speculate that our studies with CCL5 and CCR5 may have broader implications for other chemokine/chemokine receptor interactions purported to be important in neoplasias. A recent publication implicates CCR6 in promoting breast cancer initiation and progression (Boyle et al., 2015). In the absence of consideration of metabolic regulation, their data suggest that
CCR6 influences pro-tumorigenic tumor-associated macrophage (TAM) recruitment to the tumor microenvironment (Boyle et al., 2015). Future studies should be directed towards dissecting the different contributions of CCL5–CCR5 interactions in the breast tumor microenvironment, in the context of understanding how metabolic regulation may also influence the immunophenotype–TAM recruitment—and tumor onset and progression.
Chapter 4

CCL5-CCR5 interactions modulate metabolic events during tumor onset to promote tumorigenesis
4.1. Abstract

In earlier studies we have shown that CCL5 activation of CCR5 induces the proliferation and survival of breast cancer cells in an mTOR-dependent manner and that this is in part due to CCR5-mediated increases in glycolytic metabolism. In this report we provide evidence that in the absence of CCR5, the early events associated with rapid tumor growth in the MMTV-PyMT mouse model of spontaneous breast cancer development, are diminished, as demonstrated by a delay in tumor onset. In tumor transplant studies into immunocompromised mice, we identify a direct correlation between reduced tumor proliferation and decreased metabolic activity, specifically associated with tumor expression of CCR5. Consistent with previous findings, the reduction in tumorigenesis is accompanied by decreases in glucose uptake, GLUT-1 cell surface expression, intracellular ATP and lactate levels, as well as reduced CCL5 production. Using Matrix-Assisted Laser Desorption/Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry Imaging we show that the rapid early tumor growth of CCR5\textsuperscript{+/+} triple negative breast cancer cells in vivo is attributable to increased levels of glycolytic intermediates required for anabolic processes, in contrast to the slower growth rate of their corresponding CCR5\textsuperscript{-/-} cells, that exhibit reduced glycolytic metabolism. The implications are that CCL5-CCR5 interactions in the tumor microenvironment modulate metabolic events during tumor onset to promote tumorigenesis.
4.2. Introduction

Metabolic reprogramming is critical for tumor development (Diers et al., 2012). Specifically, during conditions of either nutrient sufficiency or deficiency, tumors will reprogram their metabolic activity towards anabolic or catabolic metabolism, respectively (Elstrom et al., 2004; Gatenby et al., 2004; Zu et al., 2004). Notably, the Warburg effect of aerobic glycolysis associated with glucose uptake and increases in glycolytic flux enables tumor cells to invoke subsidiary metabolic pathways to support the energetic demands of the proliferating tumor cells (Hsu et al., 2008; Lunt et al., 2011; Vander Heiden et al., 2009).

The tumor microenvironment is characterized by inflammatory cells and soluble factors that regulate tumor development. Chemokines, including CXCL3, CXCL12, CXCL13, CCL21, CCL2 and CCL5, have been implicated in promoting the malignant phenotype in breast cancer (Biswas et al., 2014; Luboshits et al., 1999; Müller et al., 2001; See et al., 2014). While CCL5 has a role in promoting anti-tumor responses, there is accumulating evidence for CCL5 supporting cancer metastasis and progression (Karnoub et al., 2007; Khalid et al., 2015; Lv et al., 2013). Specifically, CCL5 levels are markedly higher in more aggressive forms of breast cancer and are predictive of rapid disease progression in stage II breast cancer patients (Yaal-Hahoshen et al., 2006). Moreover, CCL5 levels are notably more elevated in the sera of patients with high-grade tumors compared to those with low-grade tumors (Niwa et al., 2001). In addition, CCL5 production by tumors enhances the infiltration of tumor-associated macrophages (TAM) and myeloid derived suppressor cells (MDSCs), leading to the production of growth factors that enhance tumor cell proliferation (Lv et al., 2013; Zhang et al., 2015). TAMs spontaneously produce CCL22, which facilitates the recruitment of regulatory T cells (Tregs) to the tumor microenvironment. The presence of
Tregs and MDSCs leads to the production of IL-6 and IL-10 by macrophages, which repress T cell activity thereby resulting in poor anti-tumor immunity (Solinas et al., 2009; Ugel et al., 2015).

In earlier studies, we showed that CCL5 engagement with its cognate receptor, CCR5, results in the up-regulation of mRNA translation of pro-survival factors leading to enhanced proliferation in MCF-7 breast cancer cells (Murooka et al., 2009). More recently, we demonstrated that CCL5 activation of CCR5 results in a significant increase in cellular glycolytic activity, specifically increased glucose uptake, GLUT-1 surface expression and increases in intracellular ATP levels, while promoting a global shift towards anabolic metabolism (Gao et al., 2016). We provided evidence that CCL5 activation of CCR5 can stimulate proliferation of breast cancer cells in an mTOR-dependent manner.

These data suggest that in addition to a role for CCL5 in the tumor microenvironment influencing the recruitment of immune cells that inhibit anti-tumor immunity, CCL5 also appears to influence tumor metabolism. To interrogate these in vitro findings further, we examined the effects of these CCL5-CCR5 interactions in the context of regulating tumor metabolism, in vivo. We provide evidence that in the absence of CCR5, the early events associated with rapid tumor growth are diminished; our studies identify a direct correlation between reduced tumor proliferation and decreased metabolic activity. The novelty in our finding suggests targeting CCL5-CCR5 interactions may be a potential therapeutic strategy to limit tumor proliferation and tissue invasion.
4.3. Results

In a first series of experiments we employed the MMTV-PyMT mouse model to understand the role of CCL5-CCR5 in modulating tumor metabolism \textit{in vivo}. MMTV-PyMT mice express the polyomavirus middle T-antigen under the control of mouse mammary tumor virus LTR (Guy et al., 1992). PyMT is a potent oncogene that encodes a transmembrane protein which activates several signal transduction pathways, including those of the \textit{Src} and \textit{ras} family and PI3 kinase pathways (Lin et al., 2003). PyMT expression is also associated with increased \textit{c-myc} levels, leading to prolonged cell survival (Lin et al., 2003). Female MMTV-PyMT mice develop mammary hyperplasia and exhibit an average onset of palpable tumors by 15 weeks of age. To examine the effects of CCL5-CCR5 activation on breast cancer development, we generated MMTV-PyMT.CCR5\textsuperscript{+/−} mice by breeding MMTV-PyMT.CCR5\textsuperscript{+/+} mice with C57Bl/6.CCR5\textsuperscript{−/−} mice, as described earlier (Gao et al., 2016). In a first series of experiments we identified a delay of 12.1 days in palpable tumor detection (∼20mm\textsuperscript{3}), hereafter defined as tumor onset, in the MMTV-PyMT.CCR5\textsuperscript{−/−} mice compared with the MMTV-PyMT.CCR5\textsuperscript{+/+} mice (Figure 4.1A). Further, we observed a 4.8-day delay in tumor endpoint, hereafter defined as ∼700mm\textsuperscript{3} (Figure 4.1B). Notably, the MMTV-PyMT.CCR5\textsuperscript{−/−} mice exhibited an increase in tumor-free survival rate (Figure 4.1C).

