FUNCTIONAL ANALYSIS OF ADAPTER PROTEIN
C-ABL SRC HOMOLOGY 3 DOMAIN-BINDING
PROTEIN 2

BY

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

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3BP2 is a pleckstrin homology (PH) domain- and Src homology 2 (SH2) domain-containing adapter protein that has been linked through genetic evidence to a rare human disease called cherubism \(^{146}\). 3BP2 was originally cloned in a screen to identify c-Abl SH3 binding proteins \(^{23,24}\). In overexpression studies, 3BP2 has been implicated as a positive regulatory adapter molecule coupled to immunoreceptor on T cells \(^{67,69,70}\), B cells \(^{68}\), NK cells \(^{71-73}\) and mast cells \(^{74,75}\). It was also evident that 3BP2 forms complexes with a number of signaling molecules, such as Zap-70, LAT, phospholipase C-\(\gamma\)1 (PLC-\(\gamma\)1), Grb2, Cbl, and Fyn in Jurkat cells \(^{67}\) and Vav1, Vav2, PLC-\(\gamma\), and Syk in Daudi B cells \(^{68}\).

Despite the growing body of biochemical data to support the importance of 3BP2 in cells of the hematopoietic lineage, a clear picture of the biological function of 3BP2 has yet to emerge. To elucidate the \textit{in vivo} function of 3BP2, our laboratory has generated \textit{3BP2} gene-deficient mice through homologous recombination \(^{452}\). The 3BP2-
deficient (3BP2⁻/⁻) mice were born at the expected Mendelian frequency and were fertile and viable.

3BP2⁻/⁻ mice accumulate splenic marginal-zone (MZ) B cells, possess a reduced frequency of peritoneal B-1 B cells, and have a diminished thymus-independent type 2 (TI-2) antigen response. 3BP2⁻/⁻ B cells demonstrate diminished proliferation and cell survival following cross-linking of the B-cell receptor (BCR). Following BCR ligation, 3BP2 might be recruited to BCR complex through its inducible interaction with BCR costimulatory molecule CD19. In the absence of 3BP2, the activation of BCR downstream effectors such as MAPK Erk1/2, JNK, and c-Abl is normal; however, 3BP2 deficiency leads to defects in Syk phosphorylation and calcium flux.

In addition to defects in peripheral B cell activities, 3BP2 deficiency contributes to defects in neutrophil activities. In response to the chemotactic peptide, fMLF, 3BP2⁻/⁻ neutrophils fail to establish directional migration in vitro. There is a defect in the accumulation of filamentous actin at the leading edge of migrating 3BP2⁻/⁻ neutrophils which might be responsible for the random movement of these cells under shallow gradient of fMLF. In vivo, there is a delay in the recruitment of circulating neutrophils to the site of chemically induced inflammation in 3BP2⁻/⁻ mice. Compared to wildtype neutrophils, 3BP2⁻/⁻ neutrophils fail to properly produce superoxide anion (O₂⁻) following fMLF stimulation. Defects in both directional migration and superoxide production of 3BP2⁻/⁻ neutrophils might contribute to the reduction in bacteria clearance and the increased mortality in 3BP2⁻/⁻ mice post Listeria Monocytogenes infection.

In Chapter 1 of this thesis, I have reviewed basic structures and functions of the domain modules found in adapter proteins. In addition, I have reviewed the findings
from numerous reports on the function of 3BP2 in different cell types. A discussion of the physical appearance and some of the initial characterization of 3BP2-deficient mice (3BP2^{-/-}) we have generated in our laboratory are included in Chapter 1. The second part of Chapter 1 consists of an introduction on B cell receptor signaling pathway and B-cell development and activation. A discussion of G protein-coupled receptor-mediated neutrophil functions can also be found in Chapter 1.

Chapter 2 contains all the methods and materials used in my study.

Chapter 3 includes the characterization of peripheral B cell compartment of 3BP2^{-/-} mice as well as the role of 3BP2 downstream of B-cell antigen receptor and in T-independent immune response.

In chapter 4, I present data from experiments designed to examine the role of 3BP2 downstream of a G protein-coupled receptor, fMLF receptor, of neutrophils. I also show the requirement of 3BP2 in the clearance of Listeria Monocytogenes.

In chapter 5, I propose two models for 3BP2 action based on the findings in B cells and neutrophils and discuss future areas for investigation.
ACKNOWLEDGMENTS

I would like to thank the many people who made this work possible.

Dr. Robert Rottapel, my supervisor, for his guidance and insights. Thank you for inspiring me to become a good scientist, always reminding me to think about the “big picture” and your encouragement when dealing with negative data and all hurdles during my time in the lab.

Thanks to Drs. Michael Ratcliff, Pamela Ohashi and Wen-Chen Yeh, my committee members, for all your support and advice. Thanks to my dearest family and friends for your love and support through the good times and rough patches over the last six years.

Thanks to our laboratory members: Jose La Rose for being the “lab father” taking care of everything in the lab from ordering reagents to training new students and for putting up with me for the past six years. Drs. Liliana Clemenza Ioannis Dimitrious, Melissa Greeve, Andy Scotter, Paul Daniel Simoncic, and Oleksandr Voytyuk for your support and advice. All the students, Andrea Brunet, Jane Cullis, Noah Fine, Lisa Gabrielli, Fiona Guerra and Bryan Kim, for making the lab a fun place to work and more importantly, for tolerating my moodiness. And last but not least, Donna De Francesco for handling all my tedious administrative matters.

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# TABLE OF CONTENTS

List of Figures

List of Table

List of abbreviation

<table>
<thead>
<tr>
<th>Chapter 1</th>
<th>Introduction</th>
<th>p.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Adapter Protein</td>
<td>p.2</td>
</tr>
<tr>
<td>1.1.1</td>
<td>Domain structure and its preferred binding ligand</td>
<td></td>
</tr>
<tr>
<td>a.</td>
<td>SH2 domain</td>
<td>p.3</td>
</tr>
<tr>
<td>b.</td>
<td>SH3 domain</td>
<td>p.4</td>
</tr>
<tr>
<td>c.</td>
<td>PH domain</td>
<td>p.5</td>
</tr>
<tr>
<td>1.1.2</td>
<td>Function of SH2, SH3 and PH domain</td>
<td></td>
</tr>
<tr>
<td>a.</td>
<td>Subcellular localization</td>
<td>p.7</td>
</tr>
<tr>
<td>b.</td>
<td>Regulation of enzymatic activities</td>
<td>p.9</td>
</tr>
<tr>
<td>1.2</td>
<td>Adapter protein: SH3-domain binding protein-2 (3BP2)</td>
<td>p.10</td>
</tr>
<tr>
<td>1.2.1</td>
<td>T cell</td>
<td>p.11</td>
</tr>
<tr>
<td>1.2.2</td>
<td>B cell</td>
<td>p.15</td>
</tr>
<tr>
<td>1.2.3</td>
<td>Natural Killer (NK) cell</td>
<td>p.16</td>
</tr>
<tr>
<td>1.2.4</td>
<td>Mast cell</td>
<td>p.18</td>
</tr>
<tr>
<td>1.2.5</td>
<td>Osteoclast</td>
<td>p.20</td>
</tr>
</tbody>
</table>
1.3 3BP2-deficient mice  p.24

1.4 B cell receptor (BCR) signaling and B-cell development and activation

1.4.1 BCR and coreceptor

a. Protein tyrosine kinase  p.27
b. Lipid metabolizing enzymes: PLC-γ and PI3K  p.29
c. Guanine nucleotide exchange factor: Vav  p.30
d. Adapter protein: BLNK  p.32
e. Membrane-anchored coreceptor of BCR: CD19  p.33

1.4.2 B cell development  p.35

a. BCR signal strength and additional non-BCR signaling  p.37
b. Chemokine and integrin signals: migration and retention  p.42

1.4.3 B cell activation  p.44

a. Thymus-dependent (TD) humoral response  p.45
b. Thymus-independent (TI) humoral response  p.48

1.5 G protein-coupled receptor signaling and neutrophil function

1.5.1 Neutrophil and G protein-coupled receptor (GPCR)  p.54

1.5.2 GPCR-mediated neutrophil function

a. Chemotaxis  p.56
b. Respiratory burst  p.62

Chapter 2  Methods and Materials  p.67

Chapter 3  The 3BP2 Adapter Protein is Required for Optimal B cell Activation and Thymus-Independent Type 2 Humoral Response  p.83

3.1  Introduction  p.84

3.2  Results

3.2.1  3BP2−/− mice exhibit increased marginal zone B cells and decreased peritoneal CD5+B-1 B cells  p.85

3.2.2  Impaired TI-2 response in 3BP2−/− mice  p.91

3.2.3  Impaired proliferation and survival of 3BP2−/− splenic B cells  p.93

3.2.4  Impaired survival of 3BP2−/− MZ B cells  p.96

3.2.5  The 3BP2 signaling complex  p.100

3.3  Discussion  p.106

Chapter 4  The 3BP2 Adapter Protein is Required for Optimal Activation of Neutrophils in Response to Chemoattractants  p.110

4.1  Introduction  p.111

4.2  Results

4.2.1  3BP2−/− neutrophils are defective in chemotaxis  p.113

4.2.2  Reduced recruitment of neutrophils to the site of inflammation in 3BP2−/− mice  p.115

4.2.3  3BP2 deficiency results in a partial reduction in ROS production in neutrophils  p.117
4.2.4 Increased susceptibility of 3BP2/− mice to challenge with *Listeria monocytogenes* p.120

4.2.5 3BP2 is required for full activation of Src PTK, Vav, Rac2, Cdc42, and Erk MAPK in response to fMLF stimulation p.122

4.2.6 3BP2 forms a complex with Gβγ subunits p.127

4.3 Discussion p.129

Chapter 5 Concluding Remarks p.135

5.1 Overview p.136

5.2 Positive regulatory role of 3BP2

5.2.1 Release of intramolecular autoinhibition p.137

5.2.2 Organizing the assembly of catalytic enzymes and their substrates p.138

References p.144
LIST OF FIGURES

Chapter 1

Figure 1-1. Schematic model of 3BP2 interactions with various proteins. p.23
Figure 1-2. Generation of 3BP2<sup>−/−</sup> mice. p.25
Figure 1-3. The expression of 3BP2 transcript in normal mouse tissues. p.26
Figure 1-4. Development of conventional B cells and B-1 B cells. p.38
Figure 1-5. Localization of signaling events in a chemotaxing cell. p.61
Figure 1-6. NADPH oxidase complex. p.65

Chapter 3

Figure 3-1. 3BP2<sup>−/−</sup> mice have normal B-cell development. p.86
Figure 3-2. 3BP2<sup>−/−</sup> mice have increased marginal zone B cells. p.87
Figure 3-3. 3BP2<sup>−/−</sup> mice have increased marginal zone B cells, decreased peritoneal B-1 B cells, and reduced basal serum IgG1 ad IgA levels. p.89
Figure 3-4. 3BP2<sup>−/−</sup> mice have no detectable T-cell development abnormalities within the thymus, spleen or lymph node. p.90
Figure 3-5. Humoral response in 3BP2<sup>−/−</sup> mice. p.92
Figure 3-6. The BCR-mediated proliferation and cell viability of 3BP2<sup>−/−</sup> resting B cells are reduced. p.94
Figure 3-7. 3BP2<sup>−/−</sup> splenic B cells have accelerated apoptosis with enhanced activation of caspase-3. p.95
Figure 3-8. The cell viability of 3BP2<sup>−/−</sup> MZ B cells is reduced after BCR cross-linking but is unaffected following anti-IgM+anti-CD40, LPS and rBAFF stimulation. p.97
Figure 3-9. 3BP2−/− MZ B cells have enhanced activation of caspase-3 and fail to properly up-regulate anti-apoptotic proteins Bcl-xL and Bcl-2 following BCR cross-linking. p.99

Figure 3-10. The 3BP2 signaling complex. p.101

Figure 3-11. 3BP2−/− splenic and MZ B cells have reduced intracellular calcium concentration compared to those of 3BP2+/+ cells in response to BCR cross-linking. p.103

Figure 3-12. 3BP2 forms an inducible complex with CD19 via SH2-phosphotyrosine interaction following BCR cross-linking. p.105

Chapter 4

Figure 4-1. 3BP2 is required for neutrophil chemotaxis. p.114

Figure 4-2. 3BP2 is required for spatially-restricted F-actin assembly in chemotaxing neutrophils. p.116

Figure 4-3. Acute inflammatory response in 3BP2+/+ and 3BP2−/− mice. p.118

Figure 4-4. Effect of 3BP2 deficiency on superoxide anion production. p.119

Figure 4-5. 3BP2 is critical for the clearance of Listeria monocytogenes infection. p.121

Figure 4-6. Impaired Src activation in 3BP2−/− osteoclasts (OCs). p.123

Figure 4-7. 3BP2 is required for the full activation of Src and Vav1. p.124

Figure 4-8. 3BP2 is required for the full activation of Cdc42, Rac2 and Erk1/2 MAPK. p.126

Figure 4-9. 3BP2 forms a complex with Gβ subunit. p.128

Chapter 5

Figure 5-1. Positive role of 3BP2: Release of intramolecular autoinhibition p.139

Figure 5-2. Far-Western blots on mouse 3BP2 peptide arrays using GST-SH3 construct of Src. p.140
Figure 5-3. Positive role of 3BP2: Organizing the assembly of catalytic enzymes and their substrates
LIST OF TABLE

Chapter 1

Table 1-1. Knockout mice with alterations in peripheral B cell subsets. p.43
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbr.</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>βARK</td>
<td>β-adrenergic receptor kinase</td>
</tr>
<tr>
<td>3BP1</td>
<td>SH3 binding protein-1</td>
</tr>
<tr>
<td>3BP2</td>
<td>SH3 binding protein-2</td>
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<td>3BP2&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>3BP2-deficient</td>
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<td>Ab</td>
<td>antibody</td>
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<td>Ag(s)</td>
<td>antigen(s)</td>
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<td>APC(s)</td>
<td>antigen-presenting cell(s)</td>
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<td>APRIL</td>
<td>a proliferation-inducing ligand</td>
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<tr>
<td>Arg</td>
<td>arginine</td>
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<td>BAFF</td>
<td>B-cell-activating factor of TNF family</td>
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<tr>
<td>BCR</td>
<td>B-cell receptor</td>
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<td>BCMA</td>
<td>B cell maturation antigen</td>
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<tr>
<td>BLC</td>
<td>B-lymphocyte chemoattractant</td>
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<tr>
<td>BLNK</td>
<td>B-cell linker</td>
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<tr>
<td>BM</td>
<td>bone morrow</td>
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<tr>
<td>Btk</td>
<td>Bruton’s tyrosine kinase</td>
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<td>CD40L</td>
<td>CD40 ligand</td>
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<tr>
<td>CGD</td>
<td>chronic granulomatous disease</td>
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<td>CSF-1</td>
<td>colony-stimulating factor-1</td>
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<td>CSR</td>
<td>class switch recombination</td>
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<td>Abbreviation</td>
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<td>CXCR5</td>
<td>C-X-C receptor 5</td>
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<td>DAG</td>
<td>diacylglycerol</td>
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<td>dendritic cell(s)</td>
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<td>DH</td>
<td>Dbl-homology</td>
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<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
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<tr>
<td>Erk</td>
<td>extracellular signal-regulated kinase</td>
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<td>FcεRI</td>
<td>Fc epsilon receptor I</td>
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<td>FcR</td>
<td>Fc receptor</td>
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<tr>
<td>FDC(s)</td>
<td>follicular dendritic cell(s)</td>
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<tr>
<td>fMLF</td>
<td>N-formyl-methionine-leucine-phenylalanine</td>
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<td>FO</td>
<td>follicular</td>
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<td>FPR</td>
<td>formyl peptide receptor</td>
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<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
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<td>GC</td>
<td>germinal center</td>
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<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
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<td>glycosphingolipid-enriched membrane microdomain(s)</td>
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<td>GPCR</td>
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<td>Grb2</td>
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<td>GST</td>
<td>glutathione-S-transferase</td>
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<tr>
<td>HA</td>
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<td>Ig(s)</td>
<td>immunoglobulin(s)</td>
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<td>IgM-IC</td>
<td>IgM-containing immune complex</td>
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<tr>
<td>IL-2</td>
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<tr>
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<td>i.p.</td>
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<td>IP$_3$</td>
<td>inositol-1,4,5-triphosphate</td>
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<tr>
<td>ITAM(s)</td>
<td>immunoreceptor tyrosine-based activation motif(s)</td>
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<tr>
<td>i.v.</td>
<td>intravenous(ly)</td>
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<tr>
<td>JNK</td>
<td>Jun N-terminal kinase</td>
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<tr>
<td>LAT</td>
<td>linker for activation of T cells</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
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<tr>
<td>MZ</td>
<td>marginal zone</td>
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<td>NFAT</td>
<td>nuclear factor of activated T cells</td>
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<tr>
<td>NK</td>
<td>natural killer</td>
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<td>OC(s)</td>
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<td>p160-ROCK</td>
<td>Rho-associated coiled coil-containing kinase</td>
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<td>PH</td>
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<td>PI3K</td>
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<td>PIP$_2$</td>
<td>phosphatidylinositol-4,5-bisphosphate</td>
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<td>PKC</td>
<td>protein kinase C</td>
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PLC-β, -γ, -δ. phospholipase C-β, -γ, -δ
PMA phorbol esters
Pro-rich proline-rich
PTK protein tyrosine kinase
pTyr phospho-tyrosine
Pyk-2 protein tyrosine kinase- 2
RANKL receptor activator of NFκB ligand
ROS reactive oxygen species
S1P sphingosine 1-phosphate
SAM sterile alpha motif
SAP SLAM-associated protein
SDF-1 stroma-derived factor-1
SH2 Src-homology-2
SH3 Src-homology-3
siRNA short interfering RNA
SLC secondary lymphoid tissue chemokine
SLP-76 SH2 domain-containing leukocyte phosphoprotein of 76 kDa
SOCS suppressor of cytokine signaling
SOD superoxide dismutase
SOS Son of Sevenless
T1 transitional type 1
T2 transitional type 2
<table>
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<td>tumor necrosis factor</td>
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<td>trinitrophenyl-lipopolysaccharide</td>
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<td>TRAP</td>
<td>tartrate-resistant acid phosphatase</td>
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<td>Trp</td>
<td>tryptophan</td>
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<tr>
<td>WW</td>
<td>tryptophan-tryptophan</td>
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CHAPTER 1

Introduction

• Jose La Rose and Dr. Wen-Chen Yeh generated the 3BP2-deficient mice.
• The experiments in Figure 1-2B, C and D were performed by Jose La Rose and the experiment in Figure 1-2E was performed by me.
• Expression of 3BP2 mRNA from different tissues (Figure 1-3A) was obtained from Genomics Institute of Novartis Research foundation. The experiment in Figure 1-3B was performed by Jose La Rose.
1.1 Adapter proteins

Cellular response to extracellular stimuli usually starts with binding of extracellular signaling molecules (or ligands) to cell-surface receptors. Ligand binding initiates intracellular biochemical pathways leading to a cellular response, which can be cell cycle progression, cytoskeletal reorganization, cell migration or cell survival. These intracellular signaling pathways are governed by post-translational protein modifications (e.g. phosphorylation), subcellular relocalization of proteins and complex formation of activators and their specific substrates. An emerging class of proteins that are a major contributors to these processes are adapter proteins. Although adapter proteins themselves do not contain any enzymatic or other direct effector function, their ability to nucleate the formation of protein complexes make them indispensable in regulating cell signaling in a spatial and temporal fashion.

From a functional point of view, adapter proteins may be divided into those exerting positive regulatory functions and those possessing negative regulatory capacities. Thus, adapter proteins are best known to be able to translate the whole plethora of externally applied signals into an appropriate cellular response by dynamically modulating protein-protein, and protein-lipid interactions.

Adapter proteins contain a variety of conserved protein modules. Protein modules usually contain sequences of 50-100 amino acids in length. Some of the examples of protein modules are sterile alpha motif (SAM), PDZ, tryptophan-tryptophan (WW), FYVE, Src-homology-2 (SH2), Src-homology-3 (SH3) and pleckstrin homology (PH) domains. Each of these domains can fold into a compact and functional
module independently of surrounding sequences. This section of the thesis will focus on three protein modules, SH2, SH3 and PH domains. The preferred binding partner and the function of each of these domains will be discussed.

1.1.1 Domain Structure and its preferred binding ligand

(a) SH2 domain

SH2 domains bind to phospho-tyrosine (pTyr)-containing sites on activated receptors and cytoplasmic phosphoproteins in vivo\(^{11,12}\). SH2 domains bind to phosphopeptides of optimal sequence with relatively high affinity (\(K_d = 10\text{-}100\text{nM}\))\(^{13\text{-}15}\) and were believed to have no binding affinity for unphosphorylated peptides\(^{13}\). However, several studies demonstrated the capacity of SH2 domains to interact with their ligands in a pTyr-independent manner. Poy et al. showed that the SH2 domain of a small adapter protein SLAM-associated protein (SAP) binds to phosphorylated and nonphosphorylated lymphocyte coreceptor SLAM in a similar mode, with the Tyr or pTyr residue inserted into the pTyr-binding pocket\(^{16}\) of SAP. Our laboratory demonstrated a pTyr-independent binding between the SH2 domain of suppressor of cytokine signaling (SOCS)-1 and the Vav guanine nucleotide exchange factor (GEF) which targets Vav for ubiquitin-mediated protein degradation\(^{17}\).

The surrounding sequences of the pTyr residue influence the binding affinity between the SH2 domain and pTyr-containing peptide therefore determining the binding specificity of any given SH2 domain. The phosphopeptide binding site is bipartite\(^{18,19}\) composed of a conserved pocket that binds to pTyr residue and a more variable binding
surface that allows specific recognition of the amino acids immediately C-terminal to the pTyr.

The conserved pTyr-binding pocket of the SH2 domain lined by basic residues contains the only invariant SH2 residue, an arginine (Arg) which forms hydrogen bonds with two-pTyr phosphate oxygen atoms. The SH2 ligand binding specificity is largely determined by the three amino acid residues immediately C-terminal to the pTyr, pTyr-X-X-X, where X-X-X are amino acid residues that vary for different SH2s. For example, using the systematic approach for determining the optimal peptide ligand for specific SH2 domain, Songyang et al. has identified pTyr-Glu-Asn-X (where X can be any amino acid) as the optimal binding motif for adapter protein SH3-domain-binding protein-2 (3BP2) whereas SH2 domain of Vav GEF preferentially binds to motif pTyr-Met-Glu-Pro. Such bipartite organization of the SH2 ligand-binding site allows tyrosine phosphorylation to function as an all-or-none switch for SH2 binding while enabling the sequence context of the pTyr site to dictate which SH2 domains are bound.

(b) SH3 domain

Initial identification of an SH3 binding partner was achieved by screening a cDNA expression library with the Abl SH3 domain; during which two SH3-binding proteins, SH3-domain-binding protein-1 (3BP1) and 3BP2, were identified. Following the initial screening, Ren et al. mapped an approximate 10-amino acid proline (Pro)-rich sequence on 3BP1 and 3BP2 to be the location of the possible SH3 binding region. Since then, a number of in vivo SH3 ligands have been identified, and the binding sites have similarly been mapped to Pro-rich sequences. It is now commonly accepted
that the SH3 domain preferentially binds to Pro-rich peptide stretch and all high affinity SH3 ligands identified so far contain a PXXP (where X can be any amino acid) motif. Despite the low degree of sequence similarity, the overall topology of the SH3 structure is well conserved among the already identified SH3-containing proteins. The basic fold of the SH3 domain consists of five or six antiparallel β strands that pack to form two perpendicular β sheets. A hydrophobic patch that contains a cluster of conserved aromatic residues and is surrounded by two charged and variable loops forms the ligand-binding pocket of SH3 domain\textsuperscript{26}. It is believed that the two critical Pro residues in the PXXP motif directly pack against the SH3-containing protein, lodging themselves into a host of conserved, mostly aromatic residues; whereas several poorly conserved residues in the variable loops surrounding the hydrophobic binding surface of the SH3 domain are likely to be involved in determining binding specificity\textsuperscript{26,27}. The binding specificity is evident in that Abl SH3 domain and Src SH3 domain have highest binding affinity only to their specific ligands\textsuperscript{28}.

(c) PH domain

PH domain was first identified at the N- and C-termini of pleckstrin, a major protein kinase C (PKC) substrate in platelets\textsuperscript{29,30}. There is a low sequence homology among PH domains; however, alignment of PH domain sequences from different PH-domain containing proteins showed that all PH domains can be defined by six weakly conserved sequence blocks (i.e. 6 subdomains). Within the subdomain 6 (C-terminal α-helix), a single tryptophan (Trp) residue is conserved in all characterized PH domain sequences\textsuperscript{31}. Despite the low sequence homology, all PH domains share a similar
structural fold. Three-dimensional structures of PH domains have been determined\textsuperscript{32-35}. The core structure of PH domain consists of a \( \beta \)-barrel of seven antiparallel \( \beta \)-sheets and a long C-terminal \( \alpha \) helix\textsuperscript{32}. Ligands of PH domains range from proteins to phosphoinositides making PH domain-containing proteins capable of mediating protein-protein and protein-lipid interactions. Many of the PH domain-containing proteins including \( \beta \)-adrenergic receptor kinase (\( \beta \)ARK) and Bruton’s tyrosine kinase (Btk) have been shown to interact with the \( \beta \gamma \) subunits of G proteins\textsuperscript{36,37}. The C-terminal \( \alpha \)-helix region including the most conserved Trp residue of PH domains mediates the binding between the PH domain and the \( \beta \gamma \) subunits of G proteins (\( G\beta\gamma \))\textsuperscript{38}.

Harlan et al.\textsuperscript{39} first demonstrated that PH domains from pleckstrin and several other proteins can bind to lipid vesicles containing the phosphoinositide phosphatidylinositol-4,5-bisphosphate (PIP\(_2\)). Different from the \( G\beta\gamma \)-binding site, the site that mediates the association between PH domain and PIP\(_2\) is located in the cleft of the N-terminal \( \beta \)-barrel\textsuperscript{39}. It was later demonstrated using the \( \beta \)ARK PH domain that the conserved Trp residue is not only critical for \( G\beta\gamma \) binding but is also required for stabilization of the PIP\(_2\)-binding cleft in the N-terminus of the PH domain\textsuperscript{40}. Further analysis of the binding property between various PH domains and phosphoinositides showed that some PH domains can bind to both PIP\(_2\) and phosphatidylinositol-3,4,5-triphosphate (PIP\(_3\))\textsuperscript{41,42}. In a survey of PH domains, most were found to bind phosphoinositides with high affinity but low selectivity\textsuperscript{43} with the exception of the PH domain of phospholipase C-\( \delta \) (PLC-\( \delta \)) and the PH domain of the ARF protein exchange factor GRP1, which bind PIP\(_2\) and PIP\(_3\), respectively, with high affinity and selectivity\textsuperscript{43}. 
1.1.2  Function of SH2, SH3 and PH domains

(a) Subcellular localization

Signal propagation usually involves post-translational protein modifications such as tyrosine or serine/threonine phosphorylation, myristoylation or ADP-ribosylation. These processes are carried out by enzymes with specificity to particular substrates and are tightly regulated. In addition to directly activating or inhibiting the effector function of the enzyme, compartmentalization of an enzyme and its substrates in different cellular subregions is a useful mechanism to control signal propagation. Therefore, subcellular redistribution of proteins followed by the formation of an enzyme-substrate complex can be seen after stimulating cells with growth factors or specific receptor ligands. The redistribution of these proteins and protein complex formation are made possible by the defined protein modules SH2, SH3 and PH domains.

Through recognition of polyphosphoinositides, PH domain-mediated subcellular localization usually involves membrane targeting of cytoplasmic proteins. The PH domain at the N-terminus of PLC-δ₁ was the first shown to recognize a specific phosphoinositide ligand. The isolated PH domain from PLC-δ₁ binds strongly and specifically to both PIP₂ and its soluble headgroup, inositol-1,4,5-triphosphate (IP₃) and it is sufficient and necessary to target the host protein to the plasma membrane in vivo. In addition, several studies demonstrated that the high affinity binding between PIP₂ and the PH domain of PLC-δ₁ enhances the enzymatic activity of PLC-δ₁. It is believed that the PH domain not only mediates membrane recruitment of PLC-δ₁ and localizes PLC-δ₁ to its substrates but also influences its activation state.
An example of the SH2 domain-mediated recruitment of an effector molecule can be seen during activation and autophosphorylation of the epidermal growth factor receptor (EGFR) \(^{21}\). Tyrosine phosphorylation of EGFR following ligand binding creates the SH2 domain-binding site for an adaptor molecule growth factor receptor-bound protein 2 (Grb2) which constitutively associates with the GEF Son of Sevenless (SOS) in the cytoplasm. Recruitment of Grb2 to the membrane through pTyr-SH2 interaction brings its binding partner SOS to the membrane where the substrate of SOS, Ras GTPase, is located. The intrinsic catalytic activity of SOS in activated or quiescent cells is indistinguishable; therefore, in EGF-treated cells, Ras activation is due to the increased local concentration of SOS at the membrane \(^{21}\).

