THE ROLE OF RAP AND PHOSPHOINOSITIDE 3-KINASE DELTA IN B LYMPHOCYTE FUNCTION

by

CAYLIB DURAND

B.Sc., The University of British Columbia, 2003

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES
(Microbiology and Immunology)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

June 2009
© Caylib Durand, 2009
ABSTRACT

B cells eliminate pathogens by producing antibodies, activating other immune cells, and secreting cytokines. To carry out these functions, B cells must traffic from the bone marrow into the blood and then into secondary lymphoid organs to encounter antigens and become activated. As a result, B cell trafficking is highly regulated and critical for the activation of self-reactive B cells that contribute to autoimmunity and the spread of malignant B cells. The Rap GTPases regulate integrin activation and in chapter two I showed that Rap1 activation is required for B cell migration and adhesion. Because, lipid mediators including Sphingosine 1-phosphate (S1P) and lysophosphatidic acid (LPA) are potent regulators of cell adhesion and migration, I asked whether LPA regulates B cell adhesion and migration. I found that LPA reduces B cell migration by favoring strong integrin-mediated adhesion.

Phosphatidylinositide 3-kinase (PI3K) controls multiple proteins that regulate cell motility, survival and activation. Therefore in the third chapter I investigated the role of p110δ, the major isoform of PI3K in B cells, in the activation, migration, and function of conventional and innate-like B cells. B-2 cells are involved in T cell-dependent antibody responses while B-1 and marginal zone (MZ) B cells are innate-like lymphocytes that mediate T cell-independent responses to microbial antigens. Importantly, these cells are also responsible for many antibody-mediated autoimmune diseases. I showed that p110δ is needed for the activation and chemotaxis of B-2, B-1 and MZ B cells. I also showed that the in vitro adhesion and in vivo localization of MZ B cells is dependent on p110δ activity. Interestingly, I found that p110δ activity is needed for Rap1 activation, making p110δ a key regulator of B cell trafficking. B-1 and MZ B cells produce natural antibodies in the absence of immunization that often recognize self-antigens. We showed that the production of natural antibodies, both protective and pathogenic, depends on p110δ activity and that p110δ inhibition can reduce the levels of pathogenic auto-antibodies in collagen-induced arthritis.

This work suggests that by regulating Rap, p110δ, or LPA, it may be possible to control B cell-mediated diseases including inflammation, autoimmunity, and cancer.
TABLE OF CONTENTS

Abstract .......................................................................................................................... ii
Table of Contents .......................................................................................................... iii
List of Tables ................................................................................................................ vii
List of Figures .............................................................................................................. viii
List of Abbreviations ................................................................................................... xi
Acknowledgments ......................................................................................................... xiv
Dedications .................................................................................................................... xv
Co-Authorship Statement ............................................................................................. xvi

CHAPTER 1: Introduction .............................................................................................. 1
  1.1 B cells in the immune system ............................................................................ 2
  1.2 Importance of migration and adhesion in B cell function ................................. 2
  1.3 Multiple functions of B cells .......................................................................... 4
  1.4 Follicular B-2 cells ............................................................................................ 5
  1.5 Marginal zone B cells ....................................................................................... 6
  1.6 B-1 cells ............................................................................................................ 7
  1.7 Regulatory B cells ............................................................................................ 8
  1.8 Natural antibody production by B-1 and MZ B cells ........................................ 9
  1.9 B cells and disease ............................................................................................ 10
    1.9.1 Rheumatoid arthritis ............................................................................... 11
    1.9.2 Systemic lupus erythematosus ................................................................. 12
    1.9.3 Diabetes .................................................................................................... 13
    1.9.4 B cell leukemia and lymphoma ................................................................. 14
  1.10 Chemoattractant receptor signaling in B cells .................................................. 15
  1.11 B cell receptor signaling in B cells ................................................................. 16
  1.12 The importance of PI3K in B cells ................................................................. 17
  1.13 The role of Rap activation in B cells ............................................................... 18
1.14 Role of lysophosphatidic acid in B cells .......................................................... 20
1.15 Objectives and aims ............................................................................................ 21
1.16 References ........................................................................................................... 35

CHAPTER 2: The Rap GTPases mediate CXCL13- and Sphingosine

1-phosphate-induced chemotaxis, adhesion, and Pyk2 tyrosine phosphorylation in B cells .......................................................... 53

2.1 Introduction ........................................................................................................... 54
2.2 Results .................................................................................................................. 57

2.2.1 CXCL13 and S1P induce Rap1 activation in B cells ........................................ 57
2.2.2 CXCL13 and S1P stimulate ERK, JNK, and Akt phosphorylation, which is not blocked by RapGAPII ........................................................... 59
2.2.3 The S1P₁ and S1P₃ receptors can mediate Rap activation ............................. 59
2.2.4 CXCL13- and S1P-induced B cell migration is dependent on Rap activation .......................................................... 61
2.2.5 CXCL13- and S1P-induced adhesion to ICAM-1 and VCAM-1 is dependent on Rap activation .......................................................... 62
2.2.6 CXCL13- and S1P-induced tyrosine phosphorylation of Pyk2 is dependent on Rap activation .......................................................... 62

2.3 Discussion .............................................................................................................. 64
2.4 Materials and methods ........................................................................................ 69
2.5 References ............................................................................................................ 83

CHAPTER 3: Phosphoinositide 3-kinase p110δ regulates natural antibody production, marginal zone and B-1 B cell function, and autoantibody responses ......................................................... 88

3.1 Introduction ........................................................................................................... 89
3.2 Results .................................................................................................................. 92
3.2.1 Reduced levels of circulating natural antibodies in p110δ knock-in mice ................................................................. 92
3.2.2 IC87114 inhibits PI3K-dependent activation of Akt in splenic and peritoneal B cell subsets ................................................................. 93
3.2.3 p110δ activity is important for TLR-induced proliferation of B-1 and MZ B cells ................................................................. 94
3.2.4 IC87114 inhibits chemoattractant-induced migration of splenic B-2, B-1a and MZ B cells ................................................................. 95
3.2.5 p110δ activity is required for the activation of the Rap1 GTPase .......... 97
3.2.6 IC87114 inhibits MZ B cell adhesion in vitro and disrupts MZ B cell localization in vivo ................................................................. 97
3.2.7 IC87114 reduces in vitro antibody responses by B-1 and MZ B cells ....... 98
3.2.8 p110δ inhibitors reduce pathogenic antibody responses in vivo .......... 98
3.3 Discussion .................................................................................. 100
3.4 Material and methods ................................................................... 104
3.5 References .................................................................................. 128

CHAPTER 4: Lysophosphatidic acid regulates B cell chemotaxis and adhesion ........................................................................... 133

4.1 Introduction .................................................................................. 134
4.2 Results ......................................................................................... 137
4.2.1 B cells express several LPA receptors ............................................... 137
4.2.2 LPA is a negative regulator of B cell migration .................................. 137
4.2.3 LPA induces B cell adhesion to fibronectin, VCAM-1, and ICAM-1 ..... 138
4.2.4 LPA induces B cell spreading .......................................................... 138
4.2.5 LPA activates Rap1 and Akt in B cells ............................................. 139
4.2.6 LPA does not regulate B cell proliferation ....................................... 139
4.3 Discussion .................................................................................. 141
4.4 Material and methods ................................................................. 145
4.5 References ............................................................................. 156

CHAPTER 5: Conclusions and future directions ........................................ 162
5.1 Summary and overview ............................................................. 163
5.2 Future directions ................................................................. 166
  5.2.1 The role of p110δ in B cell function ........................................ 166
  5.2.2 The role of LPA in B cell function ........................................ 170
5.3 Conclusions ................................................................. 171
5.4 References ............................................................................. 174

APPENDIX A .............................................................................. 176
A.1 The role of Rap activation in macrophage function .................... 177
A.2 The role of Rap activation in macrophage development .......... 178
A.3 The role of Rap activation in macrophage-mediated immunity .... 179
A.4 The role of macrophages and Rap activation in disease .......... 182
A.5 References ............................................................................. 186

APPENDIX B .............................................................................. 188
Animal licenses and certificates ......................................................... 189
LIST OF TABLES

CHAPTER 1

Table 1.1 LPA receptors and corresponding cellular responses........................................... 34
LIST OF FIGURES

CHAPTER 1
Figure 1.1 B cell development and trafficking ............................................................. 23
Figure 1.2 B cell rolling adhesion in the HEV and extravasation into lymphoid tissues .................................................................................................................. 25
Figure 1.3 B cell effector functions ................................................................................ 26
Figure 1.4 B cell cancers can arise at all stages of B cell development and activation .... 27
Figure 1.5 G protein-coupled receptor signaling in B cells ........................................... 28
Figure 1.6 B cell receptor signaling ................................................................................ 29
Figure 1.7 PI3Kδ signaling in B cells ................................................................................ 30
Figure 1.8 The Rap GTPase cycle ................................................................................... 31
Figure 1.9 Rap GTPase signaling in B cells ..................................................................... 32
Figure 1.10 LPA-induced signaling ................................................................................ 33

CHAPTER 2
Figure 2.1 CXCL13 and S1P induce Rap1 activation that can be blocked by RapGAPII expression .............................................................................................................. 74
Figure 2.2 The CXCL13 and S1P induce phosphorylation of ERK, JNK, and Akt, which is not affected by RapGAPII expression ......................................................... 76
Figure 2.3 Both the S1P1 and S1P3 receptors can contribute to S1P-induced Rap1 activation .................................................................................................................... 77
Figure 2.4 Blocking Rap activation via expression of RapGAPII inhibits CXCL13- and S1P-induced B cell migration in Transwell assays .............................................. 79
Figure 2.5 Blocking Rap activation via expression of RapGAPII inhibits CXCL13- and S1P-induced adhesion of B cells to ICAM-1 and VCAM-1 ......................... 80
Figure 2.6 Blocking Rap activation via expression of RapGAPII inhibits CXCL13- and S1P-induced tyrosine phosphorylation of Pyk2 ........................................... 81
CHAPTER 3

Figure 3.1 Decreased levels of natural Abs against PPS-3 and OxLDL in p110δ KI mice .................................................................................................................................................. 108

Figure 3.2 Decreased levels of natural Abs against heart antigens in p110δ KI mice .................................................................................................................................................. 110

Figure 3.3 IC87114 inhibits chemoattractant-induced Akt phosphorylation in B-2, B-1 and MZ B cells .................................................................................................................................................. 111

Figure 3.4 IC87114 inhibits anti-Ig- and CpG DNA-induced Akt phosphorylation in B-2, B-1 and MZ B cells .............................................................................................................................................. 113

Figure 3.5 IC87114 inhibits TLR-induced proliferation of MZ B cells and peritoneal B-1 cells ...................................................................................................................................................... 115

Figure 3.6 p110δ activity is required for B cell chemotaxis but not for chemokinesis ........................................................................................................................................................................ 117

Figure 3.7 p110δ activity is required for Rap1 activation ......................................................................................................................................................................................... 119

Figure 3.8 IC87114 inhibits MZ B cell adhesion in vitro and disrupts MZ B cell localization in vivo ........................................................................................................................................... 120

Figure 3.9 IC87114 reduces in vitro Ab responses by B-1 and MZ B cells ......................................................................................................................................................... 122

Figure 3.10 p110δ inhibitors reduce anti-SRBC and anti-collagen Ab responses in vivo .................................................................................................................................................. 123

Figure S3.1 p110δ is required for production of natural Abs against oxidized epitopes ........................................................................................................................................................................ 124

Figure S3.2 IC87114 does not inhibit T cell migration towards CXCL12 ......................................................................................................................................................... 125

Figure S3.3 p110δ activity is required for chemoattractant-induced Akt phosphorylation in WEHI-231 B lymphoma cells ........................................................................................................................................ 126

Figure S3.4 Differential effect of IC87114 on T cell versus B cell proliferation ............................................................................................................................................. 127

CHAPTER 4

Figure 4.1 B cells express multiple LPA receptors .......................................................................................................................................................................................... 149
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.2</td>
<td>LPA inhibits chemoattractant-induced B cell migration</td>
<td>150</td>
</tr>
<tr>
<td>4.3</td>
<td>LPA is not a chemorepellant for B cells</td>
<td>151</td>
</tr>
<tr>
<td>4.4</td>
<td>LPA induces B cell adhesion to FN, VCAM-1, and ICAM-1</td>
<td>152</td>
</tr>
<tr>
<td>4.5</td>
<td>LPA induces B cell spreading on fibronectin</td>
<td>153</td>
</tr>
<tr>
<td>4.6</td>
<td>Effects of LPA on the activation of ERK, JNK, Akt and Rap1 in WEHI-231 cells</td>
<td>154</td>
</tr>
<tr>
<td>4.7</td>
<td>LPA does not modulate B cell proliferation</td>
<td>155</td>
</tr>
</tbody>
</table>

**CHAPTER 5**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1</td>
<td>Rap activation, PI3Kδ activity, and LPA are important regulators of B cell function</td>
<td>172</td>
</tr>
<tr>
<td>5.2</td>
<td>Heterozygous p110δ KI mice have reduced natural Abs to cardiac myosin</td>
<td>173</td>
</tr>
</tbody>
</table>

**APPENDIX A**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.1</td>
<td>LPS induces Rap1 activation in macrophages</td>
<td>183</td>
</tr>
<tr>
<td>A.2</td>
<td>Floxed RapGAP transgene</td>
<td>184</td>
</tr>
<tr>
<td>A.3</td>
<td>LysM-RapGAP transgenic mice have reduced Rap activation in macrophages and are more susceptible to infection with <em>Salmonella</em></td>
<td>185</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

Ab  antibody
Ag  antigen
ALL  acute lymphoblastic leukemia
APC  antigen-presenting cell
ATX  autotaxin
BCR  B cell antigen receptor
BL  Burkitt lymphoma
BM  bone marrow
BSA  bovine serum albumin
BTK  Bruton’s tyrosine kinase
cDNA  complementary deoxyribonucleic acid
CFSE  carboxyfluorescein diacetate, succinimidyl ester
CLL  chronic lymphocytic leukemia
CLP  common lymphoid progenitor
CML-LBC  chronic myelocytic leukemia-lymphoid blast crisis
CSR  class switch recombination
CVID  common variable immune deficiency
Da  Daltons
DAG  diacylglycerol
DC  dendritic cell
DLBCL  diffuse large B cell lymphoma
DMEM  Dulbecco’s minimum essential medium
DMF  dimethylformamide
DMSO  dimethylsulphoxide
DNA  deoxyribonucleic acid
EAE  experimental autoimmune encephalomyelitis
ECL  enhanced chemiluminescence
ERK  extracellular signal-regulated kinase
FACS  fluorescence activated cell sorting
FBS  fetal bovine serum
FDC  follicular dendritic cell
FITC  fluorescein isothiocyanate
FL  follicular lymphoma
FN  fibronectin
GAP  GTPase activating protein
GC  germinal center
GEF  guanine nucleotide exchange factor
GPCR  G protein-coupled receptor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>HCL</td>
<td>hairy cell leukemia</td>
</tr>
<tr>
<td>HEV</td>
<td>high endothelial venule</td>
</tr>
<tr>
<td>HL</td>
<td>Hodgkin’s lymphoma</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IBD</td>
<td>inflammatory bowel disease</td>
</tr>
<tr>
<td>ICAM</td>
<td>intercellular cell adhesion molecule</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IP&lt;sub&gt;3&lt;/sub&gt;</td>
<td>inositol trisphosphate</td>
</tr>
<tr>
<td>IR</td>
<td>ischemia reperfusion</td>
</tr>
<tr>
<td>ITAM</td>
<td>immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun NH2-terminal kinase</td>
</tr>
<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
</tr>
<tr>
<td>LFA-1</td>
<td>lymphocyte function-associated antigen-1</td>
</tr>
<tr>
<td>LN</td>
<td>lymph node</td>
</tr>
<tr>
<td>LPA</td>
<td>lysophosphatidic acid</td>
</tr>
<tr>
<td>LPP</td>
<td>lipid phosphate phosphatase</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MAA</td>
<td>malondialdehyde-acetaldehyde</td>
</tr>
<tr>
<td>MAdCAM</td>
<td>mucosal addressin cell adhesion molecule</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MCL</td>
<td>mantle cell lymphoma</td>
</tr>
<tr>
<td>MDA</td>
<td>malondialdehyde</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MM</td>
<td>multiple myeloma</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MSCV</td>
<td>murine stem cell virus</td>
</tr>
<tr>
<td>MZ</td>
<td>marginal zone</td>
</tr>
<tr>
<td>MZL</td>
<td>marginal zone lymphoma</td>
</tr>
<tr>
<td>NOD</td>
<td>non-obese diabetic mice</td>
</tr>
<tr>
<td>NZB</td>
<td>New Zealand black</td>
</tr>
<tr>
<td>NZW</td>
<td>New Zealand white</td>
</tr>
<tr>
<td>oxLDL</td>
<td>oxidized low-density lipoprotein</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PIP&lt;sub&gt;2&lt;/sub&gt;</td>
<td>phosphatidylinositol (4,5)-bisphosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PIP₃</td>
<td>phosphatidylinositol (3,4,5)-trisphosphate</td>
</tr>
<tr>
<td>PL</td>
<td>plasmablastic lymphoma</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
</tr>
<tr>
<td>QRT-PCR</td>
<td>quantitative reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>S1P</td>
<td>sphingosine 1-phosphate</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SHM</td>
<td>somatic hypermutation</td>
</tr>
<tr>
<td>SLE</td>
<td>systemic lupus erythematosus</td>
</tr>
<tr>
<td>SLL</td>
<td>small lymphocytic lymphoma</td>
</tr>
<tr>
<td>SRBC</td>
<td>sheep red blood cell</td>
</tr>
<tr>
<td>T1</td>
<td>transitional 1</td>
</tr>
<tr>
<td>T1D</td>
<td>type-1 diabetes</td>
</tr>
<tr>
<td>T2</td>
<td>transitional 2</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline with Tween-20</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TD</td>
<td>T cell-dependent</td>
</tr>
<tr>
<td>TI</td>
<td>T cell-independent</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>VLA-4</td>
<td>very late antigen-4</td>
</tr>
<tr>
<td>WM</td>
<td>Waldenstrom macroglobulinemia</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>XLA</td>
<td>X-linked agammaglobulinemia</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I would like to thank all of the people who have provided assistance and been supportive over the duration of my PhD. This includes my supervisor, Dr. Michael Gold, who spent endless hours training and helping with writing manuscripts, proposals, and letters to promote my career as a scientist. I would like to thank the members of the Gold and Matsuuchi labs, both past and present, as well as all of the undergraduate students that worked with me during my PhD for providing technical assistance and numerous thought provoking discussions. I would also like to thank the members of my committee (Pauline Johnson, Ninan Abraham, and Kelly McNagny) for their suggestions and support over the years. While I have been involved in several projects and met many people during my PhD, I would like to acknowledge the following:

Chapter 2: I would like to thank Fumio Takei for key reagents, Hugh Rosen and Arthur Sampaio for advice, and Sarah McLeod for reading the manuscript and providing advice.

Chapter 3: I would like to thank Lisa Osborne, Maya Poffenberger, and Ninan Abraham (University of British Columbia) for mice and reagents, Marc Horwitz, Jane Cipollone, Marcia Graves, Jeff Duenas, Andy Johnson (University of British Columbia), for technical assistance and advice. I would like to also thank Kamal Puri (Calistoga Pharmaceuticals), Karsten Hartvigsen, Linda Fogelstrand, and Joseph Witztum (UCSD) for embarking on interesting collaborations together.

Chapter 4: I would like to thank Morgan Riggan for all of her hard work as an undergraduate student on the project.

Finally, I would like to acknowledge family and friends who have been supportive while I completed this degree.
DEDICATION

I would like to dedicate this thesis to my grandparents who have waited patiently for me to finish my studies.
CO-AUTHORSHIP STATEMENT

Research design, data analysis and manuscript preparation were completed with the assistance of Dr. Michael Gold. Experimental research was conducted by the author with the following exceptions:

Chapter 2: K. W. Tse did the experiments for figure 2.6.

Chapter 3: K. Hartvigsen and L. Fogelstrand completed experiments for figures 3.1A,C-H, 3.4C, 3.9C-E, and S3.1A-D. S. Iritani and K. D. Puri did the experiments for figure 3.10A,B and figure S3.4.
CHAPTER 1

Introduction
1.1  B cells in the immune system

The host immune system is comprised of innate and adaptive immunity, both of which are needed for protection from invading pathogens. Innate immune cells respond immediately to pathogens and help control the majority of infections. Monocytes, macrophages, dendritic cells (DCs), NK cells, mast cells, eosinophils, basophils, and neutrophils are the key players in innate immunity and have restricted, germ-line encoded receptors that recognize pathogens (1). In the event that a pathogen escapes the innate immune system, the adaptive immune system can fight infections through humoral or cell-mediated immunity. Lymphocytes compose the adaptive immune system and consist of T cells, which develop in the thymus and contribute to cell-mediated immunity, whereas B cells develop in the bone marrow and mediate humoral immunity through the production of antibodies (Abs). B cells express clonally-diverse cell surface immunoglobulin (Ig) receptors that recognize specific antigenic epitopes found on pathogens but that also respond to pathogens using Toll-like receptors (TLRs) that recognize conserved microbial components (2). Upon activation, B cells terminally differentiate into Ab-secreting plasma cells, a complex process that involves highly-regulated cell trafficking and cell signaling events (3).

1.2  Importance of migration and adhesion in B cell function

Chemoattractant-directed B cell localization and subsequent integrin-mediated adhesion play important roles in B cell development, activation, survival, and differentiation (4-6). The chemokine CXCL12 (SDF-1) retains B cell progenitors in the bone marrow (BM) and promotes their adhesion to stromal cells, which provide survival and differentiation signals (7-9). B cells also need pre-B cell antigen receptor (pre-BCR) and IL-7 receptor signaling to develop into immature B cells. Once they reach the immature state, they can leave the BM and home to the spleen, where they become mature naïve B cells (10). Mature B cells can then leave the spleen and enter circulation (10, 11) (Fig. 1.1).

Antigen (Ag) encounter, which usually occurs in the secondary lymphoid tissues, causes mature naïve B cells to differentiate into Ab-secreting plasma cells. The entry of mature B cells into lymph nodes (LN) is mediated by rolling and then firm adhesion to the high endothelial
venules (HEV) (12) (Fig. 1.2). L-selectin is important for B cell rolling, which brings the B cell into close contact with the endothelium where the chemokines CXCL12, CXCL13 (BLC) and CCL21 (SLC) are displayed on the surface of the HEV cells (12-15). These chemokines stimulate lymphocyte function-associated antigen-1 (LFA-1, αLβ2 integrin) activation. LFA-1 binds to intercellular adhesion molecule-1 (ICAM-1) and ICAM-2, leading to firm adhesion (16, 17). These interactions allow B cells to extravasate into the LN (11, 18).

By stimulating integrin activation and chemotaxis, CXCL12, CXCL13, and CCL21 also direct the trafficking of B cells within the lymphoid organ (Fig. 1.1). Gradients of CXCL13 and CCL21 direct B cells to the B cell follicle or to the T cell zone, respectively (19). These chemoattractants allow follicular B-2 cells to localize to the edge of the T cell zone where they can interact with Ag-specific T cells (4, 5, 20-22). By receiving co-stimulatory signals from T cells via CD40-CD154 (CD40L) interactions (22, 23), B cells activated through the B cell antigen receptor (BCR) undergo proliferation and differentiation. Once the B cell receives a BCR signal plus T cell help, it can form primary follicles in extrafollicular sites and differentiate into a short-lived plasma cells that secretes Abs.

Alternatively, depending on the Ag or co-stimulatory signaling strength, CXCL13 directs B-2 cells into lymphoid follicles (4, 24) to establish germinal centers (GCs) (25-27). B cells can also encounter Ag in the follicle that is presented by follicular dendritic cells (FDCs). This upregulates the expression of CCR7, the receptor for CCL21, and causes the B cell to migrate to the T cell zone to look for Ag-specific T cell help (13, 19). While in the GC, integrin-mediated adhesion to FDCs provides survival signals for both naïve and activated B-2 cells (28-31). With the help of follicular T helper cells (32) and FDCs (28, 31), B cells undergo clonal expansion, class switch recombination (CSR), somatic hypermutation (SHM) of Ig variable region gene segments, and affinity maturation (33, 34). During affinity maturation, B cells undergo selection for increased affinity of the BCR for Ag, thereby generating high affinity Ab-producing B cells (2, 33, 35, 36). This GC reaction also leads to the differentiation of B cells into memory cells or long-lived Ab-secreting plasma cells.

Sphingosine 1-phosphate (S1P) plays a key role in allowing both naïve and activated B cells to exit lymphoid organs and return to the circulation (37). After exiting secondary lymphoid organs, plasma cells can then be directed to the BM by CXCL12 (38), where they survive and secrete antibodies for long periods of time (3, 39). The role of S1P in directing B
cell localization has only recently been appreciated. There are at least five different receptors for S1P, S1P$_{1-5}$ (40, 41), with S1P$_1$ and S1P$_3$ being important for lymphocyte trafficking (37). S1P$_1$ plays a key role in the exit of T and B cells from peripheral lymphoid organs and the thymus (37, 42) and S1P$_1$ overexpression causes B cells to relocalize from the splenic white pulp to the outer red pulp (43), possibly by overriding CXCL13-induced migration towards the lymphoid follicles (44). It has been suggested that S1P in the blood and lymph causes rapid downregulation of S1P$_1$ on circulating lymphocytes, allowing them to enter lymphoid organs without immediately exiting (43). After a certain period of time in the lymphoid organ, re-expression of S1P$_1$ allows the lymphocyte to re-enter the circulation and traffic to other lymphoid organs.

The importance of B cell migration and adhesion is highlighted by the complex interactions between multiple chemoattractants that direct B cells from the BM and spleen to the correct locations in secondary lymphoid organs. Correct localization is needed for the B cell to encounter Ag and interact with other immune cells, to be activated, to differentiate and to carry out effector functions. In addition to being important for normal B cell development and function, chemoattractant-regulated B cell trafficking also underlies the formation of ectopic GCs in autoimmune diseases such as rheumatoid arthritis (RA) (45, 46) as well as the homing of malignant B cells to the BM (47-49). As a result, B cell trafficking and localization can contribute to autoimmunity or cancer.

1.3 Multiple functions of B cells

B cells are typically known for their role in Ab-mediated immunity. B cells are involved in three types of Ab responses: natural Ab production, T cell-independent (TI) IgM Ab responses, and T cell-dependent (TD) high affinity Ab production (2, 3). However, Ab production is not the only function that B cells carry out to maintain immune homeostasis (2) (Fig. 1.3). B cells can bind specific Ag via their BCR such that internalized proteins are processed into peptides that are presented to T cells on major histocompatibility complex (MHC) proteins (50-52). Antigen presentation and costimulation by CD40, CD80, and CD86, which are expressed on activated B cells, is important for the activation of T cells that mediate adaptive immune responses. B cells are also important for the optimal activation of CD4$^+$ T
cells during immune responses to low doses of foreign Ags and autoAgs and secrete cytokines that influence Th1 versus Th2 T cell differentiation (53, 54). Thus, effector B cells regulate both humoral and cellular immunity (55).

The importance of B cells in the immune system is illustrated by μMT mice, which have a genetic defect that prevents B cell development (56). In these mice, which are missing the μM exon, B cell development is blocked at the pre-B cell stage and there are no mature B cells. This results in significant abnormalities in the immune system with decreased thymocyte numbers and diversity (57), defects within spleen DC and T cell compartments (58-60), the absence of Peyer Patches, lymphoid follicles, FDC networks (61, 62), and the absence of marginal zone (MZ) metallophilic macrophages in the spleen. Many of these defects are due to the absence of B cell-derived cytokines such as lymphotoxin-α (60, 61). Thus, B cells regulate lymphoid tissue organization and neogenesis, wound healing, transplanted tissue rejection, and tumor immunity (2). In addition, B cells produce both proinflammatory and immunosuppressive cytokines that can regulate immune responses (2). B cells are typically thought to be part of the adaptive immune response, but it is now recognized that B cells express TLRs and can respond rapidly to bacterial and viral components, much like innate immune cells (63-66). This suggests that B cells provide an important link between innate and adaptive immune responses through their numerous effector functions. Given the diverse roles that B cells play in the immune system it is not unexpected that there are distinct B cell populations, which are described in the next sections.

1.4 **Follicular B-2 cells**

The most prevalent B cell subset in both mice and humans are the well characterized circulating follicular B-2 cells. B-2 cell progenitors are generated in the BM, enter the blood after differentiating into transitional 1 (T1) B cells, and then migrate to the periarterial lymphatic sheath of the spleen, where they are characterized by the expression of CD23 and IgD (1, 10, 67). These cells then differentiate further into transitional 2 (T2) B cells and finally into recirculating follicular B-2 cells (Fig. 1.1), which are IgMloIgDhiCD21loCD23hi (1, 10). As a result of their ability to enter the circulation, B-2 cells are located in the blood, gut, peritoneal cavity, spleen and peripheral LNs (1). Follicular B-2 cells are dependent on T cell help and are
known for their major contribution to humoral immune responses via the production of high affinity Abs (2, 39). When B-2 cells encounter Ag, they remain in peripheral lymphoid organs, and after receiving T cell help, they move into lymphoid follicles and form GCs (Fig. 1.1). Once there they undergo SHM, CSR, and affinity maturation, they develop into high affinity Ab-secreting B cells or memory B cells (39). However, follicular B-2 cells are not the only B cells that are important for protection from invading pathogens.