In order to reduce the effects of CCL5-CCR5 mediated immune cell recruitment to the tumor microenvironment that would influence tumor proliferation, we harvested tumors from MMTV-PyMT.CCR5\textsuperscript{+/+} and MMTV-PyMT.CCR5\textsuperscript{−/−} mice, prepared tumor cell suspensions, then injected these cell suspensions into mammary fat pads of NSG mice, as described in the Materials & Methods section. NSG mice lack T, B and NK cells and have
Figure 4.1. MMTV-PyMT.CCR5\(^{-/-}\) mice have delayed tumor onset and increased tumor free survival compared to MMTV-PyMT.CCR5\(^{+/+}\) mice.

A-B MMTV-PyMT.CCR5\(^{+/+}\) and MMTV-PyMT.CCR5\(^{-/-}\) mice were monitored daily for palpable tumors, using calipers. Tumor onset is defined as detection of a palpable tumor, volume \(\sim 20\text{mm}^3\). Tumor endpoint is defined as a palpable tumor with volume \(\sim 700\text{mm}^3\). Mice were euthanized when tumors reached this size. Each data point represents a single mouse. Means and SE are identified as horizontal lines. C Tumor-free survival rate is the percentage of the mice in the colony that are tumor-free at each specified age. **\(p<0.01\).
defective antigen presenting cells and functionally immature macrophages (Shultz et al., 2005). This experimental strategy allows for the investigation of the direct effects of CCL5-CCR5 activation on the tumor cells, specifically in our context of metabolic activation.

In a first series of experiments we observed a delay in the onset of palpable tumors (~20mm$^3$) when MMTV.PyMT.CCR5$^{-/-}$ cells are transplanted into NSG mice compared with transplanted MMTV.PyMT.CCR5$^{+/+}$ cells, of the order of 4.9 days (Figure 4.2A). Moreover, we consistently observed a delay of >2 days to tumor endpoint (~700mm$^3$) for the MMTV.PyMT.CCR5$^{-/-}$ tumors compared with the MMTV.PyMT.CCR5$^{+/+}$ tumors (Figure 4.2B). Furthermore, tumor volume remained lower when MMTV.PyMT.CCR5$^{-/-}$ cells were transplanted compared with MMTV.PyMT.CCR5$^{+/+}$ cells (Figure 4.2C). Onset tumors were harvested, fixed in 10% formalin, thin sections prepared and stained with H&E. Both MMTV.PyMT.CCR5$^{+/+}$ and MMTV.PyMT.CCR5$^{-/-}$ tumors were identified as high grade adenocarcinomas with nuclear pleomorphism, high mitotic count and low tubule formation (Figure 4.2D). Adenocarcinomas often form glandular structures. Typically, a lack of tubules signifies a more advanced grade of cancer. In addition, vesicular nuclei, often large and irregularly shaped, and high mitosis rates are classical hallmarks of high grade tumors (Rakha et al., 2010).

We next compared MMTV.PyMT.CCR5$^{+/+}$ and MMTV.PyMT.CCR5$^{-/-}$ tumor cells by examining their metabolic status ex vivo. We found that both MMTV.PyMT.CCR5$^{+/+}$ and MMTV.PyMT.CCR5$^{-/-}$ tumors were more metabolically active during onset (~20mm$^3$) compared to endpoint (~700mm$^3$), as demonstrated by higher glucose uptake (Figure 4.3A) and surface GLUT-1 expression (Figure 4.3B). Notably, at onset, MMTV.PyMT.CCR5$^{+/+}$
Figure 4.2. MMTV-PyMT.CCR5-/- tumor cells transplanted into NSG mice exhibit delayed tumor growth compared with MMTV-PyMT.CCR5+/+ tumor cells.

Tumors (100-700mm$^3$) from MMTV-PyMT.CCR5+/+ and MMTV-PyMT.CCR5-/- mice were harvested and injected into lower mammary fat pads of NSG recipients (n=12), as described in Materials & Methods. Tumor growth was monitored externally using calipers. A Tumor volume was measured over time to determine the rate of tumor growth B Time to palpable tumor onset (~20mm$^3$) and C tumor endpoint (~700mm$^3$). For B and C, each data point represents a single tumor. Means and SE are identified as horizontal lines. D Onset tumors were harvested, fixed, thin sections prepared and stained with H&E. Arrows show indicated features. * $p<0.05$ and *** $p<0.001$
tumors were more metabolically active than MMTV.PyMT.CCR5−/− tumors, given the same tumor volume. MMTV.PyMT.CCR5+/+ tumors exhibited higher cellular lactate levels than MMTV.PyMT.CCR5−/− tumors at onset, potentially due to a higher rate of glycolysis (Figure 4.3C). However, by endpoint, lactate levels have increased and no differences were identified between MMTV.PyMT.CCR5+/+ and CCR5−/− tumor cells. MMTV.PyMT.CCR5+/+ tumors also produced the highest levels of CCL5 ex vivo when harvested at both onset and endpoint, with consistently higher CCL5 levels detectable at onset compared with endpoint (Figure 4.3D).

One of the most aggressive and malignant types of breast cancer is the triple negative (estrogen receptor- (ER-), progesterone receptor- (PR-), human epidermal growth factor receptor 2- (HER2-)) basal-like adenocarcinoma. MDA-MB-231 is a widely used triple negative human breast cancer cell line that expresses CCR5 (Gao et al., 2016; Velasco-Velázquez et al., 2012). Accordingly, to further interrogate the contribution of CCR5 to breast cancer metabolism, we generated MDA-MB-231.CCR5−/− tumor cells using CRISPR/Cas9 technology (Figure 4.4 A-C). In an earlier publication we provided evidence that CCL5 treatment enhances the proliferation of MDA-MB-231.CCR5+/+ cells, which is dependent on glucose and glutamine metabolism (Gao et al., 2016). The MDA-MB-231.CCR5−/− cells exhibit a lower basal proliferation rate compared to MDA-MB-231.CCR5+/+ and their growth rate is unaffected by CCL5 treatment (Figure 4.5A). Specifically, in medium deficient in glutamine and in medium containing 2-DG, CCL5-inducible increased cell proliferation is diminished in MDA-MB-231.CCR5+/+ cells and absent in MDA-MB-231.CCR5−/− cells. In addition, MDA-MB-231.CCR5+/+ cells secrete CCL5 at physiological levels (Azenshtein et al., 2002; Luboshits et al., 1999), while CCL5
secretion was undetectable from the MDA-MB-231.CCR5<sup>−/−</sup> cells, likely due to a lack of CCL5-CCR5 autocrine signaling (Figure 4.B). MDA-MB-231.CCR5<sup>+/+</sup> and CCR5<sup>−/−</sup> cells were further analyzed for their rates of glucose uptake (Figure 4.C), surface GLUT-1 expression (Figure 4.D),
Figure 4.3. MMTV-PyMT.CCR5−/− tumor cells harvested from NSG mice exhibit lower glucose uptake, reduced GLUT-1 expression and lower levels of cellular metabolites than MMTV-PyMT.CCR5+/+ tumor cells.