The SH3 domain has also been shown to be involved in subcellular relocation and complex formation of cytoplasmic proteins at the cell membrane. For example, formation of the NADPH oxidase system of phagocytic cells such as neutrophils depends on SH3 action \(^{52}\). A detailed description of NADPH oxidase complex can be found in section 1.5.2b (also see Figure 1-6) of this thesis. Briefly, a functional NADPH oxidase system contains two transmembrane proteins, gp91\(^{phox}\) and p22\(^{phox}\), three cytoplasmic SH3-domain-containing proteins, p47\(^{phox}\), p67\(^{phox}\), and p40\(^{phox}\), and a small GTP-binding protein, Rac. Subunits p47\(^{phox}\) and p67\(^{phox}\) inducibly translocate to the membrane and associate with membrane subunit p22\(^{phox}\) following stimulation. The SH3 domains of p47\(^{phox}\) and p67\(^{phox}\) have been shown to be responsible for the redistribution of these two subunits and the assembly of the functional oxidase \(^{25,53,54}\). In addition, Dinauer and coworkers demonstrated the requirement of SH3 domain-mediated binding in the formation of a functional multicomponent NADPH oxidase system. They have presented
evidence showing that a missense mutation in the Pro-rich region of p22\textsubscript{phox}, which possibly prevents the binding of p47\textsubscript{phox} SH3 domain to p22\textsubscript{phox}, is associated with a nonfunctional NADPH oxidase and chronic granulomatous disease (CGD) \textsuperscript{55}.

(b) Regulation of enzymatic activities

Domain modules also function in regulating enzymatic activities of certain proteins. Through intramolecular binding between these domains and their binding ligands such as pTyr-SH2 and Pro-rich-SH3 associations, these domains negatively regulate the enzymatic activities either by blocking the access of the catalytic domain to its substrate or by keeping the catalytic domain in a closed, inactive conformation. Examples of such intramolecular inhibition can be seen in protein tyrosine kinases (PTKs) such as Src and Abl. In the inactive form, a phosphorylated tyrosine in the C-terminal tail of the Src protein binds to the protein’s own SH2 domain \textsuperscript{56}. The pTyr-SH2 binding triggers the association of the SH3 domain with sequences in the linker region that lies between the SH2 domain and the catalytic domain, which in turn keeps the catalytic domain to adopt a conformation incompatible with catalysis \textsuperscript{57,58}. Both SH2 and SH3 domains play a central role in regulating Src PTK catalytic activity by keeping the Src kinase domain in a closed, inactive conformation \textsuperscript{57-59}. The regulation of Abl PTK appears to be more complicated than that of Src PTK and involves inputs from more than its SH2 and SH3 domains. Earlier work on Abl regulation demonstrated that Abl is activated by mutations in the SH3 domain that interfere with its ligand binding, suggesting that Abl-SH3 mediates negative regulation of Abl \textsuperscript{60} by either interaction with inhibitory proteins \textsuperscript{61,62} or an intramolecular autoinhibitory mechanism. More recent
studies suggested that the N-terminal myristate-attached cap domain of Abl binds to the C-terminal lobe of the catalytic domain and locks Abl in an inactive state, indicating its involvement in Abl autoinhibition. Although the mechanisms for the autoinhibition of Src and Abl catalytic activities are different, ligand binding to their SH2 and SH3 domains shares a common influence on their activation state. Studies have shown that activation of Src and Abl can occur when high-affinity ligand for their SH3 and/or SH2 domains break the intramolecular contacts. Therefore, the SH3 and/or SH2 ligand-containing binding partners of these PTKs are sometimes viewed as a part of the multilevel regulation for Src and Abl enzymatic activity.

### 1.2 The adapter protein: SH3 binding protein-2 (3BP2)

As described in section 1.1.2b, one of the functions of the protein modules SH3 and SH2 domains is the regulation of enzymatic activities. Catalytic activities of PTKs such as Src and Abl can be modulated by their SH3 and/or SH2 domains through autoinhibitory mechanisms and/or association with SH3/SH2 ligand-containing proteins. In order to further delineate the regulatory mechanisms of these PTKs, a search for SH3 and SH2 ligand-containing binding partners of these PTKs has been conducted. Baltimore and coworkers isolated two proteins that bind specifically to the SH3 domain of Abl in a cDNA expression library screen with a fusion protein containing glutathione-S-transferase (GST) fused to the SH3 domain of Abl. Two proteins identified in the screen were named 3BP1 and 3BP2 for SH3 binding protein-1 and -2, respectively. Despite having a similar amino acid sequence in a region containing the SH3 binding
site, there is no overall similarity in both amino acid sequence and function of 3BP1 and 3BP2. While 3BP1 was later identified to display GTPase activating protein (GAP) activity specifically for the small GTPase Rac, 3BP2 was recognized as a PH domain- and SH2 domain-containing adapter protein. In addition to its potential binding to Abl, 3BP2 was found to interact with other signaling molecules including Zap-70, LAT, PLCγ1, Grb2, Cbl, and Fyn in Jurkat T cells and Vav1, Vav2, PLCγ and Syk in Daudi B cells. The function of 3BP2 has been studied extensively by overexpressing 3BP2 in different cell lines. In these studies, 3BP2 has been implicated as a positive regulatory adapter molecule coupled to immunoreceptor on various cell lines including T cells, B cells, NK cells and mast cells. More recently, a role of 3BP2 in osteoclast function has been demonstrated using 3BP2 mutant knock-in mice.

1.2.1 T cell

T cell receptor (TCR) recognizes antigens (Ags) bound to major histocompatibility complex (MHC) molecules found on the antigen presenting cells (APCs). When a T cell interacts with an APC, a specialized structure known as an immunological synapse forms at the point of cell-cell contact. The immunological synapse is composed of a central cluster of TCRs/Ags/MHC surrounded by a peripheral ring of adhesion molecules. TCR is a multisubunit complex that consists of the clonotype-specific α/β heterodimers noncovalently associated with the invariant CD3-γ, -δ, -ε and -ζ chains. The intracellular portion of the CD3-γ, -δ, -ε and -ζ subunits contain copies of immunoreceptor tyrosine-based activation motifs (ITAMs). The engagement of the TCR with MHC-Ag complexes on APCs leads to the activation of proximal PTKs.
including members of the Src, ZAP-70/Syk, Tec and Csk families. Activation of these
PTKs results in the tyrosine phosphorylation of ITAMs and multiple membrane-bound
and cytosolic proteins 78 such as phospholipase C-γ (PLC-γ), linker for activation of T
cells (LAT), SH2 domain-containing leukocyte protein of 76 kDa (SLP-76) and Vav
GEFs. All of these proteins contribute to the optimal TCR-induced calcium mobilization
which results in an increase in intracellular calcium concentration ([Ca^{2+}]) of the
activated T cells 79-82. An increase in [Ca^{2+}] is critical for nuclear factor of activated T
cells (NFAT) to translocate to the nucleus where it interacts with activator protein-1 (AP-1) and other transcriptional partners to promote gene transcription 83.

Several adapter proteins such as LAT 84,85 and SLP-76 86,87 have already been
shown to play an essential role in the TCR signal transduction. Deckert et al. identified
3BP2 as an important adapter in the assembly of antigen receptor-induced signaling
complexes leading to gene transcription in the Jurkat T cell line 67. Since engagement of
the TCR induces the accumulation of activated signaling molecules in the lipid rafts, the
intracellular localization of 3BP2 in T cells was examined. Both endogenous 3BP2 88
and ectopically expressed NH2-terminal hemagglutinin (HA) epitope-tagged 3BP2 67
were shown to redistribute from cytoplasm to membrane following TCR stimulation.
The Pro-rich region of 3BP2 was shown to mediate the translocation of 3BP2 to the lipid
raft. In addition, it was shown in Jurkat T cells that the endogenous 3BP2 is inducibly
tyrosine phosphorylated at sites Tyr174, Try183, and Tyr446 in response to TCR engagement
88 (Figure 1-1). The stimulus-dependent membrane translocation and tyrosine
phosphorylation of 3BP2 implied a role of 3BP2 as a cytoplasmic adapter protein that
potentially modulates TCR. Studies using Jurkat T cells that involved either transient
3BP2 overexpression\textsuperscript{67} or suppression of endogenous 3BP2 with short interfering RNA (siRNA)\textsuperscript{88} demonstrated a positive regulatory role of 3BP2 in TCR-mediated transcriptional activation of the interleukin-2 (IL-2) gene promoter and its NFAT or AP-1 elements. The SH2 domain and the putative phosphorylation sites Tyr\textsuperscript{183} and Tyr\textsuperscript{446} of 3BP2 are required for this positive regulatory role as the SH2-truncated mutant of 3BP2 or a mutant with substitution of either Tyr residues exhibited reduced ability to activate NFAT, AP-1 or the IL-2 promoter\textsuperscript{67,88}. The SH2 domain of 3BP2 was found to associate with Syk PTK in yeast and mammalian two-hybrid systems and with Tyr phosphorylated Zap-70, an essential signaling molecule for T cell development and activation\textsuperscript{89-92}, in a pull-down assay using a glutathione S-transferase (GST)-3BP2-SH2 fusion protein. Since overexpression of 3BP2 in a Jurkat variant cell line that lacks Syk/Zap-70\textsuperscript{93} no longer induces NFAT activation, it was proposed that the complex formation between 3BP2 and Syk/Zap-70 through the 3BP2-SH2 domain allows functional cooperation between the interacting molecules in enhancing TCR-dependent signals leading to activation of the IL-2 promoter\textsuperscript{67}. It was later demonstrated that the associated Zap-70 phosphorylates Tyr\textsuperscript{446} of 3BP2 which mediates the recruitment of the Src family PTK, Lck. Lck is involved in phosphorylation of Tyr\textsuperscript{183} of 3BP2 which then becomes the docking site for GEF Vav1 recruitment\textsuperscript{88} (Figure 1-1). These results suggested that 3BP2 is a critical scaffold protein that organizes the assembly of Src family kinase, Syk, Zap-70 and Vav1 required for TCR-mediated transcriptional activation of NFAT and the IL-2 promoter.

The direct interaction between 3BP2 and the chaperone protein 14-3-3 mediated by two serine residues (Ser\textsuperscript{225} and Ser\textsuperscript{277}) (Figure 1-1) of 3BP2 has been shown to
negatively regulate 3BP2-induced NFAT activation in lymphocytes. Foucault et al. provided evidence that 3BP2 interacts with 14-3-3 through a serine phosphorylation-dependent mechanism and that PKC is one of the serine/threonine kinases phosphorylating 3BP2 to facilitate this association. Mutation at Ser^277 abrogates the binding of 3BP2 to 14-3-3 and the mutant 3BP2 was shown to be a more potent activator of NFAT in both T and B cells. These results implicated a negative regulatory role of 14-3-3 on 3BP2 adapter function on the signaling pathways leading to NFAT transcriptional activities. Subsequent events leading to inhibition of 3BP2 function in the NFAT pathway by 14-3-3 require further investigation.

In addition to its positive influence on TCR-mediated cytokine synthesis, a more recent study demonstrated a potential role of 3BP2 in endocytic and cytoskeletal regulation in T cells. Le Bras et al. added two more potential interacting proteins of 3BP2 to a growing list of binding partners that have already been identified, an endocytic scaffold protein CIN85 and an actin-binding protein HIP-55. Yeast two-hybrid interaction analysis and biochemical assays revealed that 3BP2 directly interacts with the SH3 domains of either CIN85 or HIP-55, through different Pro-rich sequences (Figure 1-1). In addition, a significant fraction of 3BP2, CIN85 and HIP-55 were found to co-localize in the immunological synapse formed at the T cell and APC interface and in glycosphingolipid-enriched membrane microdomains (GEMs), a region where the initiation of signal transduction via immunoreceptors takes place. Both CIN85 and HIP-55 have been implicated in the process of ligand-dependent receptor internalization. CIN85 was found to be a part of the multimeric complex involved in receptor internalization and down-regulation of PTK signaling in different cell types.
HIP-55 was originally identified involving in signaling events connected to endocytic trafficking and cytoskeletal regulation\textsuperscript{100,101}. More recently, HIP-55 has been shown to redistribute to the T cell-APC synapse in an antigen-dependent manner and localize with endosomes where it may regulate TCR down-regulation\textsuperscript{96}. With a similar distribution and a direct association between 3BP2 and these proteins, it was proposed that 3BP2 may participate in the assembly of multimolecular complexes containing CIN85 and HIP-55 and regulate actin cytoskeleton-dependent endocytic trafficking in T cells.

1.2.2 B cell

3BP2, which is abundantly expressed in various B-cell lines including Daudi, BJAB, Raji and RPMI8866 cells, is rapidly and transiently Tyr phosphorylated, possibly by the Src family PTK Fyn and/or Syk following B cell receptor (BCR) aggregation\textsuperscript{68}. The high expression and inducible Tyr phosphorylation suggested a functional role of 3BP2 in B cells. Indeed, interfering 3BP2 expression in Raji B cells with siRNA resulted in a significant reduction of BCR-mediated NFAT activation which suggested that 3BP2 participates in BCR-mediated transcriptional activation. 3BP2 was found to constitutively interact with GEF Vav proteins through its Pro-rich region in B cells (Figure 1-1), and the interaction is enhanced following B cell activation. Since Vav proteins are important regulators of Rho GTPases and NFAT activities in lymphoid cells\textsuperscript{102,103}, 3BP2 might participate in BCR-mediated NFAT activation through its interaction with Vav. It was found that 3BP2 overexpression leads to an enhanced activation of Rho GTPase Rac1 and 3BP2-induced NFAT activation in B cells was blocked by GEF-inactive forms of Vav1 or Vav2, and by dominant-negative mutant forms of the GTPases.
RhoA, Rac1 and Cdc42. Therefore, it was suggested that 3BP2 acts upstream of Vav and Rho GTPases in the signaling pathways required for gene activation by BCR. These results established a positive regulatory role of 3BP2 in BCR functionality.

1.2.3 Natural Killer (NK) cell

One of the multiple functions of NK cells is the direct killing of virus-infected cells. Such NK cell-mediated cytotoxicity can be carried out by surface Fc receptors (FcRs) recognizing an antibody (Ab)-coated target cell. As a result of the FcR-Ab interaction, the NK cell activates proximal Src and Syk family PTKs, which in turn stimulate downstream molecules such as PLC-γ and the Rho family of GTP-binding proteins. Two adapter proteins, LAT and SLP-76, have previously been described to participate in NK cell-mediated cytotoxicity. Adding to the list, Jevremovic et al. demonstrated that 3BP2 is biochemically and functionally linked to the activating receptors on NK cells. Upon stimulation of NK cells through the FcR, 3BP2 is rapidly and transiently Tyr phosphorylated and associates with several Tyr-phosphorylated molecules including LAT, Vav-1 and PLC-γ. Whereas the SH2 domain of 3BP2 is required for optimal Tyr phosphorylation of 3BP2 and for its ability to associate with LAT following FcR cross-linking, the binding between 3BP2 and Vav proteins and PLC-γ is direct and is mediated by phosphorylated Tyr of 3BP2 (Figure 1-1). Functionally, overexpressing 3BP2 enhanced NK cell-mediated cytotoxicity suggesting a positive regulatory role of 3BP2 in this process. During the development of NK cell-mediated cytotoxicity, Tyr of 3BP2 mediates complex formation between 3BP2, Vav and PLC-γ which critically influences 3BP2-mediated enhancement of NK
cell cytotoxicity\textsuperscript{71}. These results have demonstrated 3BP2 as an active component of the activation cascade initiated by FcR ligation that leads to NK cell-mediated cytotoxicity.

NK cell-mediated cytotoxicity can also be activated by engagement of CD244 by antibody or by its ligand CD48\textsuperscript{115-118}. CD244 (also known as 2B4) is a member of the CD150 subfamily of the CD2 family of receptors\textsuperscript{119}. CD244 positively regulates NK cell-mediated cytotoxicity and IFN-\(\gamma\) production by activating an intracellular multiprotein signaling network that involves a small adapter protein SAP\textsuperscript{119,120}. SAP binds to Src family PTK Fyn and increases the phosphorylation status and activation of its associated proteins\textsuperscript{121,122}. CD244 function is related to SAP expression\textsuperscript{123,124}. In cells lacking SAP, CD244 is no longer phosphorylated following receptor ligation\textsuperscript{125}. In patients with SAP deficiency, which causes X-linked lymphoproliferative (XLP) syndrome, NK cells are defective in their activation through CD244\textsuperscript{126-128}.

In addition to SAP, 3BP2 has been shown to bind to CD244 and positively regulates its signaling strength. Saborit-Villarroya et al. demonstrated that 3BP2 colocalizes and specifically and directly interacts with CD244 in a phosphorylation-dependent manner in the human NK cell line (YT cell) and in primary human NK cells. 3BP2 is Tyr phosphorylated and forms a signaling complex with Vav-1 after stimulation of YT cells by CD244 engagement\textsuperscript{72}. Overexpression of 3BP2 enhances the overall protein Tyr phosphorylation of YT cells following CD244 ligation suggesting a positive regulatory role of 3BP2 in CD244 signaling. Among the molecules involved in CD244 signaling, Vav-1\textsuperscript{129,130}, PLC-\(\gamma\)\textsuperscript{131,132} and PKC-\(\delta\) (a downstream effector enzyme of PLC-\(\gamma\) activation)\textsuperscript{133} were found to be hyperphosphorylated as a result of 3BP2 overexpression\textsuperscript{73}. This suggested that 3BP2 acts upstream of and positively regulates CD244-dependent
activation of these proteins. Functionally, it was found that 3BP2 increases CD244-induced cytotoxicity of YT cells without affecting CD244-mediated IFN-γ production of these cells. 3BP2-dependent enhancement of CD244-mediated cytotoxicity was blocked by an inhibitor specific for PKC which indicated that 3BP2 enhances CD244-mediated lysis by increasing PKC activity. Saborit-Villarroya and coworkers proposed that in response to CD244 stimulation, 3BP2 is responsible for the recruitment and activation of essential signaling molecules such as Vav-1 and PLC-γ, and leads to activation of PKC-δ which in turn promotes CD244-mediated NK cell cytotoxicity \(^72,73\). Although 3BP2 interacts with human CD244 and positively mediates its activation status, it does not interact with murine CD244. The biological relevance of 3BP2 in murine CD244-dependent NK cell functions awaits further analysis.

1.2.4 Mast cell

Mast cells are best known for their role in allergy and anaphylaxis but they also play an important protective role in wound healing and defense against pathogens \(^134\). Aggregation of the high-affinity IgE receptor (FcεRI) on mast cells mediates the allergic response by the release of inflammatory mediators through a process known as degranulation and by production of cytokines and arachidonic acid metabolites \(^135\). FcεRI is a tetrameric receptor complex consists of one α (FcεRIα), one β (FcεRIβ) and two γ chains (FcεRIγ) \(^136\). The FcεRIα is responsible for binding to IgE. The FcεRIβ and FcεRIγ possess ITAMs \(^136,137\) within their cytoplasmic domains. The cross-linking of FcεRI with IgE-coated Ag initiates a signal cascade through the Tyr phosphorylation of ITAMs by the Src family PTK Lyn \(^135,138\). This leads to recruitment of Syk to the
phospho-ITAMs of FceRIγ where it is activated to phosphorylate various substrates in the downstream cascade. Another non-receptor PTK, Btk, is membrane targeted by PIP3 through its PH domain following aggregation of FceRI. Syk and Btk contribute to Tyr phosphorylation and activation of PLC-γ and calcium mobilization. Mobilization of calcium leads to elevation of \([\text{Ca}^{2+}]_i\) which is critical for FceRI-mediated degranulation.

Several adapter proteins have been implicated in FceRI signaling. Mice deficient in the adapter proteins LAT, SLP-76 or Grb2 were resistant to IgE-mediated systemic anaphylaxis. All these adapter proteins have been demonstrated to be essential in FceRI-mediated degranulation and/or cytokine production. Using the rat basophilic leukemia RBL-2H3 mast cell line, Sada et al. demonstrated the expression of 3BP2 in mast cells and functional importance of the 3BP2-SH2 domain in FceRI-mediated signal transduction. The FceRI aggregation-induced Tyr phosphorylation of 3BP2 peaks at 1 minute and is dephosphorylated to the basal level by 10 minutes after stimulation. 3BP2-SH2 domain overexpression that inhibits the function of endogenous 3BP2 leads to suppression of Tyr phosphorylation of PLC-γ, FceRI-mediated elevation of \([\text{Ca}^{2+}]_i\) and degranulation. These observations suggested that 3BP2 modulates FceRI signal transduction by positively regulating PLC-γ activation which in turn is necessary for the elevation of \([\text{Ca}^{2+}]_i\) to induce mast cell degranulation.

A more recent study on the role of 3BP2 in FceRI signal transduction in RBL-2H3 cells demonstrated that Syk PTK mediates FceRI-dependent phosphorylation of Tyr174, Tyr183 and Tyr446 on 3BP2 (Figure 1-1). Syk-mediated phosphorylation of Tyr446 creates the major site on 3BP2 that contributes to its interaction with the Lyn-SH2
domain upon FcεRI aggregation in mast cells (Figure 1-1). Additionally, it was found that 3BP2 constitutively interacts with the Lyn-SH3 domain through its Pro-rich region independent of Syk activity and FcεRI aggregation (Figure 1-1). Functionally, it was shown that overexpression of 3BP2 enhances the autophosphorylation of Lyn to increase its kinase activity. It was suggested in this study that in unstimulated cells, a Pro-rich region may guide 3BP2 close to Lyn, and then the receptor aggregation creates the additional high-affinity binding between phosphorylated Tyr^{446} of 3BP2 and Lyn. Interaction between Lyn-SH2 domain and phosphorylated Tyr^{446} of 3BP2 could induce conformational alteration of Lyn leading to autophosphorylation of Lyn and hence 3BP2 positively regulates the kinase activity of Lyn^{75}.

Combining the observations from both studies, 3BP2 was found to be an essential adapter protein in the early FcεRI signal transduction where it is a part of a positive feedback loop that enhances the catalytic activity of Lyn and Syk PTKs. Together, 3BP2, Lyn and Syk function upstream of PLC-γ activation which then acts to elevate [Ca^{2+}]_{i}, leading to mast cell degranulation.

1.2.5 Osteoclasts

Osteoclasts (OCs) are the cells responsible for bone resorption^{143}. OCs are derived from the monocyte lineage, which originate from hematopoietic stem cells (HSCs) in the bone marrow (BM). Differentiation of monocytes into multinucleated, tartrate-resistant acid phosphatase (TRAP) positive OCs depends on signals provided by colony-stimulating factor-1 (CSF-1) and the receptor activator of NfκB ligand (RANKL). OC differentiation is critically dependent upon expression of the transcription
factor NFATc1, which drives the expression of several genes required for OC maturation. Mature OCs are highly motile and form a characteristic F-actin ring when placed on bone, where bone resorption takes place. Excessive OC production and/or uncontrollable OC activity can lead to too much bone resorption that causes a decrease in bone mass or osteoporosis, which increases the risk of fractures.

Genetic evidence has linked 3BP2 to a rare human disease called cherubism. Cherubism is an autosomal dominant disorder characterized by erosion of maxillar and mandibular bone, with resultant dental and facial deformity due to excessive OC activity and giant cell granuloma formation. Mutations leading to single amino acid substitution in 3BP2 have been identified in cherubism patients and map to a six amino-acid stretch, R415SPP418DG420, lying between the PH and SH2 domain. To investigate the mechanism by which amino acid substitutions of 3BP2 contribute to the phenotypes observed in cherubism patients, Ueki et al. introduced the most common mutation found in cherubism, a Pro-to-Arg substitution (P418R in human; P416R in mice), into the mouse 3BP2 gene ("cherubism" mice). Mutant mice generated are osteoporotic with increased numbers of OC in bone as a result of an enhanced OC differentiation of progenitor cells in response to CSF-1 and RANKL. The resultant mutant OCs are unusually large and possess elevated bone-resorbing activity. Examination of the expression and phosphorylation levels of intracellular signaling components in CSF-1- and RANKL-dependent differentiating OCs revealed no significant differences in a number of signaling intermediates and transcription factors between wildtype and mutant cells. However, the level of Syk Tyr phosphorylation was found increased in cells carrying either homozygous or heterozygous 3BP2 mutation compared to wildtype cells.
Similar results were obtained overexpressing wildtype 3BP2 in wildtype, BM-derived osteoclastic progenitors combined with stimulation with CSF-1 and RANKL. These observations suggested that the P416R mutation is a gain-of-function mutation of 3BP2, when it replaces the wildtype protein, it amplifies the signal transduction necessary for OC differentiation and its bone-resorbing activity by enhancing Syk activity in a manner similar to having excess amount of 3BP2 in these cells.

In addition to being osteoporotic, mice carrying homozygous 3BP2 mutation develop systemic inflammation with massive infiltration of macrophages into skeletal elements and internal organs and have increased serum levels of TNF-α. Peritoneal macrophages isolated from homozygous mutant mice show a dramatic increase in the proportion of TNF-α-expressing cells, and these purified mutant macrophages produce an increased amount of TNF-α following CSF-1 stimulation in culture. The cherubism mutation in 3BP2 enhances CSF-1-mediated TNF-α production of macrophages by increasing both levels and duration of phosphorylation of extracellular signal-regulated kinase 1/2 (Erk 1/2). The enhanced CSF-1-mediated signaling was determined to partially contribute to the 3BP2 mutant phenotypes. Most of the disease phenotypes such as increased cortical bone resorption and inflammation of the stomach mucosa were eliminated by crossing homozygous mutant mice with mice lacking CSF-1. However, serum TNF-α levels were still higher and macrophage numbers were still increased when compared to wildtype mice, and infiltration of macrophages into liver was reduced but not eliminated, suggesting involvement of other factor(s) besides CSF-1. Eliminating TNF-α expression by crossing 3BP2 mutants with Tnf-α null mice rescued mutant mice
Figure 1-1. Schematic model of 3BP2 interactions with various proteins. The 561-amino acid protein consists of three modular domains (PH domain, SH3-binding domain and SH2 domain). All mutations found in cherubism patients are located within a 6-amino acid sequence, lying upstream of the SH2 domain. Three tyrosine residues, Tyr174, Tyr183 and Tyr446 were found to be phosphorylated by Syk and Src PTK. Proteins binding to pTyr183 and pTyr446 of 3BP2 via their SH2 domains have been identified. The SH3-binding domain and SH2 domain of 3BP2 are responsible for interactions between 3BP2 and a number of signaling proteins. Two 14-3-3 binding sites on 3BP2 (Ser225 and Ser277) have been identified. The interaction between 14-3-3 and 3BP2 is serine phosphorylation-dependent and several serine/threonin kinases including PKC have been demonstrated to mediate 3BP2 serine phosphorylation.
from systemic inflammation and prevents bone loss suggesting cherubism is a TNF-α-dependent disorder.

The present study on cherubism mutation of 3BP2 led to the proposed pathogenesis of cherubism. In the presence of mutant 3BP2, myeloid progenitor cells are hyperresponsive to CSF-1 and RANKL. When exposed to CSF-1, these myeloid cells differentiate into hyperactive macrophages with enhanced production of TNF-α, which in turn promotes the systemic inflammation phenotype seen in cherubism mice. When stimulated with CSF-1 and RANKL, these progenitor cells differentiate and fuse to large OCs with high bone-resorbing activity. Since TNF-α has been shown to stimulate CSF-1 and RANKL production by stromal cells\textsuperscript{148}, increased levels of TNF-α can therefore further enhance macrophage and OC formation in these mice.

1.3 3BP2-deficient mice

Studies using various cell lines and cherubism mice implicate an important role of 3BP2 in cells of the hematopoietic lineage; however, a clear picture of the biological function of the wildtype 3BP2 has yet to emerge. In order to elucidate the \textit{in vivo} function for 3BP2 we used homologous recombination to generate 3BP2-deficient (3BP2\textsuperscript{-/-}) mice (Figure 1-2A). Germline transmission of the disrupted 3BP2 allele was confirmed by genomic Southern blotting (Figure 1-2B) as well as by PCR (Figure 1-2C). 3BP2 messenger RNA was absent in 3BP2\textsuperscript{-/-} tissues (Figure 1-2D). Absence of 3BP2 protein in 3BP2\textsuperscript{-/-} mice was verified by Western blotting using an antibody directed
Figure 1-2

(A) Targeting construct

Wild-type allele

Targeted allele

BglII

BglII

-/- +/+ -/

2.7kb

4.5kb

-/- +/+ -/

4.5kb (mt)

2.7kb (wt)

B.

C.

D.

E.

