CHARACTERIZATION OF TWO SURFACE PROTEINS EXPRESSED THROUGHOUT THE LIFE OF A B CELL, AA4.1 AND CD45.

by

Tania Claire Benatar

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

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ABSTRACT

Characterization of two surface proteins expressed throughout the life of a B cell, AA4.1 and CD45.

Degree of Doctor of Philosophy (1998)

Tania Claire Benatar

Graduate Department of Immunology, University of Toronto.

B cell development occurs through discrete developmental stages that are associated with the expression of a variety of cell surface molecules. In this thesis, I describe two cell surface molecules, AA4.1 and CD45, that are expressed from the earliest stages of B cell development. Using CD45 exon 6-deficient mice, I have demonstrated that in the absence of the B220 isoform of CD45, B cell development is blocked at the transition from the immature to the mature B cell stage. Previous work had demonstrated that B cells from CD45 exon 6-deficient mice are unable to proliferate upon BCR aggregation. I have attempted to discern the biochemical mechanism responsible for this deficiency. Important downstream signaling pathways such as the induction of tyrosine phosphorylation of CD79α, PLCγ2, and activation of the MAP kinase, ERK2, are unaffected by the lack of CD45 expression. The one pathway that appears affected by the lack of CD45 is the induction of calcium influx. While BCR aggregation results in the release of calcium from intracellular stores, calcium influx is abrogated in B cells from CD45−/− mice. These results suggest a novel biochemical pathway, i.e. calcium influx, mediated by CD45 in B cells. The
influx of extracellular calcium may also play a role in the selection of B cells into the mature B cell pool.

Previous reports had suggested that the AA4.1 determinant is lost upon B cell maturation. I have demonstrated that the AA4.1 determinant is expressed at high levels on developing B cells in the bone marrow, and decreases to low levels upon B cell maturation. I demonstrate for the first time that mature peripheral B cells express the AA4.1 determinant on their surfaces at low levels. The expression decreases upon stimulation of mature B cells with stimuli that mimic T-dependent responses. In contrast, T-independent stimuli, such as LPS, result in increased AA4.1 expression levels. Finally, I have identified for the first time the protein recognized by AA4.1 as a 115-130 kD glycoprotein, rich in O-linked sugars. The protein does not exist in a covalent-linked complex.
Acknowledgements

I gratefully acknowledge my supervisor Dr. Christopher J. Paige for providing me with the opportunity to work in his laboratory. Chris has provided me with the environment to become an independent scientist, and I thank him for that. I would also like to express my appreciation to the members of my supervisory committee, Dr. Michael Julius and Dr. Jenny Jongstra-Bilen for their time and support over the years, and helpful criticisms. I also want to thank Dr. Gillian Wu for her support and continual faith in my abilities.

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I would also like to acknowledge Mrs. Dorothy Ashley for her time and input in editing portions of this thesis.