1.5 Marginal zone B cells

In addition to circulating follicular B-2 cells, MZ B cells play an important role in helping clear infections. B-2 and MZ B cells are thought to come from a common precursor T2 cell. Some T2 cells migrate to the MZ of the spleen and differentiate into MZ B cells, which are IgM^hi^IgD^lo^CD21^hi^CD23^lo^ (1, 55) (Fig 1.1). The level of BCR signaling is thought to play an important role in B-2 versus MZ B cell fate as is Notch signaling (68). Strong BCR signaling facilitates follicular B-2 maturation (69, 70) while low-affinity BCR signaling may result in MZ B cell maturation (1). In support of this idea, MZ B cells have a lower activation threshold than conventional recirculating B-2 cells (55). Further work is needed to understand the cell fate decisions that lead to MZ B cell development.

MZ B cells are innate-like lymphocytes that help clear pathogens by producing natural Abs in the absence of Ag stimulation and by generating rapid TI Ab responses against multivalent microbial Ags (71, 72). The BCRs of MZ B cells possess short CDR3 regions and lack N sequences (73) giving them a limited, germline-encoded Ig repertoire that is distinct from that of B-2 cells. In contrast to conventional follicular B-2 cells, MZ B cells do not circulate but are localized proximal to the marginal sinus of the spleen, where they act as the first line of defense against blood-borne encapsulated bacteria (1, 2, 72, 74). B-2 and MZ B cells also differ in their relative half life. Mouse B-2 cells have a half life of 4.5 months while MZ B cells can survive for more than 1 year and are capable of self-renewal (75).

MZ B cells secrete natural Abs (76) but can also be activated by Ag to become short lived Ab-secreting plasma cells (74). Both of these Ab responses are usually IgM and are often directed against polysaccharide Ags on encapsulated bacteria. While MZ B cells don’t recirculate, they do migrate to B cell follicles after exposure to bacterial products and can
transport blood-borne Ags to FDCs to facilitate the GC reaction in which B-2 cells proliferate and differentiate (76, 77). In this capacity they transport Ag captured in the marginal sinus to GCs in the LNs, acting as an antigen presenting cell (APC) that helps initiate the adaptive immune response (78). MZ B cells, and the Abs that they produce protect against infections (79-82), highlighting the important contribution MZ B cells make to host immunity. As a result of their ability to participate in both TI and TD responses (77), MZ B cells are a unique population of splenic B cells (72).

Migration and adhesion are critical for the localization and function of MZ B cells. The chemoattractant S1P, and its receptor S1P3, are required for the correct localization of MZ B cells in the splenic MZ. The S1P3 receptor is important for MZ B cells to migrate towards S1P (44) and S1P3-deficient mice have a disordered splenic marginal sinus, resulting in defective MZ immune responses (83). The importance of cell migration and adhesion for MZ B cell function is also illustrated by the observation that Pyk2-, Rac-2-, DOCK2-, and Lsc-deficient mice, all lack MZ B cells. These molecules are all important for actin polymerization, membrane remodeling, and cell movement (84-88). In addition, the integrins LFA-1 and very late antigen-4 (VLA-4, α4β1 integrin), which are expressed on MZ B cells, allow the retention of MZ B cells in the MZ by binding ICAM-1 and VCAM-1, respectively (89). This highlights the critical involvement of chemoattractant-mediated migration and adhesion in the correct localization and function of MZ B cells. However, it is likely that other signaling proteins that regulate cell migration and adhesion are also involved in the retention of MZ B cells in the MZ.

1.6 B-1 cells

Like MZ B cells, B-1 cells are innate-like B cells. They are found in the peritoneal cavity and the spleen. B-1 cells are thought to develop from a distinct progenitor cell from B-2 cells (90), although this is still under investigation. B-1 cells differ from B-2 cells in terms of their phenotype, anatomical location, self-renewing capacity, and natural Ab production (91-93). B-1 cells constitutively produce natural Abs in the absence of Ag stimulation and also make rapid TI Ab responses against multivalent microbial antigens such as bacterial polysaccharides (94). Interestingly, B-1 cells are capable of Ag presentation (95, 96) and can interact with invariant natural killer T cells, an innate-like T cell population (97, 98).
B-1 cells can be further subdivided into B-1a (CD5+) and B-1b (CD5−) cells. B-1a cells produce natural Abs that provide immediate innate protection against bacterial infections in naïve hosts (99). In contrast, B-1b cells are the source of long-term adaptive Ab responses to polysaccharides and other TI Ags that are encountered during infection (99). Once activated by TLR ligands, B-1a cells can migrate to the spleen in response to chemoattractants, where they differentiate into plasma cells (100, 101). The origin of B-1 cells and whether B-1a and B-1b cells are derived from the same or distinct progenitor cells, is not known. B-1 cells may be derived from fetal liver progenitors that migrate to the peritoneal cavity, where they can undergo self-renewal (93).

### 1.7 Regulatory B cells

In addition to conventional B-2 cells and the innate-like B-1 and MZ B cells, there are regulatory B cells (102, 103). It is still unclear if regulatory B cells develop from a distinct progenitor or are derived from activated B cells. Some evidence suggests that regulatory B cells only appear under inflammatory conditions and are not found under normal conditions (102). There are two types of regulatory B cells, the “acquired type”, which are thought to be follicular B-2 cells activated by self-Ag and CD40, and the “innate type”, which are thought to arise from lipopolysaccharide- (LPS) or CpG-activated MZ B cells (103). Accordingly, there are regulatory B cells that have a similar phenotype to B-2 cells (CD11b+CD5−IgD+) whereas other regulatory B cells share features with splenic MZ B cells (102, 104). Recently, Yanaba et al. identified IL-10-producing regulatory B cells, which they termed B10 cells (CD1dhiCD5+). These cells share cell surface markers with B-1a and MZ B cells (105). However, the origin of B10 cells is not known.

Much like regulatory T cells, B10 cells can regulate T cell-mediated inflammatory responses (105). In mice, they can enhance the recovery from experimental autoimmune encephalomyelitis (EAE) (106), prevent arthritis (107), suppress ulcerative colitis (102, 108, 109), and inhibit the progression of inflammatory bowel disease by suppressing macrophage-mediated proinflammatory responses (110, 111). In addition to producing the immunosuppressive cytokine IL-10, some regulatory B cells produce TGF-β1 and participate in the induction of low-dose oral tolerance (112, 113). TGF-β1-producing B cells generated in
vitro protect NOD mice from diabetes (114). However, whether such regulatory B cells develop in vivo is still unclear (103). The discovery of regulatory B cells provides an additional set of functions for B cells, not just contributing to immunity or autoimmunity, but also suppressing immune responses.

1.8 Natural antibody production by B-1 and MZ B cells

The Ig repertoires of B-1 and MZ B cells are skewed towards the recognition of both microbial Ags and self-Ags. Natural Abs are Abs produced in the absence of immunization or Ag exposure. They are made by innate-like B cells and often recognize cross-reactive epitopes on encapsulated Gram-positive bacteria, pathogenic viruses, apoptotic cells, and oxidized low-density lipoproteins (OxLDL) (79, 115, 116). As a result, natural Abs provide immediate protection against viral and bacterial infections (79-82) and prevent inflammation by facilitating the clearance of oxidized lipids, oxidized proteins, and apoptotic cells (115, 117). In addition to their important immunoprotective and homeostatic functions, B-1 and MZ B cells are also a major source of Abs that contribute to acute inflammation as well as chronic autoimmune diseases.

When ischemia-reperfusion (IR) injury or other types of acute tissue damage expose intracellular proteins, natural Abs against specific intracellular self-Ags can initiate complement-mediated inflammation that exacerbates IR injury of the heart, mesentery, and brain (116, 118, 119). Similarly, natural Abs that recognize microbial Ags during infection can cross-react with self-Ags and cause heart damage, as is seen in rheumatic fever (120, 121).

In chronic inflammatory diseases such as RA and systemic lupus erythematosus (SLE), there are expanded populations of B-1 and MZ B cells (122-127) and Abs produced by these cells contribute to disease symptoms (128, 129). Natural Abs produced by B-1 and MZ B cells may then be able to activate other autoreactive B cells by forming immune complexes (130). Specifically, complement activation initiated by immune complexes may promote the activation of self-reactive B-2 cells because Ag and C3d bridge the BCR to the CD21/CD19 complex and amplifies BCR signaling (131). Thus, modulating the activation of MZ and B-1 B cells could be a useful approach for treating Ab-mediated inflammation and autoimmune diseases.
1.9 B cells and disease

B cell development, activation, trafficking, and differentiation need to be tightly controlled because aberrant B cell functions can lead to disease. There are three types of B cell mediated diseases; congenital immunodeficiencies, leukemia and lymphoma, and autoimmunity (2). When B cell functions fail this can lead to immunodeficiencies. B cell immunodeficiency diseases include X-linked agammaglobulinemia (XLA) (132, 133). This disease is a result of a mutation in Bruton’s tyrosine kinase (BTK) gene that disrupts B cell development and results in reduced Ab production (134, 135). Other B cell immunodeficient diseases include common variable immune deficiency (CVID) and hyper-IgM syndrome. Both result in increased susceptibility to infections, and are associated with reduced memory B cells, and impaired CSR, SHM, and B cell activation resulting from a mutation in costimulatory ligands or receptors (136-139).

Unregulated B cell functions can also lead to pathogenic autoAb production and the development of autoimmunity (140-142). Normally, central and peripheral tolerance mechanisms prevent the development of autoreactive B cells. In the peripheral lymphoid tissues, there are multiple mechanisms that establish and maintain B cell tolerance, including clonal deletion (apoptosis), functional silencing (anergy), or further BCR rearrangement (receptor editing) to eliminate autoreactive B cells (143-145). However, if the normal process of removing autoreactive B cells fails, this can lead to an immune response that is directed against self-tissues (146). The MZ B cell compartment is often enriched in self-reactive B cells (147-149) possibly because the Ig repertoire of MZ B cells recognizes common bacterial components that crossreact with self-Ags. B cells may also contribute to autoimmune diseases by presenting auto-antigen to self-reactive T cells or through the production of pro-inflammatory cytokines (150).

B cells may also be involved in limiting autoimmunity either via cytokine production or via natural Ab secretion. B cells have been linked to EAE, an autoimmune disease of the central nervous system that models human multiple sclerosis (151, 152) as well as to chronic colitis and inflammatory bowel disease (IBD). In these autoimmune diseases B cells are thought to be required for EAE and IBD recovery but not for the initiation of disease (55). This may reflect a regulatory role for B cells.
The deleterious role of B cells in autoimmune diseases such as RA, SLE, and type-1 diabetes (T1D) suggests that regulating B-1 and MZ B cell localization, migration and activation could allow us to limit the development or progression of autoimmune diseases as well as the spread of malignant B cells.

1.9.1. Rheumatoid arthritis

RA is an autoimmune, chronic progressive joint disease that is mediated in part by the production of autoAbs (124, 153, 154), with collagen-induced arthritis (CIA) in rodents being recognized as a model of human RA (155, 156). B cells capable of Ag presentation are essential for autoreactive T cell activation in RA (157). After the activation of autoreactive T cells there is production of autoAbs to collagen, glucose-6-phosphate isomerase (GPI), and citrullinated protein (124, 158). Immune complex-mediated recruitment and activation of inflammatory cells then leads to leukocyte invasion of the synovial lining and hyperplasia of the resident synoviocytes. The resulting inflammation and invasion of joints by leukocytes causes neovascularization, cartilage destruction, bone erosion, and remodeling of the joint structures (159).

Both autoAb production and the ability of B cells to present Ag to autoreactive T cells is needed to cause severe arthritis, indicating that both pathways contribute to RA. K/BxN mice that have transgenic T cell receptors for GPI generate anti-GPI autoAbs that cause disease (158, 160). The GPI-specific autoAbs alone can transfer destructive arthritis to mice lacking lymphocytes, highlighting the contribution of autoAbs to RA (161). B cell-deficient mice and mice with only membrane bound IgM on B cells do not develop arthritis after immunization. However, if given Ag, these B cells can activate T cells without secreting Ab and only cause mild arthritis (162). Given the importance of B cells in RA, B cell depletion reduces the severity of disease (163) and anti-CD20 monoclonal Ab (Rituximab) treatment has become a standard therapy for RA (164).

Ectopic GCs, or GCs located in synovial tissue, are also found in RA. B cell migration and adhesion play a critical role in B cell homing and in the formation of ectopic GCs in joint tissue (124). In ectopic GCs, AutoAbs are made by plasma cells that undergo clonal expansion, SHM, and affinity maturation (45). Lymphotoxin-β, produced by B cells, and the chemokine
CXCL13, are important for lymphoid neogenesis in RA (165). This shows that B cells can also be the source of cytokines that contribute to the development of RA and that chemokines are important for B cells to form ectopic GCs.

B cell activation in RA synovitis can occur through TD (CD40-CD40L) and TI pathways (TLRs, BAFF) (124), suggesting the involvement of both B-2 and innate-like B cell subsets. Furthermore, the levels of BAFF, a cytokine that is important for the survival and differentiation of B-2 and MZ B cells (166), are elevated in the sera and synovial fluid of RA patients. This may promote the survival of autoreactive B cells (167). The relative contributions of B-1 and MZ B cells to the development of RA is still being investigated. Nevertheless, regulating the activation and trafficking of B-2, B-1 and MZ B cells may be one approach to prevent the onset of RA or treat existing disease.

1.9.2. Systemic Lupus Erythematosus

SLE results from B cells that respond excessively to immune stimulation, leading to the generation of autoAbs that cause tissue damage (146). The excessive activation of B cells in SLE is due to abnormalities in B cell signaling and migration, increased levels of costimulatory molecules and cytokines, and disrupted positive and negative selection of B cells. As a result, B cells generate anti-DNA Abs that cause immune complex deposition and glomerulonephritis (146). In addition, B cells can also generate anti-cardiolipin Abs involved in thrombosis and anti-Ro Abs involved in congenital heart blockage (146). The unregulated control of B cell functions leads to the development of the multiple autoimmune reactions associated with SLE.

The role that B cells play in SLE is illustrated by MRL-lpr/lpr mice, which have mutations in Fas or FasL that allow B cells to avoid negative selection and apoptosis. These mice spontaneous develop an autoimmune disease resembling SLE (168). New Zealand Black (NZB) mice also develop a lupus-like syndrome (169) that is mediated by expanded B-1 populations (170, 171). MZ B cells in NZB mice also expanded and hyperactivated (172, 173), resulting in the production of anti-DNA Abs (174, 175). In NZB x New Zealand White (NZW) mice, B-1 cell depletion delays disease onset and reduces disease severity (176, 177). Depletion of B-1 and MZ B cells using B cell superAgs also reduces disease (1). This suggests that B-1
and MZ B cells are involved in SLE, although their direct involvement is still being investigated.

In some autoimmune diseases, B-1 and MZ B cells home aberrantly to other lymphoid organs and cause disease (178, 179). B-1 cells typically migrate towards CXCL13 (180) but fail to home to the peritoneal cavity in aged NZB mice (179). This aberrant homing can then result in the production of autoAbs involved in lupus. Regulating the chemoattractant-regulated localization and activation of B cells might be a useful approach for Ab-mediated autoimmune diseases.

Further support for the involvement of B cells in SLE comes from mice overexpressing BAFF. These mice have expanded B cell populations, elevated levels of anti-DNA Abs and immune complexes in the kidney (181, 182). These mice spontaneously develop SLE which is associated with a dramatic increase in MZ B cells (181, 183). Human SLE patients also have elevated levels of BAFF (2). Furthermore, mice deficient in SPA-1 (a Rap GTPase-activating protein) have elevated numbers of B-1a cells that produce anti-dsDNA Abs, and develop lupus-like nephritis (122). Regulating B-1 and MZ B cell populations may therefore be an effective way to control SLE.

1.9.3. Diabetes

B cells have been implicated in the initiation of T1D in non-obese diabetic (NOD) mice (184). Autoreactive B cells generate islet-specific autoAbs, including anti-insulin autoAbs, that play a role in the pathogenesis of autoimmune diabetes (185). Interestingly, NOD mice have expanded populations of MZ B cells and the onset of diabetes correlates with the accumulation of MZ B cells in the pancreatic LN. Once in the pancreatic LN, MZ B cells can activate autoreactive T cells by presenting self-Ags to effector T cells (186-188). Upregulation of the costimulatory molecules CD80 and CD86 and the production of pro-inflammatory cytokines by MZ B cells (188) can help activate autoreactive T cells (150). The activated autoreactive T cells then move into the pancreas and destroy insulin-producing islet cells, leading to diabetes. In addition, B cells promote the survival of intra-islet CD8+ cytotoxic T cells through direct cell-cell contact, which leads to further destruction of islets (189).
B cell-deficient NOD mice do not develop diabetes, supporting the idea that B cells are involved in disease progression (190). B cells that cannot secrete Abs can still mediate the development of diabetes, supporting the idea that B cell-mediated Ag presentation and the subsequent activation of autoreactive T cells is a critical step (190). It is also interesting that the \textit{in vivo} neutralization of BAFF reduces the level of insulin autoAbs, islet inflammation, and the incidence of spontaneous diabetes (191). Neutralizing Abs to BAFF cause the depletion of MZ and follicular B cells, disrupts T cell activation, and prevents islet cell destruction in NOD mice. Thus, regulating MZ B cell localization, migration and activation might allow for the control or development of T1D.

\subsection{1.9.4. B cell leukemia and lymphoma}

Since B cells are continually developing and being activated during immune responses, critical regulatory mechanisms are needed to ensure that lymphoproliferative disorders don’t result. However, mutations that lead to the loss of growth control frequently occurs in B cells, leading to the development of B cell leukemias and lymphomas. There is also a close link between autoimmunity and the development of cancer because of uncontrolled B cell activation. For example, uncontrolled activation of B cells through the BCR can lead to malignancy in RA patients as demonstrated by the increased occurrence of diffuse large B cell lymphoma (DLBCL) in patients with RA (192).

Most stages of B cell development and different B cell subsets can give rise to lymphomas or leukemias (2) (Fig. 1.4). There are a high frequency of B cell cancers that are due to the genetics of Ig gene rearrangements. Hodgkin’s lymphoma (HL) is a well characterized B cell lymphoma (193). There are also several non-Hodgkins lymphomas that are derived from B cells. Interestingly, a majority of B cell lymphomas originate from the GC as a result of mutations and chromosomal translocations caused by aberrant SHM and CSR (194, 195).

Pre-GC cancers arise from B cells developing in the bone marrow and give rise to B-lineage acute lymphoblastic leukemia (ALL) and chronic myelocytic leukemia-lymphoid blast crisis (CML-LBC) cells. The primary B cell cancer arising from circulating B cells is chronic lymphocytic leukemia (CLL) whereas B cells in primary follicles give rise to small lymphocytic
B cell cancers derived from GC B cells include Burkitt lymphoma (BL), DLBCL, follicular lymphoma (FL), and HL, all of which result from aberrant SHM (194, 195). Finally, lymphomas derived from post-GC B cells include hairy cell leukemia (HCL), multiple myeloma (MM), marginal zone lymphoma (MZL), plasmablastic lymphoma (PL), and Waldenstrom macroglobulinemia (WM) (2). Given the large number of different B cell leukemias and lymphomas, it is essential to understand B cell activation, differentiation, and trafficking in order to develop therapies that target these processes and thereby interfere with the spread and survival of malignant B cells.

Chemoattractants and their receptors play an important role in B cell lymphoma localization and dissemination (48). CXCR5 (CXCL13), CXCR4 (CXCL12), and CCR7 (CCL21) are highly expressed on B-CLL cells and are important for the dissemination of these cells throughout the body (47). CXCR5 and CXCR4 are expressed on all B cell cancers including CLL, HCL, MCL, MZL, SLL, and FCL (48). In addition, MM cells express CXCR4 and migrate readily to CXCL12 (49). Since CXCL12 is important for selective plasma cell homing back to the BM, MM cells can use CXCL12-induced homing to localize to the BM. By regulating chemoattractant-induce migration and adhesion it might be possible to control B cell-derived cancers.

### 1.10 Chemoattractant receptor signaling in B cells

B cell localization is dependent on cues from chemoattractants. Chemoattractants bind G protein-coupled receptors (GPCRs) on the cell surface and initiate intracellular signaling that promotes cell migration and adhesion (Fig. 1.5). GPCRs can activate different G proteins depending on the specific receptor (196). The activation of G proteins leads to phospholipase C (PLC) activation, causing phosphatidylinositol (4,5)-bisphosphate (PIP$_2$) hydrolysis and further signaling through diacylglycerol (DAG) and inositol trisphosphate (IP$_3$). Chemoattractants activate Rho, which controls cell contraction and rounding (196, 197). Phosphoinositide 3-kinase (PI3K) can be activated by chemoattractants, resulting in the activation of Rac which controls cell spreading and migration (197, 198). PI3K also activates Akt, which regulates cell survival. GPCR signaling also activates Ras leading to extracellular signal-regulated kinase (ERK) activation which controls cell proliferation and survival (198, 199). Finally,
chemoattractants activate Rap1, a GTPase important for cell migration and integrin-mediated adhesion (199). By acting via diverse signaling pathways, chemoattractant receptors control cell migration and adhesion.

1.11 B cell receptor signaling in B cells

Signaling through the BCR is needed for B cell development and activation. The BCR is a multiprotein structure and consists of an Ag binding subunit, the membrane Ig, and a signaling subunit (Igα and Igβ) (200) (Fig. 1.6). BCR aggregation initiates signaling by causing the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) on Igα and Igβ by Src family tyrosine kinases (Lyn) (69, 200, 201). These phosphorylated sites allow for the recruitment of proteins with SH2 domains. These include the Syk tyrosine kinase as well as adaptor proteins such as B cell linker protein (BLNK) and B cell adaptor for PI3K (BCAP) (69). Syk and the adaptor proteins activate the important signaling enzymes, PLCγ and PI3K resulting in the activation of subsequent signaling pathways that control B cell activation, proliferation and development.

PLCγ activation and the resulting production of DAG and IP₃, leads to the activation of PKC and the transcription factors NF-κB and NFAT (69). NF-κB and NFAT regulate the expression of multiple proteins involved in B cell survival and differentiation. PLCγ also activates the Rap GTPase through the production of DAG (202). Rap activation controls cytoskeletal rearrangements and integrin activation (203). PLCγ-dependent activation of the Rac GTPase by Vav, promotes the activation of the p38 and c-Jun NH2-terminal kinase 1/2 (JNK1/2) mitogen-activated protein (MAP) kinases (201). The activation of p38 and JNK mediates the activation of multiple transcription factors including ATF-2, Creb, and Jun which are important for cell survival. Rac activation is also important for regulating actin polymerization and cytoskeleton reorganization (69, 200).

PI3K activation and recruitment to the BCR coreceptor, CD19, occurs after Syk phosphorylates BCAP and CD19 (69). Lyn also directly phosphorylates CD19 allowing for the recruitment of PI3K (200). Once activated PI3K generates PIP₃ and recruits PH domain-containing proteins (204). One protein with a PH domain is Akt, which is responsible for cell survival and proliferation. RasGRP is also recruited to the plasma membrane after PI3K
activation and activates the Ras GTPase. Ras activation results in the activation of Erk1/2 (201). Erk is important for cell proliferation and survival (200).

1.12 The importance of PI3K in B cells

Given the multiple roles of B cells in immunity and disease, it is important to understand the signaling pathways that regulate B cell trafficking and activation. PI3K regulates a diverse range of biological processes including differentiation, survival, and migration in most cell types (204). PI3K generates the lipid second messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP$_3$) at the cell membrane by phosphorylating PIP$_2$ in B cells activated by BCR, co-stimulatory, or cytokine receptors (204) (Fig. 1.7). PIP$_3$ generated by PI3K can then bind and regulate the subcellular localization and activity of intracellular enzymes that control cell migration, adhesion, survival, growth, and proliferation (204). The lipid phosphatases, phosphatase and tensin homologue (PTEN) and inositol polyphosphate 5’ phosphatase (SHIP) (205), attenuate PI3K signaling by hydrolysing PIP$_3$.

PI3K is made up of two subunits, a regulatory p85 subunit and a catalytically active p110 subunit (204). There are four p110 subunits with p110$\alpha$ and p110$\beta$ ubiquitously expressed, while p110$\delta$ and p110$\gamma$ all expressed in the immune system. PI3K is associated with the CD19 B cell co-receptor and is involved in amplifying BCR signaling and lowering the threshold for B cell activation (206, 207). PI3K plays a critical role in early B cell development by activating Akt and by contributing to the activation of key components of the BCR signalosome such as PLC$\gamma$2, the Btk tyrosine kinase, and Vav, an upstream activator of the Rac GTPase (208-210). PI3K also generates high concentrations of PIP$_3$ at the leading edge of cells after chemokine stimulation in order to establish cell polarity that leads to migration (211). For mature B cells, PI3K is important for chemokine-induced migration that controls their localization and for Ag-induced activation (209, 212).

Two different isoforms of PI3K have been shown to be important for immune cells. The isoform p110$\gamma$ is important for T cell development and function, macrophage and neutrophil migration, and mast cell function (213-216), while p110$\delta$ is important for B cell development and activation (88, 208, 210). Mice deficient for p110$\delta$ (p110$\delta^{-/-}$), or that have a catalytically inactive version of p110$\delta$ (p110$\delta$ knock-in [KI]), lack MZ and B-1 B cells (88, 210), indicating
that development of these cells is dependent on p110δ. In p110δ KI mice, the production of mature circulating B-2 cells is reduced and the B-2 cells that do develop exhibit impaired chemokine-induced migration, BCR-induced proliferation, and differentiation into Ab-producing cells (208-210, 212, 217). However, because B-1 and MZ B cells are missing in p110δ−/− and p110δ KI mice, the role of p110δ in the localization and activation of B-1 and MZ B cells in adult mice is not known. It is also not known if p110δ is important for regulating the production of natural Abs made by innate-like B cell subsets.

The localization, survival, and activation of B-1 and MZ B cells is regulated by multiple receptors that activate PI3K including chemokine receptors, the BCR, and TLRs. Therefore, I hypothesized that p110δ plays a critical role in the functions of these cells in adult animals. IC87114, a highly selective inhibitor of p110δ enzymatic activity (218, 219), has been widely used to inhibit p110δ in cells that have developed normally in wild type animals (219-227). Unlike LY294002 and wortmannin, the broad-spectrum PI3K inhibitors which act on all cell types, p110δ inhibitors selectively block PI3K signaling in B cells. IC87114 treatment of murine splenic B cells, which are predominantly B-2 B cells, inhibits anti-Ig-induced proliferation, IL-4-dependent survival, and multiple PI3K-dependent signaling events (220), reproducing the effects seen in p110δ KI mice. However, this study did not address the role of p110δ in B-1 and MZ B cells, important targets for the modulation of Ab-mediated autoimmune diseases. By specifically regulating the activity of p110δ, it might be possible to influence the pathogenesis of many autoimmune diseases as well as the trafficking and survival of B cell lymphomas and leukemias.

1.13 The role of Rap activation in B cells

The Rap1 GTPase, a member of the Ras superfamily, functions as a molecular switch that cycles between an inactive GDP-bound state and an active GTP-bound state (228) (Fig. 1.8). Guanine nucleotide exchange factors (GEFs) facilitate the exchange of GDP for GTP, leaving Rap in an active GTP-bound state. Once activated, Rap can be turned off by GTPase activating proteins (GAPs) which activate Rap so that the rate of GTP hydrolysis is increased, leaving Rap in a GDP-bound off state. Rap1 can be activated by chemokine receptors, antigen receptors, and receptor tyrosine kinases in most cell types (229, 230) (Fig. 1.9). The GEFs that
activate Rap1 include RasGRP2, C3G, Epac, PDZ-GEF, and Dock4. The GAPs that inactivate Rap1 include RapGAP and Spa1 (229, 231-234). It is still unclear which of the GEFs and GAPs are responsible for regulating Rap1 activation in B cells.

Rap1 exerts its effects by binding several different effector proteins that are linked to migration and adhesion events (229) (Fig. 1.9). Active Rap1 binds effector proteins such as RapL and Rap1-interacting adaptor molecule (RIAM), which promote integrin activation and reorganization of the actin cytoskeleton, processes that underlie cell migration (229, 235, 236).

In terms of cell migration, Rap1 plays an essential role in the proper localization of cells during *Drosophila* embryogenesis (237). Similarly, activation of Rap1 is important for CCL21-induced extravasation and adhesion of T cells (238). The finding that T and B cells from RapL-deficient mice have impaired homing to peripheral lymphoid tissues suggests that Rap, and its effector RapL, are central regulators of CCL21- and CXCL12-mediated entry of lymphocytes into lymphoid organs (239).

A number of studies have shown that Rap1 activation is essential for integrin activation in leukocytes (203, 240-242). We have shown that activation of the Rap GTPases is required for CXCL12-induced B cell migration and for the integrin-dependent adhesion of B cells to purified adhesion molecules and to BM stromal cells (203, 243). However, it was not known whether other chemoattractant receptors induce cell migration via Rap1 activation. In particular it is not known whether CXCL13, which directs B cells into B cell follicles, or S1P, which retains MZ B cells in the MZ and promotes the egress of B cells from lymphoid organs, activate Rap1 or promote cell migration and adhesion via Rap1.

Because there are five Rap GTPases, Rap1A, Rap1B, Rap2A, Rap2B, and Rap2C, it has been difficult to study the effects of Rap activation in vivo. One approach is to overexpress dominant negative Rap proteins or RapGAPs to interfere with normal Rap activation. Mice expressing dominant negative Rap, Rap1A17, in B cell lineages have defects in early B cell development in the BM, highlighting the important of Rap in B cells (244, 245). More recently, individual Rap deficient mice have been made. Interestingly, *Rap1a*−/− mice have relatively normal development of B cells (246). However, Rap1b-deficient mice have impaired mature B cell trafficking and development of MZ B cells, suggesting that Rap1b is the dominant isoform of Rap1 in B cells (247, 248). This suggests that the different Rap GTPases may not be functionally redundant but may differentially regulate cellular events in different cell types. In
Spa-1<sup>−/−</sup> mice (a RapGAP) there is enhanced development of B-lineage cell leukemia that have B-1 cell phenotypes (122). These mice also have expanded B-1 cell populations in the peritoneal cavity that are associated with the generation of anti-dsDNA Abs and lupus-like glomerulonephritis (122). These studies show that regulation of Rap activation is essential for the development and function of B cells and if disrupted can lead to cancer and autoimmunity.