Figure 1-1. Generation of 3BP2^-/- mice. (A) Schematic diagram of the wild-type 3BP2 gene locus, the targeting vector, and the integrated 3BP2 locus. Wild-type 3BP2 gene is composed of 13 exons. Exon 2 contains the start codon, exons 2 to 5 encode the PH domain and exons 10 to 13 encode the SH2 domain of 3BP2. Part of exon 4 and 5 and the intervening intron were deleted. The disrupted region lies within the PH domain coding region. The location of the neo probe and the flanking probe used for genomic Southern analysis and the BglII restriction digest sites in the wild-type and mutant alleles are shown as part of the diagram. (B) A Southern analysis of genomic DNA from 3BP2 wild-type (+/+), heterozygous (+/-), and mutant (-/-) mice. The expected BglII restriction fragment size is shown on the right (mt, mutant; wt, wild-type). (C) Germline transmission of the disrupted 3BP2 allele was confirmed by PCR with genomic DNA. (D) RT-PCR was performed on mRNA extracted from different organs of wild-type (+/+), heterozygous (+/-), and mutant (-/-) mice. PCR was performed using a primer set flanking exon 5. (E) Western analysis of 3BP2 protein expression in purified 3BP2^+/+ and 3BP2^-/- splenic B cells and neutrophils. 3BP2 protein was detected by an antibody directed against the SH2 domain of 3BP2.
Figure 1-3. The expression of 3BP2 transcript in normal mouse tissues. (A) Expression of 3BP2 mRNA from different tissues (source: Genomics Institute of Novartis Research foundation)\(^\text{129}\). (B) Northern blot analysis demonstrated that 3BP2 mRNA is predominantly expressed in spleen, thymus, lymph nodes, bone marrow and lungs with slightly lower expression in organs such as large intestine, kidney, brain and ovary. L32 (ribosomal protein mRNA) was used as an internal control for loading.
against the C-terminus of the protein (Figure 1-2E). 3BP2−/− mice were born at the expected Mendelian frequency (n = 292) and were fertile and viable.

The expression of 3BP2 transcripts in normal mouse tissues as determined by GenAtlas using Affymetrix chip hybridization demonstrated that 3BP2 mRNA expression was restricted to bone, oocytes, lungs, lymph nodes and was most highly expressed in B lymphocytes (Figure 1-3A). This pattern of expression was verified by Northern blot analysis (Figure 1-3B).

With 3BP2−/− mice, we have demonstrated for the first time the in vivo function of 3BP2 downstream of BCR in B cells (Chapter 3) and downstream of G protein-coupled receptor in neutrophils (Chapter 4).

1.4 B cell receptor signaling and B cell generation and activation

1.4.1 B cell Receptor and Coreceptors

B cell receptor (BCR) signaling mediates both B-cell development by transmitting signals that regulate B-cell fate decisions (section 1.4.2) and B-cell activation in both T-cell-dependent and T-cell-independent manners (section 1.4.3). In this section, major components that contribute to BCR signal propagation and the role of the coreceptor CD19 will be discussed.

(a) Protein Tyrosine Kinase (PTK)

The BCR complex is made up of immunoglobulin heavy (IgH) and light (IgL) chains associated with two signaling components, Igα and Igβ. Immediately following
BCR ligation by antigen, Src-family PTK is activated. Activation of the Src-family kinase Lyn leads to the phosphorylation of ITAMs characterized by YXX[L/V]X\(_7\). YXX[L/V] (where Y is tyrosine, L is leucine, V is valine and X represents any amino acid) in the cytoplasmic tails of Ig\(\alpha\) and Ig\(\beta\). The importance of Lyn-mediated Tyr phosphorylation downstream of BCR ligation was demonstrated by the Lyn-deficient B cell line DT40 where there were profound defects in overall tyrosine phosphorylation upon BCR cross-linking \(^{149}\). Although B cells also express other members of Src-family kinases such as Fyn, Blk, Fgr, and Hck, mutations of Fyn, Blk and Fgr show little effect on B-cell function \(^{150,151}\) which is possibly due to redundancy among individual Src PTKs. More recent analysis using Blk, Fyn-, and Lyn triple-deficient mice demonstrated an essential role of Blk, Lyn and Fyn in pre-BCR-mediated pro- to pre-B cell development in the BM \(^{152}\). Phosphorylated tyrosine residues on ITAMs are important docking sites that recruit and facilitate activation of downstream effectors such as Syk PTK and TEC-family PTK Btk \(^{153}\). Syk is a key B-cell signaling molecule. Syk-deficiency prevents most downstream BCR signaling \(^{149,154}\), resulting in a block in B-cell development at pro-B to pre-B cell transition in the bone marrow (BM) and a failure to produce and maintain a mature B-cell population \(^{155}\). On the other hand, either a complete loss of Btk expression or point mutations in subdomains of Btk results in X-linked agammaglobulinemia (XLA) in humans \(^{156,157}\). XLA patients have a block in B cell development at the pre-B cell stage \(^{158}\), resulting in a deficit of mature B cells and serum immunoglobulin (Ig) \(^{159}\). A point mutation in the Btk PH domain leads to X-linked immunodeficiency (xid) in mice \(^{160,161}\). Xid mice have 30-50% decrease in peripheral B cell numbers with the most pronounced loss in the mature B cell population.
Collectively, these reports demonstrated an absolute requirement of Syk and an important role of Btk for BCR signal during B cell-development.

(b) Lipid Metabolizing Enzymes: PLC-γ and PI3K

In addition to the protein components, membrane phospholipids are phosphorylated and dephosphorylated in response to BCR ligation and play an essential role in signal propagation. Two enzymes that function to modify membrane lipids are PLC-γ and phosphatidylinositol 3-kinase (PI3K). B cells express PLC-γ1 and PLC-γ2 and both are Tyr phosphorylated following BCR cross-linking. Activation of PLC-γ upon BCR ligation requires its recruitment to the membrane and its Tyr phosphorylation. Membrane recruitment of PLC-γ is mediated by its PH domain binding to the PI3K product PIP₃, making its PH domain and PIP₃ indispensable for BCR induced activation of PLC-γ. Studies using Syk- and Btk-deficient DT40 B cells suggested that Syk and Btk play non-redundant roles in PLC-γ activation, perhaps by phosphorylating distinct Tyr residues on PLC-γ. In addition to these kinases, activation of PLC-γ depends on a non-enzymatic adapter protein B-cell linker (BLNK). BLNK is Tyr phosphorylated by Syk and efficiently connects Syk and Btk to their substrate PLC-γ. Active PLC-γ hydrolyzes PIP₂ to generate soluble IP₃ and membrane-anchored diacylglycerol (DAG), which are required for BCR-induced release of intracellular calcium and activation of PKC, respectively.

Class I PI3Ks, comprised of a p110 catalytic unit and a p85 regulatory subunit, appear to be the most important class among the family of PI3K enzymes for BCR-mediated B-cell responses. PI3K is recruited to the membrane through its p85 regulatory
domain binding to phosphotyrosines either on the receptor tails or on other membrane-associated signaling molecules such as CD19. CD19 is a B-cell coreceptor which amplifies the BCR signal and is important in the activity of PI3K by recruiting PI3K in close proximity to its substrate PIP2 (the role of CD19 in BCR signal will be discussed later). PI3K phosphorylates PIP2 to generate PIP3 which recruits a number of PH-domain containing signaling molecules including Btk172,173 and PLC-γ165 to the membrane170,174,175. In addition, PIP3 has been reported to stimulate enzymatic activity of PLC-γ directly in vitro176 which may provide an additional mechanism by which PI3K activity impacts on PLC-γ activation. The ability to generate docking sites for numerous PH-domain containing signaling molecules to the membrane makes PI3K a crucial signal transducer in the BCR signaling cascade.

(c) Guanine Nucleotide Exchange Factor (GEF): Vav

The Vav family of GEFs has three known members in mammalian cells: Vav1, Vav2 and Vav3. Expression of Vav1 is restricted to hematopoietic cells177 whereas Vav2 and Vav3 are more widely expressed178,179. All Vav family members are characterized by similar structures: a Dbl-homology (DH) domain, which catalyses GTP exchange on small Rho GTPases, a PH domain, and an SH2 domain which is flanked by two SH3 domains. Activation of Vav is mediated by Src- and Syk-PTK-dependent Tyr phosphorylation129,180,181. Gene-targeted studies have established roles for Vav proteins in B-cell development and function. Vav1−/− B cells proliferate poorly in vitro and Vav1−/− mice have a diminished B-1 B cell population (section 1.4.2) with normal numbers of BM, spleen and lymph node B-2 B cells81,182 (section 1.4.2). The relatively normal B-cell
development of Vav1−/− mice raised the possibility that other members of Vav family might compensate for the loss of Vav1. Hence Vav2−/− and Vav1/Vav2 double deficient mice were generated to test this possibility. In mice deficient in Vav2, although B-1 B cell-development and B-2 B-cell development are normal, there is a clear defect in T cell-independent type 2 (TI-2) immune responses. There were also modest defects in BCR-mediated responses in vitro, including calcium elevation and proliferation. In Vav1/2 double deficient mice, the numbers of B cells (both B-2 and B-1 cells) were greatly reduced and B cells from these mice were unable to proliferate in vitro or to produce antibodies normally after antigenic challenge. These results suggested that Vav2 can partially compensate for Vav1 in B-cell development and function and Vav1 and Vav2 play specific roles in signaling from BCR.

Two known Vav downstream targets, Rac and Rho small GTPases have been shown to interact with phosphatidylinositol 4-phosphate 5-kinase (PIP5K) which synthesizes PIP2 from phosphatidylinositol 4-phosphate (PI4P). By activating Rac and Rho, Vav promotes continuous production of PIP2 which supplies the rapid depletion of PIP2 by PLC-γ allowing continued generation of IP3 and maintaining the BCR-induced elevation of $[Ca^{2+}]$. In addition, active Rac promotes actin polymerization, which may lead to aggregation of lipid rafts and prolong retention of BCR within the lipid rafts. This results in sustained Tyr phosphorylation of proteins within the rafts and therefore, prolongs the BCR signaling. The cytoskeletal effect bought about by Vav may be another mechanism of this protein to contribute to the strength of BCR signaling.
(d) Adapter Protein: BLNK

Several adapter proteins have been identified to function downstream of BCR. One of the most extensively studied, and found to link many signaling events following BCR stimulation is the adapter protein BLNK (also known as SLP-65\textsuperscript{191} or BASH\textsuperscript{192}). Following BCR cross-linking, BLNK is rapidly Tyr phosphorylated by Syk\textsuperscript{167} and couples Syk activation to PLC-\(\gamma\) that is associated with intracellular Ca\(^{2+}\) mobilization\textsuperscript{167}. BLNK mediates membrane recruitment of PLC-\(\gamma\) which not only allows the activation of PLC-\(\gamma\) but also position it in direct proximity with its substrate PIP\(_2\) for cleavage to IP\(_3\) and DAG. BLNK has also been implicated in activation of Ras pathway and downstream mitogen-activated protein kinase (MAPK) Erk through its association with another adapter molecule Grb2. Complex of Grb2 and Sos GEF is constitutively associated with BLNK and such association increases upon BCR cross-linking\textsuperscript{167,191}. Membrane recruitment of Sos permits it to directly activate Ras GTPase by providing GEF function\textsuperscript{193}. However, since downstream activation of Erk MAPK was only partially diminished by either overexpression of Tyr-mutated BLNK or ablation of the gene for this adapter in B cells\textsuperscript{167,191}, there must exist alternative Ras activation pathways. One of which might be mediated by another adapter protein Shc\textsuperscript{194-197}.

Another function of BLNK in B cells is the membrane recruitment of Vav GEF. BLNK-mediated co-recruitment of Vav and PLC-\(\gamma\) provides critical linkage for the activation of other MAPKs, \textit{Jun N-terminal kinase} (JNK) and p38. The stimulation of both JNK and p38 was virtually abolished in BCR-stimulated BLNK-deficient DT40 B cell-lines\textsuperscript{198}. Together with Grb2, BLNK acts to promote Tyr phosphorylation of Vav contributing to optimal activation of Vav and ensuing GEF activities on the GTPase Rac1.
Both Syk-mediated PLC-γ activation and Vav-dependent Rac1 activation have been shown to participate in and are necessary for the full activation of JNK and p38.

Even though BLNK does not possess any enzymatic activity, its presence facilitates the formation of a B cell signalsome promoting the activation of downstream effectors such as PLC-γ and Vav at the same time positioning these enzymes close to their substrates. These properties make BLNK indispensable for BCR signal propagation which is not only necessary for B cell activation during immune response but is also essential for B cell development.

(e) Membrane-anchored coreceptor of BCR: CD19

CD19 is an essential coreceptor for the BCR, and is expressed by early pre-B cells from the time of heavy chain rearrangement until plasma cell differentiation. Despite its early appearance during ontogeny, deletion of CD19 does not have a significant effect on early B-cell development in the BM suggesting CD19 is dispensable for the normal generation and maturation of BM B cells. In contrast, CD19-deficiency leads to a significant reduction in the numbers of peripheral B cells, especially those of splenic marginal zone (MZ) B cells and peritoneal CD5+ B-1 B cells (section 1.4.2a). CD19 is expressed on the B cell surface as a noncovalent complex with CD21 (the C3d complement receptor 2 or CR2), CD81 (TAPA-1) and Leu-13. Since CD21 recognizes C3d complement, it has been proposed that the association between CD19 and CD21 provides a mechanism for bridging BCR with the CD19 complex through covalent complexes of C3d and Ag. CD19 functions to synergistically enhance signaling...
through the BCR, therefore reducing the threshold for B cell activation. Coligation of CD19 to BCR lowers the number of BCR required for inducing increases in \([\text{Ca}^{2+}]_i\) and the proliferation of B cells. CD19 also cooperates with BCR to enhance the activation of MAPKs Erk2, JNK/SAPK, and p38. Therefore, at least two general BCR-mediated signaling pathways are amplified by CD19: elevated \([\text{Ca}^{2+}]_i\) and the MAPK cascades.

The costimulatory effect of CD19 on \([\text{Ca}^{2+}]_i\) is associated with the enhanced generation of IP\(_3\). O’Rourke et al. demonstrated that the mechanism for this function of CD19 involves the activity of Vav. Vav has previously been shown to associate with Tyr-phosphorylated CD19, and ligation of CD19 or coligation of BCR and CD19 induces Tyr phosphorylation of Vav. O’Rourke et al. showed that Vav was required for CD19- (alone or coligate with BCR) mediated long-term elevation of \([\text{Ca}^{2+}]_i\) and cross-linking CD19 alone or with the BCR led to synthesis of PIP\(_2\) by PIP5K which was reduced by half in the absence of Vav. These results concluded that CD19 acts through Vav to promote activation of PIP5K for PIP\(_2\) synthesis which in turn gets hydrolyzed by PLC-\(\gamma\) to form IP\(_3\), resulting in elevated \([\text{Ca}^{2+}]_i\). The conclusion is consistent with the proposed stimulatory role of Vav on PIP5K activation (section 1.4.1c).

CD19 is rapidly Tyr phosphorylated by Src PTK Lyn and c-Abl following ligation of BCR. The phosphorylated sites provide binding sites for SH2-domain-containing cytoplasmic proteins. By recruiting the downstream effector molecules to the membrane and in close proximity to BCR, CD19 functions as a membrane-anchored adapter protein to amplify BCR signaling. CD19 has been shown to regulate Src PTK
activation in B cells possibly by a processive amplification mechanism in which one Lyn molecule first binds to and phosphorylates Tyr residue on CD19 followed by recruitment of a second Lyn molecule to that phosphorylated Tyr residue. The two Lyn molecules juxtaposed by binding to two different phosphorylated Tyr residues are likely to reciprocally transphosphorylate and activate each other. Consistent with a Lyn-regulatory role of CD19, BCR-induced Lyn kinase activity was significantly lower in CD19−/− B cells than that of wildtype B cells. CD19 also binds to PI3K through its phosphorylated Tyr residues. The membrane recruitment of PI3K allows it to generate 3′-phosphorylated inositides which in turn recruit other downstream effectors as described earlier.

1.4.2 B cell Development

Three subsets of mature B cells categorized on the basis of phenotypic, topographic and functional characteristics exist in mice and humans. They are follicular (FO) (also called B-2), marginal zone (MZ) and B-1 B cells. FO and MZ B cells originate in the BM from hematopoietic stem cell precursors. The developing B cells progress through various stages of differentiation such as pro-B and pre-B stages before becoming immature B cells ready to leave the BM. The earliest phases of B cell development start when multipotent lymphoid progenitors yield B lineage-committed pro-B cells. Both Flt3 ligand and the cytokine interleukin 7 (IL-7) have been shown to be indispensable for B cell lineage commitment. The pro-B cells undergo ongoing variable-joining recombination of the Ig heavy-chain locus (Igh) to generate a functional rearranged µ heavy chain. Growth and survival of pro-B cells and Igh recombination are
each regulated by IL-7\textsuperscript{225-227}. Nascent \(\mu\) heavy chains pair with surrogate light chains to form the pre-BCR, which work together with IL-7 receptor to promote pro-B cell proliferation and differentiation into large pre-B cells\textsuperscript{227}. From the large proliferative pre-B cells soon arise the non-dividing pre-B cells characterized by rearrangement of Ig light chain, which in turn yield immature B cells positive for surface BCR. With the expression of BCR, these immature B cells are ready to undergo antigen specific positive and negative selection events in the BM which will influence the generation of the peripheral mature B cell repertoire\textsuperscript{228}. Immature B cells exit the BM and enter the peripheral lymphoid tissues, where they continue the selection and maturation processes. In mice, maturation of these newly formed B cells occurs in the spleen and involves a series of intermediate stages\textsuperscript{229}. Immature B cells enter the spleen as transitional type 1 (T1) B cells, which can differentiate into transitional type 2 (T2) B cells\textsuperscript{230}. Within the T2 B cell population, two subsets have been identified, T2 follicular B cells that give rise to mature FO B cells and T2 MZ B cells which are precursors of MZ B cells\textsuperscript{231,232}. A third intermediate subset of splenic immature B cells has been identified and designated as transitional type 3 (T3), but these cells do not give rise to mature B cells\textsuperscript{233,234}. FO B cells represent most recirculating B cells in adult animals and reside in follicles of secondary lymphoid organs whereas MZ B cells represent a minor population of mostly non-circulating B cells enriched primarily in the marginal zone of the spleen\textsuperscript{235,236}.

It has been long believed that in contrast to the adult BM progenitor cell-derived FO and MZ B cells, B-1 B cells develop from fetal precursors and are self replenishable. Whether or not B-1 B cells develop from a progenitor committed to the B-1 B cell lineage remained controversial\textsuperscript{237,238}. However, a recent study by Montecino-Rodriguez
et al. reported the isolation and characterization of a B-1 B cell-restricted progenitor in adult BM which raise the possibility that in addition to the self-renewal of mature cells, B-1 B cell numbers during adult life may be supplemented by B-1 progenitor cell differentiation \(^{239}\). B-1 B cells are absent from peripheral lymph nodes and variably make up about 5% of splenic B cells, but they constitute a substantial fraction of B cells in the peritoneal and pleural cavity \(^{240,241}\). Within the B-1 B cell population, two subsets of phenotypically and functionally distinct cells, B-1a and B-1b, are found. Whereas B-1a B cells express CD5, secret “natural” antibodies that recognize high-molecular-weight polymeric Ags and are essential in the early response to encapsulated extracellular bacteria, B-1b cells are CD5 negative \(^{242-244}\), produce antibodies only after exposure to Ags and are required for long-lasting protective immunity to pathogens \(^{245,246}\).

B cell development is a complex process which occurs in both the BM and spleen. Discussion in the following part of thesis will be focused on the molecular mechanisms involved in the generation of different mature B cell subsets in the periphery (Figure 1-4).

(a) BCR signal strength and additional non-BCR signaling

Hardy and coworkers provided the first direct evidence for positive selection in peripheral B-cell development by using an Ig transgenic system to show the Ag-dependent development of B-1 B cells \(^{247}\). More recently, using a similar technique, evidence for Ag-driven MZ B-cell maturation has been demonstrated \(^{248}\). However, a role for Ag in FO B cell development remains controversial. Phenotype analysis of mice with gene-targeted disruptions of BCR signaling components led to the conclusion that functional BCR signaling is required for the development of all subsets of peripheral
Figure 1-4. Development of conventional B cells and B-1 B cells. (A) Immature B cells exit the bone marrow (BM) and enter the spleen as transitional type 1 (T1) B cells, which differentiate into transitional type 2 (T2) B cells. Differentiation of T1 to T2 B cells depends on B cell receptor (BCR) signaling and the survival signal provided by tumor necrosis factor (TNF) family member B-cell-activating factor of TNF family (BAFF). T2 B cells give rise to follicular (FO) and marginal zone (MZ) B cells. A weak BCR signal together with BAFF signal and signal provided by Notch2 are essential in shaping MZ B cell population. On the other hand, FO B cell development depends on an intermediate BCR signal and BAFF survival signal. (B) B-1 B cells develop from fetal precursors and are self-replenishable. A more recent study demonstrated a B-1 B cell-restricted progenitor in adult BM which raise the possibility that in addition to the self-renewal of mature cells, B-1 B cells numbers during adult life may be supplemented by B-1 progenitor cells differentiation. Development of B-1 B cells depends on a strong BCR signal. Within B-1 B cell population, two subsets of phenotypically and functionally distinct cells, B-1a and B-1b, are found. Whereas B-1a B cells express CD5 and secrete "natural" antibodies, B-1b cells are CD5 negative.
mature B cells, but different strength of BCR signaling is required for the development of each mature B-cell subset. The development of peritoneal B-1 B cells requires the strongest BCR signal, FO B cells require an intermediate BCR signal and MZ B cells require a weaker signal \(249,250\). This model is supported by the phenotypes of various gene-knockout mice including Syk- \(251\) and Btk- \(160,252\) deficient mice. Removal of the pivotal kinase downstream of BCR, Syk, leads to the absence of all three subsets \(251\), whereas disruption of Btk, which impairs \(\text{Ca}^{2+}\) flux and PKC activation leads to the absence of only FO B and B-1 B cells \(160,252\) (Table 1-1). The “signal strength” theory is further supported by a study using a monoclonal BCR mouse line, specifically for the self-Ag Thy-1 \(248\). The study demonstrated that whereas high levels of soluble self-Ag led to B-1 B-cell development at the same time preventing MZ and FO B-cell differentiation, lower doses of the same Ag promoted preferential differentiation of MZ B cells \(248\).

However, there is compelling evidence suggesting that the “signal strength” theory alone is insufficient to explain the decision-making of peripheral B cells on shaping their developmental path. For instance, removal of a negative regulator of BCR, Aiolos, which enhances BCR signaling, led to a significant reduction in both MZ and peritoneal B-1 B cells with increased FO B cells in mice \(253\) (Table 1-1). In addition, B cells with diminished BCR signaling, including PI3Kδ- and CD19-deficient mice, lack both MZ and B-1 B cells \(208,254\) (Table 1-1). In the case of CD19, it has been suggested that signals initiated by receptors other than the BCR may potentially utilize CD19 for the recruitment and activation of PI3 kinase \(221\) and Vav \(190\). Therefore, in addition to its costimulatory role in amplifying BCR signal which supports B-1 and FO B-cell
development based on the “signal strength” theory, CD19 may favor the generation and maintenance of MZ B cells in a coreceptor-independent manner. These results clearly indicated that MZ and B-1 B cells arise as the consequence of positive selection events that may be both quantitatively and qualitatively distinct from events required for generating FO B cells.

In contrast to B-1 B-cell development, additional non-BCR signals are indispensable for FO and MZ B-cell differentiation. One of which is the survival signal provided by tumor necrosis factor (TNF) family member B-cell-activating factor of TNF family (BAFF). BAFF and its receptors are essential for peripheral B-cell survival, a crucial function that has a considerable impact on B-cell maturation. It has been shown in vitro that combining with BCR stimulation, BAFF supports the survival of T2 B cells and promotes differentiation of T2 B cells into mature B cells. Analysis of BAFF-deficient mice showed a critical role of BAFF in the transition between T1 and T2 B cells and in generation of mature FO and MZ B cells. In these mice, while T1 B cells were present in normal numbers, there were almost no cells of a T2 phenotype and a substantial reduction in FO and MZ B cell numbers (Table 1-1). However, development of peritoneal B-1 B cells appeared to be intact in BAFF-deficient mice suggesting that B-1 B cells are developed and maintained independently of BAFF. Since BAFF provides the survival signal for the peripheral T2, mature FO and MZ B cells, this signal is essential for both differentiation and maintenance of pools of FO and MZ B cells.

In comparison to BAFF, members of the Notch family provide the essential non-BCR signal for MZ B-cell lineage commitment exclusively. Notch was previously
implicated in T-cell lineage commitment. It has been shown that the inactivation of Notch1 results in the blockage of T cell development and ectopic B cell development in thymus. Conditional knockout of RBP-J, a signal transducer in the Notch signaling cascade, display a similar phenotype with an additional defect in MZ B-cell development (Table 1-1). These results suggested that signaling through members of Notch family other than Notch1 might be essential in MZ B-cell differentiation. When comparing the expression of Notch genes in various T and B cell fractions, it was found that Notch1 expression level was generally higher in T cells than in B cells and Notch2 displayed a reciprocal expression pattern to Notch1. Notch2 expression level increased with B cell maturation and was prominent in mature B cell subsets in the spleen. Analysis of Notch2 conditional knockout mice showed a substantial reduction in MZ B-cell compartment while the other mature B cell subsets, FO and B-1 B cells, were normal. Together, these reports demonstrated a regulatory role of Notch-RBP-J in the cell-fate decision between FO and MZ B cells.

The non-BCR signals act in synergy with BCR signal strength to mold the B cell populations in the spleen. One model for lineage commitment in peripheral B cells argues that nascent transitional B cells (i.e. T2 B cells) must receive a BAFF survival signal and a BCR signal sufficient in strength to initiate a FO B-cell developmental program. Those B cells that survive because of BAFF but receive a weak BCR signal then become receptive to inductive signals through Notch2 that support the development of MZ B cells.
Chemotactic responses have been shown to control many aspects of B cell compartmentalization including the chemokine C-X-C receptor 4 (CXCR4)-dependent retention of precursor B cells in the BM, the CXCR5-dependent follicular organization of the spleen and the homing of follicular B and T cells. The fact that MZ B cells occupy a distinct area within the spleen compared to FO B cells raises the possibility that the ability of these cells to migrate and remain at this site can influence the differentiation process. MZ B cells are highly motile cells. In response to chemokines such as stroma-derived factor-1 (SDF-1), B-lymphocyte chemoattractant (BLC) and secondary lymphoid tissue chemokine (SLC), MZ B cells migrate considerably faster than immature and FO B cells. In addition, treatment of a wildtype animal with minimal doses of G-αi (a subunit of G protein-coupled receptor, detailed description in section 1.5.1) inhibitor pertussis toxin (PTX) led to the selective disappearance of MZ B cells, supporting the notion that the presence of this highly motile population is chemotactic signaling-dependent. Mice deficient in protein tyrosine kinase-2 (Pyk-2), a signal transducer of various chemokine and integrin signal cascade, have normal numbers of FO and immature B cells but a selective absence of MZ B cells caused by intrinsic migration defect of these cells (Table 1-1). Gene-targeted experiments that involve GEFs dedicator of cytokinesis 2 (DOCK2) and Lsc further emphasized the regulatory role of chemokine signal in MZ B-cell migration (Table 1-1). Collectively, these results suggested that part of MZ B-cell formation depends on their correct localization to a specific niche of the spleen. Signals produced by chemokine receptors are crucial for this localization process.
The strength of B cell receptor (BCR) signaling regulates which mature B-cell subsets can develop. This idea is consistent with studies of Syk-, Btk-deficient mice; B-1 B cells require a strong BCR signal, follicular (FO) B cells require an intermediate BCR signal, and marginal zone (MZ) B cells require a weak BCR signal for development. However, the “signal strength” theory alone appears to be insufficient to explain the decision-making of peripheral B cells on shaping their developmental pathway. Removal of a negative regulator of BCR, Aiolos (Aiolos−/−), which enhances BCR signaling, leads to a significant reduction in both MZ and B-1 B cells with an increase in FO B cells. On the other hand, B cells with diminished BCR signaling, including PI3Kδ- and CD19-deficient mice, lack both MZ and B-1 B cells with normal FO B cell population. Non-BCR signal such as the survival signal provided by B-cell-activating factor of TNF family (BAFF) is essential for the development of both MZ and FO B cell population, whereas, Notch signaling is specifically required for MZ B cell-development. Chemokine and integrin signals affect the development of peripheral B cell subsets as well. Mice deficient in protein tyrosine kinase-2 (Pyk-2−/−), a signal transducer of various chemokine and integrin signal cascade, have normal numbers of FO and immature B cells but a selective absence of MZ B cells caused by intrinsic migration defect of these cells. Gene-targeted experiments that involve GEFs dedicator of cytokinesis 2 (DOCK2) and Lsc further emphasized the regulatory role of chemokine signal in MZ B-cell migration.