MMTV-PyMT.CCR5+/+ and MMTV-PyMT.CCR5−/− tumors were harvested from recipient NSG mice when they were either just palpable (~20mm³), i.e. tumor onset, or ~700mm³, endpoint. Following 16 h in culture, A glucose uptake, B GLUT-1 expression, C intracellular lactate and D CCL5 production, were measured. Values are the means +/- S.E. of technical triplicates. *p<0.05, **p<0.01, ***p<0.001.
Figure 4.4. Generation of MDA-MB-231.CCR5<sup>−/−</sup> using CRISPR/Cas9.

The knockout cassette carries puromycin resistance. A Candidate MDA-MB-231.CCR5<sup>−/−</sup> cells were first screened for viability in the presence of 1µg/mL of puromycin. Subsequently, candidate MDA-MB-231.CCR5<sup>−/−</sup> cell lines were confirmed CCR5 null by B genomic PCR and C staining with an anti-CCR5 antibody.
**Figure 4.5. In vitro,** MDA-MB-231.CCR5\(^{-/-}\) cells are less metabolically active than MDA-MB-231.CCR5\(^{+/+}\) cells.

**A** Cells were either left untreated (medium alone), treated with 10nM CCL5, or pre-treated with 2mM 2-DG for one hour prior to CCL5 treatment, or maintained in glutamine-free medium. For all, medium was changed every other day and the treatment(s) re-applied. Cell proliferation was quantified using an MTT assay as described in Materials & Methods. The proliferation index is normalized against untreated conditions (i.e. medium alone). Values are means +/- S.E. of triplicate assays and each data point combines the data from 3 independent experiments. Statistical analysis was performed comparing untreated cells with CCL5-treated cells and inhibitor-treated cells with CCL5+inhibitor treated cells, or comparing CCL5-treated with CCL5 + inhibitor-treated cells. **B** CCL5 levels were measured from culture supernatants after 16 h incubation. **C** Glucose uptake, **D** GLUT-1 expression, **E** intracellular ATP and **F** intracellular lactate were measured as described in Materials & Methods in cells treated with 10nM CCL5 or 2μM oligomycin for 3 h. Data are expressed as percent-change relative to untreated MDA-MB-231.CCR5\(^{+/+}\) cells. Values are means +/- S.E. of triplicate assays and each data point combines the data from 3 independent experiments. * \( p<0.05 \), ** \( p<0.01 \), *** \( p<0.001 \).
intracellular ATP (Figure 4.5E) and lactate levels (Figure 4.5F). MDA-MB-231.CCR5<sup>+/+</sup> cells exhibited a lower basal rate of metabolism, as evidenced by their lower glucose uptake, GLUT-1 expression and intracellular ATP and lactate levels. As anticipated, MDA-MB-231.CCR5<sup>-/-</sup> cells were not responsive to CCL5 treatment.

We next examined the proliferative capacity of the MDA-MB-231.CCR5<sup>+/+</sup> and MDA-MB-231.CCR5<sup>-/-</sup> tumor cells in vivo. As above, we employed NSG mice, to eliminate/dramatically diminish the contributions of CCL5-CCR5 interactions on an immune response that would influence tumor growth. MDA-MB-231.CCR5<sup>+/+</sup> and MDA-MB-231.CCR<sup>-/-</sup> cells were transplanted into the fat pads of NSG recipient mice. Similar to the MMTV-PyMT transplant study, NSG mice transplanted with MDA-MB-231.CCR5<sup>-/-</sup> cells exhibited a lower tumor load (Figure 4.6A) and on average a 3.96 day delay in tumor onset (Figure 4.6B). H&E staining of tumor thin sections revealed that both MDA-MB-231.CCR5<sup>+/+</sup> and MDA-MB-231.CCR5<sup>-/-</sup> tumors were high grade adenocarcinomas with nuclear pleomorphism, high mitotic count and low tubule formation (Figure 4.6C). When tumors were harvested at onset, then cultured ex vivo, MDA-MB-231.CCR5<sup>+/+</sup> cells had a higher rate of glucose uptake and GLUT-1 expression compared to MDA-MB-231.CCR5<sup>-/-</sup> cells. When tumors were harvested at endpoint, glucose metabolism was significantly lower and no differences were observed between MDA-MB-231.CCR5<sup>+/+</sup> and MDA-MB-231.CCR5<sup>-/-</sup> cells (Figure 4.7 A-B). The levels of CCL5 produced were significantly higher (approximately 4-fold) from MDA-MB-231.CCR5<sup>+/+</sup> compared with MDA-MB-231.CCR5<sup>-/-</sup> cells, when tumors were harvested at onset, yet this production diminished significantly at endpoint, albeit lower CCL5 levels were produced by MDA-MB-231.CCR5<sup>-/-</sup> compared with MDA-MB-231.CCR5<sup>+/+</sup> cells (Figure 4.7C).
Figure 4.6. MDA-MB-231.CCR5/− cells transplanted in NSG mice exhibit delayed tumor growth compared with MDA-MB-231.CCR5+/+ cells.

MDA-MB-231.CCR5/− and MDA-MB-231.CCR5+/+ cells were injected into mammary fat pads of NSG mice (n=12), as described in Materials & Methods. Tumor growth was monitored externally using calipers. A Tumor volume was measured over time to determine tumor growth B Time to palpable tumor onset (~20mm³). For B, each data point represents a single tumor. Means and SE are identified as horizontal lines. C Onset tumors were frozen, thin sections prepared and stained with H&E. Arrows show indicated features. * p<0.05.
Figure 4.7. MDA-MB-231.CCR5−/− tumor cells harvested from NSG mice exhibit lower glucose uptake, reduced GLUT-1 expression and lower levels of cellular metabolites than MDA-MB-231.CCR5+/+ tumor cells.