1.14 Role of lysophosphatidic acid in B cells

Besides chemokines and S1P, other chemoattractants may have an important role in regulating B cell migration or may modulate responses to chemokines. Many lipids like S1P are important regulators of cell motility. Lysophosphatidic acid (LPA) is a soluble, extracellular lysophospholipid mediator that regulates cell proliferation, survival, migration, morphological changes, and cytokine and chemokine secretion in T cells, neutrophils, monocytes, neurons, endothelial cells, stem cells, and cancer cells (196, 249-251). The pathways for LPA production are found in most tissues and, as a result, LPA is found in serum, the peritoneal cavity, cerebrospinal fluid, saliva, seminal fluid, and the ascites fluid of certain cancers (252-256). LPA is present in serum at 1-5 µM and is produced by platelets and activated immune cells including monocytes, neutrophils, mast cells, DC’s and B cells (253, 257, 258). LPA gradients can be generated by integral membrane lipid phosphate phosphatases (LPPs), which degrade extracellular LPA (259, 260).

Because LPA is involved in many important cellular processes, it is not surprising that there are several LPA receptors that couple to different G proteins and thereby mediate diverse biological effects. There are seven identified GPCRs for LPA (Table 1.1) (250). These LPA receptors are coupled to four different G protein α-subunits, G<sub>i/o</sub>, G<sub>q</sub>, and G<sub>12/13</sub> (Fig. 1.10). The LPA<sub>1</sub> (261), LPA<sub>2</sub> (262), and LPA<sub>4</sub> (263) receptors are coupled to G<sub>i/o</sub>, G<sub>q</sub>, and G<sub>12/13</sub> (264, 265). G<sub>i/o</sub> inhibits activation of adenylyl cyclase and prevents cAMP accumulation but leads to Ras activation and subsequent Erk activation (265-267), which is important for cell survival and proliferation. Signaling through G<sub>i/o</sub> also activates PI3K, leading to the activation of Rac and Akt (257). Rac is important for cell motility and Akt activation is important for survival and proliferation (265, 268). G<sub>q</sub> leads to PLC activation, DAG production, and intracellular Ca<sup>2+</sup> signaling, contributing to cell activation and proliferation (264, 265). Finally, G<sub>12/13</sub> leads to
activation of the Rho GTPase, which is important for cell adhesion and morphological changes (269, 270). Therefore, it is not unexpected that LPA<sub>1</sub> and LPA<sub>2</sub> induce Rho-dependent cell rounding (271), and activate PI3K and Akt thereby affecting cell survival (259, 272, 273).

LPA<sub>3</sub> (274) and LPA<sub>5</sub> (275) are coupled to G<sub>i/o</sub> and G<sub>q</sub> but are unable to activate G<sub>12/13</sub> (250, 257). As a result LPA<sub>3</sub> signals through PLC, Ras/ERK pathways but does not activate Rho (196, 257). Little is known about signaling pathways initiated by LPA<sub>5</sub> and even less is known about GPR87 (250, 276). P2Y5 (277) couples to G<sub>s</sub> and G<sub>i/o</sub> where G<sub>s</sub> is important for the accumulation of cAMP and activation of adenyl cyclase and is involved in hair growth and human hair loss (250, 263). LPA<sub>4</sub> also uses G<sub>s</sub> in addition to G<sub>i/o</sub>, G<sub>q</sub>, and G<sub>12/13</sub> and has been shown to activate adenyl cyclase (196, 264, 278) and inhibit cell migration (279).

Little is known about how LPA regulates B cell functions including migration and adhesion. It is also unclear which signaling pathways or LPA receptors are involved in regulating B cell functions. Since LPA regulates migration, adhesion, and proliferation in other cell types, regulating the activity of LPA in B cells may allow for the control of B cell-mediated diseases.

### 1.15 Objectives and aims

The overall goal of this thesis was to gain new insights into signaling proteins that control B cell activation, migration, adhesion, proliferation, and Ab production. I initially set out to test whether CXCL13 and S1P activate Rap and whether Rap activation is important for CXCL13- and S1P-induced migration and adhesion in B cells. This was based on our previous findings that Rap activation is important in CXCL12-induced migration and adhesion in B cells. Since PI3K is also important for cell motility and activation, I hypothesized that p110δ is important for these functions in B-1 and MZ B cells. Specifically, I asked if p110δ activity is required for B-1 and MZ B cell activation, migration, adhesion and Ab production. We were interested in whether p110δ was needed for B cell proliferation and survival and whether B-2, B-1, and MZ B cell chemotaxis and directional migration was dependent on p110δ. Finally, I wanted to test if p110δ activity was important for natural Ab production and if p110δ inhibitors could be used to control B cell-mediated autoimmune diseases. Specifically, we tested whether
pathogenic Ab responses in an autoimmune model of RA in vivo could be modulated by inhibiting p110δ activity.

The final aim was to test whether LPA played an important role in B cell migration, adhesion and function. Specifically I asked if LPA regulates B cell migration and adhesion. I also asked if LPA was involved in B cell spreading and whether LPA could stimulate the MAPK and PI3K pathways. Since Rap is important for B cell migration and adhesion, I asked whether LPA stimulated Rap activation, as this would suggest a possible mechanism for regulating adhesion.

The work in this thesis shows that Rap is an important regulator of B cell migration and adhesion for multiple chemoattractants and that p110δ is important for Rap activation. In addition, I showed that p110δ is essential for regulating B-2, B-1, and MZ B cell functions. Thus by regulating Rap activation or p110δ activity it might be possible to modulate B cell function and treat B cell-mediated autoimmunity and inflammatory diseases.
Figure 1.1
**Figure 1.1:** B cell development and trafficking. B cells develop from stem cells (CLP) in the bone marrow where they undergo VDJ rearrangements to express a functional BCR on the cell surface. Immature B cells then traffic to the spleen and differentiate further into follicular B-2 cells or MZ B cells. Upon Ag encounter, B-2 or MZ B cells can form primary follicles and become short-lived plasma cells, or they can move into germinal centers and, with T cell help, undergo SHM, CSR and affinity maturation. These cells can then become plasma cells and secrete antibodies or become memory B cells. Memory B cells may then traffic to the BM to become long lived plasma cells. More detailed B cell trafficking is described in section 1.2. HEV, high endothelial venules; S1P, sphingosine 1-phosphate; LN, lymph node; MZ, marginal zone; FDC, follicular dendritic cell; CLP, common lymphoid progenitor; T1, transitional 1 B cell; T2, transitional 2 B cell; SHM, somatic hypermutation; CSR, class switch recombination.
Figure 1.2: B cell rolling adhesion in the HEV and extravasation into lymphoid tissues. B cells traveling in the blood undergo rolling adhesion when L-selectin interacts with PNAD, a set of sialomucins on the HEV wall. This brings the B cell close to the HEV wall where GPCR’s can interact with immobilized chemokines to activate integrins (LFA-1). Activated integrins bind to ICAM-1 to mediate firm adhesion. B cells can then extravasate through the HEV in response to chemokine gradients in the lymphoid tissues.

HEV, high endothelial venule; GPCR, G protein-coupled receptor; PNAD, peripheral lymph node addressin; LFA-1, Leukocyte function-associated antigen-1; ICAM-1, intracellular adhesion molecule-1; LN, lymph node.
Figure 1.3: B cell effector functions. Examples of how B cells mediate immunity and influence other cells in the immune system. Natural Abs are produced in the absence of Ag challenge while adaptive Ab responses require T cell help and challenge with Ag. Autoreactive Abs can be both natural and adaptive Abs that recognize self Ags.
Figure 1.4: B cell cancers can arise at all stages of B cell development and activation. ALL, acute lymphoblastic leukemia; CML-LBC, chronic myelocytic leukemia-lymphoid blast crisis; CLL, chronic lymphocytic leukemia; SLL, small lymphocytic lymphoma; MCL, mantle cell lymphoma; FL, follicular lymphoma; DLBCL, diffuse large B cell lymphoma; BL, Burkitt lymphoma; HL, Hodgkin lymphoma; MM, multiple myeloma; WM, Waldenstrom macroglobulinemia; MZL, marginal zone lymphoma; HCL, hairy cell leukemia; PL, plasmablastic lymphoma. Adapted from (2).
**Figure 1.5:** G protein-coupled receptor signaling in B cells. Chemoattractant receptors couple to different G proteins. $G_q$ activates PLC which hydrolyses PIP$_2$ into the intracellular signaling molecules IP$_3$ and DAG. $G_{i/o}$ activates PI3K leading to Rac activation which controls cell spreading and migration. In addition, $G_{i/o}$ also activates Ras leading to Erk activation to control proliferation and survival. $G_{i/o}$ also activates Rap1 to control cell adhesion and migration. $G_{12/13}$ activates Rho to control contraction and cell rounding.

GPCR, G protein-coupled receptor; PLC, phospholipase C; PI3K, phosphoinositide 3-kinase; PIP$_2$, phosphatidylinositol (4,5)-bisphosphate; Erk, extracellular signal-regulated kinase.
Figure 1.6: B cell receptor signaling. BCR aggregation allows Src family kinases such as Lyn to phosphorylate ITAMs on the BCR. This recruits Syk which can phosphorylate downstream signaling proteins. ITAM phosphorylation recruits the adaptor proteins. BLNK recruits PLC which generates DAG and IP₃. DAG activates Rap1 to control cell migration and adhesion or PKC to activate NF-κB. IP₃ activates calmodulin and the transcription factor NFAT to control cytokine production and proliferation. PLC also activates Ras and Erk to control cell proliferation and survival. Finally, PLC and PI3K activate Rac to control spreading and migration as well as the activation of p38 and JNK. Lyn and Syk phosphorylate CD19 which recruits PI3K to CD19. PI3K activates Akt to control cell survival.

BCR, B cell antigen-receptor; BLNK, B cell linker protein; Btk, Bruton’s tyrosine kinase; ITAM, immunoreceptor tyrosine-based activation motif; CaM, calmodulin; PKC, protein kinase C.
Figure 1.7: PI3Kδ signaling in B cells. Multiple receptors can activate PI3Kδ in B cells, leading to the generation of PIP₃ (phosphatidylinositol (3,4,5)-triphosphate). Proteins containing PH domains such as Akt and PDK1 can bind PIP₃ at the plasma membrane and signal to other effectors that control B cell functions. PI3Kδ signaling is attenuated by the activation of the phosphatases SHIP and PTEN, which hydrolyse PIP₃.
Figure 1.8: The Rap GTPase cycle. Rap functions as a small molecular switch that cycles between a GDP-bound inactive state and a GTP-bound active state. The interconversion is mediated by guanine nucleotide exchange factors (GEFs) and GTPase activation proteins (GAPs). The hydrolysis activity of Rap can be enhanced by GAPs. Active Rap can then bind other effector molecules to mediate different cellular functions. Our lab manipulates this cycle by overexpressing RapGAPII to suppress Rap activation.
Figure 1.9: Rap GTPase signaling in B cells. Multiple receptors on the B cell surface can activate Rap. Active GTP-bound Rap can then regulate many different effector molecules including AF6, TIAM, and RIAM to regulate actin polymerization. Active Rap can also bind to RapL to regulate integrin activation. Ultimately, through different effector proteins, Rap activation mediates cell migration, adhesion, and spreading.
Figure 1.10: LPA-induced signaling. LPA can be produced by enzymes, including autotoxin (ATX), on the surface of immune cells. LPA can bind to seven different LPA receptors which, depending on the receptor (Table 1.1) can activate different G proteins. Signaling downstream of the G proteins can activate pathways that control the indicated cellular functions. ATX, autotoxin; LPC, lysophosphatidylcholine; LPP, lipid phosphate phosphatase; MAG, monoacylglycerol.
Table 1.1: LPA receptors and corresponding cellular responses.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Synonym</th>
<th>G Protein</th>
<th>Cellular Response</th>
<th>Physiological Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDG subgroup</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPA₁</td>
<td>EDG2</td>
<td>Gᵢₒ, Gᵣ, G₁₂/₁₃</td>
<td>Proliferation, chemotaxis, fiber stretching, anti-apoptosis</td>
<td>Brain development</td>
</tr>
<tr>
<td>LPA₂</td>
<td>EDG4</td>
<td>Gᵢₒ, Gᵣ, G₁₂/₁₃</td>
<td>Proliferation, chemotaxis, fiber stretching, anti-apoptosis</td>
<td>Unknown</td>
</tr>
<tr>
<td>LPA₃</td>
<td>EDG7</td>
<td>Gᵢₒ, Gᵣ</td>
<td>Proliferation, chemotaxis</td>
<td>Embryo implantation</td>
</tr>
<tr>
<td>P2Y subgroup</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPA₄</td>
<td>GPR23, P2Y9</td>
<td>Gₛ, Gᵢₒ, Gᵣ, G₁₂/₁₃</td>
<td>Stress fiber formation, neurite extraction, cell adhesion</td>
<td>Unknown</td>
</tr>
<tr>
<td>LPA₅</td>
<td>GPR92</td>
<td>Gᵢₒ, Gᵣ</td>
<td>Stress fiber formation, neurite extraction</td>
<td>Unknown</td>
</tr>
<tr>
<td>GPR87</td>
<td></td>
<td></td>
<td></td>
<td>Unknown</td>
</tr>
<tr>
<td>P2Y₅</td>
<td>P2RY5</td>
<td>Gₛ, Gᵢₒ</td>
<td></td>
<td>Hair growth</td>
</tr>
</tbody>
</table>
References


177. Steinberg, B. J., P. A. Smathers, K. Frederiksen, and A. D. Steinberg. 1982. Ability of the xid gene to prevent autoimmunity in (NZB X NZW)F1 mice during the course of their natural history, after polyclonal stimulation, or following immunization with DNA. *J Clin Invest* 70:587-597.


276. Tabata, K., K. Baba, A. Shiraishi, M. Ito, and N. Fujita. 2007. The orphan GPCR GPR87 was deorphanized and shown to be a lysophosphatidic acid receptor. *Biochem Biophys Res Commun* 363:861-866.


CHAPTER 2

The Rap GTPases mediate CXCL13- and sphingosine 1-phosphate-induced chemotaxis, adhesion, and Pyk2 tyrosine phosphorylation in B cells

2.1. Introduction

Chemokines and chemoattractant lipids such as sphingosine 1-phosphate (S1P) direct the trafficking and localization of B cells and thereby play key roles in B cell development, activation, survival, and differentiation (1-3). The chemokine CXCL12 retains B cell progenitors in the bone marrow and promotes their adhesion to stromal cells, which provide them with survival and differentiation signals (4-6). The entry of mature B cells into peripheral lymphoid organs is mediated by chemokines such as CXCL12 and CCL21 that are displayed on the surface of high endothelial venules (7, 8). By stimulating integrin activation and chemotaxis, these chemokines allow B cells to extravasate into the T cell region of the lymphoid organ where they can interact with antigen-specific T cells (1, 2). The chemokine CXCL13 then directs B cells into lymphoid follicles (1, 9) where integrin-mediated adhesion to follicular dendritic cells provides survival signals for both naïve B cells and for activated B cells which undergo a germinal center reaction and differentiate into memory cells or antibody-secreting plasma cells (10, 11). S1P plays a key role in allowing B cells to exit lymphoid organs and return to the circulation (12). Plasma cells can then be directed back to the bone marrow by CXCL12 (13, 14), where they survive and secrete antibodies for long periods of time. In addition to being important for normal B cell development and function, chemoattractant-regulated B cell trafficking also underlies the formation of ectopic germinal centers in autoimmune diseases such as rheumatoid arthritis (15, 16) and the homing of malignant B cells to the bone marrow (17-19).

The role of S1P in directing B cell localization has only recently been appreciated. There are at least five different receptors for S1P, S1P1-5 (20, 21), with S1P1 and S1P3 being important for lymphocyte trafficking (12). S1P1 plays a key role in the exit of T and B cells from peripheral lymphoid organs and the thymus (12, 22) and S1P1 overexpression causes B cells to relocalize from the splenic white pulp to the outer red pulp (23), possibly by overriding CXCL13-induced migration towards the lymphoid follicles (24). It has been suggested that S1P in the blood and lymph causes rapid downregulation of S1P1 on circulating lymphocytes, allowing them to enter lymphoid organs without immediately exiting (23). After a certain period of time in the lymphoid
organ, re-expression of S1P1 allows the lymphocyte to re-enter the circulation in search of the next lymphoid organ. S1P and the S1P3 receptor are also required for the correct localization of marginal zone (MZ) B cells in the splenic MZ. The S1P3 receptor is important for MZ B cells to migrate towards S1P (24) and S1P3-deficient mice have a disordered marginal sinus in the spleen, resulting in defective MZ immune responses (25).

The Rap1 GTPase, a member of the Ras superfamily, functions as a molecular switch that cycles between an inactive GDP-bound state and an active GTP-bound state. Rap1-GTP binds effector proteins including RapL and RIAM which promote integrin activation and reorganization of the actin cytoskeleton, processes that underlie cell migration (26-28). A number of studies have shown that Rap1 activation is essential for integrin activation in leukocytes (26, 29, 30). In terms of cell migration, Rap1 plays an essential role in the proper localization of cells during Drosophila embryogenesis (31) and activation of Rap1 is important for CCL21-induced extravasation and adhesion of T cells (32). We have shown that activation of the Rap GTPases is required for CXCL12-induced B cell migration as well as for the integrin-dependent adhesion of B cells to purified adhesion molecules and to bone marrow stromal cells (30, 33). Moreover, the finding that T and B cells from RapL-deficient mice have impaired homing to peripheral lymphoid tissues (34) suggests that Rap1, and its effector RapL, are central regulators of CCL21- and CXCL12-mediated entry of lymphocytes into lymphoid organs. However, it is not known whether all chemoattractant receptors induce lymphocyte migration via Rap1 activation. In particular it is not known whether CXCL13, which directs B cells into B cell follicles, or S1P, which retains MZ B cells in the MZ and promotes the egress of B cells from lymphoid organs, activate Rap1 or promote cell migration and adhesion via Rap1.

We have previously shown that the tyrosine kinase Pyk2 is a downstream target of Rap-dependent signaling (30). Pyk2 plays a key role in B cell migration and in the proper localization of B cells in vivo. Pyk2-deficient mice lack MZ B cells and isolated follicular and immature B cells from Pyk2-deficient mice exhibit reduced migration towards the chemokines CXCL12, CXCL13, and CCL21 (35). Tyrosine phosphorylation of Pyk2 on sites that are critical for its activation can be induced by receptors for
chemokines and lipid chemoattractants as well as by integrins (36). We have shown that Rap activation is required for CXCL12 to induce Pyk2 tyrosine phosphorylation (30) but it is not known if CXCL13 or S1P induce Pyk2 phosphorylation in B cells and if so, whether this is regulated by Rap activation.

In this report we show that both CXCL13 and S1P activate Rap1 in B cells and that the ability of these chemoattractants to stimulate B cell migration and the adhesion of B cells to ICAM-1 and VCAM-1 depends on the activation of Rap. We also show that CXCL13 and S1P stimulate tyrosine phosphorylation of Pyk2 in a Rap-dependent manner.
2.2. Results

2.2.1. CXCL13 and S1P induce Rap1 activation in B cells

Previous work from our lab showed that CXCL12 induces Rap activation and that Rap activation is important for CXCL12-induced B cell migration (33). Based on these findings we were interested in determining whether other B cell chemoattractants induce Rap activation and promote migration in a Rap-dependent manner. Specifically, we focused on CXCL13 and S1P, chemoattractants that play a key role in B cell trafficking and localization in vivo. We stimulated B cells with 100-400 nM CXCL13, concentrations that have been shown to promote B cell chemotaxis in vitro (14), or with 300 nM S1P. Physiological levels of S1P have been reported as 100-1000 nM in the blood and 30-300 nM in the lymph (37). Moreover, 200 nM CXCL13 and 300 nM S1P were used for a comprehensive analysis of CXCL13- and S1P-induced signaling in B cells (http://www.signaling-gateway.org/). To look at Rap activation, we used a GST-RalGDS fusion protein to selectively pull down the active GTP-bound form of Rap1, which we then detected by immunoblotting.

Figure 2.1 shows that both CXCL13 and S1P stimulated Rap1 activation in primary murine B cells (Fig. 2.1A,B) and in two murine B cell lines (Fig. 2.1D), the IgM⁺ WEHI-231 immature/transitional B cell line and the IgG⁺ A20 mature B cell line. Both CXCL13 and S1P induced rapid activation of Rap1 that was evident by 1 min and usually declining by 5 min. The amount of activated Rap1 in the cells returned to basal levels after 10-30 min (data not shown). The magnitude of CXCL13-induced Rap1 activation was similar to that induced by anti-Ig Abs that cluster the BCR (Figs. 2.1A, D), which we have previously shown to induce robust activation of Rap1 in B cells (38). S1P also induced strong Rap1 activation in the WEHI-231 and A20 cell lines (Fig. 2.1D). When splenic B cells were stimulated immediately after being purified (<1 h ex vivo), S1P was less effective than CXCL13 at activating Rap1 (Fig. 2.1A). However when splenic B cells were cultured for several hours before stimulation, S1P induced very robust Rap1 activation (Fig. 2.1B, left panel). This is consistent with reports showing that splenic B cells migrate poorly towards S1P when analyzed directly ex vivo but
acquire responsiveness after several hours of *in vitro* culture (39). This presumably reflects the recovery of S1P receptor expression that occurs when B cells are no longer exposed to *in vivo* concentrations of S1P that promote receptor internalization and downregulation (23). S1P treatment also increased the amount of activated Rap1 in purified lymph node B cells that were maintained *in vitro* for several hours before being stimulated (Fig. 2.1B, right panel). Unlike splenic B cells, which contain follicular B cells and the much less prevalent MZ B cells, lymph nodes contain only follicular B cells. Thus these data show that S1P can activate Rap1 in follicular B cells. Given the small number of MZ B cells, we were unable to test whether S1P also activates Rap1 in MZ B cells. Nevertheless, the data in figure 2.1 show that CXCL13 and S1P, which both signal through heterotrimeric G protein-coupled receptors, activate Rap1, a GTPase that has been implicated in the migration of B cells towards CXCL12.

To test whether Rap1 activation is important for CXCL13- and S1P-induced B cell migration and adhesion, we used a loss-of-function approach in which we blocked Rap activation by overexpressing RapGAPII, a Rap-specific GTPase activating protein (GAP) that converts Rap1 to its inactive GDP-bound state. This approach has been widely used to assess the role of Rap in cellular functions (40-42) and we have previously used this approach to block Rap activation in the WEHI-231 and A20 B cell lines (30, 33). RapGAPII selectively blocks the activation of Rap while having no effect on the activation of the Ras (43), Rac1 (33), or RhoA (44) GTPases. Bulk populations of WEHI-231 cells and A20 cells that stably express FLAG-tagged RapGAPII were generated by retrovirus-mediated gene transfer. The expression of FLAG-RapGAPII in WEHI-231 cells is shown in Fig. 2.1C, while expression in A20 cells has been shown previously (30). Figures 2.1D and 2.1E show that RapGAPII expression completely blocked CXCL13- and S1P-induced Rap1 activation in both WEHI-231 and A20 cells at the 1 min and 5 min time points, even when S1P concentrations as high as 3 µM were used. Moreover, no Rap1 activation was observed in the RapGAPII-expressing cells at 10, 15, or 30 min after addition of CXCL13 or S1P, indicating that RapGAPII expression prevents CXCL13- and S1P-induced Rap1 activation, as opposed to merely delaying it (data not shown). RapGAPII expression also blocked the activation of Rap1 by anti-Ig Abs, which was used as a positive control. Thus RapGAPII expression effectively
prevents Rap1 activation by CXCL13 and S1P and allows us to assess the role of Rap activation in CXCL13- and S1P-induced responses.

2.2.2. **CXCL13 and S1P stimulate ERK, JNK and Akt phosphorylation, which is not blocked by RapGAPII**

To confirm that RapGAPII expression selectively blocked the activation of Rap1 without affecting other signaling pathways initiated by CXCL13 and S1P, we used phosho-specific Abs to compare CXCL13- and S1P-induced phosphorylation of ERK, JNK, and Akt in vector control and RapGAPII-expressing cells. Phosphorylation of these kinases correlates with their activation. The ERK MAPK is a downstream target of the Ras signaling pathway, the JNK MAPK is a downstream target of the Rac GTPase, and the Akt kinase is a target of the phosphatidylinositol 3-kinase signaling pathway. Although CXCL13 and S1P signaling have not been completely characterized in B cells, ERK, JNK, and Akt are common targets of chemoattractant receptor signaling. Figure 2.2 shows that both CXCL13 and S1P induced the phosphorylation of ERK, JNK, and Akt in WEHI-231 cells and that RapGAPII expression had no effect on their ability to be phosphorylated in response to CXCL13 or S1P. Similar results were obtained when we compared CXCL13- and S1P-induced phosphorylation of ERK, JNK, and Akt in vector control versus RapGAPII-expressing A20 cells (data not shown).

2.2.3. **The S1P1 and S1P3 receptors can mediate Rap activation**

The five known S1P receptors, S1P1-5, activate distinct, but overlapping, sets of signaling pathways by interacting with different trimeric G proteins (20, 21). Using quantitative RT-PCR, Cyster and colleagues showed that murine splenic B cells express S1P1 and S1P3, with MZ B cells expressing much higher levels of S1P3 than follicular B cells (24). Therefore we characterized S1P receptor expression in the WEHI-231 and A20 cells cell lines used in this study and then assessed which of these receptors were capable of mediating S1P-induced activation of Rap1. Using quantitative RT-PCR to assess the expression of S1P1 and S1P3 mRNA, we found that both WEHI-231 cells and
A20 cells expressed mRNA for the S1P<sub>1</sub> receptor, although at lower levels than primary B cells (Fig. 2.3A). In addition, WEHI-231 cells expressed substantial S1P<sub>3</sub> mRNA, perhaps 10-fold higher than the levels of S1P<sub>1</sub> mRNA in these cells. In contrast, A20 cells did not express detectable amounts of S1P<sub>3</sub> mRNA (Fig. 2.3A). Thus with regard to S1P receptor expression, WEHI-231 cells resemble MZ B cells in that they express both S1P<sub>1</sub> and S1P<sub>3</sub> while A20 cells resemble follicular B cells that express primarily S1P<sub>1</sub>. Importantly, this analysis also showed that expressing RapGAPII in the WEHI-231 and A20 cells did not significantly alter the levels of S1P<sub>1</sub> or S1P<sub>3</sub> mRNA. To address whether the WEHI-231 and A20 cell lines express S1P<sub>2</sub>, S1P<sub>4</sub>, or S1P<sub>5</sub>, we performed semi-quantitative RT-PCR with the same primers used by Cinamon <i>et al.</i> to assess the expression of these receptors in splenic B cells (24). Similar to what this group found for splenic B cells, we found that WEHI-231 cells and A20 cells contained little or no mRNA for S1P<sub>4</sub> or S1P<sub>5</sub> (data not shown). These cell lines did however express low levels of S1P<sub>2</sub> mRNA (data not shown), which was not detected in splenic B cells (data not shown, (24)). Thus, S1P<sub>1</sub> and S1P<sub>3</sub> appear to be the predominant S1P receptors expressed by WEHI-231 cells whereas S1P<sub>1</sub> is the predominant S1P receptor expressed by A20 cells. This allowed us to address whether S1P<sub>1</sub> and S1P<sub>3</sub> are both capable of activating Rap1.

To determine whether the S1P<sub>1</sub> receptor can promote Rap1 activation, we treated WEHI-231 cells with SEW2871, a highly selective S1P<sub>1</sub> agonist. This compound does not initiate signaling via S1P<sub>2-5</sub>, even at concentrations as high as 10 µM (45). Figure 2.3B shows that treating WEHI-231 cells with 300 nM or 600 nM SEW2871 induced significant Rap1 activation, equivalent to that induced by 300 nM S1P.

Although DMSO, the solvent used to dissolve SEW2871, caused some Rap1 activation, SEW2871 caused greater activation of Rap1 than the corresponding amounts of DMSO used as the solvent control (Fig. 2.3B). These data, together with the observation that S1P induces Rap1 activation in A20 cells, which express S1P<sub>1</sub> but not S1P<sub>3</sub>, support the idea that the S1P<sub>1</sub> receptor is capable of activating Rap1.

Since S1P<sub>3</sub> is important for the <i>in vitro</i> migration of MZ B cells, we used CAY10444 (46), an S1P<sub>3</sub>-selective antagonist, to determine if S1P<sub>3</sub> contributes to S1P-induced Rap1 activation. CAY10444 has minimal inhibitory activity towards S1P<sub>1</sub> (46).
Figure 2.3C shows that pre-treating WEHI-231 cells with 10 µM CAY10444 reduced S1P-induced Rap1 activation by more than 50% when compared to cells that were pre-treated with an equivalent volume of dimethylformamide (DMF), the solvent used to dissolve the CAY10444. This suggests that the S1P₃ receptor is also capable of activating Rap1.

Having shown that RapGAPII selectively inhibits Rap1 activation without affecting the expression of the S1P₁ or S1P₃ receptors or altering other S1P- or CXCL13-induced signaling pathways, we used this loss-of-function approach to test whether Rap1 activation is important for CXCL13- and S1P-induced B cell migration and adhesion.