Table 1-1. Knockout (-/-) mice with alterations in peripheral B cell subsets. The strength of B cell receptor (BCR) signaling regulates which mature B-cell subsets can develop. This idea is consistent with studies of Syk-, Btk-deficient mice; B-1 B cells require a strong BCR signal, follicular (FO) B cells require an intermediate BCR signal, and marginal zone (MZ) B cells require a weak BCR signal for development. However, the “signal strength” theory alone appears to be insufficient to explain the decision-making of peripheral B cells on shaping their developmental pathway. Removal of a negative regulator of BCR, Aiolos (Aiolos−/−), which enhances BCR signaling, leads to a significant reduction in both MZ and B-1 B cells with an increase in FO B cells. On the other hand, B cells with diminished BCR signaling, including PI3Kδ- and CD19-deficient mice, lack both MZ and B-1 B cells with normal FO B cell population. Non-BCR signal such as the survival signal provided by B-cell-activating factor of TNF family (BAFF) is essential for the development of both MZ and FO B cell population, whereas, Notch signaling is specifically required for MZ B cell-development. Chemokine and integrin signals affect the development of peripheral B cell subsets as well. Mice deficient in protein tyrosine kinase-2 (Pyk-2−/−), a signal transducer of various chemokine and integrin signal cascade, have normal numbers of FO and immature B cells but a selective absence of MZ B cells caused by intrinsic migration defect of these cells. Gene-targeted experiments that involve GEFs dedicator of cytokinesis 2 (DOCK2) and Lsc further emphasized the regulatory role of chemokine signal in MZ B-cell migration.

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<th>MZ B</th>
<th>FO B</th>
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<td>Syk−/−</td>
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<td>Btk−/−</td>
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<td>Aiolos−/−</td>
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<td>CD19−/−</td>
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<td>BAFF−/−</td>
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<td>257</td>
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<td>RBP-J−/−</td>
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<td>260</td>
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<td>Notch2−/−</td>
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<td>Pyk2−/−</td>
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<td>DOCK2−/−</td>
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After proper localization of B cells to MZ of the spleen which is mediated by chemokine receptors, these cells rely on the contribution of adhesion molecules to lodge and remain in MZ. Elevated expression of αL and β2 integrin subunits were found on MZ B cells compared to FO B cells, suggesting that MZ B cells have higher amounts of the αLβ2 heterodimer LFA-1 integrin. In addition, MZ B cells expressed greater amounts of β1-containing integrins with higher levels of α4β1 than FO B cells. When treating mice with a combination of α4 and αL blocking antibodies which prevents cell adhesion mediated by binding of these integrin receptors to their ligands, a selective reduction of MZ B cell numbers in the spleen was identified; however, FO B cell numbers in the spleen were unchanged. This result demonstrated that the long-term retention of B cells in MZ depends on distinct molecular mechanisms than those required by FO B-cell retention; and these mechanisms involve activation of integrin receptors such as LFA-1 and α4β1.

1.4.3 B cell activation

Ab production by B cells can be divided into thymus-dependent (TD) and thymus-independent (TI) types. TD humoral response encompasses the response to most complex proteins and it requires appropriate T-cell help (section 1.4.3a). Nevertheless, humans and mice with T-cell deficiencies make Abs to many bacteria through TI humoral response. The Ags that elicit TI humoral responses are known as TI Ags which can be categorized into two classes. Ags in the first class, TI-1 Ags, contain an intrinsic activity that can directly induce the proliferation of B cells. At a high concentration, these molecules lead to the polyclonal activation of B cells. As a result of their ability to
stimulate most B cells to divide, regardless of their Ag specificity, TI-1 Ags are often called B-cell mitogens. The second class of TI Ags (TI-2) consists of molecules such as bacterial cell-wall and capsular polysaccharides that have repeating antigenic epitopes. Given the highly repetitive structures of TI-2 Ags, they are believed to activate B cells and promote Ab production in the absence of T-cell help by extensively cross-linking B-cell-surface receptors. However, in addition to BCR signaling, signaling through complement receptor and members of the TNF receptor family are required for the appropriate TI-2 Ags-mediated humoral response (section 1.4.3b).

(a) Thymus-dependent (TD) humoral response

TD humoral response is a complex process involving Ag-specific FO B and T-helper (Th) cells as well as follicular dendritic cells (FDC) which reside in B-cell follicle of peripheral lymphoid organs. In addition, the complement system, MZ B cells and preimmune serum Ig (natural IgM: Abs that are produced spontaneously in the absence of apparent stimulation by specific Ags) produced mainly by B-1 B cells contribute to TD humoral responses. The role of each participant in achieving an appropriate TD immune response will be discussed in this section.

When TD Ag is introduced into mice, it is captured and processed by professional APCs, especially dendritic cells (DCs). The Ag-primed DC localizes to the T-cell zone of the secondary lymphoid organs to recruit naïve but Ag-specific Th cells. Cytokines released by the DC and costimulatory molecules expressed on DC all contribute to the clonal expansion of Ag-specific Th cells. These Ag-primed Th cells migrate to the follicular borders of the secondary lymphoid organs in order to interact with Ag-primed
B cells \(^{270}\). Ag-specific B cells bind, process and present Ags and interact with Ag-primed Th cells. Molecules on Th cells such as CD40 ligand (CD40L) induce B cell differentiation and promote class switching on B cells. Mice deficient in either CD40 or CD40L were capable of eliciting a normal TI response but were impaired in their capacity to produce primary IgM Ab responses and completely lack the capacity to switch and produce IgG Abs in response to the TD Ags. These results demonstrated the participation of CD40-CD40L signal for the generation of TD IgM responses and the absolute requirement of this signal for TD Ag-induced class switching of Ig \(^{270,271}\). The progeny of B cells that have been activated by Th cells follow one of two fates: 1) some migrate to the medullary cords and differentiate into short-lived plasma cells secreting IgM or IgG, thus providing an early source of circulating Abs; 2) Others migrate along with the T cells that activated them into the B-cell follicles, where they proliferate further to form germinal centers (GC) \(^{272}\).

Within the GC, Ag-specific B cells undergo rapid clonal expansion and diversify their BCR by somatic hypermutation and receptor editing of Ig variable (V) region gene \(^{272}\). The variant BCRs are “tested” for Ag binding through interaction with Ags presented on FDCs within GC. Diminished binding for Ag leads to programmed cell death and rapid clearance of apoptotic B cells locally by macrophages; whereas improved binding to Ag results in positive selection of the variant. Positively selected B cells either resume the cycle of expansion, diversification, and selection or exit the GC cycle and enter the memory B-cell compartment \(^{272}\). Hence production of Abs with high affinity to Ags and generation of memory B cells depend heavily on Ag presentation by FDCs. The deposition of Ag on FDC appears to depend on MZ B cells, the complement system, and
secreted natural IgM. It was initially shown that mice deficient in secreted IgM (µs−/−) have impaired responses to limiting doses of TD Ags. These mice are more susceptible to bacterial and viral infections, and exhibit reduced GC formation in early phases of immune responses. All of which implied the involvement of secreted IgM in TD humoral immunity. In addition, mice deficient in the components of the classical complement pathway or in the complement receptor CR1/2 (CD21/35) have defects similar to those observed in µs−/− mice, suggesting that the complement system works in concert with secreted IgM to elicit a normal TD humoral immune response. Ferguson et al. later demonstrated the loading of TD Ag, in a complex with secreted IgM and activated complement component C3, onto FDCs and a direct role of these IgM-containing immune complexes (IgM-IC) in promoting rapid GC formation.

Furthermore, it was shown that IgM-IC localize first to the splenic MZ where the IgM-IC bind MZ B cells in a complement and complement receptor (CR1/2) dependent process. IgM-IC-bound MZ B cells emigrate and transport IgM-IC into the B-cell follicle for deposition onto FDCs. The requirement for MZ B cells in the deposition of IgM-IC was further confirmed with CD19−/− mice that have substantial reduction in MZ B-cell number and with PTX treatment of wildtype mice which functionally deplete their MZ B cells. Under both conditions, although IgM-IC were trapped normally within the MZ, they were not subsequently found on FDCs in the B-cell follicle. Clearly, MZ B cells are indispensable for TD Ag transportation and loading onto FDCs; however, the mechanisms which trigger the initial MZ emigration of these cells were less defined. Recently, Cinamon et al. provided strong evidence to demonstrate that instead of being a sessile cell population MZ B cells shuttle continuously back and forth between MZ and
B-cell follicle independent of BCR engagement. The propensity to shuttle between these two regions is regulated by the signaling from two G-protein-coupled receptors, sphingosine 1-phosphate (S1P) receptor and CXCR5 receptor. They further demonstrated and concluded that positioning MZ B cells in the MZ is required for their efficient Ag-capture and their transport of the Ag may be a “default” feature of their continuous shuttling activity.

(b) Thymus-Independent (TI) humoral response

As described earlier, Ags that elicit TI humoral response are grouped into two classes. TI-1 Ags are usually considered as B cell polyclonal activators and induce B cell activation via antigen nonspecific mechanisms. An example of TI-1 Ags is trinitrophenyl-lipopolysaccharide (TNP-LPS). TNP-LPS delivers two activation signals to the B cell, one via TNP-specific BCR and one by LPS and its receptor Toll-like receptor 4 (TLR4). Among all the microbial products known to activate B cells, bacterial LPS is the most well characterized TLR ligand that is able to activate both FO and MZ B cells. In the case of FO B cells, TLR4 is essential for LPS-induced activation that leads to cell division and antibody isotype switching. TLR4 is a member of the family of pattern recognition receptors (PRRs) capable of recognizing conserved pathogen-associated molecular patterns (PAMPs) constitutively expressed by broad classes of pathogens. The human TLR family is composed of at least 10 members, TLR1 through 10. Each TLR is specific in its expression pattern and PAMP sensitivities. TLR4 was the first characterized mammalian TLR. It is expressed in a variety of cell types, including B cells, macrophages and DCs. In addition to LPS, TLR4 is
involved in the recognition of several other ligands, including another common bacterial structural pattern: lipoteichoic acid (LTA) from Gram-positive bacteria\textsuperscript{297}, and a heat-sensitive cell-associated factor derived from \textit{Mycobacterium tuberculosis}\textsuperscript{298}. Key signaling networks activated in FO B cells downstream of TLR4 include the nuclear factor $\kappa B$ (NF-$\kappa B$) and MAP kinase pathways\textsuperscript{299}. These signaling cascades promote cell survival, cell proliferation, activation and antibody production of the responding B cells.

It has been proposed that the immunogenicity of TNP-LPS is apparently based on the portion of LPS which is responsible for B cell mitogenicity\textsuperscript{300} and in this case, TNP-specific BCRs appear to serve only as “passive focusing devices” to concentrate LPS on the surface of B cells\textsuperscript{301}. In other words, the presence of mitogenic stimulators such as LPS permits B cells that have an insufficient BCR signal to be activated and mount an appropriate TI-1 response. However, proof of this theory has been difficult to demonstrate experimentally. Since targeted disruption of genes involved in BCR signaling that alter the strength of the signal usually also lead to alteration in peripheral B cell composition (section 1.4.2a), it is difficult to distinguish whether the defect in TI-1 response is due to a defective BCR signal or due to the developmental loss of certain B cell subsets.

In contrast to TI-1 Ags, TI-2 Ags, which are typically derived from polysaccharides, usually consist of complex repeating units that drive B cell responses by extensively cross-linking specific membrane BCR\textsuperscript{269} (for example, TNP-Ficoll). Although TI-2 Ags may be extremely potent B-cell activators, they were shown to be unable to induce Ab secretion when cultured alone with resting B cells\textsuperscript{302}. This suggests that the microenvironment of TI-2 Ag-responding B cells provide additional signals.
necessary to achieve B-cell differentiation into plasma cells for Ab production. In addition, the primary Ig isotype of Ab induced by carbohydrate Ags is IgG₂ in human and IgG₃ in mice suggesting that the appropriate Ig class switching event must occur in the absence of T-cell help (CD40L). Based on these observations, it can be concluded that immune response against TI-2 Ags is a combinatory effort of BCR signal and other signals.

Members of TNF family provide one of the additional signals required for TI-2 humoral response. Two TNF family members: BAFF and a proliferation-inducing ligand (APRIL) have been shown in human B cells to activate class switch recombination (CSR) which mediates class switching events of Ig. Litinskiy et al. showed that in the presence of cytokines such as IL-15 and extensive BCR cross-linking, BAFF and APRIL expressed on DCs and macrophages initiate CD40-independent Ig class switching to IgG and IgA and promote B-cell differentiation to plasmacytoid cells that secrete class-switched Abs. BAFF and APRIL can each bind to more than one receptor. BAFF binds to all three distinct receptors expressed by B cells, including transmembrane activator and calcium modulator and cyclophylin ligand interactor (TACI), B cell maturation antigen (BCMA) and BAFF receptor (BAFF-R). APRIL binds to BCMA and TACI with similar affinity but not to BAFF-R. Studies using TACI-deficient mice demonstrated a requirement for TACI in mounting a robust TI-2 Ab response. It was proposed that TACI is a functional homolog of CD40 able to costimulate B cells in a similar manner as CD40 but in the absence of T cells. More recently, in vitro studies using B cells derived from TACI-, BCMA- and BAFF-R-deficient mice reinforced the argument that BAFF/APRIL and their receptors promote
appropriate TI-2 immune responses by inducing class switching events in responding B cells. In addition, these in vitro studies demonstrated that signaling through BCMA is dispensable for B-cell class switching whereas TACI mediates class switching by APRIL and both TACI and BAFF-R mediate class switching by BAFF.

Complement receptors provide the second additional signal for a robust TI-2 immune response. It is well documented that complement proteins play an important role in B cell activation and in immune responses driven by TI Ags. Complement is a plasma protein system of innate immunity that is activated in the absence of Ab. Through stepwise proteolytic events, the third complement protein C3 is cleaved into fragments. Two of these fragments, C3d and C3dg, bind to CR2 (or called CD21) on B cells. C3-deficient patients have an increased susceptibility to infections by encapsulated bacteria (TI-2 Ags) and tend to suffer from recurrent bacterial infection demonstrating the requirement of C3 for mounting TI-2 immune response. Studies with C3-deficient mice showed that these mice displayed a significant reduction in IgM and IgG response to TI-2 Ags again indicating the role of complement-mediated help in TI-2-mediated Ab production. Since CD21 associates with the BCR coreceptor CD19 (section 1.4.1e), ligand-binding to CD21 may have B cell-stimulating functions which lower the threshold of B cell activation. Hence, coligating BCR and CD21/CD19 generates sufficient signal strength to activate B cells in the absence of T-cell help. In support of this, depleting C3 or blocking the binding of ligand to CD21 raises the threshold dose of Ag required to elicit Ab production by approximately 10-fold. On the other hand, co-cross-linking of CD21 with BCR led to an enhanced B cell proliferation with concentrations of anti-BCR 10,000-fold less than was required in the absence of CD21 engagement. In
addition to its BCR costimulatory role, it's believed that complement and its receptor promote TI-2 humoral response by localizing TI-2 Ags to a specific compartment where the primary B cells participating in TI-2 immune response are found (see the discussion below).

The MZ B cell has been strongly implicated in response to TI-2 Ags. The earliest observation linking MZ B cells to TI-2 immune responses demonstrated that the time at which the human or mouse immune system acquires responsiveness to polysaccharide Ags coincides with maturation of the MZ B cell. This occurs at approximately two years in humans and 2-3 weeks after birth in mice. Studies by Guinamard et al. using Pyk-2-deficient (Pyk-2−/−) mice demonstrated a direct contribution of MZ B cells to TI antibody responses. Pyk-2−/− mice were shown to have a specific defect in MZ B cell development that was associated with reduced TI-1 and TI-2 antibody responses.

Comparing the distribution of TI-2 Ags (TNP-Ficoll) following immunization between wildtype-, Pyk-2−/−- and C3−/−-mice demonstrated that normally TI-2 Ags rapidly localize to MZ B cells and this localization is complement dependent. Since TI-2 Ags such as TNP-Ficoll are known to be capable of activating complement, these results suggested that TI-2 Ags, by their ability to activate complement, facilitate the localization of these Ags to MZ B cells that express high levels of the complement receptor. In addition, the ability of complement to activate B cells by bridging coengagement of BCR to CD21 (CR2)/CD19 may directly facilitate the stimulation of MZ B cells to induce TI Ab responses.

B-1 B cells have also been linked to TI-2 humoral responses. Xid mice (Btk mutation) or Btk-deficient mice have a normal MZ B cell compartment but diminished B-
1 B cell population and they do not respond to TI-2 Ags\(^{327,328}\). When re-introducing B-1 B cells by intravenous transfer to xid mice, responsiveness to TI-2 Ags was restored in xid recipients suggesting that B-1 B cells provide the primary response to TI-2 Ags\(^{329}\). In contrast to xid and Btk\(^{-/-}\) mice, Pyk-2\(^{-/-}\) mice have a normal B-1 B cell population but an almost complete loss of the MZ B cell population yet Pyk-2\(^{-/-}\) mice share the same defective TI-2 humoral response as xid and Btk\(^{-/-}\) mice\(^ {265}\). Based on these findings, it appears that both MZ B and B-1 B cell compartments are critical for TI-2 immune response. B cells from these compartments cooperate with each other and it is unlikely that their functions are redundant. Martin et al. used BCR transgenic (Tg) mice to demonstrate that B cells with MZ and B-1 B-cell phenotypes can both participate in TI humoral responses. In addition, they proposed that the routes of immunization as well as the types of TI Ag used for immunization might influence the responses of these two B-cell compartments where the response from one B-cell compartment dominates over that of the other\(^ {330}\).

Collectively, although additional signals are sometimes required, BCR signaling is critical for processes from B-cell development to both TD and TI B-cell activation. As described earlier in this chapter (section 1.4.1d), many adapter proteins have been demonstrated to modulate the signal emitted from BCR, and some of them are absolutely essential for BCR signal propagation. In chapter 3, a novel functional role of another adapter protein, 3BP2, downstream of BCR will be described.
1.5 G protein-coupled receptor signaling and neutrophil function

1.5.1 Neutrophil and G protein-coupled receptor (GPCR)

Neutrophils are highly motile cells and play an essential role in the innate immune system by phagocytosing, killing and digesting microbial pathogens. They are the most abundant leukocyte found in blood with approximately $5 \times 10^6$ cells per milliliter of blood in humans\textsuperscript{331}, and they are usually the first cells that are recruited to the sites of infection. The importance of neutrophils in controlling an infection is revealed by the high susceptibility to various bacterial or fungal infections in individuals suffering from severe neutropenia as a result of chemotherapies\textsuperscript{332}.

During a bacterial infection, compounds elaborated by bacteria such as $N$-formylmethionyl oligopeptides like fMet-Leu-Phe (fMLF)\textsuperscript{333}, complement fragment C5a formed in blood plasma upon complement system activation\textsuperscript{334} and interleukin-8 (IL-8) produced by phagocytes and stimulated tissue cells\textsuperscript{335} can all act as chemotactic agonists that rapidly trigger the defense function of the neutrophils, i.e., activation of motile system, exocytosis, and respiratory burst. These chemoattractants are ligands of heptahelical, heterotrimeric G protein-coupled receptors (GPCRs) on the leukocytes. G proteins are composed of $\alpha$, $\beta$, and $\gamma$ subunits. A single receptor can activate multiple G protein molecules, thus amplifying the ligand binding event. Upon receptor-ligand interaction, the G protein dissociates into the GTP-bound $\alpha$ subunit and the $\beta\gamma$ subunit complex\textsuperscript{336}. The functions of G proteins are regulated cyclically by association of GTP with the $\alpha$ subunit, hydrolysis of GTP to GDP and Pi, and dissociation of GDP. Binding to GTP is viewed as the “activation state” of the G protein. When GTP is hydrolysed to...
GDP, $G\alpha$ is inactivated along with deactivation of $G\beta\gamma$ and downstream effectors. Based on their amino acid sequence similarities, the $\alpha$ subunits can be grouped into four families: $G\alpha$s, $G\alpha$i, $G\alpha$q and $G\alpha$12/13 \(^{337}\). Functionally, $G\alpha$s and $G\alpha$i subtypes have been implicated in the regulation of adenylate cyclase and the gating of certain ion channels \(^{338}\). The use of pertussis toxin (PTX) is believed to be one of the most effective tools for implicating G proteins in specific functions in intact cells. The toxin uncouples the receptor from its G protein and thus blocks signal transduction by receptors that cause decreases in cAMP that regulate ion channels, and that activate phospholipases. Among the $\alpha$ subunit families, members of Gi class of $\alpha$ subunits contain sites susceptible to modification by PTX therefore mediate activation of the PTX-sensitive processes. PTX treatment abrogates the majority of chemoattractant responses of neutrophils; therefore, it was believed that fMLF- and C5a-stimulated responses are mediated by the Gi family of $\alpha$ subunits \(^{339-341}\). However, it was later demonstrated with differentiated HL-60 cells (differentiated human promyelocytic cells/neutrophil-like cells) that although PTX treated HL-60 cells failed to migrate towards an fMLF source they developed uropod-like structures that resemble the structure at the rear of a normal HL-60 cells polarized toward a fMLF source \(^{342}\). These results demonstrated that a response can be elicited by fMLF in the presence of PTX in neutrophil-like cells, suggesting the involvement of other $G\alpha$ subunits downstream of fMLF receptor \(^{342}\). The earliest functions established for individual subunits of G proteins such as the activation of adenylyl cyclase were carried out by $G\alpha$ subunits \(^{343}\). Based on these findings, $G\alpha$ subunits were thought to be the primary effector regulatory subunit and $G\beta\gamma$ was thought to influence the activation state of $G\alpha$ since interaction of $G\beta\gamma$ with $G\alpha$ increases the GDP affinity of $G\alpha$ \(^{344}\). However,
accumulated evidence showed that Gβγ directly bind and activate numerous effectors. Collectively, Gα and Gβγ both have the capacity to regulate the activities of intracellular effectors leading to biological functions such as directional cell migration (a process known as chemotaxis), respiratory burst and degranulation.

1.5.2 G protein-coupled receptor-mediated neutrophil function

(a) Chemotaxis

Chemotaxis is defined as a process where a cell can sense the presence of extracellular signals and guides their movement in the direction of increasing concentrations of the signaling molecules. Chemotaxis depends on the coordination of many cellular functions: (1) morphological changes to achieve cell polarity; (2) actin-dependent protrusion of the anterior lamellipodium extended in the direction of movement; (3) regulation of adhesion between adhesion receptors and ligand in the substratum, and (4) an active myosin-based contractive force needed to move the cell body forward. However, this discussion will focus on the signal transduction events involved in establishing cell polarity, particularly signaling events involved in the directional sensing mechanism at the leading edge (pseudopod) of a motile cell in the presence of a chemotactic gradient.

It has been demonstrated by numerous studies that neutrophils are capable of polarizing in the absence of a concentration gradient. The concentration gradient-independent polarization may arise from perceived spatial or temporal stimulus gradients caused by microscopic nonuniformities or by kinetic fluctuations in receptor-
ligand binding. Such self-organized polarization is critical for cell locomotion allowing cells to move in a persistent, but random direction. However, to guide their movement efficiently in the direction of a concentration gradient, cells need to combine polarization with directional sensing mechanisms. Eukaryotic cells are extremely sensitive to chemotactic gradients with the capacity of detecting the differences in chemoattractant concentration that are as small as 2-10% between the front and the back of the cell. Directional sensing can be explained by two different models. In temporal models, a cell projects random pilot pseudopodia in all directions therefore sequentially measuring the concentration of attractants at two points in the gradient but reinforcing only the ones that experience increased receptor occupancy. On the other hand, there is the spatial model, which only applies to cells with a diameter that is at least 10-20 µm. In the spatial model, cells simultaneously measure the gradient at two points and compare receptor occupancies at two ends of the cell body followed by formation of a pseudopod in the direction where there is a higher concentration of stimuli.

Neutrophils and simple amoebae such as Dictyostelium discoideum have frequently been used as the model systems to study chemotaxis. The ability to sense and respond to shallow gradients of extracellular signals is remarkably similar between the two. Chemoattractant receptors in both D. discoideum and neutrophils signal through G proteins. Combining the observations made from these two model systems, two essential components required for an efficient chemotaxis have been identified: directional sensing and self-organized polarization.

Directional sensing refers to the ability of a cell to detect an asymmetric extracellular cue and generate an internal amplified response. Directional sensing is
independent of actin cytoskeleton, because treating cells with inhibitors of actin polymerization such as latrunculin A did not block the ability of these cells to sense chemoattractant gradients. However, directional sensing is G protein-dependent as cells lacking functional G proteins are no longer capable of going through this process. The “local excitation-global inhibition” model proposed that directional sensing depends on a balance between a rapid, local “excitation” and a slower global “inhibition” process. The local excitation is brought about by an increased local receptor occupancy at an area where there is a higher concentration of chemoattractants which exceed the global inhibition determined by the average fraction of occupied receptors in all regions of the cell.

Unlike directional sensing, self-organized polarization depends on G protein signaling and actin cytoskeleton. In neutrophils, chemoattractant receptors such as those of fMLF are linked to $G \cdot \bar{\alpha}i$ and $G \cdot \bar{\alpha}12/13$. fMLF binds to its receptor which activates both $G \cdot \bar{\alpha}i$ and $G \cdot \bar{\alpha}12/13$ to generate two divergent, opposing signaling pathways to promote polarizing “frontness” and “backness”, respectively. The formation of polarity does not involve redistribution of fMLF receptors and the coupled G proteins; these upstream components are uniformly distributed along the cell perimeter at all times. However, there is a clear spatial segregation of downstream effector molecules and cytoskeletal responses between the leading and the trailing edges of a migrating cell. Following stimulation, the active effectors found in the leading edges are Rho GTPases Rac, Cdc42, PI3K, PI3K lipid product PIP$_3$ and F-actin whereas active Rho GTPase RhoA, a Rho-dependent protein kinase (p160-ROCK), and myosin are located at the trailing edges of cells. The separation of these molecules into distinct domains of the cell
is believed to be brought about by localized mechanochemical incompatibility of the two cytoskeletal responses, combined with the ability of each to damp signals that promote the other.

At the leading edge of a chemotaxing cell, the signaling activities are dominated by Gαi, PI3K, GTPase Rac and Cdc42. Activation of these molecules leads to an accumulation of PIP_3, PH-domain containing proteins and polymerized actin at the leading edge. The development of polarized pseudopodia at the leading edge of mouse neutrophils can be separated into two sequential signaling events; a PI3K independent process followed by a PI3K dependent process. Neutrophils isolated from PI3Kγ (the most highly expressed class I PI3K isoform in neutrophils)-deficient mice showed wildtype directionality and straightness of cell movement with reduced migration speed. These data suggested that PI3K is not required for the initial partial polarization of neutrophils and the existence of a PI3K-independent mechanism which allows cells to migrate up the gradient though is insufficient to generate efficient chemotaxis of these cells. Normally, the PI3K-independent pathway works in parallel with PI3K pathway and the components from the two pathways can form positive feedback loops with each other amplifying the signal to generate full polarization and efficient chemotaxis of neutrophils.

The initial establishment of the partial polarity depends on activation of small GTPases of the Rho family, Rac and Cdc42. Cdc42 seems to function as a master regulator of polarity in eukaryotic cells. Inhibition of Cdc42 activity with its dominant negative form (Cdc42N17) prevents consolidation of a single leading edge upon ligand stimulation in neutrophils, fibroblasts and macrophages. In vivo assays have
shown that Cdc42 is active towards the front of migrating neutrophils. Activated Cdc42 at the leading edge of migrating neutrophils is essential for the massive recruitment of Arp2/3 complexes following ligand stimulation to the same area. Arp2/3 binds to the side of the existing actin filament and nucleates assembly of a new filament initiating the pseudopodia formation in the front of the cell. Cdc42 has also been shown to locate upstream of a signaling pathway which promotes localized degradation of RhoA (Rho A induces contractile actin-myosin stress fibers at the trailing end); hence excluding RhoA from the pseudopodia. Furthermore, active Cdc42 appears to be necessary to induce the spatial segregation of PIP₃ and its degrading enzyme PTEN which might be essential in maintaining the intracellular gradient of PIP₃. Like Cdc42, Rac GTPase is essential for the early polarity establishment and actin polymerization of neutrophils as demonstrated by complete random movements of Rac1- and Rac2-deficient neutrophils in the in vitro chemotaxis assays. However, Rac GTPase plays a distinct role from those of Cdc42 in regulating polarity during neutrophil chemotaxis. Rac GTPase is required for the chemoattractant-induced accumulation of PIP₃ and PH-domain containing proteins at the leading edge of migrating neutrophils. Rac, working together with PI3K, confines PIP₃ to the prospective leading edge which was initially formed independent of PI3K. The accumulated PIP₃ further enhances Rac activity by locally activating GEFs such as P-Rex1 and DOCK2 for Rac. The F-actin formed in response to Rac activation has been shown to positively regulate PI3K activities possibly by stabilizing the association of PI3K with the membrane and thereby stimulate more PIP₃ production. These positive feedback loops formed between Rac, F-actin and PI3K/PIP₃ provide the amplification mechanisms necessary for the
Figure 1-5. Localization of signaling events in a chemotaxing cell. The formation of polarity of a chemotaxing cells does not involve redistribution of chemoattractant receptors and the coupled G proteins; these upstream components are uniformly distributed along the cell perimeter at all time. However, there is a clear spatial segregation of downstream effector molecules and cytoskeletal responses between the leading and trailing edges of a migrating cell. The active effectors found in the leading edges are Rho GTPases Rac, Cdc42, PI3K, PI3K lipid product PIP\textsubscript{3} and F-actin whereas Rho GTPase RhoA, a Rho-dependent protein kinase (ROCK), ad myosin are located at the trailing end of cells. The separation of these molecules into distinct domains of the cell is brought about by localized mechanochemical incompatibility of the two cytoskeletal responses. Activated Cdc42 leads to a massive recruitment of Arp2/3 complex which nucleates assembly of new actin filaments from the existing ones. Cdc42 has also been shown to locate upstream of a signaling pathway which promotes localized degradation of RhoA. In addition, active Cdc42 appears to be necessary to induce the spatial segregation of PIP\textsubscript{3} and its degrading enzyme PTEN which is needed to maintain the intracellular gradient of PIP\textsubscript{3}. Rac is the other GTPase localized specifically at the leading edge of a chemotaxing cell. Active Rac, working together with PI3K, confines PIP\textsubscript{3} to the prospective leading edge. The accumulated PIP\textsubscript{3} enhances Rac activity by locally activating Rac GEFs such as P-Rex1 and DOCK2. The F-actin formed in response to Rac activation positively regulates PI3K activity. The positive feedback loops formed between Rac, F-actin and PI3K/PIP\textsubscript{3} provide the amplification mechanisms necessary for the conversion of a shallow extracellular gradient of chemoattractant into a steep intracellular second messenger gradient in the chemotaxing cells.
conversion of a shallow extracellular gradient of chemoattractant into a steep intracellular second messenger gradient in neutrophils. These processes facilitate the assembly of a “complete” leading edge and full polarization, which are required for efficient chemotaxis (Figure 1-5).