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Ag                     antigen
AGM                   aorta-gonads-mesonephros region
A-MuLV                Abelson murine leukemic virus
ADP                   adenosine diphosphate
AP-1                  activating protein-1
ATF                   activating transcription factor
ATP                   adenosine triphosphate
BCR                   B cell receptor
BM                    bone marrow
BSAP                  B cell specific activator protein
btk                   Bruton's tyrosine kinase
Ca²⁺                  calcium
[Ca²⁺]i               concentration of intracellular calcium
cdk                   cyclin dependent kinase
Con-A                 Concanavalin A
CR2                   complement receptor 2
CREB                  cAMP response element binding factor
cμ                    cytoplasmic mu heavy chain protein
D                     immunoglobulin diversity region
DAG                   diacylglycerol
DNA                   deoxyribonucleic acid
EBF                   early B cell factor
EBV                   Epstein-Barr virus
ECM                   extracellular matrix
EGF                   epidermal growth factor
egr-1                 early growth response gene
ER                    endoplasmic reticulum
ERK                   extracellular signal-regulated kinase
EDTA                  ethylenediamine-tetraacetic acid
EGTA                  ethyleneglycol-tetraacetic acid
FCS                   fetal calf serum
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<td>FL</td>
<td>fetal liver</td>
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<td>FSC</td>
<td>forward scatter</td>
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<td>GDP</td>
<td>guanosine diphosphate</td>
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<td>grb2</td>
<td>growth factor receptor binding protein 2</td>
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<td>GTP</td>
<td>guanosine trisphosphate</td>
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<td>GNEF</td>
<td>guanine nucleotide exchange factor</td>
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<td>GSK-3</td>
<td>glycogen synthase kinase-3</td>
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<td>h</td>
<td>hour</td>
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<td>HA</td>
<td>hyaluronic acid</td>
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<tr>
<td>HBSS</td>
<td>Hank's Balanced Salt Solution</td>
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<td>HC</td>
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<td>hen egg lysozyme</td>
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<td>HIM</td>
<td>hyper-IgM syndrome</td>
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<td>HRP</td>
<td>horse-radish peroxidase</td>
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<tr>
<td>HSA</td>
<td>heat stable antigen</td>
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<td>HSC</td>
<td>hematopoietic stem cell</td>
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<td>HSP</td>
<td>heat shock protein</td>
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<tr>
<td>( I_{Ca^{2+}}^{\text{CRAC/DAC}} )</td>
<td>( Ca^{2+} ) release-activated or depletion-activated current</td>
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<td>ICAM</td>
<td>intracellular adhesion molecule</td>
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<td>Ig</td>
<td>immunoglobulin</td>
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<td>interleukin</td>
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<td>IP3</td>
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<td>IRS</td>
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<td>ITAM</td>
<td>immunoreceptor tyrosine-based activation motif</td>
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<td>ITIM</td>
<td>immunoreceptor tyrosine-based inhibitory motif</td>
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<td>J</td>
<td>immunoglobulin joining region</td>
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<tr>
<td>JAK</td>
<td>Janus kinase</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<tr>
<td>kDa</td>
<td>kilodalton</td>
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<td>LC</td>
<td>light chain</td>
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<td>lymphocytic choriomeningitis virus</td>
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<td>LPS</td>
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<tr>
<td>mIg</td>
<td>membrane Ig</td>
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<td>mAb</td>
<td>monoclonal antibody</td>
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<td>MBP</td>
<td>myelin basic protein</td>
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<td>MAPK</td>
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<td>MAPKAP-2</td>
<td>MAPK-activated protein kinase-2</td>
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<td>MEK</td>
<td>MAPK/ERK kinase</td>
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<tr>
<td>MGF</td>
<td>mast cell growth factor</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
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<tr>
<td>ml</td>
<td>millilitre</td>
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<td>NCAM</td>
<td>neural cell adhesion molecule</td>
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<tr>
<td>NFAT</td>
<td>nuclear factor of activated T cells</td>
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<tr>
<td>NF-κB</td>
<td>nuclear factor-κB</td>
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<tr>
<td>NP</td>
<td>nitrophenyl</td>
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<tr>
<td>Na3VO4</td>
<td>sodium orthovanadate</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PDGF</td>
<td>platelet derived growth factor</td>
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<td>PE</td>
<td>phycoerythrin</td>
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<td>PH</td>
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<td>PHSC</td>
<td>pluripotent hematopoietic stem cell</td>
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<td>PI</td>
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<tr>
<td>PKB</td>
<td>protein kinase beta</td>
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<td>PKC</td>
<td>protein kinase C</td>
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<tr>
<td>PLC</td>
<td>phospholipase C</td>
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<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
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<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
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<td>PTB</td>
<td>phosphotyrosine binding</td>
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<td>PTK</td>
<td>protein tyrosine kinase</td>
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<tr>
<td>P-Sp</td>
<td>paraaortic splanchnopleura</td>
</tr>
<tr>
<td>PTP</td>
<td>phosphotyrosine phosphatase</td>
</tr>
<tr>
<td>R</td>
<td>receptor</td>
</tr>
<tr>
<td>RAG</td>
<td>recombinase activating gene</td>
</tr>
<tr>
<td>RF</td>
<td>reading frame</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
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<td>SAPK</td>
<td>stress activated protein kinase</td>
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<td>SCID</td>
<td>severe combined immunodeficiency disease</td>
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<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<td>SERCA</td>
<td>sarco/endoplasmic reticulum Ca\textsuperscript{2+} ATPase</td>
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<td>SH</td>
<td>Src homology</td>
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<td>SHIP</td>
<td>SH2-domain-containing inositol polyphosphate 5-phosphatase</td>
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<td>SHP</td>
<td>SH2-domain-containing phosphotyrosine phosphatase</td>
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<td>sIg</td>
<td>surface immunoglobulin</td>
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<td>SL</td>
<td>surrogate light chain</td>
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<td>SRE</td>
<td>serum response element</td>
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<td>SRF</td>
<td>serum response factor</td>
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<td>SSC</td>
<td>side scatter</td>
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<td>STAT</td>
<td>signal transducer and activator of transcription</td>
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<td>TAPA-1</td>
<td>target of anti-proliferative antibody</td>
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<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TD</td>
<td>thymus-dependent</td>
</tr>
<tr>
<td>Tdt</td>
<td>terminal deoxynucleotide transferase</td>
</tr>
<tr>
<td>TH</td>
<td>Tec homology</td>
</tr>
<tr>
<td>TI</td>
<td>thymus-independent</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TNFR</td>
<td>TNF receptor</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNFR-associated factor</td>
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<td>V</td>
<td>immunoglobulin variable region</td>
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<td>VCAM</td>
<td>vascular cell adhesion molecule</td>
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<td>VLA</td>
<td>very late antigen</td>
</tr>
<tr>
<td>VSV</td>
<td>vesicular stomatitis virus</td>
</tr>
<tr>
<td>xid</td>
<td>X-linked immunodeficiency</td>
</tr>
<tr>
<td>XLA</td>
<td>X-linked agammaglobulinemia</td>
</tr>
<tr>
<td>4PS</td>
<td>IL-4-induced phosphotyrosine substrate</td>
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CHAPTER 1