### 2.2.4. CXCL13- and S1P-induced B cell migration is dependent on Rap activation

Cell migration is important for B cells to localize to secondary lymphoid organs where they can receive survival signals and differentiate into plasma cells. To test whether Rap activation is important for B cells to migrate towards CXCL13 or S1P, we used Transwell migration assays to compare the ability of vector control and RapGAPII-expressing WEHI-231 cells to migrate towards CXCL13 or S1P. Figure 2.4 shows that both CXCL13 and S1P induced robust migration of vector control WEHI-231 cells whereas RapGAPII-expressing WEHI-231 cells exhibited greatly reduced migration towards these chemoattractants. Both CXCL13- and S1P-induced migration were reduced by more than 70% in the RapGAPII-expressing cells. This inhibition was consistently observed over a wide range of chemoattractant concentrations (50-200 nM CXCL13; 3 nM to 3 µM S1P), including concentrations that induced very strong chemotactic responses (40-55% of the cells migrating into the lower chamber) in the vector control cells. We did not observe inhibition of the chemotactic response at high S1P concentrations, a phenomenon associated with desensitization and downregulation of G-protein coupled receptors such as S1P₁ and S1P₃. This could reflect the involvement of additional low affinity receptors for S1P. However, these data support the idea that activated Rap is important for B cells to efficiently migrate towards CXCL13 and S1P, chemoattractants that play a key role in directing the trafficking and localization of B cells.
2.2.5. **CXCL13- and S1P-induced adhesion to ICAM-1 and VCAM-1 is dependent on Rap activation**

In addition to promoting cell migration, chemokines and other chemoattractants induce the activation of integrins. This plays a key role in the entry of lymphocytes into lymphoid organs and the proper localization of lymphocytes within those organs. The major integrins on B cells are LFA-1 (α4β2 integrin) and α4 integrins such as VLA-4 (α4β1 integrin). LFA-1 mediates the adhesion of lymphocytes to the ICAM-1 and ICAM-2 adhesion molecules while α4 integrins mediate adhesion to the VCAM-1 adhesion molecule and to fibronectin, an extracellular matrix (ECM) component. Since Rap activation has been implicated in integrin activation, we asked whether blocking Rap activation would prevent CXCL13- and S1P-induced adhesion to ICAM-1 and VCAM-1. Figure 2.5 shows that both CXCL13 and S1P increased the ability of A20 cells to bind to immobilized ICAM-1 (Fig. 2.5A) and VCAM-1 (Fig. 2.5B) to a similar extent as anti-IgG Abs, which we had used as a positive control. The binding of the cells to ICAM-1 and VCAM-1 was mediated entirely by the LFA-1 and α4 integrins, respectively, as pre-treating the cells with the anti-LFA-1 blocking mAb TIB213 or with the anti-α4 integrin blocking mAb PS/2 completely abrogated binding to ICAM-1 and VCAM-1. Importantly, CXCL13- and S1P-induced adhesion to ICAM-1 and VCAM-1 was substantially decreased in RapGAPII-expressing A20 cells in which Rap activation is blocked. RapGAPII expression also decreased the basal and anti-IgG-induced adhesion of A20 cells to ICAM-1 and VCAM-1, as we had shown previously (30). Thus, Rap activation is clearly required for CXCL13 and S1P to activate the LFA-1 and α4 integrins on B cells.

2.2.6. **CXCL13- and S1P-induced tyrosine phosphorylation of Pyk2 is dependent on Rap activation**

The Pyk2 tyrosine kinase is involved in cell migration, integrin-mediated signaling events, and cytoskeleton dynamics (35, 36, 47). In particular, Pyk2 is required
for B cells to migrate towards CXCL13 and other chemokines (35). The phosphorylation of Pyk2 on several tyrosine residues is required for its activation (48) and we have previously shown that both anti-Ig- and CXCL12-induced phosphorylation of Pyk2 on tyrosines 579 and 580 is dependent on Rap activation (30). To test whether other chemoattractants induce Pyk2 phosphorylation in a Rap-dependent manner, we stimulated vector control and RapGAPII-expressing A20 cells with CXCL13 or S1P, then immunoprecipitated Pyk2 and assessed its tyrosine phosphorylation using the 4G10 anti-phosphotyrosine (anti-P-Tyr) mAb. Figure 2.6 shows that both CXCL13 and S1P induced transient tyrosine phosphorylation of Pyk2 in the vector control cells. Similar to the kinetics we observed for CXCL13- and S1P-induced Rap1 activation, the induction of Pyk2 tyrosine phosphorylation by these stimuli was maximal at 1 min and decreased by 5-10 min. Maximal S1P-induced Pyk2 phosphorylation was observed at 300-600 nM S1P (Fig. 2.6B). Other experiments showed that 800 nM and 1.2 µM S1P did not induce greater Pyk2 phosphorylation (data not shown). Importantly, blocking Rap activation by expressing RapGAPII significantly reduced both CXCL13- and S1P-induced tyrosine phosphorylation of Pyk2. Longer time courses showed that there was no Pyk2 tyrosine phosphorylation after 15 or 30 min in either the vector control or RapGAPII cells (data not shown), indicating that preventing Rap activation inhibited CXCL13- and S1P-induced Pyk2 phosphorylation, as opposed to merely delaying it. Thus, Rap activation is critical for CXCL13 and S1P to induce the phosphorylation of Pyk2 on sites that regulate its activation and function. Since Pyk2 plays an essential role in B cell migration, the Rap-dependent regulation of Pyk2 by CXCL13 and S1P may be essential for these chemoattractants to induce B cell migration.
2.3. Discussion

In this report we show that CXCL13 and S1P activate the Rap1 GTPase and that Rap activation is required for these chemoattractants to promote B cell migration as well as LFA-1- and α4 integrin-mediated adhesion. We also show that Pyk2, a tyrosine kinase that is essential for B cell migration, is a downstream target of both CXCL13 and S1P signaling and that Rap activation is important for CXCL13 and S1P to stimulate tyrosine phosphorylation of Pyk2, a modification that augments Pyk2 kinase activity. Finally, we have characterized CXCL13- and S1P-induced signaling in B cells and shown that both CXCL13 and S1P stimulate transient activation of the ERK, JNK, and Akt kinases. This suggests that these chemoattractants signal via the upstream activators of these kinases, Ras, Rac, and phosphatidylinositol 3-kinase, respectively. In contrast to cell migration and integrin-mediated adhesion, the phosphorylation of ERK, JNK, and Akt by CXCL13 and S1P was not affected when we blocked Rap activation by expressing RapGAPII. This demonstrates the specificity of using RapGAPII as a probe to study Rap-dependent processes and indicates that CXCL13 and S1P activate ERK, JNK, and Akt in a Rap-independent manner.

S1P_1 and S1P_3 are the two major S1P receptors expressed by B cells and recent work has clearly pointed out that these receptors have distinct functions in B cells (24). The S1P_1 receptor, which is highly expressed by both follicular and MZ B cells, is required for B cells to exit secondary lymphoid organs and for the proper positioning of MZ B cells. In contrast, S1P_3, which is expressed at much higher levels on MZ B cells than on follicular B cells, is the major mediator of MZ B cell chemotaxis, at least _in vitro_. Our data indicate that both S1P_1 and S1P_3 can promote Rap1 activation in B cells and that Rap activation is important for these receptors to promote B cell adhesion and migration _in vitro_.

By mediating CXCL13- and S1P-induced migration and adhesion, Rap activation may be critical for these chemoattractants to regulate the trafficking of B cells through lymphoid organs and to specific compartments within lymphoid organs where they receive survival, activation, and differentiation signals. CXCL13 recruits B cells into the follicular zones (1, 9) where they receive survival signals from follicular dendritic cells.
and, if activated by antigen, undergo somatic hypermutation and isotype switching (10, 11, 49). S1P-induced integrin activation plays a key role in maintaining the localization and function of splenic MZ B cells (24), a unique B cell population with a restricted Ig repertoire focused on microbial antigens. S1P may also control the emigration of B cells from lymphoid follicles both by inducing chemotaxis and by inhibiting migration towards CXCL13 produced in the follicle (24). S1P also plays an important role in the egress of lymphocytes from lymphoid organs, returning naïve B cells to the circulation and allowing plasma cells to traffic to the bone marrow. Together with previous work showing that Rap activation is important for CXCL12-induced migration and adhesion in B cells (30, 33) and for CCL21-induced migration and adhesion in T cells (32), our current findings suggest that activation of Rap may be a general requirement for G protein-coupled chemoattractant receptors to stimulate lymphocyte migration and adhesion. In this way Rap activation may regulate the trafficking of B cells in vivo and play a key role in both the initiation and effector phases of the immune response.

The activated GTP-bound form of Rap1 appears to promote cell adhesion and migration by binding a number of different effector proteins. Kinashi and colleagues identified a Rap effector called RapL that binds to and activates LFA-1, in addition to directing LFA-1 to the leading edge of the cell (27). RapL-deficient lymphocytes show impaired homing to target organs (34), consistent with the idea that Rap activation is important for lymphocyte migration in vivo. Rap-GTP also appears to regulate cell adhesion, actin polymerization, and cytoskeletal organization via the RIAM adaptor protein. (28). RIAM interacts with Ena/VASP and profilin, regulators of actin dynamics. Reducing RIAM expression in Jurkat T cells using siRNA inhibits Rap1-mediated integrin activation and reduces the amount of F-actin in the cells. Rap1-GTP may also promote reorganization of the actin cytoskeleton and the formation of lamellipodia by binding Tiam1 and Vav2, upstream activators of the Rac1 GTPase (50). Expressing activated Rap1 in HeLa cells has been shown to cause local activation of Rac1 at the edges of the cell (50). We have shown that blocking Rap activation by expressing RapGAPII has no effect on the activation of Rac1 in WEHI-231 cells (33) or on the activation of JNK (Fig. 2), which is dependent on Rac1 activation (51). Thus, it is not clear if Rap-dependent activation of Rac1 occurs in B cells or if it affects only a subset of
Rac1 molecules at the periphery of the cell. Other Rap1-binding proteins that could mediate the effects of Rap1-GTP on the actin cytoskeleton include ARAP3 (52), a regulator of the RhoA GTPase, and AF-6, which binds profilin, a regulator of actin polymerization (53, 54). It is not known which of these Rap1 effectors are important for CXCL13- and S1P-induced migration and adhesion in B-lymphocytes.

Another intracellular signaling protein that may link Rap-GTP to cell migration and changes in cell morphology is the Pyk2 tyrosine kinase, which has been implicated in integrin-mediated signaling and cytoskeleton rearrangements (35, 36, 47). Pyk2-deficient mice lack MZ B cells (35), suggesting that Pyk2 might mediate the chemokine-induced integrin activation that is required for MZ B cells to be retained in the marginal zone (55). In addition, Pyk2-deficient follicular B cells exhibit a significantly decreased ability to migrate towards CXCL12, CXCL13, and CCL21 in vitro (35). Pyk2 also regulates cell morphology and actin remodeling and Pyk2-deficient macrophages fail to undergo changes in morphology when stimulated with chemokines (47). Although the pathways by which Pyk2 regulates cell migration, adhesion and morphology are not completely understood, these data suggest that Pyk2 may function by regulating both integrin activation and the actin cytoskeleton.

Since Pyk2-deficient B cells exhibit similar defects in cell migration as RapL-deficient B cells and B cell lines in which Rap activation is blocked, we hypothesized that Pyk2 might be a downstream target of Rap-GTP signaling. Indeed, we showed that anti-Ig- and CXCL12-induced phosphorylation of Pyk2 on tyrosine residues that are required for its activation and function is dependent on Rap activation (30). In this report we show for the first time that CXCL13 and S1P stimulate the tyrosine phosphorylation of Pyk2 and that this is dependent on Rap activation. CXCL13- and S1P-induced tyrosine phosphorylation of Pyk2 was significantly reduced in RapGAPII-expressing A20 cells. These experiments were performed on A20 cells plated on a collagen/fibronectin ECM. Plating A20 cells on ECM is required to obtain maximal Pyk2 tyrosine phosphorylation (30), suggesting that integrin engagement is involved. Thus, our model is that CXCL13- and S1P-induced Rap1 activation leads to integrin activation, the binding of ECM ligands to integrins, and subsequent outside-in integrin
signaling that leads to Pyk2 phosphorylation. Thus Pyk2 may indirectly link Rap-GTP to integrin-mediated signaling events that regulate cell migration.

The signaling pathways that couple the receptors for CXCL13 and S1P to the activation of Rap1, and subsequently to the downstream effectors of Rap1 such as Pyk2, remain to be elucidated. Rap1 can be activated by a variety of guanine nucleotide exchange factors that are regulated by distinct signaling pathways. These Rap exchange factors include (i) EPAC, which is activated by cAMP, (ii) C3G, which is regulated by phosphotyrosine/SH2 domain-mediated recruitment, and (iii) members of the RasGRP family that are regulated by diacylglycerol produced by phospholipase C (26). Since CXCL13 and S1P do not cause significant increases in cAMP in B cells, but do signal via phospholipase C (http://www.signaling-gateway.org/), they may activate Rap1 via a phospholipase C-dependent pathway, as has been shown for CXCL12 (SDF-1)-induced Rap1 activation in WEHI-231 cells (33). Both the S1P1 and S1P3 receptors have been reported to activate phospholipase C in fibroblasts (20, 21).

In addition to Rap1 and Pyk2, we also identified ERK, JNK, and Akt as downstream targets of CXCL13 and S1P signaling. CXCL13 can induce Akt phosphorylation in B cells (56) and the S1P1 receptor agonist SEW2871 has been shown to activate Akt and ERK in S1P1-transfected CHO cells (45). To our knowledge this is the first report that CXCL13 stimulates the phosphorylation of ERK and JNK and that S1P stimulates the phosphorylation of Akt, ERK, and JNK in B-lymphocytes. In neutrophils and Dictyostelium discoideum, activated Akt moves from the cytosol to the leading edge of the cell during chemotaxis (57), suggesting a possible role for Akt in chemoattractant-induced migration. Recent work has shown that Akt-mediated phosphorylation of Girdin/APE plays a key role in reorganization of the actin cytoskeleton during cell migration (58). Activation of Akt (59) and ERK (60) is also important for chemoattractant-mediated cell survival, suggesting that CXCL13 and S1P could promote B cell survival through the activation of these two kinases. JNK regulates apoptosis and proliferation (61-63), raising the possibility that chemoattractant-induced JNK phosphorylation could modulate B cell proliferation.

In summary, we have shown that activation of the Rap GTPase is required for CXCL13 and S1P to induce B cell migration, adhesion to ICAM-1 and VCAM-1, and
Pyk2 tyrosine phosphorylation. This suggests that the ability of CXCL13 and S1P to direct the trafficking and localization of B cells is dependent on Rap activation and, therefore, Rap activation may be important for B cell survival, activation and differentiation into Ab-secreting cells.
2.4. Materials and Methods

Cells

Primary mature B cells were isolated from the spleens of C57BL/6 mice or from the peripheral and mesenteric lymph nodes of C57BL/6 mice. After lysing erythrocytes with Tris-buffered NH₄Cl, splenic B cells were purified using the MACS B cell isolation kit and a MACS LS column (Miltenyi Biotec, Auburn, CA) to deplete non-B cells. The resulting cell population was >95% B cells, as determined by flow cytometry of cells stained with anti-CD19-FITC (BD Pharmingen, San Diego, CA). The WEHI-231 IgM⁺ immature B cell line and A20 IgG⁺ mature murine B cell line were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, 50 µM 2-ME, 2 mM glutamine, 1 mM pyruvate, 15 U/ml penicillin, and 50 µg/ml streptomycin (complete medium). cDNA encoding FLAG-tagged RapGAPII in the pMSCVpuro vector (BD Biosciences Clontech, Palo Alto, CA) was expressed in both the WEHI-231 and A20 B cell lines. Bulk populations of WEHI-231 or A20 cells stably expressing either RapGAPII or the empty pMSCVpuro vector were generated by retrovirus-mediated gene transfer (64), followed by selection with 0.25 µg/ml puromycin for WEHI-231 cells or 4 µg/ml puromycin for A20 cells. FLAG-RapGAPII expression was detected by immunoblotting with the M2 anti-FLAG monoclonal antibody (Sigma-Aldrich, St. Louis, MO).

Chemoattractants and inhibitors

Recombinant mouse CXCL13 (R&D Systems, Minneapolis, MN) was resuspended to 25 µM in PBS containing 1 mg/ml BSA and stored in aliquots at -80°C. S1P (Biomol, Plymouth Meeting, PA) was resuspended to 0.5 mM in 37°C PBS containing 4 mg/ml BSA, dissolved according to the manufacturer’s instructions by repeated cycles of vortexing and mild heating, and then stored at -80°C. The S1P₁-specific agonist SEW2871 (45) (Calbiochem, La Jolla, CA) was resuspended to 10 mM in DMSO and stored at -20°C. The S1P₃-selective antagonist CAY10444 (46) (Cayman Chemicals, Ann Arbor, MI) was resuspended to 1.5 mM in DMF and stored at -20°C.
Immediately before use, CXCL13, S1P, SEW2871, and CAY10444 were diluted in modified HEPES-buffered saline (65) (HBS).

**Rap1 activation assays**

Prior to stimulation, WEHI-231 cells and A20 cells were cultured in FBS-free medium for 5 h. Primary B cells were used immediately after being isolated (<1 h *ex vivo*) or after being maintained *in vitro* for several hours. The cells (10⁷ per point for WEHI-231 and A20 cells, 1.5 x 10⁷ per point for lymph node B cells, 2 x 10⁷ per point for splenic B cells) were resuspended in 0.5 ml PBS and stimulated with goat anti-mouse IgM or IgG Abs (Jackson ImmunoResearch Laboratories, West Grove, PA), CXCL13, S1P, or SEW2871. Where indicated, cells were pre-treated for 5 min with 10 µM CAY10444 or an equivalent volume of DMF. Reactions were stopped by adding 500 µl of Rap lysis buffer (50 mM Tris-HCl, pH 7.5, 10% glycerol, 1% Igepal (ICN, Costa Mesa, CA), 200 mM NaCl, 2 mM MgCl₂, 1 mM PMSF, 1 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM Na₃VO₄). Rap1 activation was assessed as described previously (30) by using a GST-RalGDS fusion protein to selectively precipitate the active GTP-bound form of Rap1, which was detected by immunoblotting with a rabbit anti-Rap1 Ab (0.4 µg/ml; Santa Cruz Biotechnology, Santa Cruz, CA).

**ERK, JNK and Akt phosphorylation**

Ten million cells in 0.5 ml HBS were stimulated with anti-Ig Abs, CXCL13, or S1P, then washed with HBS and solubilized in RIPA buffer (30 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 1 mM PMSF, 10 µg/ml leupeptin, 1 µg/ml aprotinin, 1 mM Na₃VO₄, 25 mM β-glycerophosphate, 1 µg/ml microcystin-LR). Cell extracts (20 µg protein for ERK and Akt, 35 µg for JNK) were analyzed by immunoblotting with phospho-specific Abs for the active forms of ERK, JNK, or Akt (Cell Signaling Technologies, Beverly, MA). Blots were then stripped and reprobed with Abs to ERK, JNK (Santa Cruz Biotechnology), or Akt (Cell Signaling Technologies).
S1P receptor mRNA expression

For quantitative RT-PCR analysis of S1P₁ and S1P₃ mRNA levels, RNA was prepared using the RNAeasy kit with QIAshredder columns (Qiagen Inc., Mississauga, Ontario, Canada) and converted into cDNA using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). Equivalent amounts of cDNA were combined with TaqMan Fast Universal PCR Master Mix (Applied Biosystems) and with TaqMan Gene Expression Assay primers and probes (Applied Biosystems) specific for S1P₁ (assay # Mm00514644_m1), S1P₃ (assay # Mm00515669_m1), or 18S rRNA (assay # Hs99999901_s1). The quantitative PCR reaction and quantitation was performed using an Applied Biosystems 7500 Fast Real-Time PCR system. The data are expressed as the amount of S1P₁ or S1P₃ mRNA divided by the amount of 18S rRNA for the same sample. For detection of S1P₂, S1P₄, and S1P₅ mRNA by semi-quantitative RT-PCR, RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA) and PCR reactions were performed using the S1P₂-, S1P₄-, and S1P₅-specific primers described by Cinamon et al. (24) and puReTaq Ready-To-Go PCR beads (Amersham Biosciences, Piscataway, NJ).

Chemotaxis assays

Transwell chemotaxis assays were performed using 24 well plates and 5 µm polycarbonate Transwell inserts (Costar, Cambridge, MA), as described previously (33). The lower chamber contained 0.6 ml of CXCL13 or S1P diluted in chemotaxis medium (RPMI 1640/0.5% BSA/10 mM HEPES). Cells were diluted to 5 x 10⁶/ml in chemotaxis medium and 0.1 ml (5 x 10⁵ cells) was added to the top chamber. After 3 h at 37°C the cells that migrated into the lower chamber were collected and counted with a FACScan for 30 sec. Cells were gated using forward and side scatter to exclude dead cells and debris. To determine the percentage of cells that migrated, a 100% migration control was done in which 5 x 10⁵ cells were added directly to the bottom chamber at the beginning of the assay. WEHI-231 B cells were cultured to high density before chemotaxis assays and only early passage cells from the ATCC were used (less than 8 weeks). MSCV and RapGAPII expressing cells were also used within 1 month of being thawed for migration assays.
Adhesion assays

Adhesion assays were performed using a modification of the method described previously (30). Nunc Maxisorp 96-well plates (Nunc, Rochester, NY) were coated at room temperature for 90 min with 30 µg/ml of either soluble ICAM-1 (sICAM-1) or a VCAM-1-Fc fusion protein diluted in Hank’s balanced salt solution (HBSS). The wells were washed three times with HBSS and blocked with 0.5 mg/ml BSA in HBSS for 30 min at room temperature. A20 cells were resuspended to 1.25 x 10^6/ml in HBSS containing 10 µg/ml 5-chloromethylfluorescein diacetate (CMFDA; Molecular Probes, Eugene, OR) and incubated for 30 min at 37°C. After labeling, the cells were washed and resuspended at the same concentration in HBSS/10% FCS (binding buffer) except for cells to be treated with neutralizing antibodies to either LFA-1 or α4 integrins. These cells were resuspended at 1.25 x 10^7/ml in 50 µl binding buffer containing 50 µg/ml of either the TIB-213 anti-LFA-1 mAb (ATCC) or the PS/2 anti-α4 integrin mAb (a gift from Dr. B. Chan, Univ. of Western Ontario, London, ON, Canada), incubated for 5 min at room temperature, and then diluted to 1.25 x 10^6/ml in binding buffer. Anti-IgG Abs, CXCL13, or S1P were added to the cell suspension before transferring 1.25 x 10^5 cells in 0.1 ml binding buffer into triplicate wells coated with either sICAM-1 or VCAM-1-Fc. The cells were incubated at 37°C for 30 min and the total fluorescence signal (excitation at 485 nm, emission at 530 nm) from each well was measured using a Bio-Tek FL600 microplate fluorescence reader (Bio-Tek Instruments Inc., Winooski, VT). The wells were then washed manually 7-9 times with 37°C binding buffer to remove non-adhering cells and the fluorescence signal from each well was measured again. The post-wash fluorescence (remaining adhered cells) was divided by the pre-wash fluorescence (total input cells) for each well to yield the percent of cells that remained adhered after washing.

Pyk2 tyrosine phosphorylation

Pyk2 tyrosine phosphorylation assays were done as described previously (30). In short, 6-well tissue culture plates were coated with a collagen/fibronectin ECM prepared by adding 1.5 ml of a 2% gelatin solution (type B from bovine skin, Sigma) to each well of the plate and incubating the plate overnight at 37°C. The gelatin was aspirated and 1.5
ml of non-heat inactivated FBS was added to each well for 1 h at 37°C. The wells were then washed with PBS and vector control or RapGAPII-expressing A20 cells (1.5 x 10^7 cells in 1 ml HBS) were added to the collagen/fibronectin ECM-coated wells. After the cells were rested at 37°C for 30 min, the cells were stimulated with CXCL13 or S1P and lysed by adding 0.25 ml of cold 5X Pyk2 lysis buffer (50 mM Tris-HCl, pH 7.2, 5% Triton X-100, 140 mM KCl, 10 mM EDTA, 2.5 mM Na_3VO_4, 1 mM Na_2MoO_4, 1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin). To immunoprecipitate Pyk2, the cell extracts were mixed with 1 μg of goat anti-mouse Pyk2 Ab (sc-1514, Santa Cruz Biotechnology) for 1 h at 4°C and then transferred to tubes containing 10 μl of protein G-Sepharose beads (Sigma) for 45 min. Anti-Pyk2 immunoprecipitates were analyzed by immunoblotting with the 4G10 anti-phosphotyrosine (anti-P-Tyr) mAb (Upstate Inc., Charlottesville, VA). The filters were then stripped and reprobed with the goat anti-mouse Pyk2 Ab. To quantitate normalized Pyk2 phosphorylation, band intensities were determined using the ImageJ analysis program and the phospho-Pyk2 value was divided by the value for the total amount of immunoprecipitated Pyk2 for a given sample.
Figure 2.1
**Figure 2.1:** CXCL13 and S1P induce Rap1 activation that can be blocked by RapGAPII expression. (a) CXCL13 and S1P induce Rap1 activation in splenic B cells. Splenic B cells from C57BL/6 mice were stimulated immediately after isolation with anti-IgM Abs, CXCL13, or S1P for the indicated times. The activated GTP-bound form of Rap1 was selectively precipitated from cell lysates using a GST-RalGDS fusion protein and detected by immunoblotting with anti-Rap1 Abs. (b) B cells isolated from the spleen (left panel) or peripheral and mesenteric lymph nodes (right panel) of C57BL/6 mice were maintained for several hours in vitro and then stimulated with 50 µg/ml anti-IgM or the indicated concentrations of S1P for 1 min before being assayed for Rap1 activation (upper panels). A fraction of each cell extract was analyzed by immunoblotting with anti-Rap1 antibodies (lower panels) to show that similar amounts of Rap1 were extracted from stimulated and unstimulated cells. (c) Retroviruses were used to establish bulk populations of WEHI-231 transduced with the pMSCV vector containing FLAG-RapGAPII cDNA or with the empty pMSCV vector. Expression of FLAG-RapGAPII in cell lysates was detected by immunoblotting with an anti-FLAG mAb. (d) RapGAPII blocks CXCL13- and S1P-induced Rap1 activation in WEHI-231 cells and A20 cells. Vector control (MSCV) or RapGAPII-expressing cells were stimulated with anti-Ig Abs, CXCL13, or S1P for the indicated times before assaying for Rap1 activation as in Fig. 1a. The amount of Rap1 present in the cell extracts was not altered by CXCL13 or S1P stimulation of the cells (data not shown). (e) Vector control (MSCV) or RapGAPII expressing cells were stimulated with the indicated concentrations of S1P for 1 min before assaying cell extracts for activated Rap1. A fraction of the cell extract was analyzed by immunoblotting with anti-Rap1 antibodies (lower panel) to show that the amount of Rap1 that was recovered from the cells was not affected by either RapGAPII expression or S1P stimulation. For each panel, similar results were obtained in at least two independent experiments.
Figure 2.2: CXCL13 and S1P induce phosphorylation of ERK, JNK, and Akt, which is not affected by RapGAPII expression. Vector control or RapGAPII-expressing WEHI-231 cells were stimulated with (a) CXCL13 or (b) S1P for the indicated times and the phosphorylation of ERK, JNK, and Akt was detected by immunoblotting with phospho-specific Abs. The filters were then stripped and total ERK, JNK, and Akt was detected using the respective Abs. The data in each panel are representative of three independent experiments.
Figure 2.3

A

![Graph showing relative amount of mRNA for S1P$_1$ and S1P$_3$.