(b) Respiratory Burst

One of the critical functions of neutrophils is their ability to generate reactive oxygen species (ROS) that are involved in the regulation of cellular activities ranging from host defense to inflammation to intracellular signaling and transcription. ROS are molecules like hydrogen peroxide (H₂O₂), ions like the hypochlorite ion (OCl⁻), radicals like the hydroxyl radical (•OH) and superoxide anion (O₂⁻) which is both an ion and a radical. The high concentration of ROS is generated through the assembly of a membrane-associated NADPH oxidase complex. In the resting neutrophils, 10% of the oxidase complex is localized at the plasma membrane and 90% is found in two separate intracellular compartments: the secretory vesicles and specific granules. In both human and murine neutrophils, NADPH oxidase activation in the plasma membrane results in superoxide release to the extracellular milieu, and activation in intracellular granules leads to the production of intracellular oxidants. In mouse neutrophils, it is believed that both phorbol esters (PMA) and fMLF-induced activation results exclusively in extracellular release of ROS and no intracellular production could be detected.

The chemoattractant receptor-induced respiratory burst can be blocked by PTX treatment suggesting that the Gi family of α subunits mediates the signal transduction
Activation of ROS production of neutrophils relies on both Ca\textsuperscript{2+}-dependent and Ca\textsuperscript{2+}-independent signaling pathways. Upon receptor-ligand interaction, the dissociated G\textgreek{b}\textgreek{y} complex activates PLC-\textbeta\textsuperscript{382,383} which results in the breakdown of PIP\textsubscript{2} to form IP\textsubscript{3} and DAG. IP\textsubscript{3} induces Ca\textsuperscript{2+} mobilization from intracellular storage organelles, leading to a transient rise in cytosolic free Ca\textsuperscript{2+} which works in conjunction with DAG to activate PKC\textsuperscript{385}. PKC and various Ca\textsuperscript{2+}-sensitive protein kinases participate in respiratory burst\textsuperscript{386}. However, this rise in intracellular Ca\textsuperscript{2+} appears only to be required for the activation of these kinases especially PKC. Once these kinases are turned on, the agonist-induced activation of the NADPH oxidase is independent of Ca\textsuperscript{2+} mobilization\textsuperscript{387-389}.

NADPH oxidase is a functional multi-component electron-transfer system that catalyzes the reduction of molecular oxygen at the expanse of NADPH. The redox center is a heterodimeric flavocytochrome \textit{b}\textsubscript{558} comprised of two integral membrane proteins, gp91\textsuperscript{phox} and p22\textsuperscript{phox}\textsuperscript{376}. The gp91\textsuperscript{phox} subunit is a flavocytochrome containing an NADPH-binding site\textsuperscript{390}. The p22\textsuperscript{phox} subunit has a regulatory role which is essential for oxidase activation and recruitment of additional cytosolic subunits\textsuperscript{55}. Upon stimulation, four cytosolic proteins, p40\textsuperscript{phox}, p47\textsuperscript{phox}, p67\textsuperscript{phox}, and the GTPase Rac, translocate to the plasma membrane to assemble a fully active oxidase complex\textsuperscript{376}. The p47\textsuperscript{phox} subunit drives assembly of the complex by binding to p67\textsuperscript{phox} and facilitating the translocation of p67\textsuperscript{phox} to the membrane\textsuperscript{391}. Membrane translocation of p47\textsuperscript{phox} is made possible by phosphorylation-induced conformational changes of p47\textsuperscript{phox}. In the resting state, p47\textsuperscript{phox} exists in an autoinhibitory closed conformation formed through intramolecular binding of the two SH3 domains in the N-terminal region to a poly-basic sequence in its C-terminus.
Phosphorylation of \( p47^{\text{phox}} \) C-terminal region by PKC, Akt, Erk1/2 MAPK relieves the intramolecular inhibition and the SH3 domain is free to bind to the Pro-rich domain of \( p22^{\text{phox}} \). In addition, the freed SH3 domains unmask the phagocyte oxidase homology (PX) domain of \( p47^{\text{phox}} \) which in turn interacts with 3’-phosphorylated phosphoinositides, the products of activated PI3K.

The failure of \( p47^{\text{phox}} \)-deficient individuals to translocate NADPH oxidase cytosolic subunits demonstrated the essential role of this subunit to NADPH complex assembly. However, \( p47^{\text{phox}} \) is not intrinsically required for ROS production, because full oxidase activity can be reconstituted in vitro without \( p47^{\text{phox}} \) by increasing the concentration of \( p67^{\text{phox}} \) and Rac2. The \( p67^{\text{phox}} \) subunit interacts with \( gp91^{\text{phox}} \) directly and appears to activate catalysis by cytochrome \( b_{558} \) in a Rac-GTP dependent manner. Rac translocates to plasma membrane independently of \( p67^{\text{phox}} \) and \( p47^{\text{phox}} \). Although both Rac1 and Rac2 are expressed in neutrophils, the hematopoietic cell-restricted Rac2 is the preferred isoform to regulate neutrophil NADPH oxidase activity in response to most agonists. The selectivity for Rac2 is at least partially due to distinct subcellular distribution of Rac2 which is in turn determined by the C-terminal sequence and the aspartic acid at position 150 of Rac2. Given the importance of Rac2 in superoxide production, Rac-specific GEFs such as P-Rex1 and Vav1 are required for the full activation of NADPH oxidase. In addition to its GEF activity, Vav1 has been shown to interact directly with \( p67^{\text{phox}} \) and this interaction reinforces its GEF activity on Rac2. Vav1 appears to be a part of the positive feedback loop involving \( p67^{\text{phox}} \) for local amplification of Rac2 activation and subsequently NADPH oxidase activity (Figure 1-6).
Figure 1-6. NADPH oxidase complex. (A) A functional NADPH oxidase system contains two transmembrane protein gp91phox and p22phox, three cytoplasmic SH3-domain-containing proteins, p47phox, p67phox and p40phox, and a small GTP-binding protein, Rac (binding to the Rho GDP-dissociation inhibitor (RhoGDI)). The gp91phox subunit is a flavocytochrome containing an NADPH-binding site. The p22phox subunit has a regulatory role which is essential for oxidase activation and recruitment of cytosolic subunits. Upon stimulation/activation, four cytosolic proteins p47phox, p67phox, p40phox and Rac GTPase, translocate to the plasma membrane. The p47phox subunit drives assembly of the complex by binding to SH3 domain of p67phox through its proline-rich (Pro-rich) sequence and facilitating the translocation of p67phox to membrane. The p67phox subunit interacts with gp91phox and Rac GTPase, translocate to the plasma membrane. The p47phox subunit drives assembly of the complex by binding to SH3 domain of p67phox. Upon stimulation/activation, four cytosolic proteins p47phox, p67phox, p40phox and Rac GTPase, translocate to the plasma membrane. The p47phox subunit drives assembly of the complex by binding to SH3 domain of p67phox through its proline-rich (Pro-rich) sequence and facilitating the translocation of p67phox to membrane. The p67phox subunit interacts with gp91phox and Rac GTPase, translocate to the plasma membrane. (B) Membrane translocation of p47phox is made possible by phosphorylation-induced conformational changes of p47phox. In the resting state, p47phox exists in an autoinhibitory closed conformation formed through intramolecular binding of the SH3 domain in the N-terminal region to a Pro-rich sequence in its C-terminus. Phosphorylation of p47phox by PKC, Akt, and Erk1/2 MAPK relieves the intramolecular inhibition and the SH3 domain is free to bind to the Pro-rich domain of p22phox. In addition, the freed SH3 domains unmask the phagocyte oxidase homology (PX) domain of p47phox which in turn interacts with 3′-phosphorylated phosphoinositides in the membrane.
As described earlier in this chapter (section 1.2), the adapter protein 3BP2 contains binding sites for Vav1 and Src family PTK which have both been implicated in chemoattractant-mediated signaling. In addition, our and other laboratories have already identified 3BP2 as a positive modulator for some of its binding partners (section 1.2 and Figure 4-6). In chapter 4, the functional role of 3BP2 in chemoattractant-induced neutrophil activation will be discussed.
CHAPTER 2

Methods and Materials
2.1 Methods and Materials

3BP2 Gene-Targeted Mice. The wild-type 3BP2 gene is composed of 13 exons. Exon 2 contains the start codon, exons 2 to 5 encode the PH domain and exons 10 to 13 encode the SH2 domain of 3BP2. Part of exon 4 and 5 and the intervening intron were deleted. The 3BP2 targeting vector (Figure 1-2A) consisted of a 4 kb homology arm, a 0.9 kb short arm, and a PGK-neo cassette (neoR). The targeting vector was linearized with SalI and electroporated in the 129/Sv embryonic stem (ES) cell line, E14. ES cells were cultured in selection medium containing G418. The targeted clones were selected and injected into blastocytes from C57BL/6 mice. The chimeric animals were mated with C57BL/6 mice to yield mice that are seven-backcross generation (F7). Mice used in all experiments were in the F7 backcross generation and were maintained at the animal facilities of the Ontario Cancer Institute under specific pathogen-free conditions according to University Health Network animal care committee guidelines.

Plasmids. The expression vector of full-length murine 3BP2 was constructed by first amplifying full-length murine 3BP2 by PCR and TA-cloning the purified amplified product into PCR2.1 vector (Invitrogen, Burlington, ON). The 3BP2∆SH2 construct encoded amino acids 1-461 and lacked sequence encoding the final 98 amino acids, which includes the SH2 domain. The 3BP2∆PR plasmid was constructed by cutting pcDNA3.1-full-length 3BP2 with SmaI and BspEI and religating the vector to delete amino acids 189-290. The 3BP2 SH2 R486K construct was generated by overlapping PCR using full-length 3BP2 vector as a template.
Plasmid constructs for G protein subunits, Gαi, Gβ1 and Gγ2, were gifts from J. Sondek (University of North Carolina, USA).

**Cell Culture and Transfection.** Daudi cells expressing the ΔEC and Y9F, Y403/443F CD4:CD19 chimeras were gifts from R. Carter (University of Alabama, USA). A20 murine B lymphoma cells were purchased from American type Culture Collection (ATCC, Manassas, VA) and maintained in RPMI medium containing 10% FBS, 50 µM β-mercaptoethanol, and 10 mM HEPES. Raw264.7 macrophage cells (ATCC) were grown in DMEM containing 10% FBS. Osteoclast (OC) differentiation of Raw264.7 cells was performed by plating cells at 10,000 cells/cm² in the presence of 40µg/ml RANKL (R&D Systems, Minneapolis, MN) for 5 days. 293T cells (ATCC) were transfected with equal amount (1 or a 4 µg depending on the experiment) of the indicated plasmids using Polyfect Transfection Reagent (QIAGEN, Mississauga, ON), according to manufacturer’s recommendations, and maintained in medium containing 10% cosmic calf serum (CCS) (Thermo Fisher Scientific HyClone, Ottawa, ON), 50 µM β-mercaptoethanol, and 10 mM HEPES. Cells were cultured at 37°C in an incubator with a humidified atmosphere containing 5% CO₂.

**Reagents and Antibodies.** For CD19 and 3BP2 co-immunoprecipitation, the polyclonal antibodies against 3BP2 were prepared following sheep immunization with purified GST-3BP2 SH2 protein. To verify the absence of protein in 3BP2⁻/⁻ splenic B cells, we used affinity purified rabbit polyclonal anti-3BP2 antibody which was prepared by immunizing rabbits with purified GST-3BP2 SH2 protein (a gift from M. Deckert, Hôpital de l'Archet, Nice, France). The antibodies used in GST-pull down immunoprecipitation and immunoblotting assays were anti-GST (B14, monoclonal
antibody (mAb), Santa Cruz Biotechnology, Santa Cruz, CA), anti-Flag mAb (M2, Sigma, Oakville, ON), anti-human CD4 (Caltag, Burlington, ON), anti-mouse CD19 (MB19-1, eBioscience, San Diego, CA). The antibody used in Syk immunoprecipitation was a gift from A. Veillette (McGill University, Montreal). Anti-PLCγ2 (Q-20), anti-Myc (9E10), anti-phospho-Tyr (PY99) and anti-Vav1 (C-14) were purchased from Santa Cruz Biotechnology. Mouse anti-Flag antibody was purchased from Sigma. 4G10 antibodies were purified from hybridoma supernatant and were used to detect phosphotyrosine residues. Antibodies against phospho-Erk1/Erk2, Erk1/Erk2, phospho-Akt (Ser473), Akt, phospho-JNK/SAPK, JNK/SAPK, phospho-Src (Tyr416) and Src were purchased from Cell Signaling Technology (Danvers, MA). Antibody against pTyr173 Vav3 was purchased from Abcam. The biotin-, fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, and allophycocyanin (APC)-labeled antibodies (Abs) used for flow cytometry (all antibodies were from Pharmingen unless otherwise indicated) were anti-B220 (RA3-6B2), anti-CD43 (S7), anti-BP.1 (6C3), anti-HSA (M1/69), anti-CD4 (GK1.5), anti-CD8 (53-6.72), anti-CD25 (7D4), anti-CD44 (1M7), anti-CD3ε (145-2C11), anti-IgD (SBA.1), anti-μHC (33.60), anti-CD21 (7G6), anti-CD23 (B3B4), anti-CD5 (53-7.3), and anti-Gr1 (RB6-8C5). Recombinant BAFF (rBAFF) used for BAFF-mediated survival assays was purchased from Apotech Corporation (Switzerland).

**Isolation of Primary Lymphocytes.** Single-cell suspensions were prepared from spleen. Erythrocytes were lysed in ACK solution (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, 1M HCl). B lymphocytes were enriched by magnetic cell sorting with anti-CD43 and anti-CD11b MACS microbeads (Miltenyi Biotec, Auburn, CA) which yielded
~98% B220+ cells. Purified cells were cultured in RPMI medium containing 10% FBS, 50 µM β-mercaptoethanol, and 10 mM HEPES.

Isolation of bone marrow neutrophils. Mouse bone marrow (BM) neutrophils were isolated from femurs and tibias as described by Lowell et al. Marrow cells were flushed from bones using Hanks balanced salt solution (HBSS) (without Ca2+/Mg2+) + 0.1 % BSA followed by lysing RBCs ACK media. The remaining leukocytes were washed twice with Ca2+/Mg2+-free HBSS and resuspended in 3 ml of a 45% Percoll (Amersham Bioscience, Piscataway, NJ) solution in Ca2+/Mg2+-free HBSS. Cell suspensions were loaded on top of a Percoll density gradient prepared in a 15-ml polystyrene tube by layering successively 2 ml each of 62, 55, and 50% Percoll solutions on top of 3 ml of an 81% Percoll solution. Cells were then centrifuged at 2500 rpm for 30 minutes at room temperature. The cell band formed between the 81 and 62% layer was harvested using a Pasteur pipette, and washed twice with Ca2+/Mg2+-free HBSS + 0.1% BSA. The isolated mature neutrophils were resuspended in Ca2+/Mg2+-containing HBSS before each of the experiments unless otherwise indicated. FACS analysis of cell preparations revealed high expression of murine granulocyte marker Gr-1 on ~80-90% of the cells isolated from 62-81% interface.

Isolation of osteoclasts. BM cells were isolated from mice and treated with ACK buffer to remove red blood cells. Cells were plated at 10^6 cells/ml in α-MEM containing 10% FBS, 50U/L penicillin and 50 µg/L streptomycin, and 10 ng/ml of recombinant human CSF-1 (a gift from Chiron Corp., Emeryville, CA) for two days. The resulting BM monocytes were suspended in the same media with the addition of 40 µg/ml
RANKL. Cells were plated at $5 \times 10^5$ cells/cm$^2$ in 12 well plates. Media was changed after three days of culture. At day 5 after the addition of RANKL cells were processed.

**Flow Cytometry Analysis and Sorting.** Single-cell suspensions of thymi, lymph nodes, bone marrow and spleens from 3BP2$^{+/+}$ and 3BP2$^{-/-}$ mice were incubated with Fc block Abs (2.4G2) at 4°C to minimize nonspecific binding and then stained with a combination of biotin-, FITC-, PE, APC-labeled Abs on ice. Biotinylated Abs were visualized using streptavidin-PE-Cy5. For detection of apoptosis, cultured cells were stained with annexin V and propidium iodide (PI) using the Apoptosis Detection Kit (Pharmingen, Mississauga, ON), according to manufacturer’s recommendations. All samples were analyzed by a FACSAn™ or a FACcalibur flow cytometer and analyzed using CellQuest™ (Becton Dickinson, Mississauga, ON) and Flowjo software.

Splenic B cells were stained with anti-B220-APC (RA3-6B2), anti-CD21-FITC (7G6) and anti-CD23-PE (B3B4) antibodies, then sorted for marginal zone B and follicular B cells using a MoFlo high-speed cell sorter (Cytomation, Inc., Fort Collins, CO) which yielded ~98-99% of MZ B and ~94-97% of FO B cells.

**Immunohistochemistry.** Spleens were removed and snap frozen in OCT compound (Thermo Shandon, Pittsburgh, PA). 5 µm frozen spleen sections were then generated using a Leica 3050S cryostat and frozen sections were fixed in ice-cold acetone for 10 minutes. Spleen sections were first incubated with blocking solution (10% rabbit serum, 10% mouse serum, Fc block Abs in TBS/0.05% Tween-20) then stained with biotinylated anti-MAdCAM-1 clone MECA-367 (eBioscience) and were counter-stained with FITC-anti-B220. Sections were then stained with secondary antibodies streptavidin-conjugated horseradish peroxidase (Prozyme, San Leandro, CA) and anti-FITC.
conjugated alkaline phosphatase (Roche Diagnostics Canada, Laval PQ). Sections were then developed with Vector HRP development kit according to manufacturer’s instructions followed by Vector alkaline phosphatase substrate kit III according to manufacturer’s instructions (both Vector Laboratories Inc., Burlington, ON). Sections were mounted with Crystal/Mount™ (Biomeda corp., Foster City, CA) and visualized on a Leica upright DMRA2 microscope.

**Immunization.** 3BP2<sup>+/+</sup> and 3BP2<sup>−/−</sup> mice of 9 weeks of age were immunized intraperitoneally (i.p.) with 25 µg TNP-Ficoll or 50 µg TNP-LPS (both from Biosearch Technologies, Novato, CA) in sterile PBS and blood was collected at 0, 7, and 14 days post-immunization.

3BP2<sup>+/+</sup> and 3BP2<sup>−/−</sup> mice of 8 weeks age were immunized i.p. with 100 µg TNP-OVA (Biosearch Technologies) in sterile PBS, preincubated in Alu-Gel-S (Serva). Blood samples were collected at 0, 7, 14, and 21 days post-immunization.

**ELISA.** For measurement of the concentrations of isotype-specific immunoglobulins, each well of 96-well plates was coated with goat anti-mouse Ig as a capture reagent, and developed with isotype-specific goat sera directly conjugated with horseradish peroxidase (Southern Biotech, Birmingham, Alabama).

For anti-TNP and anti-NP ELISA, each well of 96-well plates was coated with 100 µl of 5 µg/ml BSA-TNP in PBS for 1 hr at 37°C followed by incubation at 4°C overnight. Plates were blocked with 5% BSA in PBS/0.05% Tween-20 for 2 hr at 37°C. The relative quantity of TNP- or NP-specific Abs in each blood-derived serum sample was determined by isotype-specific ELISA with horseradish peroxidase-conjugated anti-isotype Abs (Southern Biotech).
Cell proliferation Assay. Splenocytes and purified B cells from 8- to 12-week-old mice were stimulated for 48 hr with 10 µg/ml F(ab’)2 fragments of anti-mouse IgM (Jackson Immunoresearch, West Grove, PA) ± 10 µg/ml anti-CD40 clone 1C10 (Southern Biotech) or 10 µg/ml LPS (E.coli 055:B5; Sigma). The cells were harvested onto glass fiber filter mats, and the incorporated radioactivity was measured in a TopCount® liquid scintillation counter (Canberra/Packard, Mississauga, Ontario, Canada).

CMFDA Labeling of B Cells. Purified B cells were loaded with CellTracker Green CMFDA (5-chloromethylfluorescein diacetate; Molecular Probes Burlington, ON), according to manufacturer’s recommendations.

Detection of caspase-3 activity. Immediately after splenocytes were isolated from 3BP2+/- and 3BP2−/− mice, a portion of total splenocytes was incubated with FITC-DEVD-FMK (caspase-3 inhibitor) according to manufacturer’s recommendations and then stained with antibodies against surface markers, B220, CD21, and CD23. B cells were purified from the rest of splenocytes as described earlier. Purified B cells were stimulated for 12 and 24 hr with 10 µg/ml F(ab’)2 fragments of anti-mouse IgM (Jackson Immunoresearch). At each time point, cells were harvested, incubated with FITC-DEVD-FMK (caspase-3 inhibitor) and stained for B220, CD21, and CD23.

Intracellular staining of Bcl-xL, Bcl-2 and Mcl-1. Splenocytes were first stained with antibodies against surface markers, B220, CD21, and CD23 then were permeabilized with permeabilization buffer (Pharmingen) and stained with FITC-anti-Bcl-xL, PE-anti-Bcl-2 antibody (Pharmingen), or anti-Mcl-1 antibody (rabbit polyclonal; Santa Cruz). PE-anti-rabbit IgG (Jackson Immunoresearch) was used as the secondary
antibody to detect Mcl-1. After washing off the unbound antibodies, cells were fixed with 1% PFA (in PBS). B cells were purified from unstained splenocytes as described earlier. Purified B cells were stimulated for 12 and 24 hr with 10 µg/ml F(ab’)_2 fragments of anti-mouse IgM (Jackson Immunoresearch). At each time point, cells were harvested and stained for B220, CD21, CD23 and intracellular Bcl-xL, Bcl-2 or Mcl-1.

**Calcium Flux Assay.** Resting B cells were purified from 3BP2+/+ and 3BP2−/− mice as described earlier. Purified B cells were washed twice with HBSS. Cells were resuspended at 10x10^6 cells/ml with HBSS (Ca^{2+}/Mg^{2+}/0.5% BSA). Stock (5mM in DMSO) Indo-1AM (Molecular Probes) was diluted to 6 µM, and mixed with equal volume of Pluronic F-120 (20% solution in DMSO; Molecular Probes). Resuspended cells were mixed with diluted Indo-1 AM and Pluronic F-120 in 1:1 ratio, and the mixture was incubated in a 37°C water bath in the dark for 45 minutes. After incubation, cells were washed with HBSS twice, and stained for surface markers, CD21 and CD23, as previously described. Cells were then resuspended at 1x10^6 cells/ml with HEPES-buffered media (20 mM HEPES (pH 7.4), 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM glucose) for [Ca^{2+}]_i determination. Measurements were performed using a laser tuned to 338 nm while monitoring emissions at 405 and 450 nm. The concentration of intracellular Ca^{2+} was calculated according to following formula.

\[
[Ca^{2+}]_i = K_d \times \left(\frac{F_{min}}{F_{max}}\right) \times \left(\frac{R - R_{min}}{R_{max} - R}\right)
\]

Where \(K_d\) is the dissociation constant for indo-1 (250 nM at 37°C, pH 7.05); \(R\) is the fluorescence intensities measured at 405 and 450 nm during the experiments and \(F\) is the fluorescence intensity measured at 450 nm. \(R_{min}, R_{max}, F_{min}, F_{max}\) were determined from
in situ calibration of unlysed cells using 4 µM ionomycin in the absence ($R_{\text{min}}$ and $F_{\text{min}}$; 2.5 mM EGTA) and presence ($R_{\text{max}}$ and $F_{\text{max}}$) of Ca$^{2+}$

**Abl Kinase Assay.** Purified 3BP2$^{+/+}$ and 3BP2$^{-/-}$ B cells were stimulated with 10 µg/ml F(ab’)$_2$ fragments of anti-mouse IgM (Jackson Immunoresearch) for indicated time intervals and were lysed with buffer (25 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EGTA, 3 mM EDTA, 10% glycerol, 1% Triton X-100, protease inhibitor tablet, 5 mM Na orthovanadate, 10 mM β-glycerophosphate). After 30 min on ice, samples were spun for 10 min at 17000 × g in a microfuge at 4°C. To the supernatants, 2 µg of c-Abl (K12, Santa Cruz Biotechnology) antibody and 20 µl of protein A-Sepharose beads (Amersham) were added and incubated overnight at 4°C. Immune complex were washed three times with ice-cold lysis buffer and twice with ice-cold kinase wash buffer (20 mM HEPES (pH 7.4), 10 mM MgCl$_2$, 1 mM DTT, 5 mM Na orthovanadate, 10 mM β-glycerophosphate). After the last addition of kinase wash buffer, half of the sample was removed to a fresh tube which was used for Western blot to normalize Abl as loading control. To the rest of sample, 20 µl of hot kinase buffer (20 mM HEPES (pH 7.4), 10 mM MgCl$_2$, 1 mM DTT, 1 mM ATP, 1 µg/ml GST-Crk-mCTD (a gift from J. Wang, University of California, La Jolla), 20 µl [γ$^{32}$P] dATP) was added. The kinase reaction was stopped by addition of 5 µl of 6X sample buffer and boiled for 5 min at 100°C. Samples were resolved on a 12 % SDS Tris-glycine gel and then transferred onto PVDF membranes. Membranes were fixed for 30 min at room temperature in 30% methanol and 10% acetic acid with gentle shaking and dried under vacuum for 2 hr at 80°C before being exposed to film to detect radioactivity incorporated into the substrate.
**Surface expression of fMLF receptor.** BM neutrophils were resuspended at $1 \times 10^6$ cells/ml in PBS. 10 nM of N-formyl-Nleu-Leu-Phe-Nleu-Tyr-Lys-fluorescein (Molecular Probe) was added to the cell suspension and the samples were incubated for 20 minutes at 4°C followed by analyzing with FACScan flow cytometer (Becton Dickinson) and Flowjo software for fluorescent intensity. Debris and dead cells were excluded with a gate on forward and side scatter. Nonspecific binding was determined in the presence of 1 µM non-labeled N-formyl-Nleu-Leu-Phe-Nleu-Tyr-Lys.

**Chemotaxis assays.** BM neutrophils were resuspended in HBSS + 1% gelatin at $1 \times 10^6$ cells/ml. Cell suspension was allowed to attach to bovine serum albumin (BSA)-coated glass coverslips (22×40 mm) at 37°C for 20 minutes. The coverslip was inverted onto a Zigmond chamber, followed by addition of 100 µl of HBSS media to the left chamber and 100 µl of HBSS media containing 10 µM fMLF to the right chamber. Neutrophil movement in Zigmond chambers was recorded with time-lapse video microscopy (Nikon upright OPTIPHOT). Images were captured at 60-second intervals with a Nikon coolSnap Pro color camera. Cell-tracking software (Retrac version 2.1.01 Freeware) was used to characterize neutrophil chemotaxis from the captured images.

**Actin polymerization and F-actin localization.** BM neutrophils were resuspended in HBSS and stimulated with 10 µM fMLF for different time intervals. Cells were immediately fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained with Alexa Fluor 488-conjugated phalloidin (1 U/500 µl cell suspension) (Molecular Probes) to detect F-actin content. Samples were analyzed with a FACSCalibur flow cytometer.
For visualization of F-actin localization, chemotaxing neutrophils (under the 10 µM fMLF gradient) on coverslips were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 in PBS. Cells were then incubated in PBS containing 3% BSA and stained with Oregon Green 514-conjugated phalloidin (Molecular Probes). All images were taken with a laser scanning confocal microscope (Zeiss LSM510).

**Thioglycollate-induced peritonitis.** 8-10-week-old male mice were injected intraperitoneally (i.p.) with 700 µl of 4% sterile thioglycollate broth (gift from J. Jongstra-Bilen, University of Toronto) and killed 4 and 18 hours later. Total leukocytes elicited into the peritoneal cavity were harvested by peritoneal lavages and counted with a hemocytometer. Antibodies against Gr-1 and F4/80 were used to stain cells to distinguish between neutrophils (Gr-1⁺F4/80⁻) and macrophages (Gr-1⁻/F4/80⁺). Stained cells were analyzed with a FACSCalibur flow cytometer.