MDA-MB-231.CCR5+/+ and MDA-MB-231.CCR5−/− tumors were harvested from recipient NSG mice when they were either just palpable (~20mm³), i.e. tumor onset, or ~700mm³, endpoint. Following 16 h in culture, A glucose uptake, B GLUT-1 expression, C Intracellular lactate and D CCL5 production, were measured. Values are the means +/- S.E. of technical triplicates. ** p<0.01 and *** p<0.001.
To further explore the metabolic differences in MDA-MB-231.CCR5−/− and MDA-MB-231.CCR5+/+ tumors, MALDI-FTICR-MS imaging was performed. MS imaging provides the in-situ distribution profiles of metabolites within tissues and FTICR-MS provides the highest level of mass resolution necessary for identification of low molecular weight metabolites (Cornett et al., 2008). A broad spectrum of analytes ranging from proteins, peptides, small molecule drugs and their metabolites, as well as endogenous cell metabolites, and lipids are identifiable by this technology (Gessel et al., 2014). Following transfer of MDA-MB-231.CCR5−/− and MDA-MB-231.CCR5+/+ tumors into the mammary fat pads of NSG mice and a growth period of 23-40 days, intact tumors were harvested and immediately frozen in liquid nitrogen. Frozen thin sections were mounted onto slides and coated uniformly with a matrix solution of 9-AA (10mg/ml in 70% MeOH). This matrix formulation ensures robust efficiency for the ionization of important cellular metabolites in tissue samples (Dekker et al., 2015). Serial sections were stained with H&E and examined to ensure absence of necrotic cells. FTICR-MS data were collected (Lisa Cazares, USAMARIID, USA) and metabolite ions identified using a metabolite database (Smith et al., 2005). Only those peaks detected within 1 ppm of the expected m/z of the [M–H]− metabolite ions were assigned metabolite identities. Metabolite images displaying the relative abundance and box/cloud-plots depicting normalized peak intensities for metabolites identified from 3 MDA-MB-231.CCR5−/− and 3 MDA-MB-231.CCR5+/+ transplanted tumor tissues are shown in Figure 4.8. ATP is significantly reduced (p=0.044) in the MDA-MB-231.CCR5−/− tumors, and CTP reduction approaches significance (p=0.053). We also observed a marked reduction in levels of 6-phosphogluconate in the MDA-MB-231.CCR5−/−
tumors. Conversely, glycerol phosphate, a molecule reduced from DHAP, is increased in abundance in MDA-MB-231.CCR5<sup>−/−</sup> tumors.
Figure 4.8. MALDI-FTICR-MSI analysis confirms that MDA-MB-231.CCR5⁻/⁻ cells are less metabolically active than MDA-MB-231.CCR5⁺/+ cells.

Tumor tissue was harvested at tumor onset from NSG mice that had received MDA-MB-231.CCR5⁻/⁻ or MDA-MB-231.CCR5⁺/+ tumor cell transplants, rapidly frozen, then sectioned in a cryostat, sprayed with 9AA matrix and subjected to MSI using MALDI-FTICR and a raster width of 50 µm over a m/z range of 150-1100. Each panel (A-D) represents an identified metabolite. Each box-plot contains a rectangle divided by a horizontal line, which represents the median intensity of that metabolite. Lower and upper bounds of the box represent the second and third quartile. Lines extending vertically from the box represent lower and upper quartiles (0% and 99% respectively). The cloud part of the plot shows how spectra from a given region are spread by intensity for each metabolite and region. Blue dots represent the spectra in which intensities are between the lower and upper quartiles. Red dots represent outliers. Images below the box plots are show the relative abundance of each metabolite for each tissue (normalized to TIC) and based on the intensity scale provided.
A. Glycerol 3-phosphate  
\[ m/z 171.0063 \quad p = 0.059 \]

B. ATP  
\[ m/z 505.9882 \quad p = 0.044 \]
C. 6-Phosphogluconate  m/z 275.0177  p = 0.002

D. CTP  m/z 481.9775  p = 0.053
4.4. Discussion

The metabolic phenotype of cells in the tumor microenvironment is heterogeneous, wherein the majority of cells mobilize glucose by glycolysis and others via mitochondrial oxidative phosphorylation (Martinez-Outschoorn et al., 2016). Glycolysis and mitochondrial metabolism are important both for ATP generation and for the biosynthesis of cellular components. Indeed, given the aggressive growth requirements of tumor cells, they exhibit higher metabolic activity rates compared with normal cells; tumor cells metabolize glucose, glutamine and other nutrients at much higher rates than differentiated cells. Notably, tumor cells predominantly utilize aerobic glycolysis over oxidative phosphorylation (Warburg, 1956). While glycolysis generates 2 moles of ATP per mole of glucose, the complete oxidation of glucose through the electron transport chain produces 18 times more. Despite being the less efficient form of oxidative breakdown of glucose, glycolysis will produce ATP 100 times faster than oxidative phosphorylation, while also generating key metabolic intermediates for anabolic processes, mediated by activating the pentose phosphate pathway and amino acid biosynthesis (Pfeiffer et al., 2001).

Onset of tumorigenesis is a critical period when a tumor-supportive microenvironment is established, often facilitated by an unresolved inflammatory response (Quail et al., 2013). According to a published meta-analysis, tumor onset can be directly attributed to infection and inflammation in approximately 15% of cancers (Kuper et al., 2000; Rakoff-Nahoum, 2006). Various cell types accumulate during onset of tumorigenesis, including stromal cells, which contribute to the development of a pro-tumorigenic niche (Mantovani et al., 2008). Abnormal stromal cells can directly induce tumor promotion (Karnoub et al., 2007; Simoes et al., 2015). Stromal cells produce enzymes, growth factors
and cytokines to enable tumor growth (Mezawa et al., 2016; Roy et al., 2016). Fibroblasts, the most common types of stromal cells found in the tumor microenvironment, actively participate in tumorigenesis and are responsible for the production of many factors, including extracellular matrix proteins and matrix metalloproteinases (Gascard et al., 2016; Mezawa et al., 2016). Once the tumor microenvironment is established, cancer-associated fibroblasts (CAFs) become distinctly identifiable. These CAFs induce additional alterations in tumor cells that allow them to metastasize more efficiently (Gascard et al., 2016). Stromal cells, along with mesenchymal stem cells (MSCs), are the major sources of chemokines, including CCL5, in the tumor microenvironment. It is now clear that chemokines exert important roles in regulating inflammation, proliferation, survival and migration of tumor cells during the neoplastic process (Gao et al., 2016; Soria et al., 2008). CCL5 and CCR5 (but not CCR1 and CCR3) are overexpressed in the basal and HER-2+ breast cancer subtypes (Velasco-Velázquez et al., 2012). The oncogenic transformation of human breast epithelial cells is accompanied by an upregulation in the expression of CCR5, further supporting a role for CCL5-CCR5 interactions in the regulation of tumor development (Velasco-Velázquez et al., 2012; Zhang et al., 2009). CCL5 has a crucial role in the metastasis of breast cancer cells (Robinson et al., 2003) and expression of CCR5 on breast cancer cells enhances cellular invasion by 40-fold (Velasco-Velázquez et al., 2013).