B

WEHI-231 Cells

<table>
<thead>
<tr>
<th></th>
<th>S1P</th>
<th>SEW2871</th>
<th>DMSO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>300 nM</td>
<td>300 nM</td>
</tr>
<tr>
<td>Relative Amount of mRNA</td>
<td>500 nM</td>
<td>600 nM</td>
<td>0.006%</td>
</tr>
</tbody>
</table>

21 kDa Rap1-GTP
21 kDa Rap1

C

WEHI-231 Cells

<table>
<thead>
<tr>
<th>Pretreat</th>
<th>S1P</th>
<th>DMF</th>
<th>CAY10444</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>300 nM</td>
<td>300 nM</td>
</tr>
<tr>
<td>Relative Amount of mRNA</td>
<td>0</td>
<td>300 nM</td>
<td>0</td>
</tr>
</tbody>
</table>

21 kDa Rap1-GTP
21 kDa Rap1
Figure 2.3: Both the S1P₁ and S1P₃ receptors can contribute to S1P-induced Rap1 activation. (a) Quantitative RT-PCR analysis of S1P₁ and S1P₃ expression in vector control (V) and RapGAPII-expressing (R) WEHI-231 and A20 cells, as well as murine splenic B cells (Sp) and peripheral lymph node B cells (LN). The data are expressed as the amount of S1P₁ or S1P₃ mRNA relative to the amount of 18S rRNA in the same sample. The absolute values for the 18S rRNA levels were within 10% of each other for the WEHI-231 and A20 cells whereas the 18S rRNA values for the primary B cells were approximately 50% of those for the cell lines. Each sample was assayed in duplicate within a single experiment and the data are presented as the mean and SD for these determinations. A representative experiment is shown. Similar results were obtained for 3 independent RNA preparations for each cell line. (b) WEHI-231 cells were stimulated for 1 min with S1P, 300 nM or 600 nM of the S1P₁ agonist SEW2871, or as a solvent control, volumes of DMSO (0.003% and 0.006%, respectively) equivalent to that delivered with the SEW2871. Rap1 activation assays were performed as in Figure 1. Three representative experiments are shown. Although DMSO treatment activated Rap1 to some extent, quantitation of band intensities followed by statistical analysis using Student’s one-tailed paired t-test showed that the Rap1 activation caused by SEW2871 was significantly greater than that caused by the equivalent volume of DMSO (for 300 nM SEW2871 compared to 0.003% DMSO, p = 0.014, n = 9 independent experiments; for 600 nM SEW2871 compared to 0.006% DMSO, p = 0.031, n = 4 independent experiments). (c) WEHI-231 cells were pre-treated for 5 min with 10 µM of the S1P₃-selective antagonist CAY10444 or an equivalent volume of DMF (0.67% final concentration) and then incubated with or without 300 nM S1P for 1 min. Rap1 activation assays were performed as in Figure 1. The data are representative of three independent experiments.
Figure 2.4: Blocking Rap activation via expression of RapGAPII inhibits CXCL13- and S1P-induced B cell migration in a Transwell assay. Vector control or RapGAPII-expressing WEHI-231 cells were placed in the top chamber and the indicated concentrations of (a) CXCL13 or (b) S1P were placed in the bottom chamber. After 3 h, the cells that had migrated into the bottom chamber were collected and counted using a FACScan. The data are expressed as the percentage of total input cells that migrated into the bottom chamber. The error bars represent the mean ± SD for triplicate wells. For each panel, the data are representative of at least three independent experiments. The CXCL13- and S1P-induced migration of the vector control cells was identical to that of the parental non-transduced WEHI-231 cells (data not shown).
Figure 2.5: Blocking Rap activation via expression of RapGAPII inhibits CXCL13- and S1P-induced adhesion of B cells to ICAM-1 and VCAM-1. Vector control or RapGAPII-expressing A20 cells were stimulated with 10 µg/ml anti-IgG, 200 nM CXCL13 or 300 nM S1P for 30 min before being assayed for their ability to adhere to immobilized (a) ICAM-1 or (b) VCAM-1. Where indicated, the cells were pre-treated with anti-LFA-1 or anti-α4 integrin neutralizing Abs to demonstrate that the adhesion to ICAM-1 and VCAM-1 was mediated by LFA-1 and α4 integrins, respectively. The data are expressed as the percent of cells that bound firmly after washing and represent the mean ± SD for triplicate wells. For each panel, the data are representative of at least three independent experiments.
Figure 2.6: Blocking Rap activation via expression of RapGAPII inhibits CXCL13- and S1P-induced tyrosine phosphorylation of Pyk2. Vector control and RapGAPII-expressing A20 cells were plated for 30 min on wells coated with a collagen/fibronectin ECM before being stimulated with the indicated concentrations of (a) CXCL13 or (b, c) S1P. In panel b, A20 cells were stimulated with a range of concentrations of S1P for 1 min. Pyk2 was immunoprecipitated and analyzed by immunoblotting with the 4G10 anti-phosphotyrosine (anti-P-Tyr) mAb. The filters were then stripped and reprobed with a goat anti-mouse Pyk2 Ab to show total Pyk2 protein levels. The relative levels of Pyk2 phosphorylation in each sample were determined by dividing the band intensity for phosphorylated Pyk2 by the band intensity for the total amount of immunoprecipitated Pyk2 for a given sample. The levels of Pyk2 phosphorylation observed in unstimulated vector control (MSCV) cells were defined as 1 and the normalized Pyk2 phosphorylation for other samples is expressed relative to this value. The graphed data in panel a represent the average and range for two independent experiments. The data in panel b are from a single representative experiment. The graphed data in panel c represent the mean and SEM for three independent experiments. S1P-induced Pyk2 phosphorylation was significantly lower in the RapGAPII-expressing cells than in the vector control cells. Student's one-tailed paired t test yielded p values of 0.0042 for the 1 min time point and 0.041 for the 5 min time point.
2.5. References


CHAPTER 3

Phosphoinositide 3-kinase p110delta regulates natural antibody production, marginal zone and B-1 B cell function, and autoantibody responses

3.1. Introduction

B-1 and marginal zone (MZ) B cells are innate-like lymphocytes. These cells constitutively produce natural Abs in the absence of antigen stimulation and also make rapid T-independent Ab responses against multivalent microbial antigens. In contrast to conventional B-2 cells, B-1 and MZ B cells do not circulate but reside in specialized locations (1, 2). B-1 cells are found in the peritoneal cavity and the spleen, whereas MZ B cells reside near the marginal sinus of the spleen. The Ig repertoires of B-1 and MZ B cells are skewed towards the recognition of both microbial antigens and self-antigens.

Natural Abs made by these innate-like B cells often recognize cross-reactive epitopes on encapsulated Gram-positive bacteria, pathogenic viruses, apoptotic cells, and oxidized low-density lipoproteins (OxLDL) (3-5). In this way, natural Abs provide immediate protection against infection and also prevent inflammation by facilitating the clearance of oxidized lipids, oxidized proteins, and apoptotic cells (3, 6).

In addition to their important immunoprotective and homeostatic functions, B-1 and MZ B cells are also a major source of Abs that contribute to acute inflammation as well as chronic autoimmune diseases. When ischemia-reperfusion injury or other types of acute tissue damage expose intracellular proteins, natural Abs against specific intracellular self-antigens can initiate complement-mediated inflammation (7-9). Moreover, in chronic inflammatory diseases such as rheumatoid arthritis and lupus, there is an expansion of B-1 and MZ B cell populations and increased production of self-reactive Abs that contribute to autoimmunity (10-14). Thus, modulating the activation of MZ and B-1 B cells could be a useful approach for treating Ab-mediated inflammation and autoimmune diseases.

Lipids generated by phosphoinositide 3-kinase (PI3K) regulate the subcellular localization and activity of proteins that control cell migration, survival, growth, and proliferation (15). For conventional B-2 cells, activation of the PI3K signaling pathway by chemokine receptors, the BCR, cytokine receptors, and TLRs plays an important role in their development, their trafficking and antigen presentation functions, their activation and proliferation, and their ability to differentiate into Ab-producing cells (16-20). Although B cells express all four isoforms of the class I PI3K p110 catalytic subunit (21),
the p110δ isoform, which is expressed primarily in hematopoietic cells, appears to be the most important for B cell development and activation. In mice lacking a functional p110δ protein, the production of mature circulating B-2 cells is reduced and the B-2 cells that do develop exhibit impaired chemokine-induced migration, BCR signaling, impaired BCR-induced proliferation, and impaired differentiation into Ab-producing cells (16, 19, 20, 22). Interestingly, mice in which the gene encoding the p110δ subunit has been disrupted (p110δ−/−) or replaced with a catalytically inactive version (p110δ knock-in [KI]) have very few B-1 and MZ B cells (20, 22), indicating that the development of these cells is strongly dependent on p110δ. However because B-1 and MZ B cells are missing in p110δ−/− and p110δ KI mice, it is not known whether p110δ plays a critical role in the function of B-1 and MZ B cells in adult animals.

IC87114 is a highly selective inhibitor of p110δ enzymatic activity that has been widely used to inhibit p110δ in cells that have developed normally in wild type (WT) animals (21, 23-30). IC87114 has an IC50 of 0.13-0.5 µM for p110δ (23, 31) whereas its IC50 values for p110α, p110β, and p110γ, are at least 200-, 150-, and 60-fold higher, respectively (23, 31). Moreover, IC87114 has no inhibitory activity towards a number of other kinases including Akt1, PKCα, PKCβII, c-Src, p38 MAPK, casein kinase I, checkpoint kinase 1, and DNA-PK (23). Unlike the broad-spectrum PI3K inhibitors LY294002 and wortmannin, which act on all cell types, p110δ inhibitors may selectively block PI3K signaling in B cells where p110δ appears to be the most important isoform. IC87114 treatment of murine splenic B cells, which are predominantly B-2 B cells, inhibits anti-Ig-induced proliferation, IL-4-dependent survival, and multiple PI3K-dependent signaling events (21), reproducing the effects seen in p110δ KI mice. However, this study did not address the role of p110δ in B-1 and MZ B cells, which are important targets for the modulation of Ab-mediated autoimmune diseases.

We now show that inhibiting p110δ activity with IC87114 reduces the migration and activation of B-1 and MZ B cells. Moreover, we show that in vivo natural Ab production requires p110δ activity and that treating animals with a p110δ inhibitor can reduce Ab responses in a model of collagen-induced arthritis. Thus targeting p110δ may be a novel approach for modulating the functions of innate-like B cells in vivo and for
treating Ab-mediated autoimmune diseases.
3.2. Results

3.2.1. Reduced levels of circulating natural antibodies in p110δ knock-in mice

MZ and B-1 B cells are a major source of natural Abs that recognize microbial antigens and self-antigens. Because p110δ activity is required for the development of MZ and B-1 B cells, we asked whether the production of natural Abs was impaired in p110δ KI mice. As shown by Okkenhaug et al (22), total serum IgM levels were lower in p110δ KI mice than in WT mice (Fig. 3.1A). However, the levels of specific IgM natural Abs with protective functions were reduced to a much greater extent. Compared to wild type mice, p110δ KI mice had significantly decreased serum titers of IgM Abs to S. pneumoniae type 3 polysaccharides (PPS-3) (Fig. 3.1B), natural Abs that protect against encapsulated Gram-positive bacteria. The p110δ KI mice also had significantly reduced levels of IgM anti-OxLDL natural Abs, which protect against atherosclerosis. Compared to WT mice, p110δ KI mice had much lower levels of IgM natural Abs against copper-oxidized LDL (Fig. 3.1C), as well as malondialdehyde (MDA)- and malondialdehyde-acetaldehyde (MAA)-adducted LDL (Fig. 3.1, D and E), models of oxidation-specific epitopes found on LDL in atherosclerotic lesions (3, 32). Serum titers of the atheroprotective germline-encoded E06 IgM natural Ab were also reduced in the p110δ KI mice (Fig. 3.1F). E06 recognizes the phosphorylcholine headgroup of oxidized phospholipids found in OxLDL and on the surface of apoptotic cells (33, 34). E06 also binds phosphorylcholine moieties attached to the cell wall polysaccharide of many bacteria and plays a major role in protecting mice against lethal infection with S. pneumoniae (3, 34).

MZ B cells also produce IgG1 Abs (35, 36), and in mice, IgG1 Abs to MDA-LDL can have an atheroprotective role (37). Unimmunized p110δ KI mice had decreased titers of total serum IgG1 compared to WT mice, as shown previously (22) (Fig. 3.1G), and also had much lower levels of IgG1 Abs against MDA-LDL (Fig. 3.1H). For other IgG subtypes, both total Ig levels and OxLDL-specific Ab titers were only slightly reduced in p110δ KI mice (data not shown). Thus the loss of p110δ activity selectively
reduced the levels of total IgM and IgG1, and had an even more dramatic effect on the levels of IgM and IgG1 Abs to OxLDL.

In addition to their protective roles, natural Abs against intracellular proteins can initiate inflammatory reactions when acute tissue injury exposes these antigens. Compared to WT mice, we found that unimmunized p110δ KI mice had significantly reduced serum titers of both IgM natural Abs and IgG Abs against antigens present in mouse heart extract (Fig. 3.2A). Moreover, p110δ KI mice had dramatically reduced titers of IgM natural Abs against cardiac myosin (Fig. 3.2B), an abundant intracellular protein that is exposed in damaged heart tissue.

The decreased levels of natural Abs in p110δ KI mice presumably reflects the essential role of p110δ in the development of B-1 and MZ B cells, which are the major source of natural Abs. However the role of p110δ in B-1 and MZ B cells in adult mice, potential targets for modulation of natural Ab production, has not been investigated. To address this, we made use of the p110δ-selective inhibitor IC87114.

3.2.2. IC87114 inhibits PI3K-dependent activation of Akt in splenic and peritoneal B cell subsets

Phosphorylation-dependent activation of the Akt kinase mediates many of the effects of PI3K on cell survival, growth, proliferation and directional cell migration (38, 39). Therefore we assessed the role of p110δ in chemokine receptor-, BCR- and TLR-induced activation of Akt in MZ and B-1 B cells from WT mice.

The chemokine CXCL13 and the lipid chemoattractant sphingosine 1-phosphate (S1P) regulate B cell trafficking and localization (40, 41) and their receptors activate the PI3K/Akt signaling pathway (42). Intracellular staining and FACS analysis showed that CXCL13 increased Akt phosphorylation in splenic B-2, MZ B cells, and B-1a cells, and in peritoneal B-2 and B-1 B cells (Fig. 3.3, A and B). For all of these B cell populations, pre-treating the cells with 2-5 μM of the p110δ-selective inhibitor IC87114, or with 10 μM of the broad-spectrum PI3K inhibitor LY294002, completely inhibited CXCL13-induced Akt phosphorylation (Fig. 3.3, A and B). Immunoblot analysis also showed that IC87114 inhibited CXCL13-induced Akt phosphorylation in a highly enriched
population of MZ B cells, as well as in B-2 cells (Fig. 3.3C). Unlike CXCL13, which induced Akt phosphorylation in all B cell subsets, S1P induced Akt phosphorylation in splenic MZ B cells (Fig. 3.3D), but not in splenic B-2 or B-1a cells (data not shown). Importantly, S1P-induced Akt phosphorylation in MZ B cells was also completely blocked by IC87114 (Fig. 3.3D).

Clustering the BCR with anti-IgM Abs induced strong Akt phosphorylation in splenic B-2 and B-1a cells, but not in MZ B cells (Figure 3.4A). In contrast, the TLR 9 ligand CpG DNA induced strong Akt phosphorylation in splenic B-2 and MZ B cells, but not in B-1a cells (Figure 3.4B). In all cases, both IC87114 and LY294002 completely blocked BCR- and TLR9-mediated Akt phosphorylation (Fig. 3.4, A and B). Thus p110δ appears to be the main p110 isoform linking chemoattractant receptors, the BCR, and TLR9 to Akt phosphorylation in B-2, MZ, and B-1 B cells.

3.2.3. p110δ activity is important for TLR-induced proliferation of B-1 and MZ B cells

TLR ligands are potent activators of innate-like B cells. The TLR ligands LPS and CpG DNA increased the expression of the early activation markers CD69 and CD86 on purified MZ B cells (Fig. 3.5A) but this was not dependent on PI3K since it was not blocked by either IC87114 (Fig. 3.5A) or LY294002 (data not shown). In contrast, the ability of LPS to promote the survival and proliferation of MZ B cells was substantially reduced by IC87114 treatment (Fig. 3.5B). In the absence of IC87114, when MZ B cells were cultured with LPS or CpG DNA, most of the cells were viable after 48 h and had undergone cell division. However, when the cells were cultured with IC87114 plus LPS, only ~5% of the cells remained viable after 48 h, similar to what was observed when the cells were cultured without LPS as a mitogenic stimulus. CpG DNA-induced MZ B cell survival and proliferation was partially reduced by IC87114 treatment (Fig. 3.5B). In addition, IC87114 treatment reduced the number of cell divisions that purified peritoneal B-1 cells underwent in response to TLR4 ligands (LPS, purified lipid A) and to the TLR9 ligand CpG DNA (Fig. 3.5C). Thus p110δ activity contributes to the ability of both MZ B cells and peritoneal B-1 cells to proliferate in response to TLR ligands, key
physiological activators of these innate-like B cells. Note that BCR clustering alone did not induce activation marker expression or proliferation in MZ or B-1 B cells (data not shown).

An interesting observation was that the relative importance of p110δ in B cell proliferation depended not only on the receptor that was stimulated but also on the B cell subpopulation. For splenic B cells, which are ~80% B-2 cells after MACS isolation, BCR-induced proliferation was most sensitive to inhibition by IC87114, followed by TLR4-induced proliferation, and TLR9-induced proliferation, which was the least sensitive (Fig. 3.5D). The differential role of p110δ in different B cell subsets is illustrated by the finding that LPS-induced survival and proliferation was much more dependent on p110δ activity in MZ B cells (Fig. 3.5B) than in peritoneal B-1 cells (Fig. 3.5C) or in splenic B-2 cells (Fig. 3.5D).

3.2.4. IC87114 inhibits chemoattract-induced migration of splenic B-2, B-1a, and MZ B cells

CXCL13 and S1P regulate the in vivo localization of B cells. CXCL13 allows circulating B-2 cells to enter lymphoid follicles (43) and is a potent chemoattractant for B-1 cells (43). For MZ B cells, S1P is critical for their retention in the splenic MZ (44) whereas CXCL13 allows MZ B cells to shuttle into the lymphoid follicles, where they deliver blood-borne antigens to B-2 cells (45). Although splenic B cells from p110δ KI mice exhibit impaired migration to CXCL13 (16), the role of p110δ in the migration of MZ and B-1 B cells is not known and the ability of IC87114 to modulate the trafficking of these cells has not been investigated.

Using Transwell migration assays, we found that IC87114 treatment reduced CXCL13-induced migration of splenic B-2 cells, splenic MZ B cells, and splenic B-1a cells by 40-60% (Fig. 3.6A). IC87114 reduced the migration of these cells to the same extent as LY294002, suggesting that p110δ is the main PI3K isoform that mediates CXCL13-induced migration. IC87114 also inhibited S1P-induced MZ B cell migration by >50% (Fig. 3.6B). The inhibition of B cell migration by IC87114 and LY294002 was
not due to toxicity, since the cells remained viable in the presence of these inhibitors for at least 12 h. Moreover, T cell migration towards CXCL12, which is dependent primarily on p110γ (16), was not significantly inhibited by IC87114 (supplemental Fig. S3.2), indicating that IC87114 does not have off-target effects on p110γ or other enzymes involved in lymphocyte migration. Thus p110δ activity is important for innate-like B cells to migrate efficiently towards CXCL13 and S1P. Consistent with this finding, IC87114 treatment substantially reduced the ability of WEHI-231 murine B lymphoma cells to migrate towards CXCL13 and S1P (Fig. 3.6C). This cell line may represent a transformed B-1 cell since it expresses CD5 and was isolated from the peritoneal cavity of a mouse.

In neutrophils, p110δ activity is critical for directional migration along a chemokine gradient but not for random cell motility (23). Thus the partial inhibition of splenic B cell migration by IC87114 and LY294002 could reflect PI3K-independent chemokinesis, a chemoattractant-induced increase in random cell motility, which would not be inhibited by IC87114 or LY294002. To assess the contribution of chemokinesis to CXCL13-induced migration, we placed CXCL13 in both the upper and lower Transwell chambers such that the cells were exposed to CXCL13 but there was no gradient. We found that the number of cells migrating from the upper chamber to the lower chamber under chemokinesis conditions was ~50% of that observed for chemotaxis conditions in which CXCL13 was present only in the bottom chamber only. Strikingly, chemokinesis was only slightly inhibited by IC87114 whereas chemotaxis was reduced to ~50% of control values (Fig. 3.6D), the amount of migration that could be attributed to PI3K-independent chemokinesis. Similar results were obtained with S1P (data not shown). Thus p110δ activity appears to be critical for chemoattractant-induced directional B cell migration but is dispensable for chemokinesis. These data also indicate that IC87114 treatment did not downregulate expression of the receptors for CXCL13 and S1P since both of these chemoattractants stimulated robust chemokinesis in the presence of IC87114.
3.2.5. **p110δ activity is required for activation of the Rap1 GTPase**

We have previously shown that activation of the Rap1 GTPase is essential for CXCL13- and S1P-induced B cell migration, as well as for chemoattractant- and anti-Ig-induced adhesion (42). PI3K activity is required for BCR-induced Rap1 activation (46) but the relevant p110 isoform has not been identified. Moreover, it is not known whether PI3K activity is required for chemoattractant-induced Rap1 activation and TLR-induced Rap1 activation had not been demonstrated in B cells. Figure 3.7 shows that the TLR9 ligand CpG DNA induced robust Rap1 activation in splenic B cells, and importantly, we found that treating splenic B cells with IC87114 blocked anti-Ig-, CXCL13-, S1P-, and CpG DNA-induced Rap1 activation (Fig. 3.7). Thus in B cells, p110δ links the BCR, chemoattractant receptors, and TLRs to the activation of Rap1, a key regulator of B cell migration and adhesion.

3.2.6. **IC87114 inhibits MZ B cell adhesion in vitro and disrupts MZ B cell localization in vivo**

The retention of MZ B cells in the MZ surrounding the lymphoid follicles of the spleen depends on strong integrin-mediated adhesion (44). Therefore we asked whether activation of the LFA-1 integrin in MZ B cells was dependent on p110δ activity. We found that CXCL13 increased the ability of MZ B cells to adhere to immobilized ICAM-1 and that this LFA-1-dependent adhesion was reduced when the cells were treated with either IC87114 or LY294002 (Figure 3.8A). To test whether p110δ activity is required for maintaining the in vivo localization of MZ B cells, we treated mice with IC87114 for 9 days and then imaged the organization of B cells in the spleen (Fig, 3.8, B-G). Spleens of vehicle-treated wild type mice had thick rings of IgMhiIgDlo MZ B cells surrounding almost every B cell follicle (Fig. 3.8, D and F), similar to the spleens of untreated mice (Fig. 3.8B). In contrast, mice treated with IC87114 had substantially reduced numbers of MZ B cells surrounding the follicles (Fig. 3.8, E and G), and in many cases the MZ B cells did not completely surround the follicle or were lacking altogether. Although the reduction in MZ B cells in mice treated with IC87114 for 9 days was not as severe as in
the p110δ KI mice (Fig. 3.8C), these data indicate that p110δ activity is important for maintaining the correct localization of MZ B cells in the spleen. Interestingly, the organization of the B-2 cells in the lymphoid follicles (Fig. 3.8, E and G), as well as the organization of the T cell zone (data not shown) were not affected by IC87114 treatment, indicating a specific requirement for p110δ in the in vivo localization of MZ B cells in the spleen.

3.2.7. IC87114 reduces in vitro antibody responses by B-1 and MZ B cells

The role of PI3K, and specifically p110δ, in the differentiation of MZ and B-1 B cells into Ab-producing cells has not been studied. Using purified MZ B cells from WT mice, we found that IC87114 treatment substantially reduced the ability of CpG DNA to stimulate the production of IgM Abs (Fig. 3.9A), including potentially pathogenic Abs against cardiac myosin (Fig. 3.9B). Similarly, the addition of IC87114 to peritoneal B-1 cell cultures reduced the ability of the TLR ligands LPS, lipid A, and CpG DNA to increase total IgM secretion (Fig. 3.9C) as well as the production of Abs against OxLDL epitopes and cardiac myosin (Fig. 3.9, D through F). Thus inhibiting p110δ activity with IC87114 can reduce the production of both protective and potentially harmful self-reactive Abs by innate-like B cells.

3.2.8. p110δ inhibitors reduce pathogenic antibody responses in vivo

The requirement for p110δ activity in Ab production by B-2 cells (22) and by innate-like B cells (Fig. 3.9) suggests that p110δ inhibitors could be used to reduce the production of autoimmune Abs. To test this, we initially asked whether administering p110δ inhibitors could reduce in vivo Ab responses to SRBC, a T-dependent antigen. Although B-2 cells are primarily responsible for T-dependent Ab responses, MZ B cells make significant contributions to the early IgM and IgG1 responses to T-dependent antigens (35, 36). We found that treating rats with IC87114 significantly decreased the production of IgM anti-SRBC Abs as and reduced the production IgG anti-SRBC Abs (Fig. 3.10A). We then asked whether p110δ inhibitors could reduce the production of
auto-Abs in a rat model of collagen-induced arthritis, an Ab-mediated autoimmune reaction. In this case we used IC490194, a close structural analogue of IC87114. IC490194 is a potent and selective PI3Kδ inhibitor that has an IC₅₀ of 12 nM for p110δ, 8 µM for p110α, 940 nM for p110β, and 800 nM for p110γ (data not shown). Importantly, IC490194 is metabolized less rapidly than IC87114 and its pharmacokinetic properties are more suitable for chronic dosing of rats. We found that pretreating rats with IC490194 significantly reduced the circulating levels of IgG anti-collagen Abs that were present 17 and 21 days after collagen injection (Fig. 3.10B). Thus, p110δ inhibitors may be of therapeutic value for B cell-mediated autoimmune diseases.
3.3. Discussion

IC87114 is a highly selective inhibitor of p110δ (23, 47) that phenocopies many of the effects of the disrupting the p110δ gene or replacing it with a catalytically inactive version (i.e. p110δ KI) (21, 22). Since B-1 and MZ B cells do not develop in p110δ−/− mice or p110δ KI mice, we used IC87114 to show for the first time that the enzymatic activity of p110δ is important for the function of normal B-1 and MZ B cells from WT adult mice. We found that p110δ signaling contributes to chemoattractant-induced directional migration as well as TLR-induced proliferation and Ab production in these innate-like B cells. We also show for the first time that the BCR, TLRs, and chemoattractant receptors activate the Akt pro-survival kinase in MZ and B-1 B cells and that this is completely dependent on p110δ activity. Importantly, we showed that p110δ activity is critical for the in vivo production of both protective and potentially harmful natural Abs. The ability of p110δ inhibitors to reduce the activation of both innate-like and adaptive B cells suggests that such inhibitors could be useful for treating Ab-mediated inflammatory diseases. In support of this idea, we demonstrated that treating animals with p110δ inhibitors reduced the in vivo production of pro-inflammatory autoAbs in a rat model of autoimmune arthritis. Finally, we showed that IC87114 inhibited migration of the WEHI-231 B cell lymphoma cell line towards the B cell chemoattractants CXCL13 and S1P, suggesting that p110δ inhibitors might be useful for limiting the spread of B cell lymphomas.

Natural Abs produced by B-1 and MZ B cells protect against microbial infection and carry out important homeostatic functions including clearing apoptotic cells and oxidized lipoproteins that can initiate inflammatory and autoimmune diseases. Indeed, in mouse models of atherosclerosis, a more severe disease is observed in mice after splenectomy and this can be reversed by the adoptive transfer of B cells into these mice (48). We found that the in vivo production of IgM natural Abs against pneumococcal polysaccharides and OxLDL was greatly decreased in p110δ KI mice, indicating that the p110δ-dependent development of B-1 and MZ B cells is critical for the production of these protective natural Abs. This also implies that p110δ inhibitors, like other B cell-
directed therapies such as rituximab, would need to be used in a manner that achieves therapeutic reduction of pro-inflammatory and autoimmune Abs without compromising the protective and homeostatic functions of natural Abs.

B-1 and MZ B cells have been implicated in the production of self-reactive Abs that cause acute inflammatory responses. Natural Abs against intracellular antigens (e.g. myosins) that are exposed by tissue damage can activate complement and cause inflammation that exacerbates ischemia-reperfusion injury of the heart, mesentery, and brain (5, 7, 8). Similarly, Abs to cardiac myosin can cause heart damage subsequent to bacterial infections associated with rheumatic fever (49, 50). We found that p110δ KI mice had significantly decreased levels of natural Abs against heart antigens and cardiac myosin. Moreover, IC87114 treatment inhibited the ability of both B-1 and MZ B cells to produce Abs against cardiac myosin in vitro. Thus p110δ inhibitors could be useful for reducing the production of Abs that cause acute inflammatory responses associated with conditions such as rheumatic heart disease and heart ischemia-reperfusion injury.

Self-reactive Abs made by innate-like B cells also play a major, and sometimes causative role in chronic autoimmune diseases. B cell-specific deletion of the SHP1 phosphatase in mice leads to B-1a cell activation, increased production of self-reactive Abs, and autoimmune disease identical to that in motheaten mice, a model for lupus in humans (11). A role for p110δ in B-1 cells homeostasis is supported by the finding that disrupting p110δ activity reverses the expansion of B-1 cells seen in PTEN knockout mice, which have excessive PI3K signaling (51). In addition to regulating B-1 cell homeostasis, we found that p110δ activity was important for TLR-induced proliferation of B-1 and MZ B cells, as well as TLR-induced production of potentially pro-inflammatory Abs by these cells. Interestingly, microbial infections have been implicated as a trigger for autoimmunity. Thus using p110δ inhibitors to limit the production of self-reactive Abs by innate-like B cells could be an important strategy for treating both acute inflammatory responses and chronic autoimmune diseases.

Chemoattractant-induced migration and adhesion controls the trafficking and localization of B cells, processes that are critical for B cells to encounter antigen and become activated. This is the first report that p110δ activity is important for MZ and B-1 B cell migration and for MZ B cell adhesion. Importantly, our finding that p110δ
signaling is important for CXCL13- and S1P-induced activation of the Rap1 GTPase provides a mechanism by which p110δ could regulate B cell chemotaxis and adhesion. Rap1 activation is critical for chemoattractants to stimulate B cell migration and adhesion (42), as well as for lymphocyte polarization (52), which is essential for directional migration. Akt activation also promotes cell migration via the actin-binding protein girdin (39) and we showed that inhibiting p110δ activity completely blocked CXCL13- and S1P-induced Akt activation in B-1 and MZ B cells.

The localization of MZ B cells to the MZ surrounding lymphoid follicles in the spleen positions them to rapidly detect blood-borne microbial infections and depends on counterbalanced gradients of CXCL13 and S1P as well as strong integrin-mediated adhesion (44, 45). We found that treating mice with the p110δ inhibitor IC87114 resulted in decreased numbers of MZ B cells surrounding the primary follicles in the spleen. It is not clear whether inhibition of p110δ activity leads to MZ B cell death, their migration to other sites, or to a change in surface marker expression such that they can no longer be identified as MZ B cells. However these findings, together with the observation that Rap1b-deficient mice have greatly reduced numbers of MZ B cells (53), suggests that p110δ-dependent activation of Rap1 is important for the proper localization of MZ B cells.