**Superoxide anion production.** BM neutrophils (1×10⁵) resuspended in HBSS were stimulated with 10 µM fMLF or 1 µM PMA. The chemiluminescence was counted with an enhancer-containing, lumino-based detection system (National Diagnostics, Atlanta, Georgia) using a luminometer. Superoxide dismutase (SOD) was added to some samples which were used as negative controls.

**Listeria monocytogenes infection.** For the bacterial burden assay, 8-10-week-old mice were infected intravenously (i.v.) with 2×10⁴ cfu of *L. monocytogenes* (expressing recombinant ovalbumin) and killed by cervical dislocation 48 hours after infection. Numbers of viable *L. monocytogenes* in livers and spleens of infected animals were determined by plating serial dilutions of organ homogenates in PBS on brain-heart
infused agar. For survival assay, 8-10-week-old mice were infected i.v. with $2 \times 10^5$ cfu of *L. monocytogenes*. The end point of the assay is 14 days post infection.

**GST precipitation, immunoprecipitation and immunoblotting.** Purified B cells were lysed in ice-cold 1% NP-40 buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1 mM Sodium orthovanadate, 2 mM EDTA, 50 mM NaF, and protease inhibitors) for 20 minutes on ice. Isolated BM neutrophils were lysed in ice-cold 1% Triton X-100 lysis buffer (50 mM HEPES [pH 7], 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl$_2$, 1 mM EGTA, 1 mM Na orthovanadate, 1 mM phenylmethylsulfony fluoride, and other protease inhibitors) for 20 minutes on ice. Nuclei were pelleted by centrifugation for 10 min at 16,000 × g, 4°C. For GST precipitations, 10 µg of GST-3BP2 SH2 fusion protein was incubated with glutathione-sepharose 4B beads (Amersham) for 1 hr at 4°C, followed by incubation with lysates for 2 hr. Samples were washed 4 times in lysis buffer. For immunoprecipitation, lysates were incubated for 2 hr at 4°C with the indicated Abs followed by incubation with either protein A- or protein G-Sepharose™ beads (Amersham) for 1 hr. For CD19-phosphotyrosinepeptide precipitation, biotinylated peptides (5 µM) were incubated with 0.2 ml of a 25% slurry of streptavidin-agarose in PBS for 1 hr at 4°C followed by washing. The beads were incubated with NP-40 lysates of $2.5 \times 10^7$ Daudi cells for 30 min at 20°C. After incubation, pellets were washed 3 times with ice-cold lysis buffer and resuspended in SDS sample buffer. For Syk and PLCγ2 immunoprecipitation, splenic B cells were purified as described earlier and lysed with cold 1% Triton-X buffer (50 mM HEPES (pH 7), 150 mM NaCl, 10% Glycerol, 1% Triton X-100, 1.5 mM MgCl$_2$, 1 mM EGTA, 1 mM Na orthovanadate, 1 mM PMSF and other protease inhibitors). Antibodies
were added to cell lysates followed by addition of protein A-Sepharose beads (Amersham). Eluted immunoprecipitates or whole-cell lysates were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes, probed with the indicated primary Abs and horseradish peroxidase (HRP)-conjugated secondary Abs and developed using an enhanced chemiluminescence kit (Amersham) following the manufacturer’s instructions.

**Co-immunoprecipitation.** For CD19/3BP2 co-immunoprecipitation from purified splenic B cells, Affigel 10 beads (Bio-Rad, Mississauga, ON) were used and cells were stimulated with 40 µg/ml of anti-IgM F(ab’)_2 in the presence of 10 mM pervanadate.

To determine the domain(s) of 3BP2 required for its binding to CD19, 293T cells transfected with indicated plasmids were lysed with 1% NP-40 lysis buffer. Lysates were incubated with anti-Flag mAb for 1 hr at 4°C followed by incubation with protein G-Sepharose beads (Amersham) for 2 hr. Precipitated products were washed 4 times with lysis buffer and then added to A20 cell lysates (as a source of endogenous CD19) after A20 cells were stimulated with 20 µg/ml of anti-IgG F(ab’)_2. After an 1-hr-incubation at 4°C, samples were washed and resuspended in SDS sample buffer. Associated proteins were analyzed through immunoblotting as described earlier.

To determine if 3BP2 forms complex with G protein subunits, 293T cells transfected with indicated plasmids were lysed with 1% Triton X-100 lysis buffer (30 mM Tris [pH 7.5], 150 mM NaCl, 1% Triton X-100, 2 mM EGTA, 0.2% Na deoxycholate, 10 mM NaF, 1 mM Na orthovanadate, 1 mM phenylmethylsulfony fluoride, and other protease inhibitors). Lysates were incubated with anti-Myc Ab and
protein G-Sepharose beads (Amersham) for 2 hr at 4°C. Precipitated products were washed 4 times with lysis buffer. Associated proteins were analyzed through immunoblotting as described earlier.

**Pull-down assay.** BM neutrophils were lysed as described above. Aliquots of the cell extracts were kept for total lysate controls, and the remaining extracts were incubated with GST-fusion, Cdc42/Rac-binding domain of PAK1 (GST-PAK1-PBD) (Cytoskeleton Inc., Denver, CO) at 4°C for 1 hour preceded by a 1-hour-incubation of glutathione agarose beads with GST-PAK1-PBD. The bound proteins and the same amount of total lysates were analyzed by SDS-PAGE, and blots were probed with Rac1-specific antibody (BD Transduction Laboratories), Rac2-specific antibody (Upstate Biotechnology, Lake Placid, NY) or Cdc42-specific antibody (BD Transduction Laboratories).

**Preparation of peptide SPOTS array.** Peptide SPOTS arrays were prepared as previously reported by synthesis of 13-mer peptides covering the entire sequence of mouse 3BP2 with a 5 amino acid overlap between one peptide and the next in the array. This resulted in the 3 central amino acids being unique in each 13-mer. Synthesized peptides were spotted onto membranes in 3 columns of 30 spots. Spots 1 to 70 covered the entire length of 3BP2. Spot 72 represents the SH3 binding site of 3BP1 (APTMPPPLPPGGK) while spots 73-76 represent generic RxPxxP peptides (73: ARALPPLPRYA, 74: ARALAPLPRYA, 75: ARALPPLARYA, 76:ARALAPLARYA) and spots 77-80 represent generic PxxPxR peptides (77: AAFAPPLPRRA, 78: AAFAAPLPRRA, 79: AAFAPPLARRA, 80: AAFAAPLARRA). Spots 82-83 represent SH3 binding sequences of dynamin (82: PTPQRRAPAVPPG, 83: GGAPPVPSRP GAS),
spots 85-86 represent SH3 binding sites of PI3K p85 subunit (85: PRPPRPLPVAPGS, 86: RQPAPALPPKPPK), and spots 88-90 represent SH3 binding sites of Sos1 (88: PVPPPVPPRRPE, 89: ESPPLLPPREPVR, 90: IAGPPVPPRQSTS). Spots 71, 81, 84 and 87 are blank. Spotted arrays were stored at –20°C until required.

**Far-Western.** 3BP2 peptide SPOT array was wet briefly in ethanol then washed 3 times in 1 × TBST and was blocked at room temperature for 2 hours with 5 % w/v BSA. Purified recombinant GST or GST fusions of the c-Src SH3 domain were used to probe the peptide arrays at 0.5 µM in Overlay Binding Buffer (1 x TBST, 2 mM MgSO4, 1% w/v BSA) overnight at 4 °C. The arrays were washed extensively in 1 x TBST before incubation with mouse anti-GST antibody (Santa Cruz) diluted in Overlay Binding Buffer for 1 hour at room temperature. The array was thoroughly washed in 1 x TBST followed by incubation with ECL-anti-mouse antibody (raised in sheep, GE Healthcare UK) diluted in Overlay Binding Buffer for 1 hour at room temperature. The array was washed again in 1 x TBST and then briefly in water before detection using ECL western blot detection system (GE Healthcare UK).

**Statistical Analysis.** Averaged numerical data were represented as mean ± standard deviation. Unless otherwise indicated, student’s t test was used to determine the statistical significance of differences between groups. Statistical significance was defined as *p < 0.05; **p < 0.01; ***p < 0.001.
CHAPTER 3

The 3BP2 Adapter Protein Is Required for Optimal B-cell Activation and Thymus-Independent Type 2 Humoral Response

A report of the same title by Grace Chen, Ioannis D. Dimitrious, Jose La Rose, Subburaj Ilangumaran, Wen-Chen Yeh, Gina Doody, Martin Turner, Jennifer Gommerman, and Robert Rottaple is published in Molecular and Cellular Biology, 27: 3109-3122 (2007)

• The experiment in Figure 3-1 was performed by me.
• The experiment in Figure 3-2 was performed by me.
• The experiment in Figure 3-3A was performed by Dr. Jennifer Gommerman and the experiments in Figure 3-3B and C was performed by me.
• The experiment in Figure 3-4 was performed by me.
• The experiment in Figure 3-5 was performed by me.
• The experiment in Figure 3-6 was performed by me.
• The experiment in Figure 3-7 was performed by me and Dr. Ioannis D. Dimitrious
• The experiment in Figure 3-8 was performed by me.
• The experiment in Figure 3-9 was performed by me and Dr. Ioannis D. Dimitrious
• The experiment in Figure 3-10A was performed by Jose La Rose and the experiments in Figure 3-10B, C and D were performed by me.
• The experiment in Figure 3-11 was performed by me.
• The experiment in Figure 3-12A, B and D were performed by me and the experiment in Figure 3-12C was performed by Dr. Gina Doody. The constructs of 3BP2 used in the experiment in Figure 3-13B and D were made by Dr. Subburaj Ilangumaran.
3.1 Introduction

The B cell antigen receptor (BCR) plays an essential role in the generation and activation of B lymphocytes. The BCR signaling leads to various cellular responses including maturation of developing B cells and activation, proliferation, and differentiation of mature B cells (section 1.4.2 and section 1.4.3). The engagement of BCR is known to activate non-receptor PTKs such as Syk, Src family kinase Lyn and Btk (section 1.4.1a). The substrates of these activated PTKs include effector enzymes such as PLC-γ (section 1.4.1b) and Vav family of GEFs (section 1.4.1c). PLC-γ is associated with intracellular calcium mobilization and PKC activation (section 1.4.1b), whereas Vav is a GEF for Rac GTPase which in turn mediates the activation of JNK and p38 MAPKs. Each of these downstream components play a pivotal role in BCR signal transduction, therefore mutations or gene-targeted deletions of these proteins often lead to defects in B-cell activation during an immune response (section 1.4.3) as well as alterations in the peripheral B-cell composition (section 1.4.2 and Table 1.1).

BCR signaling is also regulated by its membrane-anchored coreceptor CD19. CD19 is considered a positive regulator of BCR signaling by amplifying the Src family PTK activation and MAPK activation. Coligation of CD19 and BCR greatly augments BCR-induced calcium mobilization and dramatically lowers the threshold for B-cell activation. In addition to its role in amplifying BCR signaling, CD19 signaling contributes to the development of peripheral B-cell subsets, MZ and B-1 B cells (section 1.4.2 and Table 1.1).
Accumulated evidence has suggested a role of the adapter proteins in BCR signal propagation. One of the most extensively studied, and found to link many signaling events following BCR stimulation is the adapter protein BLNK. BLNK is a substrate of Syk and couples Syk activation to PLC-γ by mediating PLC-γ membrane recruitment. Other functions of BLNK downstream of BCR include activation of Ras pathway, activation of Erk MAPK and optimal activation of Vav GEFs (section 1.4.1d). Our laboratory and others have identified a novel adapter protein, 3BP2, functions as a positive regulator downstream of immunoreceptors on many different cell types including B cells in overexpression studies (section 1.2). The expression of 3BP2 transcripts is restricted to only a few tissues and is most highly expressed in B lymphocytes (Figure 1-3). In addition, Foucault et al. demonstrated that 3BP2 form complexes with a number of signaling proteins including PLC-γ, Vav GEFs and Syk in Daudi B cells (section 1.2.2). Given the high expression of 3BP2 mRNA in B cells and its complex formation with several essential signaling molecules downstream of BCR, we hypothesized that 3BP2 plays a role in BCR signal transduction in primary B cells.

### 3.2 Results

3.2.1 3BP2−/− mice exhibit increased marginal zone B cells and decreased peritoneal CD5+ B1 B cells

Given the restricted high expression of 3BP2 in B cells, mice lacking 3BP2 were analyzed for possible defects in B cell development. The frequencies of pre-, immature and mature re-circulating B cells in the bone marrow of 3BP2−/− mice were comparable to
Figure 3-1. 3BP2$^{-/-}$ mice have normal B-cell development. (A) Bone marrow cells from 3BP2 (+/+ and mutant (-/-) mice were analyzed by flow cytometry after staining for B220, CD43, BP.1 and HSA. (B) Frequency (top panel) and the absolute number (lower panel) of splenocytes express B220 marker was obtained by FACS analysis of cells from 3BP2$^{+/+}$ (solid circle) and 3BP2$^{-/-}$ (open circle) mice. Each data point is from an individual mouse and means are indicated by horizontal lines ($n = 6$).
Figure 3-2. 3BP2<sup>−/−</sup> mice have increased marginal zone B cells. (A) Summary of the surface markers used to distinguish T1, T2, mature and MZ B cells (B) A representative plot of FACS analysis of subsets of splenic B cells from 3BP2<sup>+/+</sup> (+/+) and 3BP2<sup>−/−</sup> (-/-) mice. Antibodies against surface markers, B220, IgM, CD23, CD21, were used to distinguish between different subsets of splenic B cells. 3BP2<sup>−/−</sup> mice show 2.5-fold increase in the frequency of MZ B cells. (C) Frequency (top panels) and absolute numbers (lower panels) of subsets of splenic B cells were obtained based on FACS analysis of cells from 3BP2<sup>+/+</sup> (solid circles) and 3BP2<sup>−/−</sup> (open circles) mice. Each data point is from an individual mouse and means are indicated by horizontal lines (n=8; * p < 0.05; ** p < 0.01).
wild-type controls whereas we observed a slight decrease of intermediate pro-B cells (B220⁺CD43⁺BP.1⁺HSA⁺) in mutant mice (~40% vs. 34% of the B220⁺CD43⁺ population) (Figure 3-1A). Flow cytometric analysis of spleen revealed that the frequency and the absolute number of B220⁺ splenocytes in the 3BP2⁻/⁻ mice were comparable to wild-type controls (Figure 3-1B). We next examined the frequency of splenic B subsets, transitional-1 (T1: B220⁺CD23⁻CD21⁺IgM⁺), transitional-2 (T2: B220⁺CD23⁺CD21⁺IgM⁺), mature (B220⁺CD21⁺CD23⁺IgM⁺), and marginal zone (MZ: B220⁺CD23⁻CD21⁺IgM⁺) B cells (Figure 3-2A and 3-2B). 3BP2⁻/⁻ mice had decreased frequency (2.2% ± 0.46% 3BP2⁻/⁻ vs. 3.8% ± 1.2% control) and absolute numbers (1.7x10⁶ ± 0.67x10⁶ 3BP2⁻/⁻ vs. 2.7x10⁶ ± 1.1x10⁶ control) of T1 cells and a modest increase in frequency (2.3% ± 0.71% 3BP2⁻/⁻ vs. 1.3% ± 0.5% control) and cell numbers (1.8x10⁶ ± 0.78 x10⁶ 3BP2⁻/⁻ vs. 1.1x10⁶ ± 0.66 x10⁶ control) of MZ B cells. T2 and mature B cells were not perturbed in 3BP2⁻/⁻ mice (Figure 3-2C).

We performed immunohistochemistry to determine if splenic architecture was altered in 3BP2⁻/⁻ mice. Many follicles manifested enlargement of the MZ compartment as delineated by B220⁺ cells populating the region outside of the marginal sinus (Figure 3-3A). Many of the cells within the marginal zone stained positive for IgM⁻IgD⁻, indicative of increased numbers of MZ B cells in this expanded compartment (Figure 3-3A).

In distinction to MZ B cells, CD5⁻IgM⁺ peritoneal B1 B cells were significantly diminished in the 3BP2⁻/⁻ mice compared to 3BP2⁺/+ mice (6.3% ± 2% 3BP2⁻/⁻ vs. 16% ± 0.57% control) (Figure 3-3B). Serum antibody levels from naïve mice were measured by enzyme-linked immunosorbent assay (ELISA), demonstrating significantly reduced
Figure 3-3. 3BP2−/− mice have increased marginal zone B cells, decreased peritoneal B-1 B cells, and reduced basal serum IgG1 and IgA level. (A) Cryosections of spleens from 3BP2+/+ (+/+, to p panels) and 3BP2−/− (−/−, lower panels) were stained with antibodies against MAdCAM-1 (red) + B220 (blue) and antibodies against IgM (red) + IgD (blue). Anti-MAdCAM-1 stains the marginal sinus of spleen 303. The data shown are representative from three independent experiments. (B) Cells recovered from peritoneal washes were stained with antibodies against IgM and CD5 to compare the relative number of B-1 B cells (IgM+CD5+) between 3BP2+/+ and 3BP2−/− mice. The data shown are representative from three independent experiments. (C) Sera were collected from 9- to 11-week-old 3BP2+/+ and 3BP2−/− mice and measured for the concentrations of each Ig isotype by standard ELISA (n=6; * p < 0.05).
Figure 3-4. 3BP2<sup>−/−</sup> mice have no detectable T-cell development abnormalities within the thymus, spleen or lymph node. (A) Developing T cells isolated from thymus of 3BP2 (+/+ ) and mutant (−/−) mice were analyzed after staining for CD4, CD8, CD25 and CD44. (B) Mature T cells isolated from lymph nodes were examined for surface expression of CD4 and CD8. (C) Ratio of B cells to T cells in secondary lymphoid organs of 3BP2 and mutant mice was examined with monoclonal antibodies against B220 (B cells) and against CD3 (T cells).
levels of IgG1 and IgA, but not the other isotypes of Igs in 3BP2−/− mice (Figure 3-3C). No T cell developmental abnormalities were detected within the thymus or lymph node in 3BP2−/− mice (Figure 3-4).

These data suggest that 3BP2 deficiency gave rise to B cell-specific abnormalities characterized by diminished numbers of immature T1 B cells, an expanded MZ B cell pool, contracted CD5+ peritoneal B1 B cell population and reduced basal serum IgG1 and IgA levels.

3.2.2  Impaired TI-2 Response in immunized 3BP2−/− mice

MZ B cells and B1 B cells have been linked to immune responses to TI-2 multivalent antigens. We tested the capacity of the 3BP2−/− mice to elicit a TI-2 humoral response. 3BP2−/− mice were challenged with trinitrophenol conjugated to Ficoll (TNP-Ficoll), a classical T-cell-independent antigen. Serum anti-TNP antibodies in 3BP2−/− and wild-type controls were measured by ELISA at 7 and 14 days post-immunization with intraperitoneal injection of TNP-Ficoll. Compared to wild-type mice, 3BP2−/− mice produced significantly lower titers of anti-TNP IgM and IgG3 antibodies following immunization (Figure 3-5, top panels). In distinction to the TI-2 immune response, 3BP2−/− mice were fully competent to elicit TI-1 and TDs immune responses to TNP-LPS or TNP-Ova, respectively (Figure 3-5, middle and lower panels). These data show that 3BP2, as an adapter protein, is required for the functional response of B cells to TI-2 antigen challenge.
Figure 3-5. Humoral response in 3BP2−/− mice. 3BP2+/+ and 3BP2−/− mice were immunized with the TI-2 antigen TNP-Ficoll and TI-1 antigen TNP-LPS. Serum anti-TNP antibodies in 3BP2+/+ (solid circle) and 3BP2−/− (open circle) mice were measured by ELISA at 0, 7 and 14 days post-immunization (n = 5; * p < 0.05; ** p < 0.01; *** p < 0.001). 3BP2+/+ and 3BP2−/− mice were immunized with the T-dependent antigen TNP-OVA. Serum anti-TNP antibodies in 3BP2+/+ (solid circle) and 3BP2−/− (open circle) mice were measured by ELISA at 0, 7, 14 and 21 days post-immunization (n = 4).
3.2.3 Impaired proliferation and survival of 3BP2<sup>−/−</sup> splenic B cells

We next tested the requirement of 3BP2 in sorted resting splenic B cells to mediate proliferation and survival following BCR cross-linking with anti-IgM antibody and LPS exposure. Purified splenic B cells were stimulated with either anti-IgM, anti-IgM+anti-CD40, or LPS. Forty-eight hours after stimulation the proliferation index of the B cells was measured by [³H]thymidine incorporation. 3BP2<sup>−/−</sup> resting B cells showed a lower proliferative response following cross-linking with anti-IgM, or anti-IgM+anti-CD40 antibodies compared to control 3BP2<sup>+/+</sup> B cells while the LPS response was normal in 3BP2<sup>−/−</sup> B lymphocytes (Figure 3-6A). To determine if the diminished incorporation rates of [³H]thymidine observed in the 3BP2<sup>−/−</sup> B cells resulted from their decreased capacity to undergo cell division or an increased tendency to undergo cell death, cells were analyzed by the CFSE dilution assay or annexin V/propidium iodide staining following stimulation with anti-IgM, anti-IgM+anti-CD40, or LPS. Forty-eight hours after stimulation a smaller fraction of 3BP2<sup>−/−</sup> B cells completed the first cell division compared to 3BP2<sup>+/+</sup> B cells in response to BCR cross-linking (23% vs. 41%) (Figure 3-6B). This delay in cell division was less prominent following CD40 co-stimulation or treatment with LPS. The viability of 3BP2<sup>−/−</sup> B cells was also significantly diminished in medium alone and following BCR cross-linking relative to 3BP2<sup>+/+</sup> B cells at 48 hr (Figure 3-6C). The 3BP2<sup>−/−</sup> B cell survival defect was largely corrected by anti-CD40, LPS or BAFF stimulation (Figure 3-6C and 3-6D).

To determine whether the defect in cell survival of 3BP2<sup>−/−</sup> B cells was due to accelerated apoptosis, we examined the activation of caspase-3 following BCR cross-linking. Purified B cells were stimulated with anti-IgM antibodies for 12 and 24 hr and
Figure 3-6. The BCR-mediated proliferation and cell viability of 3BP2−/− resting B cells are reduced. (A) Purified B cells from 3BP2+/+ (solid bars) and 3BP2−/− (open bars) mice were treated as indicated. Wells of a 96-well plate were pulsed with [3H]thymidine (1 μCi/well) and DNA synthesis during the final 12 hr of culture was measured by liquid scintillation counting. The data presented are the means of triplicate wells and are representative of three independent experiments (** p < 0.01). (B) Purified B cells were labeled with CFSE and treated as indicated. Cell division was determined for viable cells. The data shown are representative of three independent experiments. (C) Purified 3BP2+/+ (solid bars) and 3BP2−/− (open bars) B cells were treated as indicated and the percentage of cells negative for both annexin V-binding and propidium iodide (PI) staining was measured after 48 hr. The values on the graph were the average values of four individual animals of each genotype (n = 4; * p < 0.05) from four independent experiments. (D) Purified 3BP2+/+ and 3BP2−/− B cells were treated with different concentration of recombinant BAFF (0.2, 12.5 and 200 ng/ml) for 72 hr. The percentage of the viable cells negative for both annexin V-binding and PI staining was shown. The data shown are representative of three separate experiments.
Figure 3-7. 3BP2⁺⁺ splenic B cells have accelerated apoptosis with enhanced activation of caspase-3. Enhanced activation of caspase-3 in 3BP2⁺⁺ splenic B cells ex vivo, 12 hr and 24 hr after BCR cross-linking compared to 3BP2⁺⁻ cells. The data shown are representative of five separate experiments. Number indicated the percentage of B220⁺ cells with activated caspase-3. The lower panels show decreased up-regulation of anti-apoptotic molecules, Bcl-xL molecules in 3BP2⁺⁻ B cells (open bars) at 24 hr after BCR cross-linking compared to 3BP2⁺⁺ B cells (solid bars). In contrast, Mcl-1 and Bcl-2 expression following BCR cross-linking was comparable in control and mutant cells (*p < 0.05). A.U., arbitrary units.
the percentage of cells with active caspase-3 was determined by a FITC-conjugated cell-permeable, non-toxic inhibitor (FITC-DEVD-FMK) that binds irreversibly to activated caspase-3 in apoptotic cells. At 24 hr, 3BP2−/− B cells demonstrated a two-fold increased frequency of caspase-3 activation compared to the 3BP2+/+ B cells (Figure 3-7).

The regulation of the intrinsic cell death pathway in B cells is controlled by the balanced expression of anti-apoptotic proteins, Bcl-xL 421, Bcl-2 422, Mcl-1 423 and A1 424. We examined the capacity of 3BP2+/+ and 3BP2−/− B cells to appropriately up-regulate Bcl-xL, Bcl-2, and Mcl-1 in comparison to the 3BP2+/+ B cells following BCR cross-linking. Purified resting B cells were stimulated with anti-IgM antibodies for 12 or 24 hr, fixed and stained with anti-Bcl-xL, anti-Bcl-2, or anti-Mcl-1 antibodies. When compared to 3BP2+/+ B cells, there was a lag in Bcl-xL up-regulation in 3BP2−/− B cells 24 hr following BCR cross-linking whereas both 3BP2+/+ and 3BP2−/− B cells were equally competent to up-regulate other anti-apoptotic molecules such as Bcl-2 and Mcl-1 in response to BCR cross-linking (Figure 3-7). These experiments show that resting B cells derived from 3BP2−/− mice demonstrate both a proliferation and survival defect in response to stimulation through BCR and suggest that 3BP2 is required for optimal BCR signal transduction.

3.2.4 Impaired survival of 3BP2-deficient MZ B cells

FO and MZ B cells respond differently to BCR signals. Cross-linking of the BCR induces proliferation and survival in FO B cells while MZ B cells undergo programmed cell death-induction in in vitro studies 413 suggesting that these related cell types may be wired differently. To test whether 3BP2 plays a selective role in BCR-mediated
Figure 3-8. The cell viability of 3BP2-/- MZ B cells is reduced after BCR cross-linking but is unaffected following anti-IgM+anti-CD40, LPS and r BAFF stimulation. (A and B) Purified 3BP2+/+ (top panels) and 3BP2-/- (lower panels) MZ B (left panels) and FO B (right panels) cells were treated as indicated and the percentage of cells negative for both annexin V-binding and propidium iodide staining was measured after 24 hr. The data presented are representative of three separate experiments.
outcomes in peripheral B cell subsets, we assessed the susceptibility of FO and MZ B cells from 3BP2^{+/+} and 3BP2^{-/-} mice to undergo antigen receptor-induced death. In concert with previous findings, we observed an increased sensitivity of wild-type MZ B cells to undergo antigen receptor-induced cell death compared to FO B cells (Figure 3-8A, top panels) (8% vs. 36% viable cells respectively at 24 hrs). However, we observed a differential sensitivity to antigen receptor-induced cell death in the MZ B cell compartment compared to FO B cells in the absence of 3BP2. Specifically, MZ B cells experienced a 42% decrease in viability compared to FO B cells whose viability diminished by only 28% in the absence of 3BP2 compared to wild-type controls (Figure 3-8A). Moreover, by 24 hours nearly all of the non-viable 3BP2^{-/-} MZ B cells had entered the late apoptotic stage (Annexin V^{+}\text{PI}^{+}) compared to wild-type MZ B cells suggesting that in the absence of 3BP2 MZ B cells undergo an enhanced rate of apoptosis (Figure 3-8A, left panels). Addition of anti-CD40 monoclonal antibodies (mAbs), LPS, or BAFF into the culture system rescued the death phenotype of wild-type and knockout B cells from both compartments (Figure 3-8B).