Immune cell infiltration is vital for establishing the tumor microenvironment (Man et al., 2013). Recruitment of inflammatory cells to tumors can facilitate disease progression and promote metastasis. CCL5 has been implicated in the recruitment of CD4+CD25+Foxp3+ Tregs, leading to the generation of a suppressive environment that supports tumor tolerance (Wang et al., 2016). MDSCs, a cell population with immune-suppressive properties, secrete
large amounts of CCL5, further promoting the recruitment of Tregs (Schlecker et al., 2012; Zhang et al., 2013). Additionally, CCR5 expressing-TAM (Soria et al., 2008) accumulation occurs in hypoxic regions in growing tumors and their accumulation correlates with angiogenesis, a prerequisite to the subsequent invasive phenotype of carcinoma (Quail et al., 2013). The tumor microenvironment is more acidified at endpoint, presumably due to an accumulation of lactate, which can stabilize hypoxia-inducible factor 1-α (HIF-1α) and leads to vascular endothelial growth factor (VEGF) expression, triggering angiogenesis and invasion (Semenza, 2010). Clearly, CCL5-CCR5 activation in the tumor microenvironment is critical.

Employing the MMTV-PyMT mouse model of spontaneous breast tumor development, we generated MMTV-PyMT.CCR5−/− mice and identified a 12 day delay in tumor onset in these mice compared to the MMTV-PyMT.CCR5+/+ mice, yet no discernible differences in tumor endpoint. These findings are consistent with the notion that the pro-survival and metabolic benefits of CCL5-CCR5 signaling are more critical during the onset of tumorigenesis, prior to the formation of a fully established tumor microenvironment, compared to tumor endpoint, when the lack of CCL5-CCR5 signaling may be compensated by other factors as a result of a fully acidified and hypoxic tumor (Balliet et al., 2011; Hitosugi et al., 2010; Semenza, 2008). When tumors from MMTV-PyMT.CCR5+/+ and MMTV-PyMT.CCR5−/− mice were transplanted into NSG mice, we likewise observed a delay in tumor onset in recipient mice that received MMTV-PyMT.CCR5−/− cells. The delay in tumor onset employing this model was approximately 4 days, compared with the 12 days in the spontaneous MMTV-PyMT mouse model, likely reflective of the more aggressive
progression of tumorigenesis in the immunocompromised NSG mice. Importantly, our data confirm that CCR5 expression on tumor cells directly contributes to tumor proliferation.

Our studies with the triple negative MDA-MB-231.CCR5−/− cells revealed that in the absence of CCR5 expression MDA-MB-231 cells do not secrete and are unable to respond to CCL5, have lower glucose uptake and GLUT-1 expression, and lower intracellular ATP and lactate levels. Indeed, the MDA-MB-231.CCR5−/− cells are more sensitive to inhibitors blocking glucose uptake and glutamine catabolism and have a lower proliferation rate than MDA-MB-231.CCR5+/+ cells, in further support of a role for CCL5 activation of CCR5 contributing to tumor cell metabolism. Comparing the metabolic status of MDA-MB-231.CCR5+/+ and MDA-MB-231.CCR5−/− tumor cells during onset and endpoint we provide evidence that cells are more metabolically active during the onset of tumorigenesis, characterized by lower GLUT-1 expression and lower glucose uptake.

MALDI-FTICR MSI analysis revealed an increase in 6-phosphogluconate, CTP and ATP levels in MDA-MB-231.CCR5+/+ tumors compared to MDA-MB-231.CCR5−/− tumors. 6-phosphogluconate is a key intermediate in the pentose phosphate pathway and is converted to ribulose 5-phosphate by phosphogluconate dehydrogenase catalysis. Accumulation of 6-phosphogluconate enables the shuttling of intermediates into anabolic pathways for macromolecule biosynthesis, a metabolic signature that supports cell proliferation and invasion. In addition to serving as high energy molecules, ATP and CTP are also involved with RNA and glycerophospholipid synthesis. High ATP levels further serve to antagonize the AMP-activated protein kinase (AMPK) while promoting the activation of mTOR and energy-intensive protein biosynthesis, another hallmark of anabolic metabolism. MDA-MB-231.CCR5−/− tumors had higher levels of glycerol 3-phosphate. Conversion of
dihydroxyacetone phosphate to glycerol 3-phosphate – the glycerol 3-phosphate shuttle – is a mechanism whereby NADH that accumulates in the cytosol during glycolysis may be utilized to generate ATP by mitochondrial oxidative phosphorylation, thereby coupling glycolysis and mitochondrial ATP production. Given the slower rate of glycolysis in MDA-MB-231.CCR5<sup>−/−</sup> tumors, we infer that the increase in glycerol 3-phosphate levels in the MDA-MB-231.CCR5<sup>−/−</sup> cells could be a compensatory mechanism to increase ATP production through oxidative phosphorylation by generating additional high potential equivalents via the glycerol 3-phosphate shuttle.
4.5. Conclusion

Viewed altogether, the data indicate that CCL5 activation of CCR5 in breast cancer cells is associated with increased metabolic activity during tumor onset. This increase in glucose uptake and ATP generation fuels the energy and biosynthetic demands of tumor cells, enhancing cell proliferation and tumorigenesis. Our findings are consistent with published studies that have identified the importance of CCR5 in breast tumor progression (Velasco-Velázquez et al., 2012; Zhang et al., 2013). Our results suggest that, even in the absence of immune cells, the expression of CCR5 on tumor cells enables tumor growth in an environment where CCL5 is produced, mediated by upregulation of metabolic events, specifically in the context of a complex immune cell microenvironment.
Chapter 5

Discussion and Future Directions
5.1. CCL5-CCR5 Interactions Regulate Cell Metabolism