TLR-induced activation can release MZ B cells from the marginal sinus (54) and may allow them to migrate to other sites, guided by CXCL13, which is a potent chemoattractant for MZ B cells. The migration of self-reactive MZ B cells to other organs could contribute to autoimmunity either via localized Ab production or the ability of MZ B cells to act as antigen-presenting cells. MZ B cells appear to traffic to secretory glands in Sjogren’s disease (55). Moreover, the migration of MZ B cells to the pancreatic lymph node, where they present self-antigens to auto-reactive T cells, has been implicated in the development of diabetes in NOD mice (56). A recent report showed that IC87114 effectively blocks the ability of B cells to present antigens and activate T cells (17). These data, together with our findings that IC87114 inhibits CXCL13-induced MZ B cell migration and adhesion, as well as TLR-induced MZ B cell proliferation and Ab production, suggest that p110δ inhibitors could reduce the ability of MZ B cells to traffic to other organs and contribute to autoimmune diseases.
A key finding was that treating animals with p110δ inhibitors reduced the \textit{in vivo} production of collagen Abs in a model of collagen-induced arthritis. Because IC87114 inhibits the activation of conventional B-2 cells, as well as B-1 and MZ B cells, the utility of p110δ inhibitors would not be limited to situations in which autoimmunity is mediated by innate-like B cells. The more stringent requirement for p110δ in B cells than in T cells (16, 19), coupled with the restricted expression of p110δ in hematopoietic cells, suggests that p110δ inhibitors could reduce autoAb production while leaving T cell responses intact and having minimal effects on other organ systems. Indeed, we found that IC87114 did not inhibit T cell migration (supplemental Fig. S3.2) and that the IC\textsubscript{50} for inhibition of splenic T cell proliferation by IC87114 was 200-times higher than that for splenic B cell proliferation (supplemental Fig. S3.4), consistent with the much more severe effects of p110δ deficiency on B cell proliferation versus T cell proliferation (19). Thus the use of p110δ-selective inhibitors such as IC87114 may be a new approach for reducing the production of pro-inflammatory self-reactive Abs in patients with chronic autoimmune diseases, for preventing acute Ab-mediated inflammation and tissue damage after ischemia-reperfusion injury, and for limiting the dissemination of B cell lymphomas.
3.4. Material and Methods

Animals, cells, and reagents

WEHI-231 cells were obtained from the ATCC. C57BL/6 mice and p110δ KI mice (p110δD910A/D910A; backcrossed to C57BL/6 for >10 generations) (22) were used at 6-12 weeks of age. Female Lewis rats (7-8 weeks old) were obtained from Charles River Laboratories. The UBC Animal Care Committee approved all protocols. Murine splenic B were purified by negative selection using a MACS cell isolation kit (Miltenyi Biotec). Subsequent FACS sorting was used to enrich CD23-lowCD21-high MZ B cells to >97% purity. Peritoneal B-1 cells were enriched to >95% purity by CD23 depletion and CD19 positive selection using MACS reagents. For analysis of Akt phosphorylation in MZ B cells by immunoblotting, mature B cells were isolated by negative selection using a B cell isolation kit from StemCell Technologies that was supplemented with Abs to CD93. MZ B cells were then enriched by further positive selection using limiting amounts of CD21-PE Abs and anti-PE-conjugated magnetic beads (StemCell Technologies). The MZ B cell-depleted population was used as an enriched population of B-2 cells. All enrichment steps were performed using a RoboSep automated cell isolation system (StemCell Technologies). B cell populations were treated with IC87114 (23), LY294002 (Calbiochem), goat anti-mouse IgM (Jackson Immunoresearch), CXCL13 (R&D Systems), sphingosine 1-phosphate (S1P) (BioMol), LPS (Sigma-Aldrich), lipid A (Avanti Polar Lipids), or CpG DNA (ODN1826; InvivoGen).

ELISA

Mouse serum was diluted in PBS containing 0.05% Tween-20 (plus 15 μg/ml PPS-22F (ATCC) to measure PPS-3-specific Abs) and added to Maxisorp 96-well plates (Nunc) coated with 5 μg/ml PPS-3 (ATCC), 5 μg/ml porcine cardiac myosin (Sigma-Aldrich), 100 μl mouse heart extract (from M. Horwitz, Univ. of British Columbia), or BSA. Bound Abs were detected with HRP-conjugated goat anti-mouse IgM or IgG and visualized with tetramethylbenzidine (Sigma-Aldrich). Relative A₄₅₀ values were calculated by subtracting BSA control values for each sample. Abs against OxLDL, MDA, and MAA, as well as the EO6 Ab, were quantified by chemiluminescent ELISA.
Akt phosphorylation

For intracellular staining and phospho-flow analysis, cells (10⁶) were stimulated in 0.25 ml modified HEPES-buffered saline (42), then fixed with 4% paraformaldehyde, permeabilized with 90% methanol for 10 min on ice, blocked with PBS containing 10% donkey serum and 1% BSA, and incubated overnight with a phospho-Akt (S473) Ab (Cell Signaling Technologies). Fc receptors were blocked with the 2.4G2 mAb (ATCC) and the cells were stained with anti-rabbit IgG-Alexa647 (Molecular Probes), anti-CD45R-pacific blue, anti-CD23-FITC, anti-CD21-PE, and anti-CD5-PECy7 (eBiosciences). Data acquired using an LSRII flow cytometer was analyzed with FlowJo software (TreeStar). Immunoblotting was performed as described (42).

B cell activation and proliferation

FACS-sorted MZ B cells (10⁵ per well) were cultured in RPMI-1640 with 10% heat-inactivated FCS, 50 μM 2-ME, 2 mM glutamine, and 1 mM pyruvate (complete medium), stained with anti-CD69-PECy7 or anti-CD86-APC (BD Biosciences), and analyzed by flow cytometry. For proliferation assays, total splenic B cells, FACS-sorted MZ B cells, or purified peritoneal B-1 cells (10⁵/well) were labeled with CFSE and then cultured in complete medium (plus 10 mM HEPES for B-1 cells) for 48-72 h. After blocking Fc receptors with the 2.4G2 mAb, cells were stained with anti-CD45R-pacific blue or anti-CD19-PE and analyzed by flow cytometry.

Chemotaxis and chemokinesis

Transwell migration assays were performed as described (42, 57), with 10⁶ total mouse splenocytes added to the upper chamber. After 3 h at 37°C, cells that migrated into the lower chamber were stained with anti-CD45R-pacific blue, anti-CD23-FITC, anti-CD21-PE, and anti-CD5-PECy7 and counted for 30 s using an LSRII flow cytometer. The percent migration for each cell type was determined by comparison to the 100% control in which 10⁶ cells were added directly to the bottom chamber.
Rap1 activation

Activated Rap1 that was precipitated using a GST-RalGDS fusion protein, as well as total Rap1 in cell lysates, was visualized by immunoblotting with a Rap1 Ab (Cell Signaling Technologies), as described previously (58).

Adhesion assays

Adhesion assays were performed as described (57). Maxisorp 96-well plates were coated with 30 µg/ml soluble ICAM-1 (StemCell Technologies) for 1.5 h and then blocked with BSA. Cells ($10^6$) were stimulated in suspension and then added to the wells for 20 min at 37°C. After washing the wells, adherent cells were detached by adding cold RPMI-1640 with 5 mM EDTA for 20 min. Fc receptors were blocked with the 2.4G2 mAb and cells were stained with anti-CD45R-pacific blue, anti-CD23-FITC, and anti-CD21-PE. The cells were analyzed by flow cytometry, collecting cells for 30 s. The percent adhesion for each cell type was determined by comparison to the 100% control in which $10^6$ splenocytes were analyzed directly by FACS.

Oral gavage and immunostaining

C57BL/6 mice were administered 0.1 ml of PEG400 (Hampton Research) or 0.1 ml of 7.5 mg/ml IC87114 (25 mg/kg) in PEG400 twice daily for 9 days by oral gavage. Spleens were preserved in frozen tissue embedding medium (Fisher) and frozen at -80°C before cutting 7-µm sections using a cryotome (Thermo Electron). The sections were fixed with ice-cold acetone, washed with PBS, and blocked with 10% rat serum and the 2.4G2 mAb for 20 min before being stained with anti-IgM-FITC (Jackson ImmunoResearch) and anti-IgD-PE (eBioscience) for 1 h. Sections were washed, mounted with Prolong Gold (Molecular Probes), and imaged using an Olympus FV1000 confocal microscope.

SRBC and collagen Ab responses

Rats were administered IC87114 or vehicle (PEG400) by oral gavage twice daily starting 3 days before they were injected i.p. with $10^8$ SRBC, and for 10 days afterwards, at which time blood was collected. SRBC Abs were measured by ELISA using plates
coated with 50 μl lysed SRBC suspension and HRP-conjugated anti-rat IgM or IgG for detection. For anti-collagen responses, rats were administered IC490194 or vehicle (Suspendol-S; Paddock Laboratories) by oral gavage twice daily starting 3 days before they were injected intradermally with 0.2 mg porcine type II collagen (Chondrex) emulsified in Freund’s incomplete adjuvant. A second intradermal injection of collagen was given on day 14. Blood was collected on days 0 (first collagen injection), 7, 14, 17, and 21. Anti-collagen IgG titers were determined with an ELISA kit (Chondrex) and converted to anti-collagen units using a standard curve.

**Statistics**

Student’s t-test was used to compare sets of matched samples.
Figure 3.1

A. Total IgM

B. IgM anti-PPS-3

C. IgM anti-Copper Oxidized-LDL

D. IgM anti-MDA-LDL

E. IgM anti-MAA-LDL

F. E06/T15

G. Total IgG1

H. IgG1 anti-MDA-LDL
Figure 3.1: Decreased levels of natural Abs against PPS-3 and OxLDL in p110δ KI mice.  
A, Total serum IgM levels from unimmunized C57BL/6 WT and p110δ KI mice were determined using a chemiluminescent ELISA. Data are reported as relative light units (RLU) per 100 ms (mean ± SD for 3 mice).  
B, Serum samples (1:100 dilution) from unimmunized C57BL/6 WT and p110δ KI mice were assayed by ELISA for IgM Abs to PPS-3. Values for the mean (horizontal bars) and for individual mice (dots) are shown.  
C-E, Serum samples from unimmunized WT and p110δ KI mice were assayed for IgM Abs against copper-oxidized LDL (C), MDA-LDL (D), or MAA-LDL (E) using a chemiluminescent ELISA. Each point is the mean ± SD for 3 mice. Similar results were obtained using MDA-BSA and MAA-BSA (supplemental Fig. 1, A and B) and there was very low reactivity against unconjugated BSA (supplemental Fig. 1C) or native LDL (data not shown), indicating that the Abs were specific for the MDA and MAA epitopes.  
F, A chemiluminescent ELISA was used to quantify the E06/T15 natural Ab in serum from 3 WT and 3 p110δ KI mice. Dilution curves for sera from individual mice are shown.  
G, Total serum IgG1 levels from unimmunized WT and p110δ KI mice were determined by ELISA.  
H, Serum samples from unimmunized WT and p110δ KI mice were assayed for IgG1 Abs against MDA-LDL. Each point is the mean ± SD for 3 mice. Similar results were obtained using MDA-BSA (supplemental Fig. 1D).
Figure 3.2: Decreased levels of natural Abs against heart antigens in p110δ KI mice. Serum samples (1:100 dilution) from unimmunized WT and p110δ KI mice were assayed for (A) IgM and IgG1 Abs that recognize antigens present in mouse heart extract or (B) IgM Abs against cardiac myosin. Values for the mean (horizontal bars) and for individual mice (dots) are shown.
Figure 3.3: IC87114 inhibits chemoattractant-induced Akt phosphorylation in B-2, B-1, and MZ B cells. A, Total splenic B cells were pretreated with IC87114, LY294002, or an equivalent volume of DMSO (solvent for IC87114 and LY294002) for 30 min and then stimulated with 200 nM CXCL13 for 2 min. FACS analysis was used to quantify intracellular phospho-Akt levels and to distinguish splenic B-2 cells (CD45R+CD23hiCD21int), MZ B cells (CD45R+CD23loCD21hi), and B-1a cells (CD45R+CD5hi). Representative FACS plots are shown. In the corresponding graphs, the difference in mean fluorescence intensity (MFI) values between unstimulated cells and CXCL13-stimulated cells (no DMSO or IC87114) was used as the 100% value (control response). The difference in MFI values between unstimulated cells and cells stimulated with CXCL13 in the presence of DMSO, IC87114, or LY294002 is expressed as a percent of the control response. Each bar is the mean ± SEM for at least three experiments. A positive response to stimulation was determined to be more than 1.5 fold increase in the absolute value of phospho-Akt. B, Peritoneal B cells were pretreated with DMSO, IC87114, or LY294002 for 30 min and then stimulated with 200 nM CXCL13 for 2 min. FACS analysis was used to quantify intracellular phospho-Akt levels and to distinguish peritoneal B-2 cells (CD45R+CD11bloCD5) from B-1 cells (CD45R+CD11bhi). The data are presented as in panel A. C, Enriched populations of B-2 and MZ B cells from mouse spleen were incubated for 30 min in the presence or absence of IC87114 and then stimulated with 200 nM CXCL13 for 2 min. Akt phosphorylation was assessed by by immunoblotting with a phospho-Akt Ab (upper panel). The blots were then stripped and reprobed with anti-Akt Abs (lower panel). D, Total splenic B cells were pretreated with DMSO, IC87114, or LY294002 and then stimulated with 300 nM or 600 nM S1P for 5 min. FACS analysis was used to quantify intracellular phospho-Akt levels and to identify splenic MZ B cells. The data are presented as in panel A.
Figure 3.4
Figure 3.4: IC87114 inhibits anti-Ig- and CpG DNA-induced Akt phosphorylation in B-2, B-1, and MZ B cells. Total splenic B cells were pretreated with IC87114, LY294002, or an equivalent volume of DMSO for 30 min and then stimulated with 5 µg/ml anti-IgM for 2 min (A) or 1 µM CpG DNA for 30 min (B). FACS analysis was used to quantify intracellular phospho-Akt levels and to distinguish splenic B-2 cells, MZ B cells, and B-1a cells. The data are presented as in Figure 3. Each bar is the mean ± SEM for at least three experiments.
Figure 3.5: IC87114 inhibits TLR-induced proliferation of MZ B cells and peritoneal B-1 cells. A, Purified MZ B cells were cultured in medium alone for 12 h (unstimulated) or were pretreated with DMSO or IC87114 for 30 min, then cultured with LPS or CpG DNA (Stim) for 12 h. Cell surface expression of CD69 and CD86 was analyzed by FACS. B-D, Purified MZ B cells (B), purified peritoneal B-1 cells (C), or total splenic B cells (D) were labeled with CFSE and then either analyzed immediately by FACS (time 0) or cultured for 48 h (MZ B cells, splenic B cells) or 72 h (B-1 cells) with the indicated stimuli in the presence of either DMSO or IC87114 before being analyzed by FACS. For each sample, 30,000 events were collected. Representative FACS plots show the CFSE fluorescence for the viable cells, which were identified by forward and side scatter profiles. For each panel, similar results were obtained in three experiments.
Figure 3.6: p110δ activity is required for B cell chemotaxis but not for chemokinesis.

A, Spleen cells were pretreated with DMSO, IC87114, or LY294002 for 30 min before being added to Transwells containing either medium or 200 nM CXCL13 in the lower chamber. After 3 h, the percent migration for each B cell subset was determined by comparison to the input population, using FACS to identify B-2, MZ, and B-1 B cells, as in Figure 3. Each bar is the mean ± SD for triplicate samples. B, Chemotaxis of spleen cells towards 300 nM S1P was assayed as in (A). Only MZ B cells migrated towards S1P. C, WEHI-231 cells were pretreated with DMSO, IC87114, or LY294002 for 30 min before being added to Transwells containing either medium, 100 nM CXCL13, or 300 nM S1P in the lower chamber. IC87114 also inhibited CXCL13- and S1P-induced activation of Akt in WEHI-231 cells (supplemental Fig. 3). D, Transwell migration assays using spleen cells were performed as in (A) except that 200 nM CXCL13 was present either in the lower chamber (chemotaxis) or in both the upper and lower chambers (chemokinesis). The percent of cells that had migrated by 3 h (mean ± SD for triplicate samples) is shown. For each panel, similar results were obtained in three experiments.
Figure 3.7: p110δ activity is required for Rap1 activation. Total splenic B cells were pretreated with DMSO (D), 2 µM IC87114 (IC), or 10 µM LY294002 (LY) for 30 min before being stimulated with anti-IgM, CXCL13, or S1P for 5 min, or with CpG DNA for 30 min. Immunoblots show the activated GTP-bound form of Rap1 (upper blots) as well as total Rap1 in the cell lysates (lower blots). Similar results were obtained in three experiments.
Figure 3.8

(a) Graph showing % Adhesion with P<0.05 and P<0.02.

<table>
<thead>
<tr>
<th>Condition</th>
<th>CXCL13</th>
<th>DMSO</th>
<th>IC87114</th>
<th>LY294002</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>.02%</td>
<td>2 µM</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td>10 µM</td>
</tr>
</tbody>
</table>

(b) C57BL/6

(c) p110δ KI Spleen

(d) C57BL/6 + vehicle gavage

(e) C57BL/6 + IC87114 gavage

(f) F

(g) G
Figure 3.8: IC87114 inhibits MZ B cell adhesion in vitro and disrupts MZ B cell localization in vivo. A, Spleen cells were pretreated with DMSO or IC87114 for 30 min, stimulated with CXCL13, and then plated on immobilized ICAM-1 for 20 min. The percent of CD45R+CD21^hi^CD23^lo^ MZ B cell that adhered was determined by comparison to the input cell population. Each bar is the mean ± SD for triplicate samples. Similar results were obtained in three experiments. B-G, Spleen sections from untreated C57BL/6 mice (B), untreated p110δ KI mice (C) and from C57BL/6 mice treated orally for 9 days with 25 mg/kg IC87114 (E and G) or vehicle (D and F) were stained with IgM-FITC and IgD-PE to distinguish MZ B cells (IgM^hi^IgD^lo^) from B-2 cells (IgM^lo^IgD^hi^). Panels D-G are representative of multiple spleen sections from different mice (n=7 mice per group). Enlarged images are shown in panels F and G.
Figure 3.9: IC87114 reduces in vitro Ab responses B-1 by and MZ B cells. A and B, Purified MZ B cells were cultured for 48 h with 1 µM CpG DNA in the presence of 2 µM IC87114 or DMSO before culture supernatants were assayed by ELISA for total IgM (A) or IgM Abs to cardiac myosin (B). C-F, Purified peritoneal B-1 cells were cultured for 72 h with medium alone, 25 µg/ml anti-IgM, 1 µM CpG DNA, 10 µg/ml LPS, or 1 µg/ml purified lipid A in the presence of DMSO or 2 µM IC87114. Culture supernatants were assayed for total IgM (C), as well as IgM Abs specific for copper-oxidized LDL (D), MDA-LDL (E), or cardiac myosin (F). Each bar is the mean ± SD for triplicate samples. For each panel, similar results were obtained in two experiments.
Figure 3.10: p110δ inhibitors reduce anti-SRBC and anti-collagen Ab responses in vivo. A, IC87114 or vehicle (control) was administered orally to rats twice daily starting 3 days prior to i.p. immunization with SRBC (day 0). Serum was collected on day 10. IgM and IgG anti-SRBC Abs were quantified by ELISA. Each bar is the mean ± SEM for 8 rats. B, IC490194 or vehicle was administered orally to rats twice daily starting 3 days prior to intradermal injection of collagen II (day 0). IgG anti-collagen Abs in serum collected on days 0, 7, 14, 17, and 21 were quantified by ELISA. Each point is the mean ± SEM for 10 rats. *, P < 0.05 compared to vehicle control.
**Supplemental Figure S3.1: p110δ is required for production of natural Abs against oxidation epitopes.** ELISA was used to quantify IgM Abs against MDA-BSA (A), MAA-BSA (B), or unconjugated BSA (C), as well as IgG1 Abs against MDA-BSA (D), in the same serum samples from unimmunized WT and p110δ KI mice used in Figure 1. Very low levels of Abs to BSA were detected, indicating that the Abs reacting with MDA-BSA and MAA-BSA were specific for the MDA and MAA oxidation epitopes. Each data point is the mean ± SD for 3 mice.
Supplemental Figure S3.2: IC87114 does not inhibit T cell migration towards CXCL12.

Spleen cells were pretreated with DMSO, IC87114, or LY294002 for 30 min before being added to Transwells containing either medium or 37.5 nM CXCL12 in the lower chamber. After 3 h, anti-CD3 staining and FACS analysis was used to quantify T cells in the input population and the migrated population and thereby determine the percent of T cells that migrated into the lower chamber. Each bar is the mean ± SD for triplicate samples. IC87114 did not cause significant inhibition of T cell migration, which is dependent largely on p110γ (Reif et al., J. Immunol. 173:2236, 2004). Note that LY294002 also has a relatively high IC50 for p110γ, as opposed to other p110 isoforms (Bilancio et al. Blood 107:642, 2006), and caused only modest inhibition of T cell migration.
Supplemental Figure S3.3: p110δ activity is required for chemoattractant-induced Akt phosphorylation in WEHI-231 B lymphoma cells. WEHI-231 cells were pretreated with DMSO, IC87114, or LY294002 for 30 min before being stimulated with CXCL13 or S1P for 5 min. Phospho-Akt (upper panels) and total Akt (lower panels) was detected by immunoblotting. For each panel, similar results were obtained in 3 experiments.
Supplemental Figure S3.4: Differential effects of IC87114 on T cell versus B cell proliferation. Purified splenic T and B cells were pretreated with IC87114 or DMSO for 30 min, then cultured with anti-CD3 or anti-IgM Abs for 48 h. $^3$H-thymidine was added for the last 4 h of culture. The IC$_{50}$ for inhibition of B cell proliferation was 0.2 µM whereas the IC$_{50}$ for inhibition of T cell proliferation was 4 µM.
3.5. References


G. Cyster. 2004. Cutting edge: differential roles for phosphoinositide 3-kinases,
22. Okkenhaug, K., A. Bilancio, G. Farjot, H. Priddle, S. Sancho, E. Peskett, W.
Pearce, S. E. Meek, A. Salpekar, M. D. Waterfield, A. J. Smith, and B.
Essential role of phosphoinositide 3-kinase delta in neutrophil directional
Suzuki, and S. Koyasu. 2007. The p85alpha regulatory subunit of class IA
Vanhaesebroeck, and A. Khwaja. 2006. A selective inhibitor of the p110delta
isoform of PI 3-kinase inhibits AML cell proliferation and survival and increases the
p110delta isoform of PI3K differentially regulates beta1 and beta2 integrin-
mediated monocyte adhesion and spreading and modulates diapedesis.


CHAPTER 4

Lysophosphatidic acid regulates B cell chemotaxis and adhesion

4.1. Introduction

B cells are responsible for humoral immunity and protect against pathogens by producing Abs (1). Migration and adhesion events are critical for B cells to traffic to specific locations within the lymph nodes (LN s), spleen and the peritoneal cavity where they can be activated and receive survival signals (1-3). While B cell migration and adhesion contribute to normal function and activation of B cells, it can also enhance autoimmune diseases or lymphoma by promoting the spread and activation of self-reactive or malignant B cells (4-11). Identifying key chemoattractants and the effects that they have on B cell trafficking and function may therefore provide a new strategy for controlling B cell-mediated diseases.

Lysophosphatidic acid (LPA) is a soluble, extracellular lipid mediator (12, 13). It regulates cell proliferation, survival, migration, morphological changes, and cytokine and chemokine secretion in a number of different cell types including T cells, neutrophils, monocytes, neurons, endothelial cells, stem cells, and cancer cells (12, 14-16). In addition, LPA is important for platelet aggregation, smooth muscle contraction, neurite retraction, and neurogenesis (12, 13, 17, 18). Although LPA regulates many biological processes, little was known about the role of LPA in B cells when these studies were initiated. Therefore I investigated the effects of LPA on B cell migration and adhesion.

LPA is generated both in the blood and intracellularly. In the blood, lysophospholipids generated by cellular phospholipase A1 (PLA1) and PLA2 are converted to LPA by lysophospholipase D (lysoPLD), which is also known as autotaxin (ATX) (12, 19). In platelets, as well as in activated monocytes, neutrophils, mast cells, DC’s, and B cells (17, 20, 21), phosphatidic acid is generated either by the action of phospholipase D on phospholipids, or by the phosphorylation of diacylglycerol (DAG) by diacylglycerol kinase (DGK), is deacylated by PLA1 or PLA2 to yield LPA (12). LPA is then transported across the cell membrane. As a result, LPA is found in the blood, the peritoneal cavity, cerebrospinal fluid, saliva, and seminal fluid, as well as in the ascites fluid associated with certain cancers (20, 22-25). The high concentration of LPA in serum (1-5 µM) is maintained by the binding of LPA to serum albumin and other lipid-binding proteins, which protect LPA from degradation (17, 20). LPA gradients can be
generated via the action of cell surface lipid phosphate phosphatases (LPPs) that degrade LPA (26, 27). The generation of LPA gradients may be important for LPA to regulate cell migration.

There are seven G-protein-coupled receptors (GPCRs) that bind LPA (12). LPA₁ (EDG2) (28), LPA₂ (EDG4) (29), and LPA₃ (EDG7) (30) are part of the EDG subgroup while LPA₄ (GPR23) (31) and LPA₅ (GPR92) (32) are part of the P2Y subgroup (Table 4.1) (12). The remaining LPA receptors, GPR87 (33) and P2Y5 (34), are not well characterized.

LPA₁, LPA₂, and LPA₄ are coupled to three G-proteins: Gᵢₒ, Gₑ, and G₁₂/₁₃ (35, 36), which helps explain the diverse effects of LPA. Gᵢₒ inhibits the activation of adenylate cyclase but activates the Ras/ERK pathway (36-38), which is important for cell survival and proliferation. Signaling through Gᵢₒ also activates PI3K, leading to Rac and Akt activation (17). Rac is important for cell motility and Akt activation is important for survival and proliferation (36, 39). Gₑ leads to phospholipase C (PLC) activation, DAG production, and intracellular Ca²⁺ signaling, contributing to cell activation and proliferation (35, 36). Finally, G₁₂/₁₃ leads to Rho activation, which is important for cell adhesion and morphological changes (40, 41). The ability of LPA₁ and LPA₂ to induce Rho-dependent cell rounding (42) and activate the PI3K/Akt pro-survival pathway (26, 43, 44) are consistent with their activation of these three G proteins.

LPA₃ and LPA₅ signal via Gᵢₒ and Gₑ but do not activate G₁₂/₁₃ (12, 17). As a result LPA₃ signals through the PLC, Ras and MAPK pathways but does not activate Rho (14, 17). Little is known about signaling pathways initiated by LPA₅ and GPR87 (12). P2Y5 signals via Gᵢₒ and Gₘ, which activates adenylate cyclase (12, 31). LPA₄ also uses Gₘ in addition to Gᵢₒ, Gₑ, and G₁₂/₁₃ and has been shown to activate adenylate cyclase (13, 14, 35). Thus the diverse biological effects of LPA reflect the actions of multiple receptors that are each coupled to several G-protein-regulated signaling pathways.

LPA promotes the proliferation and survival of endothelial cells, epithelial cells, smooth muscle, and cells of the nervous system (14), presumably by activating the PI3K-Akt pathway (45). In the immune system, LPA stimulates proliferation and IL-2 production by Jurkat T cells (46). Similar results were found with human CD4+ T cells.
after LPA stimulation through LPA₁ (47). LPA also promotes T cell survival, preventing apoptosis, presumably via its ability to downregulate the expression of Bax, a pro-apoptotic member of the Bcl-2 family (47).

LPA regulates cell morphology and cell migration in multiple cell types. In neurons, LPA induces neurite retraction (40, 48-50). LPA also activates the Ras, Rho, and Rac GTPases, which are important regulators of cell migration and morphogenesis (36, 39). LPA has been shown to induce cell migration in T cells (51) and stem cells (16). In addition, ATX is highly expressed by endothelial cells of the HEV, where it has a role in regulating lymphocyte trafficking by enhancing the binding of T cells to the HEV and promoting migration towards CCL21 (52). LPA₄ is also involved in inhibiting cell migration (53). Thus, LPA acts as a regulator of cell migration in a variety of cell types, including lymphocytes.

The role of LPA in cell growth, survival, and migration encouraged us to look at the effects of LPA on B cells. Previous studies had shown that LPA acts as a growth factor for Epstein-Barr virus (EBV)-immortalized human B cells, stimulating increases in intracellular calcium release, ERK activation, DNA synthesis, and Ab production (15). LPA also promotes the survival of chronic lymphocytic leukemia (CLL) cells by activating the PI3K/Akt pathway (54). However, it was not known whether LPA regulated normal B cell activation, migration, and adhesion. Therefore I assessed whether LPA modulated B cell migration, survival, proliferation, adhesion, or spreading. We also examined LPA receptor expression on different B lymphoma cell lines and splenic B cells. I found that B cells express several LPA receptors and that LPA inhibits chemoattractant-induced B cell migration but stimulates integrin-mediated B cell adhesion.
4.2. Results

4.2.1. B cells express several LPA receptors

To determine whether LPA might regulate B cell function, I started by looking at whether B cells express LPA receptors. I analyzed the expression of the LPA_1-4 receptors by QRT-PCR on splenic B cells and B cell lymphoma cell lines. I found that WEHI-231 B lymphoma cells expressed mRNA for LPA_1, LPA_2, and LPA_4, with LPA_1 being the most abundant (Fig. 4.1A). A20 B lymphoma cells also expressed mRNA for LPA_1, LPA_2, and LPA_4, with LPA_2 being the most abundant. Neither cell line expressed LPA_3. Splenic B cells contained mRNA for all four LPA receptors, with LPA_1, LPA_2, and LPA_3 being very abundant.

4.2.2. LPA is a negative regulator of B cell migration

B cells traffic through lymphoid organs where they scan for antigens. The recruitment of B cells to the lymph nodes and the spleen is regulated by many chemoattractants including CXCL12, CXCL13, and S1P. Since LPA regulates the migration of many cell types, we asked whether LPA regulated the migration of WEHI-231 B murine lymphoma cells and murine splenic B cells. We had previously shown that WEHI-231 cells migrate towards multiple chemoattractants. For both WEHI-231 cells and primary splenic B cells, we found that LPA alone did not stimulate cell migration but that it significantly inhibited CXCL13-induced migration (Figs. 4.2A,B). When present at 10 µM, LPA reduced CXCL13-induced migration in WEHI 231 cells by approximately 80% (Fig. 4.2A). LPA also inhibited the migration of splenic B cells towards CXCL12 (Fig. 4.2C) and S1P (Fig. 4.2D), although S1P migration was only inhibited by approximately 50%. This inhibition was not due to toxic effects, as LPA treatment did not reduce cell viability over the course of these assays (data not shown). Thus, LPA might inhibit B cell motility or act as a chemorepellant.