Introduction
Overview

Lymphocytes are white blood cells that are essential in the recognition and elimination of foreign antigens by the immune system. B lymphocytes, in particular, constitute the specific humoral arm of the immune system, whose function is to protect the individual when exposed to microbial or viral attack. This protection is mediated by the production of molecules with diverse specificities known as antibodies or immunoglobulins (Ig).

The differentiation of B-lineage cells can be divided into 2 general stages. The first stage is antigen-independent and involves differentiation of stem cells to B lymphocytes. In mammals, this process initiates primarily in the liver of the fetus, and is subsequently maintained continuously in the bone marrow (BM) of adults. During this developmental process, B cells rearrange their Ig genes, and upon expression of a functional Ig receptor on their surface, they are positively selected to mature. Each newly generated B cell expresses a novel species of Ig on its surface with a unique set of binding specificities. After acquiring antigen receptors, B cells migrate to peripheral lymphoid organs, such as the spleen and lymph nodes, where contact occurs between lymphocytes and circulating antigens. In the absence of further stimulation, these peripheral B cells are non dividing or resting cells. The second stage of B cell differentiation is antigen-dependent, and involves specific binding of antigen to the surface Ig receptor of a resting B cell. In order for the B cell to be activated by an antigen, it requires another signal that is provided by contact with activated helper T cells. The activation of the B cell then occurs through the combined efforts of surface and intracellular B cell signaling proteins, resulting in proliferation of the B cell. The progeny of this B cell can then differentiate into plasma cells, the effector cells of the humoral immune system, which secrete into the bloodstream large amounts of an Ig molecule, with the same binding specificity as that present on the membrane of the B cell that originally encountered the antigen.

It is important to understand the processes involved in both the antigen-independent and antigen-dependent phases of B cell differentiation. Without either of these phases functioning
properly, the organism is left vulnerable to invading microorganisms. Inappropriate function may also lead to the development of autoimmune disease.

Both the antigen-independent and antigen-dependent phases are dependent upon the presence of cell surface molecules. B cell development and maturation requires not only the rearrangement of Ig molecules, but also the presence of specific cell surface molecules. Such molecules are also critical for B cell survival and function.

The objective of this thesis is to examine and characterize two B cell surface molecules, AA4.1 and CD45, that are expressed during both the antigen-independent and antigen-dependent stages of B cell differentiation.

This introduction will begin by discussing B cell development. This will include a description of ontogeny, followed by a description of the different stages of B cell development. Surface molecules associated with development will also be described, as well as selection processes that affect the development and maturation of B cells. B cell signaling will then be addressed, including the major second messenger pathways that are involved. Included in this is a section on the function of CD45 in B cells. Finally, the activation of B cells by T cells will be explored.