GPCR activation leads to signaling that regulates many effector molecules, including cAMP and PKA, thereby enabling different cellular processes, including mRNA translation, chemotaxis and proliferation. CCL5 binding to its cognate GPCR, CCR5, activates the PI3K/AKT/mTOR pathway as well as the JAK2/STAT3 cascade. Specifically, CCL5 activates the mTOR/4E-BP1 pathway to directly upregulate mRNA translation. Furthermore, CCL5 promotes T cell chemotaxis, through the generation of a chemotactic gradient as well as preferentially inducing the translation of proteins implicated in mediating migration of cells, namely, MMP-9 and cyclin D1 (Murooka et al., 2008). Given that CCL5-mediated chemotaxis and mRNA translation consume high levels of ATP, we hypothesized that CCL5 may also modulate cellular metabolism via mTOR/AKT and/or JAK/STAT signaling pathways, to meet these energy demands. Initial investigations in T cells revealed that CCL5 promotes glucose uptake in an mTOR-dependent manner, likely through an upregulation in intrinsic GLUT functions. Additionally, CCL5 activates PFK2, which catalyzes F6P into F2,6BP and directly promotes glycolysis (Chan et al., 2012). Interestingly, CCL5 also promotes the activation of AMPK in T cells. Activated AMPK inhibits acetyl-CoA carboxylase (ACC)-1, which is important in the production of malonyl-CoA and lipogenesis. AMPK also stimulates glycolysis through the translocation of GLUT4 and inhibits biosynthetic process to conserve energy. In the studies reported in this thesis, we report the effect of CCL5-CCR5 interactions on regulating breast cancer cell metabolism.

Using maraviroc and TAK-779, CCR5-specific inhibitors, and rapamycin, an mTOR inhibitor, and a panel of CCR5-expressing breast cancer cell lines, we provided evidence that CCL5-CCR5 interactions induce the phosphorylation of mTOR, AKT, 4E-BP1 and GSK-3β.
within minutes. This activation leads to increases in cellular ATP, glucose uptake, increased surface GLUT-1 expression and enhanced glycolytic capacity. Using the Metabolon platform, additional evidence was provided that CCL5 invokes a global upregulation in metabolites, including those involved in glycolysis, the pentose phosphate pathway, fatty acid synthesis and glutamine metabolism. The accumulation of various intermediates in these metabolic pathways serve as biosynthetic precursors for anabolic processes, enabling accelerated cell proliferation and tissue invasion. Interestingly, we did not observe AMPK activation in breast cancer cells, likely due to the elevated intracellular ATP levels. Moreover, cancer cells may actively downregulate AMPK and prevent its inhibition of mTOR and other downstream biosynthetic processes during tumorigenesis.

Subsequently, studies were undertaken to examine whether the CCL5-CCR5 induced regulation of metabolism observed \textit{in vitro} occurs \textit{in vivo}. Initially, the contribution of CCR5 to the growth of multifocal breast cancer adenocarcinomas was examined in the MMTV-PyMT mouse model of spontaneous breast cancer development. Specifically, we examined tumor development in MMTV-PyMT.CCR5$^{+/+}$ and MMTV-PyMT.CCR5$^{-/-}$ mice and observed both a delay in tumor onset and reduced tumor burden when CCR5 was absent. In the next series of \textit{in vivo} studies, to diminish the effects of CCL5-CCR5 interactions on immune cell infiltration that will affect tumor proliferation, we employed NSG mice, that lack T, B and NK cells, have diminished numbers of macrophages and inefficient antigen presentation by APCs. In the absence of an adaptive immune response and a severely impaired innate immune system, NSG mice would serve as a useful model to distinguish the direct effects of CCL5 on cancer cell proliferation. Using the triple-negative breast cancer cell line MDA-MB-231 and MDA-MB-231.CCR5$^{-/-}$ cells that were generated by
CRISPR/Cas9 gene editing, we provided evidence that tumor onset is delayed in the absence of CCR5. Moreover, examination of the ‘onset’ tumors revealed that in the absence of CCR5 these tumors exhibited reduced glucose uptake, diminished GLUT-1 expression and significantly reduced CCL5 secretion. Further, our studies identified that the MDA-MB-231 breast cancer cells are significantly more metabolically active during tumor onset (~20mm³) compared with late stage tumor masses (~700mm³). Using MALDI-FTICR-MS we studied the in situ levels of key metabolites and metabolic precursors in the tumors harvested at onset. Using this technology to directly visualize metabolites in freshly harvested tumor tissues in situ, we provided evidence that MDA-MB-231.CCR5⁻/⁻ tumors exhibit reduced ATP and CTP levels and reduced 6-phosphogluconate, a key intermediate in the pentose phosphate pathway, compared with MDA-MB-231.CCR5⁺/⁺ tumor tissues. This approach provided an opportunity to accurately evaluate the abundance of cellular metabolites without disrupting the tissue architecture.

NSG mice may still develop myeloid cells that could influence tumorigenesis. One approach to reduce the macrophage population in these kinds of tumorigenesis studies would be to use clodronate liposomes, which are preferentially phagocytized by macrophages. This results in the intracellular accumulation of clodronate, leading to both apoptosis and necrosis of macrophages (van Rooijen et al., 1996). However, at high concentrations, clodronate liposomes can cause cardiac complications and death, while moderate concentrations will only deplete up to 60% of macrophages (Côté et al., 2013; Li et al., 2016). Moreover, in mammalian cells clodronate is metabolized to adenosine 5'-(β, gamma-dichloromethylene) triphosphate, a toxic ATP analogue (Frith et al., 1997). These analogues cannot be hydrolyzed and are released into the microenvironment upon cell death. Given that the intent
is to interrogate the effects of CCL5-CCR5 interactions on tumor metabolism, the introduction of a toxic metabolite analogue into the tumor microenvironment would likely generate additional confounding variables. To gain insights into the tumor microenvironment in our model system, we evaluated myeloid cell infiltration by staining for F4/80 and found minimal myeloid influx. Additionally, no significant differences in myeloid infiltration were observed between CCR5 sufficient and CCR5 deficient tumors. Myeloid cells derived from NSG are immature and do not respond to LPS stimulation (L D Shultz et al., 1995). Accordingly, it is reasonable to propose that the limited myeloid infiltration observed did not contribute significantly to the differences we observed between CCR5 sufficient and deficient tumor cells.

In the current studies, we provide evidence that CCL5 activation of CCR5 invokes the AKT/mTOR pathway and influences cell metabolism. We further infer that CCL5-CCR5-mediated enhancement in cell metabolism could also influence immune cell function during tumorigenesis. As previously shown, CCL5-CCR5 signaling promotes glucose uptake, intracellular ATP and mTOR-dependent chemotaxis in T cells. The increase in cellular energetics may play a critical role in promoting T cell recruitment to the tumor microenvironment during tumor onset. In addition, after stimulation, naïve T cells undergo rapid clonal expansion. During this process, the cellular metabolic signature is highly glycolytic, in order to generate sufficient amount of metabolites for cell division (van der Windt et al., 2012). CCL5 could further activate T cells in the presence of anti-CD3, likely associated with enhanced cellular metabolism in preparation for the subsequent expansion. Different macrophage subsets have also been implicated to have different metabolic signatures. M1 macrophages preferentially invoke glycolysis and PPP while M2
macrophages favor OXPHOS (Geeraerts et al., 2017). It is possible that CCL5-CCR5 also modulates macrophage metabolism in the tumor microenvironment, while promoting a balance of pro- and anti-tumor activities.