To determine if LPA was a chemorepellant, we placed CXCL13 in the bottom chamber of the Transwell chambers and then added LPA to the top or bottom chamber.
If LPA were a chemorepellant, when placed in the top chamber of the Transwell, it should increase the migration towards CXCL13 in the bottom chamber. However, we found that LPA in the top chamber did not increase the migration of WEHI-231 cells into the bottom chamber by itself, or when CXCL13 was present in the lower chamber (Fig. 4.3A). In fact LPA in the top chamber inhibited the migration of WEHI-231 cells towards CXCL13 in the bottom chamber. Similar results were obtained using CXCL12 as the chemoattractant (Fig. 4.3B). These results indicate that LPA is not a chemorepellant but inhibits B cell migration.

4.2.3. LPA induces B cell adhesion to fibronectin, VCAM-1 and ICAM-1

One way in which LPA could inhibit B cell migration would be by inducing stable non-migratory adhesion. Since LPA stimulates integrin-mediated adhesion in monocytes, neutrophils, and endothelial cells (13, 14, 17, 55), we asked if it did so in B cells. Using a static adhesion assay with A20 B lymphoma cells, we found that 1 µM LPA increased the ability of A20 cells to adhere to fibronectin (FN) and VCAM-1, ligands for \( \alpha_4 \) integrins on B cells (Fig. 4.4A, B). Thus LPA stimulation increased the ability of B cells to adhere to FN and VCAM-1, physiological integrin ligands.

4.2.4. LPA induces B cell spreading

Cell spreading is a common post-adhesion event. B cell spreading allows B cells to scan the surface of antigen-presenting cells (APCs) for captured antigens (56-58). When plated on FN, the ability of WEHI 231 B lymphoma cells to spread at 4 hr was greatly enhanced by the addition of 100 nM LPA, with >90% of the cells adopting a spread morphology (Fig. 4.5). Spreading was defined as cells being phase-dark and elongated instead of being round phase-bright cells. Cell spreading was dependent on the concentration of LPA, with 100 nM LPA causing maximal spreading. Higher concentrations of LPA resulted in less spreading. This is likely due to receptor desensitization, a common feature of G protein-coupled receptors. Taken together, our
data indicate that LPA activates B cell integrins that bind FN and that the adhesion of B cells to FN is followed by B cell spreading.

4.2.5. LPA activates Rap1 and Akt in B cells

LPA stimulates a variety of different signaling pathways that control cell adhesion, cell migration, and survival (17, 36). To see if this occurred in B cells, we stimulated WEHI-231 cells with different concentrations of LPA and looked at the activation of the Akt pro-survival kinase, the Rap1 GTPase, which plays a key role in B cell adhesion and migration (59, 60), and the ERK and JNK MAP kinases. ERK is involved in B cell proliferation (61, 62) whereas JNK activation promotes cell survival in B cells (63). We also asked whether LPA modulated the ability of chemokines to activate these signaling pathways. We found that 1 µM LPA by itself did not stimulate the phosphorylation of ERK or JNK on sites that are required for activation of these kinases (Fig. 4.6A). However, 1 µM LPA synergized with CXCL13, resulting in enhanced phosphorylation of ERK and JNK at 1 min, compared to CXCL13 alone (Fig. 4.6A,B). Further analysis showed that higher concentrations of LPA (e.g. 10 µM) induced significant ERK activation and modest JNK activation (Fig. 4.6B). We also found that 1 µM LPA induced modest phosphorylation of Akt on serine 473, a modification required for its activation. Finally, we found that 0.1-100 µM LPA induced Rap1 activation (Fig. 4.6C). This suggests that LPA may activate integrins and induce cell spreading via its ability to activate Rap1.

4.2.6. LPA does not regulate B cell proliferation

LPA regulates the proliferation of multiple cell types. Primary splenic B cells require continual stimulation by growth factors to survive and proliferate. In the absence of such stimulation normal B cells undergo rapid apoptosis with little or no cell division. To test whether LPA modulates B cell proliferation and survival, we used CFSE dilution as a measure of proliferation and stained cells with 7-AAD to assess survival. We found that 1 µM LPA alone did not prevent B cell death (data not shown) or promote sustained
B cell proliferation (Fig. 4.7). As judged by 7-AAD staining on day 4, less than 1% of untreated or LPA-treated B cells were still alive whereas 24% of anti-IgM-treated cells and 27% of anti-CD40-treated cells were alive. Of the live cells, CFSE staining suggested that LPA-treated cells underwent one round of cell division (as seen on day 2) but that all of these cells were dead by day 4. LPA treatment neither enhanced nor inhibited the ability of anti-IgM or anti-CD40 to promote B cell proliferation (Fig. 4.7).
4.3. Discussion

LPA regulates many different cellular functions including proliferation, survival, differentiation, cell rounding, aggregation, contraction, chemotaxis, membrane depolarization, and secretion (17, 64). It is not uncommon for lipid mediators to be used by the immune system to control adaptive and inflammatory responses to pathogens. Platelet activating factor (PAF), eicosanoids, thromboxanes and leukotrienes are well known for their ability to regulate immunity (13). Another lipid, S1P, is an important chemoattractant that regulates lymphocyte trafficking in vivo (65). Thus it seemed reasonable to propose that LPA may also be an important regulator of B cell function, specifically B cell adhesion and migration. In this chapter, I showed that B cells express multiple LPA receptors and that LPA inhibits CXCL13-, CXCL12-, and S1P-induced B cell migration but increases adhesion to FN, ICAM-1 and VCAM-1, as well as post-adhesion spreading on FN.

LPA regulates migration in several cell types so we speculated that LPA might also modulate B cell migration. Indeed we found that LPA inhibited chemoattractant-induced B cell migration. LPA also negatively regulates cell migration in mouse embryonic fibroblasts from LPA4-deficient mice (53). LPA could inhibit B cell migration by acting as a chemorepellant, inhibiting B cell motility, or increasing B cell adhesion. We found that LPA did not act as a chemorepellant for B cells but did increase B cell adhesion. Since LPA can activate Rho (66), another possibility is that Rho activation regulates cell rigidity to prevent cells from moving through the pores in the Transwell migration assay.

Integrin-mediated adhesion allows B cells to exit HEV’s and home to specific sites within lymphoid organs where they can encounter antigen and differentiate into Ab-producing cells. We found that LPA stimulates B cell adhesion to the ECM component FN and to the cellular adhesion molecules ICAM-1 and VCAM-1. Our results are consistent with findings that LPA increases integrin-mediated adhesion in monocytes, neutrophils, and endothelial cells (13, 14, 17, 55). I showed that LPA-induced B cell adhesion is dependent on the activation of integrins since integrin-blocking Abs prevented LPA-induced adhesion. LPA may inhibit B cell migration and promote
adhesion to prevent B cells from leaving sites of Ag encounter and allow for a more effective immune response. We also found that LPA enhanced and accelerated B cell spreading, a common post-adhesion event that promotes firm adhesion. An intriguing possibility is that activated APCs may produce LPA, which acts on B cells to cause them to adhere to the APC. This could promote spreading and enhance the ability of the B cell to scan for Ag and become activated.

B cell trafficking from the blood into lymphoid organs in response to chemoattractants occurs by crossing high endothelial venules (HEV), which express specific adhesion molecules. The LPA produced by activated cells at sites of infection may increase adhesion molecule expression on the HEV of draining LNs and increase integrin activation on B cells, causing increased B cell adhesion to the HEV and enhancing B cell entry into the LN.

After being exposed to LPA, LPA receptors may be down-regulated to allow migration of B cells within LNs towards other chemoattractants in a mechanism similar to S1P-mediated trafficking. It has been shown that LPA1 undergoes rapid LPA-induced internalization from the plasma membrane (39, 67). Chemoattractants like CCL21, CXCL12, and CXCL13 would then direct the B cells in the LN to B and T cell areas to encounter Ag.

Since LPA activates the small GTPases Rho, Rac, and as I’ve shown, Rap1, it is not surprising that LPA can regulate B cell migration and adhesion. LPA causes neurite retraction and lamellipodia formation (14), indicating that it can regulate the formation of membrane protrusions. The G-proteins, G_{i/o} and G_{12/13}, which are coupled to several LPA receptors, activate both Rho and Rac, providing a potential mechanism by which LPA could alter cell morphology. G_{i/o} leads to the activation of Rac through PI3K and the Rac-specific guanine-nucleotide exchange factor (GEF) TIAM1, a key regulator of the actin cytoskeleton that regulates cell migration, neurite outgrowth, and cell-cell adhesion (68, 69). G_{12/13} activates Rho, which induces cytoskeletal contraction and cell rounding (40-42). However, in other cell types G_{12/13} causes cell spreading and adhesion (14). It has now been shown that activation of Rho via G_{i/o} and G_{12/13} mediates LPA-induced integrin-mediated adhesion in MZ and follicular B cells (66). In addition to Rho, we showed that LPA can stimulate Rap1 activation, which is important for integrin-
mediated adhesion in B cells (59). LPA may activate Rho, Rac, Rap1 and other GTPases in a coordinated manner to regulate cell adhesion and migration (53, 70).

Previous work showed that LPA acts as both a survival factor and a mitogen for Epstein-Barr virus-immortalized human B cells, B-cell chronic lymphocytic leukemia (CLL) cells, and Jurkat human T lymphoma cells (15, 54, 71). In contrast, I found that LPA did not promote the proliferation of normal murine splenic B cells. This suggests that transformed B cells may express a different set of LPA receptors than normal B cells. Alternatively, the same LPA receptors could be coupled to different G proteins in normal and transformed cells, resulting in the same receptors activating different signaling pathways.

Activated T cells express different LPA receptors than resting T cells and this may have important biological consequences. LPA signaling via LPA2 in resting T cells promotes cell migration. In contrast, in mitogen-activated T cells, LPA signals mainly via LPA1 to suppress cell migration while promoting proliferation and IL-2 secretion (46, 71). Thus LPA2 may be involved in the normal homeostatic trafficking of T cells whereas the switch to LPA1 expression in activated T cells may help them remain at the site of infection and produce cytokines that help clear the pathogen (21). It would be interesting to determine if resting and activated B cells also express a different set of LPA receptors and respond differently to LPA.

We found that both normal and transformed B cells expressed LPA1, LPA2, and LPA4. Interestingly, only splenic B cells expressed LPA3. Goetzl and colleagues (71) had previously shown that human CD19+ B cells express predominantly LPA2 whereas Rieken et al. showed that follicular and MZ B cells express LPA1, LPA2, and LPA3, with only MZ B cells expressing LPA4 (66). We did not separate splenic B cell subsets before doing QRT-PCR and thus cannot determine whether the LPA4 mRNA we detected was exclusively in MZ B cells. It is interesting that both normal and transformed B cells express LPA4, since LPA4 has been implicated in the inhibition of cell migration in other cell types (53) and we found that LPA inhibits B cell migration. It would be of interest to determine whether the inhibition of chemokine-induced B cell migration by LPA is mediated by LPA4.
To address some of these unanswered questions, future studies would focus on which LPA receptors mediate the inhibition of B cell migration. We are also interested in determining if different B cell subsets, specifically those found in distinct locations that may have different LPA concentrations (peritoneal B cells versus splenic B cells), express different LPA receptors and respond differently to LPA. It would also be informative to determine if knockout mice lacking LPA₁, LPA₂, LPA₃, or LPA₄ exhibit normal B cell development, trafficking, and function. It is not known if LPA receptors are important for B cell development or if LPA receptor expression changes as B cells develop and mature into B cell subsets with distinct locations and functions.

The ability of LPA to enhance B cell adhesion and inhibit B cell migration suggests that it could play a role in the interaction of B cells with APCs. It would be of interest to determine if APCs secrete LPA, whether this is increased when APCs are activated by microbial or other danger signals, and whether LPA secretion by APCs facilitates the activation of B cells by antigens that have been captured by APCs.
4.4. Materials and Methods

Cells

Primary B cells were isolated from the spleens or lymph nodes of C57BL/6 mice. After lysing erythrocytes with Tris-buffered NH$_4$Cl, splenic B cells were purified using the MACS B cell isolation kit and a MACS LS column (Miltenyi Biotec, Auburn, CA) to deplete non-B cells. The resulting cell population was >95% B cells, as determined by FACS analysis of anti-CD19-FITC staining (BD Pharmingen, San Diego, CA). The WEHI-231 IgM$^+$ immature B lymphoma cell line and the A20 IgG$^+$ mature B lymphoma cell line were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, 50 µM 2-ME, 2 mM glutamine, 1 mM pyruvate, 15 U/ml penicillin, and 50 µg/ml streptomycin (complete medium).

Chemoattractants and inhibitors

Recombinant mouse CXCL13 (R&D Systems, Minneapolis, MN) was resuspended to 25 µM in PBS containing 1 mg/ml BSA and stored in aliquots at -80°C. CXCL12 (R&D Systems) was resuspended to 100 µg/ml in PBS containing 1 mg/ml BSA and stored in aliquots at -80°C. S1P (Biomol, Plymouth Meeting, PA) was resuspended to 0.5 mM in 37°C PBS containing 4 mg/ml BSA, dissolved according to the manufacturer’s instructions by repeated cycles of vortexing and mild heating, and then stored at -80°C. LPA (Biomol) was resuspended to 10 mM in PBS containing 4 mg/ml fatty acid free BSA (Calbiochem, La Jolla, CA) and stored in aliquots at -80°C.

Chemotaxis assays

Transwell chemotaxis assays were performed using 24 well plates with 5 µm polycarbonate Transwell inserts (Costar, Cambridge, MA), as described previously (72). The lower chamber contained 0.6 ml of CXCL13, CXCL12, or S1P diluted in chemotaxis medium (RPMI 1640/0.5% BSA/10 mM HEPES) with or without LPA. Cells were diluted to 5 x 10^6/ml in chemotaxis medium and 0.1 ml (5 x10^5 cells) was added to the top chamber with or without LPA. After 3 hr at 37°C the cells that migrated
into the lower chamber were collected and counted with a FACScan for 30 sec. Cells were gated using forward and side scatter to exclude dead cells and debris. To determine the percent of cells that migrated, a 100% control was done in which $5 \times 10^5$ cells were added directly to the bottom chamber at the beginning of the assay. WEHI-231 B cells were cultured to high density ($\sim 1-2 \times 10^6$/ml) before chemotaxis assays and only early passage cells (less than 8 weeks in culture) were used.

To assess cell survival during chemotaxis assays, WEHI-231 cells ($5 \times 10^5$ in 0.1 ml chemotaxis medium) were cultured with CXCL13, S1P or LPA for 3 hr at 37°C. The cells were then transferred to FACS tubes and stained with 2 μg/mL 7-AAD (Calbiochem) for 15 min at 4°C. The cells were then washed, resuspended in FACS buffer and analyzed using a FACScan.

**Proliferation and Cell Survival Assays**

To measure proliferation, isolated C57BL/6 splenic B cells were resuspended to $1 \times 10^7$ cells per ml in PBS without any FBS and labeled with 1 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) for 10 min at room temperature. The labeling reaction was terminated by adding an equal volume of FBS and then washing the cells with complete medium. Labeled cells ($3 \times 10^5$ cells in 0.5 ml complete medium) were plated in each well of a 24 well plate and stimulated with anti-IgM, anti-CD40, LPS, or LPA. The plate was incubated at 37°C with 5% CO₂ before the cells were transferred to a FACS tube. The cells were centrifuged at 1500 rpm for 8-10 min, the supernatant was aspirated, and the cells were stained with 2 μg/ml 7-aminoactinomycin D (7-AAD; Calbiochem) for 15 min at 4°C. The cells were resuspended in FACS buffer (PBS with 2% FBS, 0.1% azide) and analyzed using a FACScan.

**Adhesion assays**

Adhesion assays were performed using a modification of the method described previously (60, 73). Nunc Maxisorp 96-well plates (Nunc, Rochester, NY) were coated at room temperature for 90 min with 30 μg/ml of soluble FN (Sigma), soluble ICAM-1 (StemCell Technologies), or a VCAM-1-Fc fusion (Calistoga Pharmaceuticals) protein
diluted in Hank’s balanced salt solution (HBSS). The wells were washed three times with HBSS and blocked with 0.5 mg/ml BSA in HBSS for 30 min at room temperature. A20 cells were resuspended to $1.25 \times 10^6$/ml in HBSS containing $10 \mu$g/ml 5-chloromethylfluorescein diacetate (CMFDA; Molecular Probes, Eugene, OR) and incubated for 30 min at $37^\circ$C. After labeling, the cells were washed and resuspended at the same concentration in HBSS/10% FBS (binding buffer). Cells to be treated with neutralizing Abs to either LFA-1 or $\alpha_4$ integrins were resuspended at $1.25 \times 10^7$/ml in 50 $\mu$l binding buffer containing $50 \mu$g/ml of either the TIB-213 anti-LFA-1 mAb (ATCC) or the PS/2 anti-$\alpha_4$ integrin mAb (a gift from Dr. B. Chan, Univ. of Western Ontario, London, ON, Canada), incubated for 5 min at room temperature, and then diluted to $1.25 \times 10^6$/ml in binding buffer. Anti-IgG Abs or LPA were added to the cells before transferring 1.25 x $10^5$ cells in 0.1 ml binding buffer into triplicate wells coated with either FN, ICAM-1, or VCAM-1-Fc. The cells were incubated at $37^\circ$C for 30 min and the total fluorescence signal (excitation at 485 nm, emission at 530 nm) from each well was measured using a Bio-Tek FL600 microplate fluorescence reader (Bio-Tek Instruments Inc., Winooski, VT). The wells were then washed manually 7-9 times with $37^\circ$C binding buffer to remove non-adhering cells and the fluorescence signal from each well was measured again. The post-wash fluorescence (remaining adhered cells) was divided by the pre-wash fluorescence (total input cells) for each well to yield the percent of cells that remained adhered after washing.

Adhesion assays using red blood cell-depleted spleen cells were done similarly except that cells were not labeled with CMFDA before plating. Cells that adhered to FN, ICAM-1, or VCAM-1-Fc were removed from the plate using 5 mM EDTA in complete medium. Fc receptors were blocked with the 2.4G2 mAb (1:100), and the cells were then stained with anti-CD45R-pacific blue (1:100) and anti-CD5-PE-Cy7 (1:100) (eBiosciences). The cells were analyzed by flow cytometer, collecting cells for 30 seconds. The percent adhesion for each cell type was determined by comparison to the 100% control in which $10^6$ splenocytes were analyzed directly by FACS.
Spreading Assay

Cell spreading assays were done by coating 24 well plates overnight at 4°C with 300 µl of 15 µg/ml FN diluted in PBS. The wells were then washed with PBS and blocked with 500 µl of 2% BSA for 1 hr at room temperature. WEHI-231 cells (0.5 ml at 3 x 10^5/ml) were added to the wells and then stimulated with LPA at 37°C. Images were captured at using a Motic AE31 microscope equipped with a Moticam 2000 digital camera and analyzed using Motic Images Plus 2.0 software (Motic Instruments Inc., Richmond, BC, Canada).

LPA receptor expression by quantitative RT-PCR

For quantitative RT-PCR (QRT-PCR) analysis of LPA₁, LPA₂, LPA₃, and LPA₄ mRNA levels, RNA was prepared using the RNAeasy kit with QIAshredder columns (Qiagen Inc., Mississauga, Ontario, Canada) and converted into cDNA using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). Equivalent amounts of cDNA were combined with TaqMan Fast Universal PCR Master Mix (Applied Biosystems), TaqMan Gene Expression Assay primers, and probes (Applied Biosystems) specific for LPA₁, LPA₂, LPA₃, and LPA₄, or 18S rRNA. The QRT-PCR reaction and quantitation were performed using an Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems). The data are expressed as the amount of LPAₓ mRNA divided by the amount of 18S rRNA for the same sample. Complete protocols are contained in appendix A.
**Figure 4.1**: B cells express multiple LPA receptors. RNA was isolated from WEHI-231, A20, and purified splenic B cells. QRT-PCR was performed as described in the Materials and Methods. Pooled cDNA from each sample was used to generate a standard curve for each experiment and 18S rRNA was used to normalize the results obtained by QRT-PCR. Error bars represent the mean ± SD for three independent experiments. ND = not detected.
Figure 4.2: LPA inhibits chemoattractant-induced B cell migration. WEHI-231 B cells or purified splenic B cells were placed in the top chamber of a Transwell migration assay with CXCL13 (A,B), CXCL12 (C), and S1P (D) in the bottom chamber along with LPA or medium only. The percent migration was determined by comparison to the input population using FACS. Error bars represent the mean ± SD for triplicate wells.
Figure 4.3: LPA is not a chemorepellant for B cells. Medium, CXCL13 (A) or CXCL12 (B) was placed in the bottom chamber while LPA was placed either in the top chamber with the cells or in the bottom chamber. The percent migration was determined by comparison to the input population using FACS. Error bars represent the mean ± SD for triplicate wells. For each panel, similar results were obtained in three experiments.
Figure 4.4: LPA induces B cell adhesion to FN and VCAM-1. (A,B) CFMDA-labeled A20 B lymphoma cells were stimulated with anti-IgG, or with LPA, added to wells coated with FN (A) or VCAM-1 (B). After 20 min, non-adherent cells were washed off and remaining adherent cells were quantified using a fluorescent plate reader. The data are expressed as the percent of input cells that bound firmly after washing and represent the mean ± SD for triplicate wells. Error bars represent the mean ± SD for triplicate samples. For each panel, the data are representative of at least three independent experiments.
Figure 4.5: LPA induces B cell spreading on fibronectin. WEHI-231 B lymphoma cells were treated with the indicated concentration of LPA and then plated in wells of a 24-well plate that had been coated with FN, BSA, or medium alone as a control. After 2-24 hr, the percent of cells that were phase dark and elongated, rather than phase bright and round, was determined. Representative results from one of three independent experiments are shown.
Figure 4.6: Effects of LPA on the activation of ERK, JNK, Akt, and Rap1 in WEHI-231 cells. (A) WEHI-231 B cells were stimulated for the indicated times with 1 μM LPA, 200 nM CXCL13, or LPA + CXCL13 before immunoblotting cell lysates with phospho-specific antibodies that recognize the activated forms of ERK, JNK, and Akt (put Akt on the bottom to reflect order in which things are discussed). The filters were then stripped and probed for total ERK, JNK, or Akt (lower panels). (B) WEHI-231 B cells were stimulated for 1 min with CXCL13 or the indicated concentrations of LPA before immunoblotting for the activated forms of ERK and JNK. Total ERK and JNK are shown in the lower panels (C) WEHI-231 B cells were stimulated for 2 min with anti-IgM or with the indicated concentrations of LPA. Rap1 activation was assessed by selectively precipitating Rap1-GTP from cell lysates using a GST-RalGDS fusion protein and detected by immunoblotting with anti-Rap1 Abs. The data in each panel are representative of three independent experiments.
Figure 4.7: LPA does not modulate B cell proliferation. Purified splenic B cells were labeled with 1 µM CFSE and cultured with medium, 40 µg/ml, or anti-IgM, or 40 µg/ml anti-CD40 in the presence of absence of 1 µM LPA. The cells were stained with 7-AAD immediately before FACS analysis and CFSE data is shown only for live cells. Decreased numbers of CFSE-labeled cells indicates that there are fewer surviving cells. The data represent one of three independent experiments with similar results.
4.5. References


33. Tabata, K., K. Baba, A. Shiraishi, M. Ito, and N. Fujita. 2007. The orphan GPCR GPR87 was deorphanized and shown to be a lysophosphatidic acid receptor. *Biochem Biophys Res Commun* 363:861-866.


CHAPTER 5

Conclusions and future directions
5.1. Summary and overview

In this thesis I showed that the Rap GTPases are critical regulators of B cell migration and adhesion and mediate the effects of both chemokines and lipid chemoattractants on these processes. I also showed that PI3Kδ regulates multiple processes in conventional follicular B-2 cells and in innate-like B-1 and MZ B cells. Because PI3Kδ is expressed mainly in hematopoietic cells and is more important in B cells than T cells, it may be a novel therapeutic target for the treatment of B cell-mediated diseases. Finally, I showed that the lipid mediator, LPA, can regulate B cell migration, adhesion, and cell spreading, suggesting that it could regulate B cell trafficking and activation in vivo.

This work focused on the roles of Rap1, PI3Kδ, and the chemoattractant LPA in regulating B cell function. In chapter two I showed that the chemoattractants CXCL13 and S1P induced Rap1 activation in B cells. Importantly I found that the S1P receptors, S1P₁ and S1P₃, can mediate Rap activation while CXCL13- and S1P-induced B cell migration and integrin-mediated adhesion was dependent on Rap activation. This suggests that key steps in B cell trafficking and localization are dependent on Rap activation.

In chapter three, I investigated the role of p110δ in B cell function. I showed that p110δ activity was needed for the production of protective natural Abs to common bacterial polysaccharides as well as the production of harmful self-reactive Abs. Protective and homeostatic natural Abs to oxidized LDL antigens were also reduced in p110δ KI mice. In addition, self reactive and proinflammatory natural antibodies to mouse heart extract and to cardiac myosin were significantly reduced suggesting that p110δ is also important for the production of pathogenic autoAbs.

Akt is important for B cell survival and activation. In chapter three, I also showed that anti-IgM-, CpG-, CXCL13-, and S1P-induced Akt activation in both splenic and peritoneal B cell subsets was inhibited by the p110δ-specific inhibitor, IC87114. S1P-induced Akt activation was inhibited in MZ B cells while anti-IgM-induced signaling was dependent on p110δ in splenic B-2 and B-1a cells. CXCL13, CXCL12, and S1P-induced Akt activation was also inhibited in WEHI-231 B cell lymphoma cells.
after treatment with the p110δ inhibitor, suggesting that p110δ might be a good target for controlling cancer cells.

I also showed that CXCL13- and S1P-induced chemotaxis, but not chemokinesis, is dependent on p110δ activity in splenic B-2, B-1a, MZ B cells, as well as WEHI-231 lymphoma cells. Consistent with previous findings that Rap activation is important for integrin-mediated adhesion and chemoattractant-induced migration, I showed that anti-IgM, CXCL13, S1P and the TLR ligand CpG all induce Rap activation and that this is dependent on p110δ.

I showed that B-2 and MZ B cells required p110δ activity for CpG-induced Akt activation while the upregulation of the activation markers CD86 and CD69 after LPS or CpG stimulation didn’t require p110δ in MZ B cells. However, MZ B cell proliferation after LPS and CpG stimulation was reduced and there were fewer viable cells after treatment with a p110δ inhibitor. Peritoneal B-1 cell proliferation in response to TLR ligands were also reduced after treatment with a p110δ inhibitor. This work shows that p110δ controls B cell activation by several different classes of receptors (BCR, TLRs, GPCRs). I also showed in vitro that B-1 and MZ B cell Ab responses to cardiac myosin and oxidized-LDL antigen were reduced after treatment with the p110δ inhibitor, IC87114. Both protective and autoimmune antibody responses were further evaluated in vivo where anti-SRBC Abs and anti-collagen Abs were inhibited after animals were treated with a p110δ inhibitor. Thus, selectively targeting the PI3Kδ pathway in B cells can reduce pathogenic Ab production.

In addition to the role that Rap activation and p110δ have on B cells, we looked at whether the chemoattractant LPA regulates B cell function. I found that B cells expressed multiple LPA receptors. I also showed LPA activated the GTPase Rap1 as well as the prosurvival kinase Akt. LPA-induced integrin-mediated B cell adhesion and spreading, important processes controlled by Rap. LPA inhibited migration to CXCL12, CXCL13, and S1P possibly by stimulating integrin-mediated adhesion. However, contrary to previous reports in other cell types, LPA had no effect on B cell survival and proliferation.
This work showed that Rap and PI3Kδ are key regulators of B cell trafficking and activation (Fig. 5.1). Chemoattractants, the BCR, and TLRs signal via PI3Kδ, to activate Akt, a kinase that controls cell survival and proliferation. PI3Kδ activation also links these receptors to the activation of Rap1, a GTPase that controls chemoattractant-induced migration and integrin-mediated adhesion. Moreover, I showed that in vivo natural Ab production requires p110δ activity, that p110δ inhibitors block the in vitro migration of B cell lymphomas, and that treating animals with a p110δ inhibitor can reduce pathogenic autoAb responses in a model of collagen-induced arthritis. This suggests that Rap and PI3Kδ could be important targets for treating autoimmune diseases and B cell malignancies.
5.2. Future Directions

5.2.1. The role of p110δ in B cell function

Since B cells play multiple roles in immunity, it is important to understand how they traffic, become activated, and carry out effector functions. In p110δ
\(^{D910A}\) KI mice, which lack functional p110δ, B-1 and MZ B cells are missing, suggesting that p110δ is critical for their development (1). In chapter 3, I showed that these mice have reduced natural Ab production to oxidized-LDL targets and cardiac myosin. I also showed using a p110δ-specific inhibitor that MZ and B-1 cells require p110δ activity for migration, adhesion and the production of natural Abs. Finally we showed that p110δ-specific inhibitors can be used to reduce the severity of RA, suggesting that p110δ inhibitors might be potential therapies for treating B cell-mediated diseases.