Given the exquisite sensitivity to antigen receptor-mediated death observed in the 3BP2^{-/-} MZ B cells, we measured caspase-3 activation in MZ B cells (B220^{+}\text{CD21}^{hi}\text{CD23}^{-}) compared with FO B cells (B220^{+}\text{CD21}^{+}\text{CD23}^{-}) derived from 3BP2^{+/+} and 3BP2^{-/-} mice. Whereas 3BP2^{-/-} FO B cells showed a moderate increase in the frequency of caspase-3 activation compared to 3BP2^{+/+} FO B cells (Figure 3-9A, panel III vs. panel I), 3BP2^{-/-} MZ B cells demonstrated a 2.5-fold increased frequency of caspase-3 activation compared to the normal MZ B cells (Figure 3-9A, panel IV vs. panel II).
Figure 3-9. 3BP2−/− MZ B cells have enhanced activation of caspase-3 and fail to properly up-regulate anti-apoptotic proteins Bcl-xL and Bcl-2 following BCR cross-linking. (A) Enhanced activation of caspase-3 in 3BP2−/− MZ B cells (panel IV) and FO B cell (panel III) ex vivo, 12 hr and 24 hr after BCR cross-linking compared to 3BP2+/+ cells (panels I and II) Number indicated the percentage of MZ B and FO B cells with activated caspase-3. The data shown are representative of five independent experiments. (B) Decreased up-regulation of Bcl-xL and Bcl-2 in 3BP2−/− MZ B cells (open bars) 24 hr after BCR cross-linking compared to that in 3BP2+/+ MZ B cells (solid bars) and decreased up-regulation of Bcl-xL in 3BP2−/− FO B cells (open bars) 24 hr after BCR cross-linking compared to that in 3BP2+/+ FO B cells (solid bars). In contrast, Mcl-1 expression following BCR cross-linking was comparable in control and mutant cells (* p < 0.05). A.U., arbitrary units.
We examined the capacity of 3BP2+/+ and 3BP2−/− MZ B cells to up-regulate the anti-apoptotic proteins Bcl-xL, Bcl-2, and Mcl-1 following BCR cross-linking by flow cytometry. 3BP2−/− MZ B cells failed to optimally express Bcl-xL and Bcl-2 at both 12 and 24 hr following BCR cross-linking compared to 3BP2+/+ controls whereas Mcl-1 protein induction was not affected by the absence of 3BP2 (Figure 3-9B, upper panels). 3BP2−/− FO B cells also demonstrated a defect in Bcl-xL induction whereas both Bcl-2 and Mcl-1 induction were comparable to controls following BCR cross-linking (Figure 3-9B, lower panels). Therefore, FO and MZ B cells demonstrate a differential requirement for 3BP2 for the proper up-regulation of the Bcl-xL and Bcl-2 anti-apoptotic proteins in response to antigen receptor stimulation.

3.2.5  The 3BP2 Signaling Complex

3BP2 interacts with a number of signaling molecules in hematopoietic cells including Abl, Syk, Vav and PLCγ. Moreover, over-expression of 3BP2 stimulates the JNK and Erk MAPK pathways. In order to determine the biochemical defect underlying the B cell abnormality observed in the 3BP2−/− mice we analyzed the phosphorylation status of several of these signaling pathways. The Abl tyrosine kinase is activated following BCR cross-linking and binds to the polyproline region of 3BP2 through its SH3 domain. Since Abl is maintained in an auto-inhibited state by the intramolecular interaction of the Abl SH3 domain and the linker region, binding of 3BP2 to the Abl SH3 might stimulate Abl kinase activity. We performed an Abl \textit{in vitro} kinase assay with the exogenous Abl substrate GST-Crk-mCTD on purified B cells from 3BP2+/+ and 3BP2−/− mice to determine if the induction of Abl kinase activity is defective in cells lacking 3BP2. The induction of Abl kinase activity
**Figure 3-10.** The 3BP2 signaling complex. (A) The in vitro kinase activity of c-Abl was determined for c-Abl immunoprecipitated from either 3BP2+/+ (WT) or 3BP2−/− (KO) splenic B cells using GST-Crk-mCTD as a substrate (top panels). Abl protein was detected with anti-Abl antibody (bottom panels). (B) Kinetics and magnitude of immunoprecipitated Syk tyrosine phosphorylation following BCR cross-linking was compared between 3BP2+/+ and 3BP2−/− splenic B cells. IB, immunoblotting (C) Cell lysates from unstimulated and 10 μg/ml of anti-IgM F(ab’)2-stimulated 3BP2+/+ and 3BP2−/− splenic B cells were resolved by SDS-PAGE gel, and probed with phosphospecific antibodies against phospho-Erk1/2, -JNK/SAPK, and -Akt. The same membrane was stripped and reprobed with anti-Erk1/2, -JNK/SAPK and -Akt. (D) Resting splenic 3BP2+/+ and 3BP2−/− B cells were either left unstimulated or stimulated with 10 μg/ml of anti-IgM F(ab’)2 for indicated time intervals at 37°C before lysis. PLC-γ2 was immunoprecipitated (IP), resolved by SDS-PAGE, and probed with anti-phospho-tyrosine antibody, 4G10, the same membrane was stripped and reprobed with anti-PLC-γ2.
following BCR cross-linking was similar in amplitude and kinetics in both 3BP2\textsuperscript{+/+} and 3BP2\textsuperscript{−/−} B cells (Figure 3-10A).

3BP2 was also identified as a Syk kinase binding protein\textsuperscript{67}. We examined the induction of Syk tyrosine phosphorylation following BCR cross-linking. Immunoprecipitated Syk was rapidly and maximally tyrosine phosphorylated one minute following BCR cross-linking in 3BP2\textsuperscript{+/+} B cells. However, Syk phosphorylation was significantly reduced in B cells lacking 3BP2 with no detectable signal after 5 minutes of stimulation (Figure 3-10B). These data demonstrate that 3BP2 is required for maximal induction and sustained duration of Syk tyrosine phosphorylation following BCR activation. Importantly, other downstream targets of BCR activation such as Erk, JNK, Akt and PLC\textgammagamma2 were not affected by 3BP2 deficiency (Figure 3-10C and 3-10D).

Lastly, we measured the absolute concentration of intracellular calcium of B cells lacking 3BP2 using the cell permeable fluorescent dye Indo-1 and observed a 30% reduction in calcium levels following BCR cross-linking compared to control cells. We observed a differential requirement for 3BP2 between FO and MZ B cells for an optimal induction of calcium influx following BCR cross-linking. Whereas there was only a slight difference in the intracellular calcium concentration between 3BP2\textsuperscript{−/−} and wild-type FO B cells, 3BP2\textsuperscript{−/−} MZ B cells showed a 2-fold reduction in maximum calcium levels following BCR cross-linking compared to that of wild-type MZ B cells (400 nM vs. 800 nM) (Figure 3-11, upper panels). We noted in response to CD19 super-cross-linking that the calcium flux in 3BP2\textsuperscript{−/−} FO and MZ B cells was reduced by more than half that observed in normal B cells suggesting that 3BP2 may be important in CD19 signal transduction (Figure 3-11, lower panels). This difference was not attributable to differences in CD19
Figure 3-11. 3BP2−/− splenic and MZ B cells have reduced intracellular calcium concentration compared to those of 3BP2+/+ cells in response to BCR cross-linking. Indo-1 loaded splenic B cells, with surface staining of CD21 and CD23, were analyzed for ~120 s before stimulation with biotinylated Fab antibody to κ light-chain of BCR at 10 μg/ml (upper graphs) or with biotinylated F(ab')2 antibody to CD19 at 10 μg/ml (lower graphs). Cross-linking of BCR or super-cross-linking of CD19 was done with addition of 20 μg/ml of streptavidin. Distinction between total B cells, FO and MZ B cells was based on CD23 and CD21 surface staining. Calculation of [Ca^{2+}], was as described in Materials and Methods. Changes of [Ca^{2+}], following BCR cross-linking (upper graphs) and CD19 super-cross-linking (lower graphs) are shown with arrows indicated the addition of each stimulus. 3BP2−/− cells are represented by solid circles, and 3BP2+/+ cells are represented by open circles. The data shown are representative of four independent experiments. Equal levels of CD19 expression on 3BP2+/+ and 3BP2−/− splenocytes were verified using FITC-conjugated anti-CD19 antibody.
surface expression as both the wild-type and 3BP2<sup>−/−</sup> B cells express similar levels of CD19 (Figure 3-11).

The cytoplasmic tail of CD19 contains four possible 3BP2 SH2 domain binding sites<sup>22,427</sup>. We investigated whether 3BP2 could form an inducible and stable complex with CD19 in splenic B cells and found that endogenous 3BP2 binds to CD19 following BCR cross-linking (Figure 3-12A). Mutants of 3BP2 lacking either the SH2 domain or harboring a loss-of-function mutation in its SH2 domain did not bind to CD19 (Figure 3-12B) which demonstrated that 3BP2 binds to CD19 through its SH2 domain in a tyrosine phosphorylation dependent manner. We used streptavidin-agarose beads coated with each of nine biotinylated 11-mer peptides containing phosphotyrosine and flanking sequences corresponding to the cytoplasmic domain of human CD19<sup>428</sup> to map the interaction between 3BP2 SH2 domain and CD19. Figure 3-12C shows that phosphotyrosine 403 and 443 are capable of mediating this interaction. To confirm this result, we utilized Daudi cells transfected with one of three chimeric CD4:CD19 receptors<sup>429</sup> which contain either: the wild-type cytoplasmic domain of CD19, the cytoplasmic domain in which all nine tyrosine residues are mutated to phenylalanine (ALL F), or the cytoplasmic domain in which tyrosines 403 and 443 have been mutated to phenylalanine (Y403/443F)<sup>215</sup>. We observed that the 3BP2 SH2 domain bound to the wild-type CD4:CD19 chimera but not to the ALL F mutant or to the Y403/443F mutant receptor (Figure 3-12D). These observations demonstrate that CD19 and 3BP2 form an inducible protein complex mediated by tyrosine 403, tyrosine 443 or both.
Figure 3-12. 3BP2 forms an inducible complex with CD19 via SH2-phosphotyrosine interaction following BCR cross-linking.  (A) Inducible binding between CD19 and 3BP2 was demonstrated by coimmunoprecipitation (IP) of endogenous CD19 and 3BP2 from stimulated (with 40 μg/ml of anti-IgM F(ab')2) 3BP2+/+ splenic B cells. IB immunoblotting; WCL, whole-cell-lysat.  (B) Untransfected 293T cells (lane 1) and 293T cells transfected with Flag-3BP2 ΔSH2 (lane 2), Flag-3BP2 SH2R486K (lane 3), Flag-3BP2 (lane 4), and Flag-3BP2 ΔPR (lane 5) were used to determine the region(s) of 3BP2 required for its inducible interaction with CD19.  A20 cells were used as the source of endogenous CD19.  (C) Streptavidin-agarose beads coated with 11-mer biotinylated phosphotyrosyl peptides corresponding to sequences in the cytoplasmic domain of human CD19 (PQNQY*330GNVLSL, TAPSY*360GNPSSD, EGEGY*391EEPDS, DSEFY*403ENDSNL, DGSGY*421ENPEDE, NAESY*443ENEDEL, GSQSY*482EDMRGI, RGILY*490AAPQLR, and DADSY*513ENMDNP) (43), were incubated with lysates of Daudi B lymphoblastoid cells and adsorbed proteins were eluted, resolved by SDS-PAGE, transferred to Immobilon membrane, and immunoblotted with antibody to 3BP2, followed by enhanced chemiluminescence.  (D) Daudi B lymphoblastoid cells expressing CD4:CD19 chimeric receptors of ΔEC, in which the extracellular domain of CD19 was replaced with that of human CD4 (26) (lane 1); Y9F, in which all nine tyrosines present in the cytoplasmic tail have been replaced with phenylalanine (lane 2); or Y403/443F, in which phenylalanine was substituted for tyrosines 403 and 443 in the cytoplasmic domain (200) (lane 3) were stimulated with F(ab')2 goat antibody to IgM.  The chimeric receptors were precipitated from cell lysates by incubation with GST-3BP2 SH2.  The chimeric receptors were detected by antibodies against human CD4.  The membrane was stripped and reprobed with anti-GST antibodies.  Presence of CD4:CD19 chimeric receptors in each cell lines was confirmed by immunoprecipitation with antibody against human CD4 and immunoblotting with antibody against CD19.
3.3 Discussion

In this chapter, a requirement for 3BP2 to achieve optimal signaling through BCR has been demonstrated. As a part of the CD19 co-stimulatory complex, 3BP2 has a distinctive function in B lymphocytes involved in TI-2 humoral responses. Mice lacking 3BP2 have diminished peritoneal B1 B cells, accumulated MZ B cells which demonstrate enhanced sensitivity to antigen receptor-induced cell death in vitro.

B1 B cells and MZ B cells control the humoral response to TI-2 multivalent antigens. TI-2 immune responses occur in the absence of T-cell help but are dependent on optimal activation of the BCR together with BAFF signals elaborated by macrophages and DCs. We have shown that 3BP2-/- splenic B cells and MZ B cells fail to proliferate, survive and signal optimally following antigen receptor activation. Consistent with these data, over-expression of 3BP2 augmented BCR-mediated NFAT activation as measured by a luciferase reporter assay while 3BP2 siRNA treatment of Raji cells attenuated BCR signaling. Our data would support a model whereby the B1 and MZ B cell populations are diminished in numbers or function or both in the absence of 3BP2 leading to a defective TI-2 response in vivo.

The modest increase in MZ B cells observed in the 3BP2-/- mice may also be a reflection of suboptimal BCR signaling. One model of MZ B cell development argues that nascent transitional B cells must receive a BAFF survival signal and a BCR signal sufficient in strength to initiate a FO B cell developmental program. Those B cells that survive because of BAFF, but receive a weak BCR signal then become receptive to inductive signals through Notch2 that support the development of MZ B cells. The
accumulation of MZ B cells observed in the 3BP2−/− mice may result from a failure to maximally activate proximal signal transduction pathways downstream of the BCR thereby promoting a developmental program favoring MZ B cell commitment. In distinction to MZ B cell development, constitutive and optimal BCR signals are required for establishing normal numbers of B1 B cells in the adult animal . In this context, the weak BCR signal of 3BP2−/− B cells likely contributes to the decreased peritoneal B1 B cells found in 3BP2−/− mice. Therefore, in the absence of 3BP2, the suboptimal BCR signal affects the developmental pathway of peripheral B cells, MZ B and B1 B cells, and impairs the in vitro survival and in vivo function of MZ B cells in response to BCR cross-linking.

In order to define the biochemical defect underlying the B cell defects observed in the 3BP2−/− mice, the activity and phosphorylation status of a number of canonical signaling molecules downstream of the BCR in 3BP2−/− B cells were examined. 3BP2 was identified as a Syk tyrosine kinase binding protein and as a Syk substrate . We observed that both the induction and duration of Syk phosphorylation was attenuated in 3BP2−/− B cells compared to control cells. However, we observed that there was no effect on the induction of Abl, Erk, JNK, Akt, or PLCγ2 in 3BP2−/− B cells following BCR cross-linking. Although PLCγ2 phosphorylation was normal, 3BP2−/− B cells had reduced calcium flux in response to BCR cross-linking. What could account for the defect in calcium flux in the face of normal PLCγ2 phosphorylation in 3BP2−/− B cells? One possibility is that the production of the PLCγ2 substrate, phosphatidylinositol-4,5-bisphosphate (PIP2), is not normal in 3BP2−/− B cells. Previous studies by O’Rourke et al. demonstrated that CD19 cross-linking induces the de novo synthesis of PIP2 required to
replenish the rapid depletion of PIP₂ by PLCγ2. We are currently investigating whether the production of PIP₂ is normal in 3BP2−/− B cells.

In attempt to determine how 3BP2 might couple to the BCR signaling machinery we have shown that endogenous 3BP2 binds inducibly to CD19 following BCR cross-linking at sites previously reported to bind to the Fyn tyrosine kinase. The CD19 coreceptor complex which includes CD21 and CD81 reduces the threshold of BCR activation by up to 10^4 fold. CD19 provides this critical signal through its engagement and activation of PI3K, Vav, Lyn, and Fyn. CD19 is also required for maximum induction of calcium flux. 3BP2 may thus be a necessary component of the CD19 signaling complex such that in the absence of 3BP2 the CD19 co-stimulatory signal is weakened leading to a diminished BCR signal.

Recently another report describing the B cell phenotype in 3BP2 deficient mice has been published. Consistent with our findings, de la Fuente et al. similarly showed that 3BP2−/− B cells demonstrated a proliferation defect and impaired calcium mobilization in response to BCR cross-linking. In distinction to our findings however, they did not observe a defect in the TI-2 humoral response or a perturbation in the B1 or MZ B cell populations. Moreover, they did not observe altered survival of B cells in the absence of 3BP2. The principal biochemical abnormalities observed in their report included defects in NFATp dephosphorylation and JNK phosphorylation. The differences in our two phenotypes may result from differences in our respective targeting strategies, generation number backcrossed onto the C57BL/6 strain and local variations in microbial environments of the animal colonies.

We have demonstrated that the 3BP2 adapter protein is a component of the CD19
co-stimulatory complex and is essential for optimum BCR signaling required for normal peritoneal B1 and MZ B cell development and TI-2 antigen response.
CHAPTER 4

The 3BP2 Adapter Protein is Required for Optimal Activation of Neutrophils in Response to Chemoattractants

- The experiment in Figure 4-1 was performed by me.
- The experiment in Figure 4-2 was performed by me.
- The experiment in Figure 4-3 was performed by me.
- The experiment in Figure 4-4 was performed by me.
- The experiments in Figure 4-5A and B were performed in collaboration with Dr. Karl Lang and Dr. Philipp Lang.
- The experiment in Figure 4-6 was performed by Dr. Paul Daniel Simoncic and is included in this chapter with permission.
- The experiment in Figure 4-7 was performed by me.
- The experiment in Figure 4-8 was performed by me.
- The experiment in Figure 4-9 was performed by me.
4.1 Introduction

Chemoattractants such as fMLF, C5a and IL-8 trigger the defense function of the neutrophils through ligation of heptahelical, heterotrimeric G protein-coupled receptors (GPCRs). G proteins are composed of α, β, and γ subunits. Ligand-bound receptors activate G proteins by catalyzing the exchange of GDP bound to the α subunit with GTP, resulting in dissociation of GTP-bound Gα subunit from the βγ subunit complex. The GTP-Gα and the free βγ subunit may interact with effector proteins that further amplify the signal. For instance, Gβγ complex stimulates phospholipase C-β (PLC-β) activation which results in the breakdown of PIP₂ to form IP₃ and DAG. IP₃ induces Ca²⁺ mobilization from intracellular storage organelles, leading to a transient rise in cytosolic free Ca²⁺ which work in conjunction with DAG to activate PKC. PKC and various Ca²⁺-sensitive protein kinases participate in different neutrophil functions including respiratory burst and exocytosis.

Additional mechanisms of chemoattractant-induced neutrophil activation that are independent of PKC and Ca²⁺ have been proposed by several studies. One of such mechanisms involves activation of tyrosine kinase. Many studies have shown that GPCRs trigger a rapid tyrosine phosphorylation of several proteins in neutrophils and treatments with inhibitors of tyrosine kinase can block neutrophil responses to chemoattractants. Among cytosolic tyrosine kinases, members of the Src family have been implicated in signal transduction by fMLF. More recently, studies using neutrophils derived from Hck/Fgr-deficient mice or human neutrophils treated with the selective Src kinase inhibitor 4-amion-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo [3,4-
d]pyrimidine (PP2) demonstrated a critical role of Src kinase in fMLF-mediated respiratory burst and formation of filamentous actin (F-actin). It was first suggested that transient expression of Gβγ subunits in COS-7 cells enhance autophosphorylation and kinase activity of c-Src which coincided with a transient association between c-Src and an adapter protein Shc. Later on, another mechanism of Src activation downstream of GPCRs was presented by Ma et al. showing that Gαi and Gαs directly stimulate kinase activities of c-Src and Hck in an in vitro kinase assay. They proposed that Gαi and Gαs interact with the catalytic domain of Src which modulates the position and conformation of the activation loop resulting in increased accessibility of the active site to substrates; in addition, the conformational changes lead to exposure of the side chain of Tyr416 making it a better substrate for autophosphorylation and thus increased Src kinase activity. Regardless of the mechanism of activation, members of the Src PTK participate in GPCR signaling and are required for activation of Vav GEF. Within the three isoforms of Vav GEFs, Vav1 is expressed predominantly in hematopoietic cells. Defects in both superoxide anion production and cell migration of Vav1-deficient neutrophils demonstrated the importance of Vav1 in fMLF-mediated neutrophil functions. Activation of Vav1 is regulated by tyrosine phosphorylation. Upon fMLF stimulation, Vav1 was quickly tyrosine phosphorylated and the phosphorylation was sensitive to the Src family kinase inhibitor suggesting that Vav1 is a substrate of Src in fMLF-mediated signaling. Given the importance of both Src and Vav1 in fMLF-mediated neutrophil functions, examining how these molecules are regulated within the pathway may shed some light on the regulation of neutrophil functions.
Our laboratory and several others have identified the adapter protein 3BP2 as a binding protein of several signaling molecules (section 1.2) in different cell types. 3BP2-deficient mice have a diminished thymus-independent type 2 humoral response and fail to propagate signals emanating from the B-cell receptor (Chapter 3) \(^{452,438}\). Recently, we have mapped the Src binding site in the Pro-rich region of 3BP2 (Chapter 5 and Figure 5-1) and we have demonstrated that 3BP2 forms a signaling complex with Src family kinase, Syk and Vav GEFs which is required for integrin based signal propagation in osteoclasts (section 4.2 and Figure 4-8). We hypothesized a similar role of 3BP2 in GPCR-mediated signaling in neutrophils.

### 4.2 Results

#### 4.2.1 3BP2\(^{-/-}\) Neutrophils are Defective in Chemotaxis

To examine the role of 3BP2 in neutrophil chemotactic properties \textit{in vitro}, we prepared freshly isolated BM neutrophils from 3BP2\(^{+/+}\) and 3BP2\(^{-/-}\) mice. We evaluated the role of 3BP2 on chemokinesis and chemotaxis by using Zigmond chambers and time-lapse videomicroscopy to track individual cell migration. In the absence of chemoattractants, both 3BP2\(^{+/+}\) and 3BP2\(^{-/-}\) neutrophils did not migrate (Figure 4-1, left panels). Addition of chemoattractants such as a formyl peptide (\(N\)-formyl-Met-Leu-Phe (fMLF)) induced rapid and random movements of both 3BP2\(^{+/+}\) and 3BP2\(^{-/-}\) neutrophils with similar speed (Figure 4-1, middle panels). However, when a stable and shallow chemoattractant gradient was created by placing fMLF only on one side of a Zigmond chamber, 3BP2\(^{-/-}\) neutrophils often displayed an indecisive wandering behavior whereas
Figure 4-1. 3BP2 is required for neutrophil chemotaxis. Chemokinesis and chemotaxis mediated by fMLP was assessed in a Zigmund chamber as described (see “Materials and methods”). 3BP2 null neutrophils display a directional defect in chemotaxis plots. Plots of neutrophil chemotaxis migration in the Zigmund chamber assay are shown. Neutrophils undergoing chemokinese (middle panels) and chemotaxis (right panels) in response to fMLP were recorded using time-lapse imaging. Tracings were used to plot the final position of cells after 30 minutes. Each data point on the plot represents one individual cell. The plots showing contain data points collected from three independent experiments. The final positions of 3BP2+/+ (+/+) and 3BP2−/− (−/−) neutrophils were subjected to Rayleigh test of uniformity (WT: p = 3.76×10⁻¹²; KO: p = 0.87). Neither 3BP2+/+ nor 3BP2−/− neutrophils display spontaneous movements in the absence of chemoattractant stimulation (left panels).
3BP2^{+/+} neutrophils moved in relatively straight paths up the fMLF gradient (Figure 4-1, right panels).

Because F-actin formation provides the primary driving force for neutrophil locomotion and the accumulation of F-actin at the leading edge of migrating neutrophils has been showed to correlate with the directional movement of neutrophils^{453,454}, we examined both the F-actin formation and the location of accumulated F-actin in fMLF-stimulated neutrophils. Measurement of the total amount of cellular F-actin assembled after fMLF stimulation showed no notable differences between 3BP2^{+/+} and 3BP2^{-/-} neutrophils (Figure 4-2A). However, under a shallow gradient of fMLF, approximately 40% of 3BP2^{+/+} neutrophils had F-actin polarized to the leading edge towards higher concentration of fMLF whereas only approximately 16% of 3BP2^{-/-} neutrophils had a clear polarization of F-actin (approximately 500 cells were counted per genotype) (Figure 4-2B). Therefore, 3BP2 is important in the asymmetrical establishment and/or maintenance of sites of actin polymerization which produces directional migration of neutrophils in response to chemotactic gradients.

4.2.2 Reduced Recruitment of Neutrophils to the Site of Inflammation in 3BP2^{-/-} Mice

The faulty chemotactic response of 3BP2^{-/-} neutrophils in vitro suggests a potential defect in neutrophil recruitment to the sites of inflammation. To test whether 3BP2^{-/-} neutrophils are defective in migrating to the inflamed sites, we chemically induced peritonitis in both 3BP2^{+/+} and 3BP2^{-/-} mice. Total cell, macrophage, and neutrophil numbers per mouse were analyzed in peritoneal exudates harvested 4 and 18 hours after thioglycollate (TG) injection (Figure 4-3). At 4 hours we observed a
Figure 4-2. 3BP2 is required for spatially-restricted F-actin assembly in chemotaxing neutrophils. (A) A representative plot of FACS analysis of fMLP-stimulated total F-actin generation of 3BP2+/+ (solid circle) and 3BP2−/− (open circle) neutrophils is shown. Freshly isolate BM neutrophils stimulated with 10 μM fMLP for the indicated time, and analyzed for the content of F-actin by staining the cells with phalloidin. The results are expressed as the mean channel fluorescence. (B) BM neutrophil chemotaxing under the fMLP gradient were stained with phalloidin, and the percentages of neutrophils with polarized F-actin localization were compared (results presented are average number from three independent experiments, on average, approximately 500 cells were counted per genotype per experiment). Cells were judged to have polarized F-actin when F-actin staining is confined to less than one third of the circumference.
significant decrease in both the total number of cells and the number of neutrophils in 3BP2−/− mice compared to 3BP2+/+ mice whereas the number of F4/80-positive macrophages was comparable between the two. At 18 hours, the number of neutrophils recruited to the peritoneal cavity of 3BP2−/− mice was still lower than that of the 3BP2+/+ mice; however, the difference between the two was not statistically significant. Interestingly, we have observed an increase in the number of macrophages with high intensity of F4/80 staining in 3BP2−/− mice compared to 3BP2+/+ mice at 18 hours post TG injection. F4/80 is highly and constitutively expressed on most resident tissue macrophages. In addition, the expression of F4/80 is tightly regulated according to the physiological status of cells, and the precursor of tissue macrophages, the blood monocyte, is known to express less F4/80 than its mature counterparts455. The biological significance of this increased F4/80^high macrophage population in 3BP2−/− mice is currently unknown, but it might suggest that there is a retention of resident macrophages in these mice when normally the peritoneal resident macrophages migrate out of peritoneal cavity within one hour following TG injection456. These experiments show that during an acute inflammation induced by TG, although monocyte recruitment is normal, neutrophil recruitment is less efficient in the absence of 3BP2.

4.2.3 3BP2 Deficiency Results in a Partial Reduction in ROS Production in Neutrophils

Neutrophils produce superoxide anion rapidly after exposing to pro-inflammatory mediator such as complement fragment C5a and N-formylmethionyl oligopeptides like fMLF381. Thus, we examined the effect of 3BP2 deficiency on fMLF-induced production of superoxide anion in primary neutrophils. Both 3BP2+/+ and 3BP2−/−
Figure 4-3. Acute inflammatory response in 3BP2+/+ and 3 BP2−/− mice. Absolute value of the number of total cells, neutrophils, and macrophages recovered from peritoneal washes of 3BP2+/+ (solid circle) and 3BP2−/− (open circle) mice 4 hours and 18 hours post TG peritoneal injection are shown. Macrophage and neutrophil populations were determined by the profiles of surface staining with antibodies against Gr-1 and F4/80 where Gr-1+F4/80− population was defined as neutrophil population, and Gr-1+/− F4/80+ population represented macrophage population. Macrophage population was further divided into F4/80low and F4/80High subpopulations. (n=5 per genotype for naïve mice; n=12 per genotype for TG injected mice; Wilcoxon-Mann-Whitney test * p < 0.05; p = 0.0358 for total cell number of WT versus KO at 4hr; p = 0.0249 for neutrophil number of WT versus KO at 4hr; p = 0.0023 for F4/80hi cell number of WT versus KO at 18hr)
Figure 4-4. Effect of 3BP2 deficiency on superoxide production. (A-C) Superoxide anion levels were determined with an enhancer-containing, lumino-based chemiluminescence assay. Mouse BM neutrophils were stimulated with 10 μM of fMLP for 20 seconds (A) and 3 minutes (B) or stimulated with 1 μM of PMA (C). Kinetic plots of chemiluminescence intensity are shown. The plots presented are representative of three individual experiments. Each point is the mean of three individual measurements and the error bars are the standard deviation of triplicate readings.
neutrophils produced superoxide anion in a superoxide dismutase (SOD)-inhibitable manner with similar kinetics following fMLF stimulation. However, compared to 3BP2+/+ cells, 3BP2−/− neutrophils consistently produced less superoxide anion with a maximum 36% reduction in the amount of superoxide anion produced at 30 seconds following fMLF stimulation (Figure 4-4A and 4-4B). 3BP2 deficiency, on the other hand, did not appear to significantly affect PMA-stimulated superoxide anion production (Figure 4-4C). These results suggest that 3BP2 is primarily involved in chemoattractant-induced superoxide anion production and is dispensable for phorbal ester PMA-elicited superoxide anion production.