In addition to activating G-protein signaling, CCL5 also promotes Jak2 and Stat3 activation which have been implicated in promoting cell proliferation and angiogenesis, through directly upregulating the transcription of cyclin D1 and VEGF (Wong & Fish, 2003). The interactions between mTOR/AKT signaling and JAK/STAT signaling downstream of CCL5-CCR5 remains largely unknown. Moreover, the contribution of G-protein coupled signaling to these metabolic events is unknown. To address the mechanism(s) whereby CCL5 activation of CCR5 directly regulates glucose uptake and glycolysis, studies that involve the use of Jak inhibitors, e.g. tofacitinib, and the G-protein inhibitor pTx should evaluate the phosphorylation levels of Stat3, Stat5b, mTOR, Akt, 4E-BP1 and GSK-3β, and the metabolic status of breast cancer cells, specifically, cell surface GLUT-1 expression, glucose uptake, ATP production and glycolytic capacity. Comparing the results from cells treated with the Jak and G-protein inhibitors will provide additional insight to the underlying mechanism by which CCL5 enhances cellular metabolism.

5.2. CCL5-CCR5 in Diseases

To examine CCL5-CCR5 interactions in breast cancer, Velasco-Velázquez et al. conducted microarray analysis on 2254 primary breast cancer samples and identified that CCL5 and CCR5, but not CCR1 or CCR3 expression, are increased in triple negative and Her2+ breast cancer cells. The use of the CCR5 inhibitor, maraviroc, was effective in reducing in vitro cell invasion and in vivo pulmonary metastasis of MDA-MB-231 cells. This
finding is consistent with previously published studies that showed circulating CCL5 levels are elevated in patients with high-grade tumors compared to those with low-grade tumors and that CCL5 is a predictor of disease progression in stage II and stage III breast cancer patients (Niwa et al., 2001; Yaal-Hahoshen et al., 2006).

Furthermore, the CCR5Δ32 mutation has been implicated in susceptibility to breast cancer. The CCR5Δ32 mutation is found in approximately 10% of the northern European population (Novembre et al., 2005). The 32-bp deletion causes a shift in the reading frame and encodes a premature stop codon during CCR5 translation. The truncated CCR5 protein is non-functional and fails to be transported to the cell surface. Individuals with homozygous CCR5Δ32 are resistant to infection from multiple strains of HIV and heterozygotes have exhibited delayed development of AIDS (Novembre et al., 2005). Given that CCL5-CCR5 interactions facilitate cancer cell invasion and metastasis, a number of meta-analyses have been conducted to evaluate the correlation between carriers of the CCR5Δ32 mutation and their rate of tumor development. One study in particular, published in 2015, focused on breast cancer patients (Span et al., 2015). Samples from a total of 414 breast cancer patients were collected from the Radboud University Medical Center, in Nijmegen, the Netherlands between 1990 to 1996. Their CCR5 locus was sequenced and their CCR5Δ32 status identified. The disease-free survival (DFS) and metastasis-free survival (MFS) scores post primary surgery between CCR5WT and CCR5Δ32 patients were evaluated. In both cases, patients with the CCR5Δ32 mutation exhibited moderate delays in tumor development and extended survival with p values of 0.172 and 0.08, respectively. This outcome further supports our findings that CCR5 expression facilitates metabolic upregulation to promote tumor development. Patients were further grouped into pre- and post-menopausal.
Interestingly, there was no discernable difference between the MFS scores of pre-menopausal CCR5Δ32 patients and CCR5WT patients (p= 0.815), but post-menopausal CCR5Δ32 patients had significantly higher MFS scores than CCR5WT patients (p=0.038), and had 100% survival past 120 months post primary surgery. Subsequently, the study was expanded to validate these findings with 1017 additional breast cancer patient samples collected by the Erasmus University Medical Center in Rotterdam, the Netherlands between 1978 to 2000. This analysis arrived at the same conclusion, namely that the MFS scores of pre-menopausal patients with the CCR5Δ32 mutation were comparable to those of patients with CCR5WT (p=0.94), while the MFS scores of post-menopausal CCR5Δ32 patients were significantly higher than those of CCR5WT patients (p=0.044). These results suggest that expression of CCR5 has a role in breast cancer progression and that an estrogen-rich tumor microenvironment in pre-menopausal women may affect immune cell function to compensate for an inactive CCR5 (Fish, 2008). Notably, both post-menopausal CCR5Δ32 and CCR5WT patients exhibited higher MFS scores than their pre-menopausal counterparts. This finding is consistent with the fact that sex hormones, including progesterone and estrogen, can directly stimulate macrophages and Tregs (Markle et al., 2014), which may promote tumorigenesis, thereby reducing survival rates.

In addition to a role in breast cancer, CCR5 expression has been implicated in many diseases, including rheumatoid arthritis, coronary diseases, viral infections and graft rejection. In rheumatoid arthritis, CCR5+ monocytes in the synovial fluid influence inflammation and a meta-analysis revealed that the CCR5Δ32 mutation reduced severity of disease (Kohem et al., 2007; Prahalad, 2006). CCR5 deficient mice had lower rates of renal transplantation rejection, due to a decrease in pro-inflammatory responses (Dehmel et al., 2009). The Δ32 mutation is
also associated with a reduced risk of myocardial infarction and coronary artery disease, associated with higher levels of HDL and lower levels of triglycerides in the circulation (Hyde et al., 2010; Szalai et al., 2001). In bone marrow transplant patients, CCR5-expressing Tregs are important in prolonging graft survival (Wysocki et al., 2005). Interestingly, there is evidence that Δ32 individuals have a reduced likelihood of developing graft vs. host disease (Bogunia-Kubik et al., 2006).

CCL5 and CCR5 exert diverse and multifaceted roles in different diseases. Their contributions are dependent on the cell type. In this thesis, we propose a novel contribution mediated by CCL5-CCR5 signaling that modulates cellular metabolism in breast cancer that facilitates and enables cellular proliferation, tumorigenesis and, potentially, invasion. Similar interactions may exacerbate disease progression in autoimmune and inflammatory diseases and, by contrast, promote disease resolution in pathogenic infections.