If p110δ inhibitor treatments are to be considered for controlling the functions of B cells, specifically innate-like B cells, \textit{in vivo}, it would be important to know to what extent p110δ would have to be inhibited to be effective. Reports by Okkenhaug \textit{et. al}. show that heterozygous p110δ KI mice, unlike homozygous p110δ KI mice, have MZ and B-1 cells (1) but they did not address whether these cells function normally. I would further characterize the heterozygous p110δ KI mice to see if the innate-like B cells in these mice are functional. While testing the homozygous mice for the production of natural Abs to cardiac myosin, I found that the heterozygous p110δ KI mice had significantly reduced natural Ab titers (Fig. 5.2), suggesting that their function may be impaired. This gene dosage effect suggests that a 50\% inhibition of p110δ activity by a therapeutic agent could substantially inhibit the function of innate-like B cells.

To further test if the innate-like B cells that do develop are functional, I would test for Ab and cytokine production, Ag presentation, and activation of other cell types in heterozygous p110δ KI mice. I would look at naïve mice and test for natural Abs to common targets including oxidized-LDL and common bacterial components including PPS-3. If there were significantly reduced Ab titers in heterozygous p110δ KI mice, it would suggest that 50\% inhibition of p110δ activity is sufficient for inhibiting B cell
function. Because chemical inhibitors only cause partial inhibition, this would support the use of p110δ inhibitors for regulating B cell diseases. Moreover, partial inhibition of PI3Kδ may leave basal functions and protective immune responses intact.

**Is p110δ activity important for responses to bacterial infections?**

One interesting area of future study would be the role of p110δ in immunity mediated by innate-like B cells. It would be expected that there would be uncontrolled infection in p110δ KI mice challenged with pathogens due to the lack of innate-like B cells. Therefore, it would be interesting to see how heterozygous p110δ mice would survive when challenged with pathogens. To assess if gene dosage affects MZ and B-1 cell function *in vivo*, I would use a bacterial infection model such as *S. pneumoniae*. Mice with impaired B-1 cell function are more susceptible to bacterial infections (2) suggesting that B-1 cells and the Abs they produce are important for protection. Because I have shown that B-1 and MZ B cell activation and natural Ab production is dependent on p110δ, I would suspect that there would be a reduction in the immediate T cell-independent responses as well as impaired T cell-dependent Ab responses in p110δ KI mice. This would indicate that impaired B-1 and MZ B cell functions in the heterozygous p110δ KI mice results in higher bacterial loads and decreased rate of survival in response to bacterial infection.

**Can inhibition of p110δ reduce heart damage subsequent to ischemia reperfusion injury?**

IR injury occurs after the blood supply is cut off and then repurfuses an organ. This can occur in heart tissue after a myocardial infarction (3-5). The initial tissue damage reveals intracellular Ags that would not normally be seen by the immune system, but which can react with natural Abs that cause inflammation. The circulating Abs would bind the exposed cardiac myosin in the heart, active complement, and recruit innate immune cells that would cause further inflammation and damage to the heart.
tissue. I showed that p110δ KI mice have significantly reduced natural Ab titers to cardiac myosin. Because natural Abs are produced by innate-like B cells and Ab production by these cells is dependent on p110δ, I would like to test if the loss of p110δ function, and eventually drugs that inhibit p110δ, could reduce the amount of heart damage after IR injury. First I would determine if IR-induced heart tissue damage caused by mechanical injury or viral infection would be less severe in p110δ KI mice, which lack anti-cardiac myosin Abs. If so, I would then determine whether treating WT mice with IC87114 would reduce the tissue damage caused by natural Abs and inflammation. This would suggest that p110δ inhibitors could be used to prevent inflammation in cardiac tissue after a myocardial infarction.

Can p110δ inhibitors reduce autoimmune reactions in rheumatoid arthritis, lupus, or type-1 diabetes?

In many cases, B cells make a significant contribution to inflammatory autoimmune diseases either by producing autoAbs or by acting as APCs. Since B cell function is strongly dependent on p110δ, it would be interesting to determine if targeting p110δ would be a good way to treat autoimmune diseases that have a B cell component.

The p110δ isoform is important for the development of rheumatoid arthritis (RA), a disease resulting in inflammation and destruction of joint tissue (6) thus probably reflects the role of p110δ in PMNs and B cells, both of which contribute to inflammation in RA. Using a collagen-induced arthritis model, we showed that p110δ inhibitors could reduce symptoms if given before initiation of disease. However, most patients are seen after disease progression and already have RA. So it would be important to see whether p110δ inhibitors can reduce RA/CIA symptoms (collagen Ab titers, joint swelling), if given after the disease is initiated and symptoms are evident. If it can, then p110δ inhibitors could be used for controlling autoimmune diseases like RA.

Lupus is characterized by the production of anti-dsDNA Abs that form immune complexes, which leads to vascular and kidney damage (7, 8). We would like to test whether the loss of p110δ activity could protect lupus-prone mice from the development
of disease. MRL/lpr mice spontaneously develop lupus-like autoimmune disease (7) and I predict that once crossed to p110δ KI mice, disease would be prevented or delayed. I would also be interested in testing whether the p110δ inhibitor could reverse the disease once it has developed. Further studies could explore the role of p110δ in the generation and activation of B-1 cells that produce the natural Abs that are involved in lupus. I could cross the p110δD910A mice to Spa-1 deficient mice (9) or to SHP-1 KO mice (10), both of which spontaneously develop systemic autoimmunity characterized by anti-DNA Abs and glomerulonephritis. If they are protected by the loss of p110δ activity, this would show the importance of regulating p110δ activity in lupus.

MZ B cells have been implicated in the development of type 1 diabetes (T1D). T1D is a disease in which insulin producing β-islet cells are destroyed by the immune system resulting in diabetes (11). It is thought that some of the BCRs expressed by MZ B cells crossreact with an antigen expressed on the islet cells, causing them to become activated. Once activated, the MZ B cells invade the pancreatic LN and activate effector T cells by presenting Ags and providing costimulatory signals (12). To test the involvement of MZ B cells in diabetes, I would cross p110δ KI mice onto the NOD background and see if this prevents the NOD mice from spontaneously developing diabetes. If so, we will administer IC87114 to pre-diabetic NOD mice by gavage to see if we could prevent the onset of diabetes. A more stringent test that is more relevant for human disease would be to see if administering the p110δ specific inhibitor to diabetic mice would “cure” them. We speculate that the NOD mouse would be protected from diabetes since our previous gavage experiments resulted in the depletion of MZ B cells. If there are no MZ B cells to present Ag, there may be reduced activation of islet-destroying effector T cells by MZ B cells and this may delay or prevent the initiation of diabetes.

**Is p110δ a good target for treating B cell leukemias and lymphomas?**

Many types of malignant B cells, including B-CLL cells, MM cells, and some B cell lymphomas need to enter lymphoid organs, especially the BM, and adhere to stromal
cells in order to receive adhesion-dependent survival signals (13). Since p110δ controls B cell adhesion and migration, it may be an important target for drugs aimed at treating these very common, and often untreated (in the case of MM which are resistant to rituximab) cancers. Moreover, PI3Kδ is important for chronic lymphocytic leukemia (CLL) survival and proliferation (14). We showed that IC87114 is effective in inhibiting chemokine-induced migration, adhesion, and activation of the Akt pro-survival kinase in the WEHI-231 murine B lymphoma cell line, suggesting p110δ may be a good target for controlling the spread and survival of malignant B cells.

To test the role of p110δ in vivo we would use a tumor model with B lymphoma cells and see if treating these mice with IC87114 would reduce the lymphoma size and prevent metastasis. This could be done by injecting lymphomas or by using εμ-Myc mice that generate spontaneous B cell lymphomas. Such experiments would determine whether targeting p110δ would be an effective treatment for B cell malignancies.

**Is p110δ required for the development and function of regulatory B cells?**

A newly identified subset called B10 suppressor B cells (regulatory B cells) produce IL-10 and suppress immune cell function, similar to Treg cells (15, 16). Their inhibitory or immunosuppressive function may be important for helping control autoimmunity. Using p110δ KI mice, we can test if the development, activation, localization, and function of B10 cells depend on p110δ. If so, it would show that p110δ is important in almost all B cell subsets.

**5.2.2. The Role of LPA in B cell function**

LPA is a lipid mediator that we, and others, have shown regulates B cell migration and adhesion. I showed that LPA acts as a negative regulator of B cell migration by increasing integrin-mediated adhesion. I showed that LPA does not regulate survival or proliferation but does induce B cell spreading. These data suggest that LPA could regulate B cell (and B cell lymphoma) trafficking. However, the role of LPA in B cell development, trafficking, and function in vivo is not known.
One way to do this would be to examine B cell development and function in existing mice where individual LPA receptors have been knocked out (17). Alternatively, I could use LPA receptor agonists/antagonists. Using agonists/antagonists would allow us to manipulate individual receptor signaling to see if it was important for the functions of B cells that developed normally. I would look at B cell subset numbers, localization, natural Ab production, and T cell-dependent and T cell-independent Ab responses. I would also be interested in determining if the LPA receptors are involved in different stages of B cell development.

5.3. Conclusion

This proposed work will extend what I have established thus far and provide insight into the role that p110δ has in B cell function and natural Ab production. Future work may suggest that inhibiting p110δ activity would be a great therapy to control autoimmunity, inflammation, and B cell lymphomas. Moreover, LPA may be an important regulator of B cell function that acts by controlling B cell migration and adhesion. Finally, I have highlighted the role of Rap1 in B cell adhesion and migration which suggest that modulating Rap activation may also be a good approach for controlling B cell-mediated inflammation and autoimmunity.
Figure 5.1: Rap activation, PI3Kδ activity, and LPA are important regulators of B cell function.
**Figure 5.2:** Heterozygous p110δ KI mice have reduced natural Abs to cardiac myosin. Serum samples (1:100 dilution) from unimmunized WT, homozygous p110δ KI mice, or heterozygous p110δ KI mice were assayed for IgM Abs against cardiac myosin by ELISA. Values for the mean (horizontal bars) and for individual mice (dots) are shown.
5.4. References


APPENDIX A
A.1. The role of Rap activation in macrophage function

In an active conformation, Rap can bind to effector molecules that regulate important cell functions including migration, integrin-mediated adhesion, and cytoskeletal organization (1-4). The role of Rap in B cells is well characterized (5-7), but its role in cells of the innate immune system are less well understood. Monocytes and macrophages are an important part of the innate immune system. Monocytes are quickly recruited to sites of infections where they differentiate into macrophages, which are involved in microbial killing as well as activation of the adaptive immune system. Rap1 activation is required for LPS-induced spreading in macrophages (8) and also controls the activation of $\alpha_{M}\beta_2$ integrins on macrophages, which are important for complement-mediated phagocytosis (3). Moreover, Rap1-deficient macrophages show increased haptotaxis (9). Rap1 activation is also important for promoting NADPH oxidase function in neutrophils. I showed using the macrophage cell line RAW 264.7 that LPS induces Rap1 activation in macrophages (Fig. A.1), confirming that TLR signaling can activate Rap1. Although TLRs are critical for macrophage activation and function, the role of Rap in macrophage functions such as phagocytosis, trafficking, and microbial killing is not known.

To look at the role of Rap1 activation in macrophages in vivo, we obtained heterozygous floxed RapGAP transgenic mice from Dr. Naoki Mochizuki (National Cardiovascular Centre Research Institute, National Institute of Biomedical Innovation, Osaka, Japan). Tissue-specific expression is needed since blocking Rap activation globally causes embryonic lethality. These mice contain a pCALNL5-RapGAP vector with a floxed neomycin selection cassette and a stop codon, followed by a Flag-tagged RapGAP coding sequence (Fig A.2 A, B). Driving gene expression is the CAG promoter which is a composite promoter consisting of the human CMV enhancer and a modified chicken $\beta$-actin promoter after Cre-mediated excision of the stop sequence. This provides strong, ubiquitous expression in all mouse tissues except erythrocytes and hair cells was observed. Dr. Mochizuki found that RapGAP was efficiently expressed in myocardiocytes after crossing the mice to myosin heavy chain (MHC)-Cre mice and in blood vessels after crossing to Tie2-Cre mice (Unpublished data).
I crossed the heterozygous floxed RapGAP mice to homozygous LysM-Cre transgenic mice (Jackson Labs, Stock 004781) to get heterozygous LysM-RapGAP mice which have lineage-specific expression of RapGAP. LysM-Cre mice were chosen because they express Cre in macrophages, monocytes and neutrophils with little impact on other immune cell types (10). Clausen and colleagues show that there is greater than 90% deletion in macrophages and 80% deletion in bone marrow-derived macrophages (10). The cross was successful in generating double transgenic mice heterozygous for both the RapGAP gene and LysM-cre (Fig. A.3 A). In these mice, Cre expression would excise the floxed stop codon and the selection cassette allowing selective overexpression of the RapGAP gene in macrophages. I found that Rap activation is reduced in these mice and that Rap activation is important for clearing Salmonella bacterial infections. These results, and potential future studies, will be discussed in the following sections.

A.2. The role of Rap activation in macrophage development

Given that Rap is involved in migration and integrin-mediated adhesion it is possible that macrophages and monocytes may not develop in RapGAP-expressing mice. This would be an interesting finding, suggesting that Rap activation is needed for a crucial step in development. In the event that macrophages fail to develop or move to the periphery then we could use BM-derived macrophages to work out a possible mechanism as to why they don’t develop. However, upon initial characterization, it appeared that splenic and peritoneal macrophages do develop (data not shown). This needs to be investigated further as it is unclear if Rap activation is completely blocked in all cells or whether macrophages expressing low levels of RapGAP developed and expanded to yield normal numbers macrophages.

To assess if the RapGAP was expressed in macrophages, we would isolate macrophages by FACS sorting and do a Western blot looking at Flag expression. Since the RapGAP has a Flag tag this would allow for the quantitation of the relative amount of RapGAP being expressed. Flag intracellular staining could also been done to assess the relative expression of RapGAP on a single cell basis. It would be important to determine if the RapGAP expressed is functional and the amount of active Rap in the macrophages.
is reduced. Preliminary data suggests that there is a reduction in the amount of active Rap in LysM-RapGAP macrophages (Fig. A.3 B).

To further determine the extent of macrophage development in the LysM-RapGAP mice, I would use FACS or immunohistochemistry to look at the development of macrophages in the periphery including the skin (Langerhans cells), the airways (alveolar macrophages), the spleen (marginal zone macrophages), the liver (Kupffer cells), and the nervous system (microglia). Since Rap is involved in B cell migration and adhesion, Rap activation may regulate macrophage development in the periphery by controlling monocyte migration or prevent monocytes from leaving the BM. Rap activation may also be important for macrophage recruitment to sites of infection. I could inject mice with LPS or TNFα and look at whether the increase in the number of macrophages in the tissues is blocked by RapGAP.

A.3. The role of Rap activation in macrophage-mediated immunity

Since my preliminary data indicates that macrophages were present in the periphery of LysM-RapGAP mice, I am interested in whether these macrophages have normal effector functions. I could look at cellular functions including migration and adhesion, which are important for macrophage and monocyte trafficking to sites of infection. It has been shown that Rap1a-deficient macrophages have reduced migration to CXCL12 and CCL21 and that their adhesion is also decreased in vitro (9). However, it is not known whether monocyte and macrophage recruitment is altered in vivo. If there is a defect in migration or adhesion, we would then do in vivo competitive homing assays. LysM-RapGAP and WT macrophages that are differentially labeled with cell tracker dyes would be injected into WT mice to see if the LysM-RapGAP macrophages exhibit impaired homing to sites such as the spleen and peritoneal cavity. To further assess the recruitment of macrophages and monocytes to site of inflammation we would inject thioglycollate into the peritoneal cavity or skin and look at macrophage and monocyte recruitment. Thioglycollate is an effective inflammatory agent that has been shown to recruit immune cells to site of injection. This would show that Rap activation is needed for monocyte and macrophage trafficking to sites of inflammation.
To assess if Rap1 activation is needed for macrophage effector responses we would look at macrophage activation, inflammatory responses, phagocytosis, and macrophage-mediated bacterial killing. We would stimulate cells with different TLR ligands including CpG DNA (TLR9), LPS (TLR4), or poly I:C (TLR3) to see if these stimuli activated Rap1 in macrophages and if this signaling was attenuated in LysM-RapGAP macrophages. Macrophage activation could be characterized by looking at NF-kB activation or the up-regulation of the activation markers, CD80 and CD86. While it has been shown that Rap1 activation is important for LPS signaling through MyD88 (8), it isn’t known if Rap acts downstream of MyD88-independent pathways or TLRs other than TLR4.

To assess the importance of Rap1 activation in macrophage activation and inflammatory responses, we would look at the production of cytokines and other inflammatory mediators. Cytokines of interest would include TNF-α, IL-8 and IL-6 as well as the anti-inflammatory cytokine IL-10. Cytokine levels could be measured by ELISA or by bead array allowing multiple cytokines to be tested at once. We would also look at the production of reactive oxygen species (ROS) and nitric oxide (NO), two important macrophage products that kill bacteria during an infection. Rap associates with NADPH oxidase and regulates superoxide production (9, 11), but it is not known if there would be impaired superoxide production in macrophages in which Rap activation is blocked.

Finally we would investigate whether macrophage phagocytosis is dependent on Rap activation since current literature reports conflicting results. Caron and colleagues demonstrated that complement-mediated phagocytosis is reduced when Rap activation is blocked (3). In contrast, Li et al. found that Rap1a-deficient macrophages have normal C3bi-mediated phagocytosis and increased Fc receptor-mediated phagocytosis (9). Using a mouse with macrophage-specific expression of RapGAP, we could ask if FcR- or complement receptor-mediated phagocytosis is impaired. Cells could then be followed by microscopy.

Specifically, we could compare the phagocytosis of BCG (a model for tuberculosis, TB) in WT macrophages and macrophages in which Rap activation is blocked. In addition to phagocytosis, I could assess the role of Rap activation in the
clearance and killing of intracellular bacteria. I speculate that with the loss of Rap1 activation, macrophages would not have efficient phagocytosis, resulting in decreased bacterial sampling and killing. This hypothesis is based on the fact that Rap regulates actin dynamics through a number of effectors including TIAM-1, RIAM, and AF-6 (1) (Fig. 1.6) and that actin remodeling is important for macrophage phagocytosis. This could also provide a possible mechanism for how the loss of Rap activation might impair bacterial phagocytosis and killing.

Finally, I would be interested in looking at whether Rap activation is required for Ag presentation in macrophages. Because Rap activation is involved in regulating actin dynamics and phagocytosis, Ag presentation may also be impaired in macrophages that are unable to activate Rap. This could be tested by feeding macrophages OVA peptide, or listeria expressing OVA, and culturing them with OVA-specific T cells and measuring IL-2 production or T cell proliferation as a readout of T cell activation.

To test the role of Rap activation in macrophage antibacterial functions in vivo, we would look at bacterial infection models using *Salmonella typhimurium* or *Citrobacter rodentium* with the help of Dr. Brett Finlay’s lab. *Salmonella* and *Citrobacter* are gut pathogens that can be used to test whether the loss of Rap1 activation in macrophages is critical for clearing bacterial infections. I proposed that the macrophages would not be able to kill bacteria efficiently, potentially leaving the LysM-RapGAP mice susceptible to bacterial infections. This could be a result of deficient recruitment of monocytes and macrophages to sites of infection. To test this idea we challenged WT and LysM-RapGAP mice with *Salmonella*. Preliminary data showed that LysM-RapGAP mice were slightly more susceptible to bacterial infection that WT mice (Fig. A.3 C). This suggests that Rap activation in macrophages is important for innate immunity and the clearance of bacterial infections. We are interested in testing other bacterial strains including *Citrobacter* to see if we observe the same susceptibility. The method of bacterial challenge would also be of interest, possibly challenging the intestine, airways or the skin with different bacterial infection models.
A.4. The role of macrophages and Rap activation in disease

Excessive macrophage activation and production of inflammatory mediators can lead to autoimmune diseases such as IBD and atherosclerosis. Macrophages play a key role in atherosclerosis. Macrophage and monocyte recruitment to areas of blood vessel damage leads to foam cell development and plaque formation (12). Crossing the LysM-RapGAP mice with apolipoprotein E (ApoE)-deficient mice (13) or LDL-receptor deficient mice (14) that are susceptible to cardiovascular disease would allow us to address whether the loss of Rap1 activation in macrophages would protect these mice from the development of disease. Mice could be fed high fat diets and then plaque formation, hypertension, cardiovascular disease, and ultimately survival would be measured. We speculate that the loss of Rap1 activation would protect these mice from atherosclerosis because macrophages and monocytes would not be recruited to sites of vascular damage and, as a result, would not form foam cells. This would suggest that targeting Rap activation would be a good way to control atherosclerosis.

Another interesting model to test the importance of Rap1 activation would be in inflammatory diseases that are mediated by macrophages. Macrophages are important for the production of inflammatory mediators but if uncontrolled can lead to disease. Possible models would be inflammatory bowel disease (IBD) or airway inflammation mediated by alveolar macrophages. It is possible that with impaired macrophage and monocyte recruitment and function there would be reduced inflammation in the gut or airways and possibly a reduction in disease or it might be prevented altogether.
Figure A.1: LPS induces Rap1 activation in macrophages. RAW 264.7 murine macrophages were serum starved for 12 hours before being stimulated with LPS. Rap activation was assessed by GST-RalGDS pull-down assays and Western blotting. Data represent three independent experiments.
**Figure A.2:** Floxed RapGAP transgene. (A-B), Transgenic mice with this vector inserted into the genome were generated by viral transduction of germ cells. Upon crossing the RapGAP mice to Cre-expressing mice, the stop codon would be excised, allowing the RapGAP protein to be expressed.
Figure A.3: LysM-RapGAP transgenic mice have reduced Rap activation in macrophages and are more susceptible to infection with *Salmonella*. (A), DNA gels showing PCR genotyping samples for LysM-Cre and RapGAP transgenic mice. Primers for human transgenic RapGAP generate PCR bands at 400 bp. (B), Splenic macrophages were isolated from C57BL/6 mice using a MACS macrophage isolation kit (Miltenyi Biotec) and Rap activation was then assessed after stimulation with LPS for 5 min. (C), WT or LysM-RapGAP mice were pretreated with antibiotics before being infected by intraperitoneal injection (i.p.) with approximately $5 \times 10^4$ CFU *Salmonella enterica* in 0.3 ml of PBS. Mouse survival was monitored for two weeks post infection. N=10 for each group of mice.
A.5. References


The University of British Columbia

Biohazard Approval Certificate

<table>
<thead>
<tr>
<th>PROTOCOL NUMBER:</th>
<th>H07-0103</th>
</tr>
</thead>
<tbody>
<tr>
<td>INVESTIGATOR OR COURSE DIRECTOR:</td>
<td>Gold, Michael</td>
</tr>
<tr>
<td>DEPARTMENT:</td>
<td>Microbiology &amp; Immunology</td>
</tr>
<tr>
<td>PROJECT OR COURSE TITLE:</td>
<td>The role of the Rap GTPases in B cell adhesion and migration</td>
</tr>
<tr>
<td>APPROVAL DATE:</td>
<td>07-09-06</td>
</tr>
<tr>
<td>APPROVED CONTAINMENT LEVEL:</td>
<td>2</td>
</tr>
<tr>
<td>FUNDING AGENCY:</td>
<td>Canadian Institutes of Health Research (CIHR)</td>
</tr>
</tbody>
</table>

The Principal Investigator/Course Director is responsible for ensuring that all research or course work involving biological hazards is conducted in accordance with the Health Canada, Laboratory Biosafety Guidelines, (2nd Edition 1996). Copies of the Guidelines (1996) are available through the Biosafety Office, Department of Health, Safety and Environment, Room 50 - 2075 Westbrook Mall, UBC, Vancouver, BC, V6T 1Z1, 822-7596, Fax: 822-6650.

Approval of the UBC Biohazards Committee by one of:
Chair, Biosafety Committee
Manager, Biosafety Ethics
Director, Office of Research Services

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required.

A copy of this certificate must be displayed in your facility.

Office of Research Services
102, 8190 Agronomy Road, Vancouver, V8T 1Z3
Phone: 604-827-5111  FAX: 604-822-5033
The University of British Columbia

Biohazard Approval Certificate

PROTOCOL NUMBER: H07-0103

INVESTIGATOR OR COURSE DIRECTOR: Gold, Michael

DEPARTMENT: Microbiology & Immunology

PROJECT OR COURSE TITLE: Signal transduction in B lymphocytes

APPROVAL DATE: 07-09-06

APPROVED CONTAINMENT LEVEL: 2

FUNDING AGENCY: Canadian Institutes of Health Research (CIHR)

The Principal Investigator/Course Director is responsible for ensuring that all research or course work involving biological hazards is conducted in accordance with the Health Canada, Laboratory Biosafety Guidelines, (2nd Edition 1996). Copies of the Guidelines (1996) are available through the Biosafety Office, Department of Health, Safety and Environment, Room 50 - 2075 Westbrook Mall, UBC, Vancouver, BC, V6T 1Z1, 822-7596, Fax: 822-6650.

Approval of the UBC Biohazards Committee by one of:
Chair, Biosafety Committee
Manager, Biosafety Ethics
Director, Office of Research Services

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required.

A copy of this certificate must be displayed in your facility.

Office of Research Services
102, 8190 Agronomy Road, Vancouver, V6T 1Z3
Phone: 604-827-5111  FAX: 604-822-6083

190
Biohazard Approval Certificate

PROTOCOL NUMBER: **H08-0077**

INVESTIGATOR OR COURSE DIRECTOR: **Gold, Michael**

DEPARTMENT: **Microbiology & Immunology**

PROJECT OR COURSE TITLE: **The role of the Rap GT Pases in B cell function and B cell lymphoma homing**

APPROVAL DATE: **08-06-25**

APPROVED CONTAINMENT LEVEL: **2/3**

FUNDING AGENCY: **Canadian Institutes of Health Research (CIHR)**

The Principal Investigator/Course Director is responsible for ensuring that all research or course work involving biological hazards is conducted in accordance with the Health Canada, Laboratory Biosafety Guidelines, (2nd Edition 1996). Copies of the Guidelines (1996) are available through the Biosafety Office, Department of Health, Safety and Environment, Room 50 - 2076 Wesbrook Mall, UBC, Vancouver, BC, V6T 1Z1, 822-7596, Fax: 822-6650.

Approval of the UBC Biohazards Committee by one of:
Chair, Biosafety Committee
Manager, Biosafety Ethics
Director, Office of Research Services

This certificate is valid for one year from the above approval date provided there is no change in the experimental procedures. Annual review is required.

A copy of this certificate must be displayed in your facility.

Office of Research Services
102, 6100 Agronomy Road, Vancouver, V6T 1Z3
Phone: 604-827-5111  FAX: 604-822-5093
The University of British Columbia

Biohazard Approval Certificate

PROTOCOL NUMBER: H08-0077

INVESTIGATOR OR COURSE DIRECTOR: Gold, Michael

DEPARTMENT: Microbiology & Immunology

PROJECT OR COURSE TITLE: Signal transduction in B lymphocytes

APPROVAL DATE: 08-06-25

APPROVED CONTAINMENT LEVEL: 2/3

FUNDING AGENCY: Canadian Institutes of Health Research (CIHR)

The Principal Investigator/Course Director is responsible for ensuring that all research or course work involving biological hazards is conducted in accordance with the Health Canada, Laboratory Biosafety Guidelines, (2nd Edition 1996). Copies of the Guidelines (1996) are available through the Biosafety Office, Department of Health, Safety and Environment, Room 50 - 2075 Wesbrook Mall, UBC, Vancouver, BC, V6T 1Z1, 822-7596, Fax: 822-6650.

Approval of the UBC Biohazards Committee by one of:
Chair, Biosafety Committee
Manager, Biosafety Ethics
Director, Office of Research Services

This certificate is valid for one year from the above approval date provided there is no change in the experimental procedures. Annual review is required.

A copy of this certificate must be displayed in your facility.

Office of Research Services
102, 6190 Agronomy Road, Vancouver, V6T 1Z3
Phone: 604-822-5111 FAX: 604-822-2383
ANIMAL CARE CERTIFICATE
BREEDING PROGRAMS

Application Number: A06-1531
Investigator or Course Director: Michael B. Gold
Department: Microbiology & Immunology

Animals:
- Mice Cre-LysM x RapGAPII/fox 240
- Mice pI delta knockout x wild type heterozygotes 40
- Mice C57Bl/6 690
- Mice fox/RapGAPII 150
- Mice CD19-Cre 120
- NOD 150
- Mice CD19-Cre x RapGAPII/fox 320
- Mice Balb/c 140
- Mice Cre-LysM 60
- Mice pI delta homozygotes 80

Approval Date: December 10, 2008

Funding Sources:

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: The role of the Rap-GTPases in B cell function and B cell lymphoma homing

Funding Agency: Cancer Research Society
Funding Title: Regulation of tumor cell migration, invasion and metastasis by the Rap1 GTPase

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: Signal transduction in B lymphocytes

Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above breeding program.

This certificate is valid for one year from the above approval date provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z1
Phone: 604-827-5111 Fax: 604-822-5993
ANIMAL CARE CERTIFICATE

Application Number: A07-0194

Investigator or Course Director: Michael R. Gold

Department: Microbiology & Immunology

Animals:

- Rabbits 12
- Mice C57BL/6, BALB/c, (RapGAPII/flox x Cre-LysM) mice, (RapGAPII/flox x Cre-CD19-Cre) mice, CD20 knockout mice, p110delta knock-in mice 820

Start Date: April 1, 2005        Approval Date: May 30, 2008

Funding Sources:

- Funding Agency: Canadian Institutes of Health Research (CIHR)
  Funding Title: The role of the Rap GTPases in B cell function and B cell lymphoma homing

- Funding Agency: Canadian Institutes of Health Research (CIHR)
  Funding Title: Signal transduction in B lymphocytes

- Funding Agency: Canadian Institutes of Health Research (CIHR)
  Funding Title: The role of the Rap GTPases in B cell adhesion and migration

Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.