4.2.4 Increased Susceptibility of 3BP2−/− mice to Challenge with Listeria monocytogenes

Defects in both fMLF-induced chemotaxis and superoxide anion production of 3BP2−/− neutrophils suggest that these cells might be inferior in clearing bacterial infections. To test the role of 3BP2 in host defense, we compared the susceptibility of 3BP2+/+ and 3BP2−/− mice to infection with Listeria monocytogenes. This organism was chosen because an adequate signaling through fMLF receptor and a functional neutrophil population are necessary to successfully clear the infection457-459. We infected mice with 2×10⁴ CFU and measured bacterial burden 2 days later, a time when non-specific immune responses control infection459. At this time, 3BP2−/− mice showed 47-fold and 76-fold more bacteria in spleen and liver, respectively, relative to wildtype control mice (Figure 4-5A).
Figure 4-5. 3BP2 is critical for the clearance of *Listeria monocytogenes* infection. (A) 9- to 11-week old 3BP2\(^{+/+}\) (solid circle) and 3BP2\(^{-/-}\) (open circle) mice were injected with 2 × 10\(^4\) CFU of *Listeria monocytogenes* in the tail vein. The bacterial burden from whole spleen and liver was determined 2 days after infection. Results are from a single experiment with sex-matched mice (n=8 in each group), and are representative of three separate experiments with a consistent pattern. The mean values are indicated as solid bars. (* p < 0.05). (B) Accelerated lethality in 3BP2\(^{-/-}\) mice challenged with *L. monocytogenes*. Mice were injected with 1 × 10\(^5\) CFU in the tail vein. Results shown are data collected from three independent experiments with -/- and +/- sex-matched mice (total n = 15 in each group).  p = 0.0404 (Gehan-Breslow-Wilcoxon test)
A defect in innate immunity against *L. monocytogenes* challenge in 3BP2−/− mice suggests a possible early mortality of these mice following the infection. To test this hypothesis, we injected 3BP2+/+ and 3BP2−/− mice with *L. monocytogenes* and recorded the percentage of death in these two groups for the duration of 14 days. 47% of 3BP2−/− mice injected with 10^5 CFU died by day 4 in contrast to 20% of 3BP2+/+ mice. Only 20% of 3BP2−/− mice survived beyond 8 days, whereas 47% of 3BP2+/+ mice remained healthy (Figure 4-5B). Results from both the bacterial burden assay and the survival assay demonstrated that 3BP2 deficiency causes increased susceptibility to *Listeria* infection.

4.2.5 3BP2 is required for full activation of Src PTK, Vav, Rac2, Cdc42 and Erk MAP kinase in response to fMLF stimulation

Src PTK and Vav1 GEF have both been identified as downstream effectors of fMLF receptor signaling where Src activates Vav by phosphorylating Tyr^{174}. Previously, we have shown that 3BP2 bound to and positively influenced Src and Vav activation in osteoclasts (manuscript under revision) (Figure 4-6). We investigated the requirement of 3BP2 in full activation of Src and Vav in neutrophils in response to fMLF. Following fMLF stimulation, Src and Vav were both hypophosphorylated in 3BP2−/− neutrophils (Figure 4-7A and 4-7B, respectively). 3BP2 itself was Tyr phosphorylated in response to fMLF and it formed a constitutive complex with Vav1 (Figure 4-7C). Binding to 3BP2 induced tyrosine phosphorylation of the bound Vav1 (Figure 4-7C). These results suggest the possibility that in neutrophils 3BP2 may be a part of a signaling complex which promotes Vav1 tyrosine phosphorylation in response to fMLF.
Figure 4-6. Impaired Src activation in 3BP2−/− osteoclasts (OCs). (A) 3BP2 nucleates an endogenous complex between Src, Syk, and Vav in Raw264.7 cells incubated with RANKL for 5 days. (B) Decreased Src activation in 3BP2−/− cells at day 5 of osteoclastogenesis. (C) Reduced Syk, Vav1 and Vav3 activation in 3BP2−/− OCs.
**Figure 4-7.** 3BP2 is required for the full activation of Src and Vav1. (A) Cell lysates from unstimulated and 10 μM of fMLP-stimulated 3BP2+/+ and 3BP2−/− BM neutrophils were resolved by SDS-PAGE gel, and probed with phosphospecific antibody against phospho-Src. The same membrane was stripped and reprobed with anti-Src. IB, immunoblotting. (B) Kinetics and magnitude of immunoprecipitated (IP) Vav1 tyrosine phosphorylation following fMLP stimulation were compared between stimulated 3BP2+/+ and 3BP2−/− BM neutrophils. (C) Resting 3BP2+/+ BM neutrophils were either left unstimulated or stimulated with 10 μM of fMLP for indicated time intervals at 37°C before lysis. 3BP2 was immunoprecipitated, resolved by SDS-PAGE, and probed with anti-phospho-tyrosine antibody, pY99, the same membrane was stripped and reprobed with anti-Vav1 and anti-3BP2. The band located slightly lower than molecular weight standard 115.5 kDa has the band size equivalent to molecular weight of Vav1 (~95 kDa) whereas 3BP2 band (band size ~82.2 kDa) is located slightly higher than the predicted 3BP2 molecular weight (predicted molecular weight ~62kDa). WCL, whole-cell lysate. (D) Cell lysates from unstimulated and 10 μM of fMLP-stimulated 3BP2+/+ and 3BP2−/− BM neutrophils were resolved by SDS-PAGE gel, and probed with phosphospecific antibody against phospho-Akt. The same membrane was stripped and reprobed with anti-Akt.
Since PI3K is indispensable for numerous functions of neutrophils in response to chemoattractants \(^{461-463}\), we investigated Akt phosphorylation as a surrogate for PI3K activation of 3BP2\(^{+/+}\) and 3BP2\(^{-/-}\) neutrophils. Akt phosphorylation were comparable between 3BP2\(^{+/+}\) and 3BP2\(^{-/-}\) neutrophils in response to fMLF stimulation (Figure 4-7D).

Several \textit{in vitro} and \textit{in vivo} studies have shown that Vav1 acts primarily as a GEF for Rac1 and Rac2 \(^{103,186}\); therefore we decided to assess the activation of Rac1 and Rac2 following fMLF stimulation. We detected a significant reduction in Rac2 activation in 3BP2\(^{-/-}\) neutrophils (Figure 4-8A, left panel) whereas Rac1 activation was unaffected in these cells (Figure 4-8A, middle panel). In addition, activation of Cdc42 was modestly diminished in 3BP2\(^{-/-}\) neutrophils compared to wildtype cells (Figure 4-8A, right panel). Interestingly, Vav1-deficient neutrophils have relatively normal levels of fMLF-stimulated Rac1-GTP or Rac2-GTP formation compared with wildtype neutrophils \(^{409}\) and Cdc42 appears not to be the preferred substrate of Vav1 \(^{103,129}\). Therefore, our data suggest that 3BP2 may be involved in regulation of other GEFs downstream of fMLF receptor signaling such as P-Rex1 and \(\alpha\)-PIX which have been demonstrated as the primary GEFs that activate Rac2 and Cdc42, respectively \(^{348,374}\).

Rac2 has been implicated previously in the activation of p38 and Erk p42/p44 MAPKs in neutrophil following chemoattractant stimulation \(^{373}\). Specific cell-permeate pharmacologic inhibitors for either MAPKs blocked fMLF-mediated superoxide anion production of neutrophils indicating the requirement of both MAPKs in this process \(^{464-466}\). While Erk p42/p44 has been shown to mediate fMLF-induced phosphorylation of p47\(^{phox}\) (a cytosolic component of NADPH oxidase complexes, \textit{section 1.5.2b}) \(^{395}\), the role of p38 MAPK in fMLF-mediated respiratory burst remains controversial \(^{395,467}\).
Figure 4-8. 3BP2 is required for the full activation of Cdc42, Rac2 and Erk1/2 MAPK. (A) The levels of active Rac2 (left panels), Rac1 (middle panels) and Cdc42 (right panels) were determined in BM neutrophils via the GST-PBD pull-down assay. BM neutrophils were stimulated with 10 μM of fMLP. IB, immunoblotting; WCL, whole-cell lysate. (B) Cell lysates from unstimulated and 10 μM of fMLP-stimulated 3BP2+/+ and 3BP2−/− BM neutrophils were resolved by SDS-PAGE gel, and probed with phosphospecific antibody against phospho-Erk1/2. The same membrane was stripped and reprobed with anti-Erk1/2.
examined the phosphorylation of these MAPKs in response to fMLF to assess their activation. Phosphorylation of Erk p42/p44 MAPK was significantly diminished in the absence of 3BP2 (Figure 4-8B); however, we failed to consistently detect a differential phosphorylation of p38 MAPK between 3BP2++/ and 3BP2−/ neutrophils. Our results closely resemble what was seen in fMLF-stimulated Rac2−/ neutrophils where when compared to wildtype neutrophils there was a pronounced and consistent decrease in phospho-Erk (p42/44) but only a less reproducible and slight decrease in phospho-p38.

4.2.6 3BP2 forms a complex with Gβγ subunits

The βγ subunits of heterotrimeric G proteins have been documented to interact with PH domains of various proteins such as βARK36, Btk37 and PLC-β2468,469. The most conserved region of the PH domain containing the sequences in and extending beyond the most C-terminal region of the PH domain appears to be responsible for Gβγ binding36. Often, direct binding to Gβγ subunits stimulates the activities of these enzymes468,470. We examined whether the PH-domain-containing 3BP2 could interact with Gβγ subunits in an overexpression study. We showed that wildtype 3BP2 binds to Gβ subunits in a manner that was Gγ-dependent but Gα subunit-independent (Figure 4-9). This data suggest that 3BP2 may be modulating the activities of effector molecules lying downstream of the G-protein-couple receptor signaling by affecting the binding affinity of these molecules to Gβγ subunits.
Figure 4-9. 3BP2 forms a complex with Gβ subunit. 293T cells transfected with (A) Gαi + Myc-Gβ1 + HA-G2 + Flag-3BP2 (lane 1), Myc-Gβ1 + HA-G2 + Flag-3BP2 (lane 2), Myc-Gβ1 + Flag-3BP2 (lane 3) and Myc-Gβ1 + HA-G2 (lane 4) were used to determined if 3BP2 binds to Gβ subunit. Cells were lysed and immunoprecipitated (IP) with anti-Flag antibody/protein G beads. Lane 5 to 8 contains samples with the same transfection as those in lane 1 to 4 (same order) but samples in lane 5 to 8 were immunoprecipitated with protein G beads alone. The precipitated proteins were resolved by SDS-PAGE, and probed with anti-Myc antibody to look for Myc-tagged Gβ subunit. The membrane was stripped and reprobed with anti-Flag antibody. (B) HA-G2 + Flag-3BP2 (lane 1 and 2). Sample in lane 1 was immunoprecipitated with anti-Flag antibody/protein G beads whereas sample in lane 2 was immunoprecipitated with protein G beads alone. The precipitated proteins were resolved by SDS-PAGE, and probed with anti-HA antibody to look for HA-tagged G2 subunit. The membrane was stripped and reprobed with anti-Flag antibody. IB, immunoblotting; WCL, whole-cell lysate.
4.3 Discussion

G protein-coupled receptor signaling is a complex pathway which involves various effector molecules from lipid kinase, protein kinase, GEFs to small GTPases\(^1\). However, the roles of adapter molecules in this signaling pathway have not yet been extensively investigated. One of the few adapter proteins that have been shown to be a part of the GPCR signaling pathway is an SH2 domain-containing protein, Shc\(^{446,450}\). Shc was described as a part of a signaling complex together with Src family kinase Lyn which couples the fMLF receptor to PI3K and, potentially, to activation of the Ras/MAPK cascade and the respiratory burst oxidase\(^{446}\). In this report, we demonstrated for the first time the requirement of another adaptor protein, 3BP2, in the fMLF-mediated neutrophil functions such as chemotaxis and superoxide anion production.

Our laboratory has mapped the Src-binding site to the Pro-rich region of 3BP2 and demonstrated 3BP2 as a component of a large multiprotein complex that includes Src, and Vav in bone cell osteoclasts. In addition, we have showed that 3BP2 is a critical regulator of Src activation downstream of integrin signaling required for bone cell differentiation and function (manuscript under revision). Both Src and Vav have been implicated in propagation of fMLF signaling where Vav1 is Tyr phosphorylated in an Src kinase-dependent manner following fMLF stimulation\(^{409}\). Targeted disruption of the members of Src family kinases such as hck and fgr led to a reduction of fMLF-induced global Tyr phosphorylation\(^{449}\). We found that, in the absence of 3BP2, both Src and Vav1 were hypophosphorylated in response to fMLF which suggested that 3BP2 is involved in fMLF-induced Tyr phosphorylation of Src and Vav1. In addition, we have
shown that 3BP2 itself is Tyr phosphorylated following fMLF stimulation and it forms a constitutive complex with Vav1. Binding to 3BP2 appears to induce the Tyr phosphorylation of associated Vav1. Activation of Vav1 exchange activity requires Tyr phosphorylation which induces conformational changes and relieves autoinhibition by the N terminus of Vav1 that otherwise blocks access of substrates to its catalytic DH domain. It is also known that Vav GEF activity can be modulated by its PH domain binding to PI3K product PIP\(_3\). Vav PH domain binding to PIP\(_3\) has been suggested either to increase accessibility of Src family kinase to the autoinhibitory Tyr residue of Vav or to initiate membrane recruitment of Vav with subsequent Tyr phosphorylation mediated by membrane-bound Src family kinase. However, PI3K inhibitors LY294002 or wortmannin produced only a slight decrease in fMLF–induced Vav1 phosphorylation suggesting that there exists other means of Vav1 membrane recruitment in fMLF-stimulated neutrophils. With the capacity of binding to both Src kinase and its substrate Vav, we proposed that 3BP2 could mediate Vav1 recruitment to its activator in response to fMLF.

Functionally, Src and Vav1 are both required for fMLF-induced production of superoxide anion by neutrophils. However, whereas Vav1 is required for neutrophil chemotaxis in a modified Boyden chamber assay, Src family kinase hck and fgr were dispensable in neutrophil migration as assessed by transwell migration assays. We have observed defects in both chemotaxis and respiratory burst with 3BP2\(^{-/-}\) neutrophils. We speculate that the discrepancy between the ability of 3BP2\(^{-/-}\) and Hck\(^{-/-}\) Fgr\(^{-/-}\) neutrophils to migrate towards high concentration of fMLF could be explained by the different assays used to assess their chemotaxis. Whereas Fumagalli et al. used transwell
migration assay to examine chemotaxis of Hck−/−Fgr−/− neutrophils, we used Zigmond chambers in this report. The integrity of the concentration gradient created by a Zigmond chamber is well preserved; the gradient is stable and steepest between 15 and 90 minutes after the start of the assay. Combining with the real-time image capture at every minute of the duration of the assay, we were able to track the movement of individual cells which allows us to assess not only the locomotion but also the direction of cell movement. In contrast, using devices such as Boyden chambers or transwells, it is not possible to observe the cells as they responding. Therefore it is difficult to determine if a given alteration in the chemotactic response is due to variations in the percentage of cells responding, the accuracy of the orientation, or the frequency and/or magnitude of the turns cells made. Hence, Hck−/−Fgr−/− neutrophils might share the similar defect in directional migration as 3BP2−/− neutrophils but such defect was masked by the limitations of transwell migration assay.

We have provided evidence to demonstrate the requirement for 3BP2 in fMLF-mediated activation of Vav1 in neutrophils; however, decreased activity of Vav1 alone was insufficient to account for the phenotypes of 3BP2−/− neutrophils. For instance, in response to fMLF, the activation of Rac2 and Cdc42 was attenuated in 3BP2−/− neutrophils; however, there was no detectable difference in the fMLF-stimulated Rac2-, Cdc42-GTP formation between Vav1−/− and wildtype neutrophils. Rac2 and Cdc42 play essential roles in chemoattractant-mediated neutrophil functions. Both are required for chemotaxis and Rac2 is also indispensable in the activation of the NADPH oxidase complex. Ming et al. have demonstrated that Vav1 may only regulate a specific pool of Rac2 in proximity to the NADPH oxidase which explains the paradox of the
normal overall level of activated Rac2 of f/MLF-stimulated Vav1<sup>−/−</sup> neutrophils in the face of their 3-fold reduction of f/MLF-elicited superoxide production<sup>410</sup>. Studies have shown that in addition to Vav1, several other GEFs are activated following f/MLF stimulation in neutrophils<sup>348,353,374,474</sup>. Some of these GEFs like P-Rex1 and α-PIX have been shown to be directly activated by Gβγ subunits<sup>347,348</sup>. In mouse neutrophils, P-Rex1 preferentially activates Rac2 whereas α-PIX primarily activates Cdc42<sup>348,374</sup>. In the case of α-PIX activation, the constitutive complex between α-PIX and PAK1 serine/threonine kinase is recruited to Gβγ subunits following chemoattractant stimulation through direct binding between PAK1 and Gβγ. The active α-PIX in this complex preferentially activates Cdc42 which subsequently activates PAK1 kinase<sup>348</sup>. Each component in Gβγ/PAK1/α-PIX/Cdc42 complex is necessary for the optimal activation of one another<sup>348</sup> suggesting any molecules that stabilize or destabilize this complex might influence the duration and/or magnitude of PAK1/α-PIX/Cdc42 activation. We have demonstrated for the first time in an overexpression study that 3BP2 can interact with a Gβγ subunit. The PH domain of 3BP2 possibly mediates its binding to Gβγ, since Gβγ has been shown to interact with the PH domain of various molecules including Btk<sup>37</sup>. The biological consequence of Gβγ/3BP2 complex is currently unknown; however, there exists a possibility that 3BP2 functions as a scaffold protein which stabilizes the complex formation between Gβγ and other molecules such as PAK1/α-PIX or P-Rex1 and therefore, modulates activation of Cdc42 and Rac2, respectively. To formally test this possibility, we are currently investigating whether the presence of 3BP2 can alter Gβγ-dependent activation of Rac2 and Cdc42 by P-Rex1 and α-PIX, respectively.
In addition to activation of various protein kinases, GEFs and small GTPases, fMLF stimulation leads to a rapid accumulation of phospholipid PIP3. PIP3 plays an essential role in fMLF-mediated chemotaxis and respiratory burst making the activities of PIP3-synthetor PI3K and PIP3-degrading enzymes, PTEN and SHIP1, indispensable in these processes. PI3Kγ-null neutrophils displayed inefficient fMLF-mediated chemotaxis and were defective in fMLF-mediated superoxide anion production whereas neutrophils lacking SHIP1 were unable to establish polarity under fMLF concentration gradient with a tendency of forming multiple leading edges hence obstructing their motility. Yet neutrophils deficient in either PI3Kγ or SHIP-1 migrated very slowly in the correct direction toward a chemoattractant source suggesting both molecules are dispensable for directional sensing of neutrophils. Although morphologically irregular, a functional leading edge is established in both PI3Kγ- and SHIP1-deficient neutrophils. We have assessed PI3K activation in fMLF-stimulated 3BP2−/− neutrophils by examining phosphorylation of its downstream effector Akt and found that the levels of Akt phosphorylation were comparable between 3BP2−/− and 3BP2+/+ neutrophils. This result suggested that the overall level of PIP3 is similar between 3BP2−/− and 3BP2+/+ neutrophils since membrane recruitment of Akt through its PH domain binding to PIP3 proceeds before its phosphorylation. However, we have not yet examined the localization of accumulated PIP3 in 3BP2−/− and 3BP2+/+ neutrophils following fMLF stimulation. Normally, PIP3 accumulates only at a single consolidated leading edge of chemotaxing cells. Cdc42 and Rac are both required for establishing this intracellular gradient of PIP3. Active Cdc42 can induce spatial segregation of PIP3 and PTEN in the leading edge and trailing end, respectively, of
chemotaxing neutrophils\textsuperscript{348} whereas Rac has a dominant role in the positive feedback loop that amplifies polarized asymmetry of PIP\textsubscript{3} and actin polymers at the leading edge\textsuperscript{352,479}. With defects in both Rac2 and Cdc42 activation, 3BP\textsubscript{2}\textsuperscript{−/−} neutrophils are probably defective in spatially-restricted PIP\textsubscript{3} accumulation and hence display randomized movements in response to chemotactic gradients. Furthermore, as Rac/PIP\textsubscript{3}/F-actin positively regulates each other’s activity at the leading edge, the regions of increased F-actin concentration on neutrophil membranes normally coincide with regions of PIP\textsubscript{3} accumulation\textsuperscript{366}. Therefore, the arbitrary F-actin assembly seen in fMLF–stimulated 3BP\textsubscript{2}\textsuperscript{−/−} neutrophils might be an indication of a non-polarized deposit of PIP\textsubscript{3} in response to chemotactic gradients. To formally examine the distribution of PIP\textsubscript{3} in these cells, we will employ immunofluorescence microscopy with antibodies specifically against PIP\textsubscript{3} and phospho-Akt.

In conclusion, we have demonstrated a novel role of 3BP2 in mediating fMLF signaling of neutrophils. Based on our results, we proposed that 3BP2 is needed in multiple sites within this signaling pathway. Further detailed analysis is required to understand the role of this adapter protein in fMLF-mediated neutrophil functions. Resolving these issues will improve the understanding of the precise mechanisms underlying chemoattractant-elicited directional sensing and superoxide production of neutrophils.
Concluding Remarks

- The experiment in Figure 5-1 was performed by Dr. Andy Scotter and is included in this chapter with permission.
5.1 Overview

In this thesis, a positive regulatory role of 3BP2 downstream of BCR and downstream of the G protein-coupled receptor, fMLF receptor (N-formylpeptide receptor, FPR), has been described. These two receptors differ in their structures, ligands, the cell types on which they are expressed and the biological outcomes following their activation. While BCRs are expressed on B cells governing B-cell development, B-cell survival, proliferation and Ab productions, FPRs are chemotactic receptors found on neutrophils and monocytes involved in chemotaxis, superoxide anion production and degranulation of these cells. Examples of adapter proteins that participate in different signaling pathways and that mediate multiple cellular functions have already been shown. One of which is SH2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76). SLP-76 is an essential part of T-cell development. In the absence of SLP-76, mice have very small thymi and they lack peripheral T cells and lymph nodes as a result of a block in thymocyte development at the CD4/CD8 double negative III stage of development, when signaling through the pre-T cell receptor (pre-TCR) is first required. The failure of T-cell development in the SLP-76-deficient (SLP-76−/−) mice demonstrated how critical this adapter molecule is to the nucleation of a functional TCR signaling complex in immature thymocytes. In addition to its role in TCR signal transduction, SLP-76 is an essential part of FcγR signaling pathway in neutrophils and it is required for the activation of mast cells through the high-affinity IgE receptor (FcεRI) cross-linking.
5.2 Positive regulatory role of 3BP2

The mechanism by which 3BP2 modulates BCR and FPR signaling possibly involves regulation of some of the common downstream effector molecules of these two signaling pathways. Here I proposed two possible models of how 3BP2 regulates the activities of catalytic enzymes.

5.2.1 Release of intramolecular autoinhibition

3BP2 was initially discovered in the search for the SH3-binding, intermolecular regulatory molecules of the Abl protein. Since mutations in the Abl SH3 domain unleashed the catalytic activity and often the oncogenic potential of the Abl protein, it is believed that SH3 domain is responsible for tightly regulating the Abl protein catalytic activity. It has been proposed that Abl is regulated at least in part by an SH3-binding cellular inhibitor, and several candidate inhibitors have been identified. Yet, other studies demonstrated an autoinhibitory regulation of Abl activity and several proteins have been shown to bind to the Abl protein and potentiate Abl kinase activity presumably by releasing Abl intramolecular autoinhibition (Figure 5-1A).

3BP2 has been shown not to influence Abl kinase activity at least in B cells (Figure 3-10A). Instead, 3BP2 appears to promote activation of Src PTK which is structurally similar to the Abl protein (Figure 4-7A). The regulatory mechanism of Src PTK kinase activity is well understood. Src and its relatives are regulated by a C-terminal Tyr residue that, when phosphorylated, can bind in cis to the SH2 domain of the kinase effectively locking it in an inactive form. This inactive closed conformation is stabilized by an
interaction between the SH3 domain of Src and the linker region between its SH2 and kinase domain. Full activation of Src kinase activity depends on the deterioration of the closed conformation which can be initiated by dephosphorylation of the regulatory Tyr residue and/or binding of a high-affinity ligand to the Src SH3 domain that displaces the intramolecular SH3-linker interaction (Figure 5-1B). Our laboratory has recently demonstrated the formation of a multiprotein complex that includes Src PTK and 3BP2 together with Vav and Syk in osteoclasts. In the absence of 3BP2, all components of the complex were hypophosphorylated (manuscript under revision) (Figure 4-6). In addition, we have mapped the binding site to Src SH3 domain in the Pro-rich region of 3BP2 (Figure 5-2). These results suggest a potential role of 3BP2 as a SH3 domain-binding activator of Src PTKs that by binding to Src with high affinity, 3BP2 releases the intracellular autoinhibition of Src and potentiates its kinase activity. A direct approach involving an in vitro kinase assay with Src and peptide substrate in the presence or the absence of 3BP2 will be required to validate an activity-enhancement role of 3BP2 in Src PTK activation.

5.2.2 Organizing the assembly of catalytic enzymes and their substrates

3BP2 is required for the proper BCR-induced Tyr phosphorylation of Syk (Figure 3-10B) and proper fMLF-induced Tyr phosphorylation of Vav (Figure 4-7B), in B cells and neutrophils, respectively. Tyr phosphorylation is a critical process required for the activation of Syk and Vav.

The regulation of Syk kinase activity is a multistep process which involves phosphorylation of Tyr residues at several sites. The phospho-Tyr at each site
Figure 5-1

A.

3BP2 binds to SH3

B.

3BP2 binds to SH3

Figure 5-1. Positive role of 3BP2: Release of intramolecular autoinhibition. (A) Abl kinase activity is regulated at least in part by its SH3 domain. 3BP2 is a binding partner of Abl-SH3 domain which could potentially activate Abl kinase activity by releasing Abl intramolecular autoinhibition. (B) We proposed that 3BP2 also functions as a SH3-binding partner of Src kinases. Binding of 3BP2 to Src-SH3 domain releases the Src intramolecular autoinhibition and therefore, potentiates Src kinase activity.
Figure 5-2. Far-Western blots on mouse 3BP2 peptide arrays using GST-SH3 construct of Src. Preparation of peptide SPOTS arrays is described in methods and materials. Each spot represents one synthesized 13-mer peptide of 3BP2. Synthesized peptides were spotted onto membrane in 3 columns of 30 spots. Spot 1 to 70 covered the entire length of 3BP2. On the left is the membrane with peptide spots. GST-Src-SH3 binds to peptide sequence at spots: 10, 16, 26, 33, 34, 51, 69 and 70.

Amino acid sequence of Src-SH3-binding spots:

26 - known Abl-binding site (PPAYPPPPVPVPR)
70 - no PXXP (SH2) (LLLRHPYGYAGPR)
51 - no PXXP (PR) (PRPENTPLPHLQR)
69 - no PXXP (SH2) (QSLLLRHPYGYAG)
16 - no PXXP (PH) (WMALLRREIGHFH)
34 - PXXPXR (PR) (PGLRVPATPRRMS)
33 - no PXXP (ALGLRRVEPGLRV)
10 - no PXXP (PH) (FSLSGYNRVMRAA)
differentially contributes to Syk kinase activity regulation. For instance, Cbl recruitment to the phospho-Tyr of Syk has been shown to negatively regulate Syk kinase activity. Whereas phosphorylation of Tyr residue located in the kinase activation loop induces conformational changes that allow substrate and ATP binding with resulting activation of Syk. Vav activation is also regulated by Tyr phosphorylation where phospho-Tyr residues induce conformational changes and unmask the substrate-binding site in its catalytic Dbl homology domain.

While Syk and Src PTKs have been shown to be responsible for Vav Tyr phosphorylation, Tyr phosphorylation of Syk results from both autophosphorylation and Src PTK-mediated reaction. Both Syk and Vav are substrates of Src PTK, yet a direct binding between Src and either of these proteins has not been demonstrated. Although ITAM-dependent Syk recruitment and PIP3-dependent Vav recruitment play a role in positioning these substrates in close proximity to membrane-bound Src PTK, it does not exclude the possibility that Src-mediated Tyr phosphorylation of Syk and Vav requires the formation of a signaling complex mediated by an scaffold protein. With the capacity to bind to Src family PTK, Vav and Syk, 3BP2 is a good candidate for mediating the formation of such multiprotein complex. Several studies have already demonstrated the physical and functional interaction between 3BP2 and these molecules. Therefore, one of the functions of 3BP2 may involve the organization of signaling complex that allows full activation of downstream effector molecules.

The analysis of 3BP2 mice revealed an important role of 3BP2 in cells of the hematopoietic lineage. Although the involvement of 3BP2 in the function of other cell
Figure 5-3. Positive role of 3BP2: Organizing the assembly of catalytic enzymes and their substrates. With the capacity to bind to Src family PTK, Vav and Syk, 3BP2 is a good candidate for mediating the formation of multiprotein complex involving these molecules. We proposed that downstream of BCR, 3BP2 mediates complex formation between Lyn and Syk and therefore, facilitates Syk Tyr phosphorylation by Lyn. On the other hand, 3BP2 complexes with Vav and facilitates Vav Tyr phosphorylation by Src PTK downstream of /MLF receptor.
types remained to be determined, the results obtained with 3BP2−/− B cells and neutrophils indicate 3BP2 as a critical scaffold molecule of signaling complexes across receptor classes and cell types.


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146


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