5.3. Targeting CCL5-CCR5 Interactions in Cancer

CCL5 and CCR5 are implicated to play a role in multiple types of cancer, especially triple negative breast cancers. Interestingly, CCL5 and CCR5 are apparently dispensable/redundant for general immunity and physiology. CCR5/− and CCL5/− mice develop and live normally, while CCR5Δ32 homozygous individuals retain a fully functional immune system (de Oliveira et al., 2014). Therefore, targeted inhibition of CCL5 and/or CCR5 as well as downstream signaling may prove efficacious in reducing tumorigenesis and improving disease prognosis with only limited side effects (Figure 5.1).

Several therapeutic approaches have been developed to target CCL5 and CCR5. Firstly, inhibitors could be used to disrupt direct binding of CCL5 to CCR5. Maraviroc and
TAK-779 are CCR5 antagonists that are used as antiviral therapies against HIV infection. In preclinical studies, CCR5 inhibitors have been shown to reduce basal breast cancer cell invasion and suppress the development of hepatocellular carcinoma (Aldinucci et al., 2014). A decoy ligand for CCR5, Met-CCL5, reduces breast cancer tumor load and tumor macrophage infiltration (Robinson et al., 2003). Consistent with the literature, we report that CCR5 antagonists abrogate CCL5-mediated glucose uptake, ATP production and metabolite generation, affecting tumor proliferation.

Another therapeutic intervention strategy would be to reduce the level of CCL5 in the tumor microenvironment. MSCs migrate towards cancer cells and secrete high levels of CCL5 during tumorigenesis. Zoledronic acid significantly reduces CCL5 secretion by MSCs and disrupts the tumor-stromal crosstalk (Niu et al., 2016). Additionally, Idelalisib or GS-1101, a PI3Kδ inhibitor, is FDA-approved for the treatment of chronic lymphocytic leukemia. Idelalisib decreases CCL5 secretion by cancer cells (Meadows et al., 2012). EGFR inhibition has also been associated with the indirect inhibition of CCL5 production (Borghese et al., 2013). Gefitinib is the first commercially available EGFR antagonist and has been approved for the treatment of breast and lung cancers. Gefitinib also reduces production of CCL5 by MSCs in the tumor microenvironment (Borghese et al., 2013). To determine whether these CCR5 and CCL5 inhibitors exert their effects on tumor growth in part by limiting the metabolic regulation invoked by CCL5 activation of CCR5, both in vitro and in vivo studies similar to those described in Chapters 3 and 4 could be conducted, specifically using the inhibitors described above.
Figure 5.1. Therapies targeting cancer metabolism.

CCL5-CCR5 interactions in the tumor microenvironment can be disrupted in several ways. CCR5 antagonists (e.g. maraviroc, TAK-779) can directly interfere with CCL5 binding to CCR5. Chemotherapeutic agents (e.g. GS-1101, zoledronic acid) can reduce CCL5 secretion by MSCs and tumor cells, which indirectly prevents CCL5-CCR5 interactions by reducing CCL5 concentration in the tumor environment. Downstream intermediates may also be inhibited, including G-proteins (pTx), Jaks (tofacitinib), PI3K (GDC-0941, BKM120), Akt (MK-2206), mTOR (rapalogues) and HIF-1α (EZN-2968, PX-478). Metabolic regulators can be inhibited, including GLUT (WZB117, 2-DG), HKII (lonidamine, 3-bromopyruvate), PFK (3-PO), MCT (α-cyano-4-hydroxy-cinnamic acid) and LDHA (oxamate, FX11). Cancer metabolism can also be targeted by activating anti-proliferation enzymes, including AMPK (metformin) and PKM2 (TEPP-46).
5.4. Cancer Metabolism Therapeutics

Altered cell metabolism is a principal hallmark of cancer. This metabolic reprogramming may be a consequence of 3 distinct effectors. Firstly, the malignant phenotype can be driven by oncogenic events indirectly affecting metabolism. The constitutive activation of growth factor pathways such as IGF and a loss of function in tumor-suppressing genes, such as PTEN, may induce aggressive cell proliferation, angiogenesis and anti-apoptosis, while fueling this proliferation with accelerated cellular anabolism. Secondly, a classical characteristic of cancer growth is deregulation of metabolism. Defects in OXPHOS can potentially contribute to apoptosis resistance, while gain-of-function mutations in glycolytic enzymes may promote migration, invasion and metastasis. Lastly, the proliferative hallmarks may have coevolved with the tumor-promoting metabolic features resulting in a feed forward cascade. Altered metabolism provides abundant precursors for proliferation, while aggressive replication drives nutrient uptake.

Understanding the events that determine tumorigenesis for each individual will provide insights for the selection of the appropriate therapeutic interventions: personalized medicine. Targeted blockade of altered metabolic enzymes and/or CCL5-CCR5 interactions may limit the supply of essential biosynthetic precursors and potentially eliminate neoplasia and/or tumor invasion of tissues. For example, HIF inhibitors are capable of suppressing angiogenesis through VEGF reduction, re-establishing the p53-dependent apoptotic pathway and inhibiting PI3K/AKT/mTOR-mediated cell proliferation, in addition to normalizing metabolic functions (Mooring et al., 2011). Moreover, targeting key enzymes in key metabolic and proliferative pathways using small molecule inhibitors have yielded promising results. For example, the HK II inhibitor, 3-bromopyruvate, promotes VDAC-mediated
apoptosis in hepatocellular carcinomas while also inhibiting glycolysis (Xintaropoulou et al., 2015). Moreover, the restoration of PDH activity through PDK1 inhibition can promote carbon flow to OXPHOS, which reduces the accumulation of biosynthetic precursors and promotes apoptosis in cancer cells (Dupuy et al., 2015).

The discovery of effective and specific cancer therapeutics remains challenging. mTOR antagonists and AMPK activators show promise in limiting cancer growth, however they are highly non-specific and have extensive side effects that are not limited to cancer cells. The current list of pharmacologic agents targeting altered metabolic pathways in cancer is growing, yet the efficacy of many, if not most of them, has yet to be determined in a clinical setting. Moving forward, it would be important to identify critical metabolic alterations in cancers that are dispensable in normal, healthy cells as potential therapeutic targets. Notably, cancers exhibit plasticity and heterogeneity that add additional confounders to this therapeutic intervention strategy. It is unlikely that inhibition of any single pathway will lead to drastically improved outcomes. Consequently, we propose anti-cancer metabolism therapy as an adjunct to be combined with other courses of treatment to maximize clinical effectiveness. Developing a comprehensive understanding of the effectors that drive cancer metabolism will provide the potential targets for these combination therapies.
Chapter 6

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