1. B Cell Development

a) Ontogeny of Hematopoiesis

Hematopoiesis is the process by which lymphoid, myeloid and erythroid cells of the immune system develop from uncommitted precursors. The process is initiated by a pluripotent hematopoietic stem cell (PHSC), that differentiates into a hierarchical array of developmental intermediate cells consisting of multipotent and monopotent progenitors (1, 2). A hallmark of hematopoietic stem cells (HSC) is their capacity for self-renewal as well as their potential to differentiate into committed progenitors (3). Hematopoiesis starts in the mouse embryo at day 7.5
of gestation. The first site of hematopoiesis is the paraaortic splanchnopleura (P-Sp). Both lymphocyte and multipotent myeloid precursors can be detected at this site. At this time the isolated yolk sac is unable to contribute to definitive hematopoiesis. After the circulation is established at day 8 of gestation, both yolk sac and P-Sp contribute multipotent precursors to hematopoiesis (4). The first HSC capable of long term reconstitution of irradiated recipients can be detected at day 10 of gestation in the aorta-gonads-mesonephros (AGM) region. The AGM is a structure derived from the P-Sp (5, 6)(Fig. 1-1).

In the mouse embryo, two waves of circulating precursors are observed. The first wave is comprised of multipotent precursors, capable of differentiating into myeloid and lymphoid lineages when cultured under appropriate conditions. These are first detected at day 10 of gestation, and continue until day 12 of gestation. The second wave appears at day 13 of gestation and is comprised of committed B cell precursors (7). At day 10 of gestation, the fetal liver (FL) is seeded by blood-born precursors, presumably derived from the P-Sp and AGM regions (7-9). The FL remains the dominant site of hematopoiesis until birth (day 19-20) (10). The majority of B lineage progenitors in FL at day 11-12 of gestation are comprised of bipotent B cell/macrophage precursors (11). Multi-lineage precursors that can give rise to erythroid, myeloid, and B lymphoid precursors can be isolated from day 13 FL based on AA4.1 expression (12). AA4.1 is a monoclonal antibody that originally identified a cell surface molecule expressed on developing B cells (13). A more detailed description of the expression of the determinant recognized by AA4.1 will be discussed later in this chapter. The embryonic thymus is colonized at about day 10.5 of gestation (14-16). The spleen has been identified as a transient site for hematopoiesis from day 18 of gestation until one month after birth (10). Hematopoietic precursors are already present in BM prior to day 15 of gestation. However, active B lymphopoiesis does not appear until day 17 of gestation. Since seeding of BM and other hematopoietic organs requires circulating progenitors, it is likely that colonization of the BM and all other hematopoietic organs occurs early in development, between days 10-12 of gestation, but active hematopoiesis begins at different times.
in these organs. BM remains the primary site of hematopoiesis after birth and throughout adult life (7, 17).

In adult BM, the expression of AA4.1 together with low levels of CD4 expression characterizes a population of cells that is not yet committed to the B lineage. The population is termed $A_0$ under the nomenclature defined by the Hardy laboratory (18). Low CD4 expression in mouse BM has been shown to characterize cells that are more differentiated than the long term reconstituting HSC, but that still contain multi-lineage capacity (19). Fraction $A_0$ is thought to generate committed B cell progenitors found in fraction $A_1$ of the Hardy nomenclature, which are B220$^+$ AA4.1$^+$ CD4$^{lo}$ (18).

b) B cell Ontogeny

The transition from multipotent stem cells to the B lineage involves the acquisition and loss of a series of traits. B lymphopoiesis is a complex process that involves a programmed cascade of gene rearrangements, whereby functional antibody variable region genes are assembled from Ig heavy (H) and light (L) chain gene segments (V, D, J) by recombination (20). B cell development occurs predominantly in FL during embryonic life and in the BM of adult animals (21, 22). In fetal B cell development, B cell progenitors have been detected as early as 9.5 to 10 days of gestation first in P-Sp and then in AGM based on the expression of RAG mRNA and Ig heavy chain (HC) D-JH rearrangements (Fig. 1-1) (23). However, no mRNA for the pre-B cell specific $\lambda_5$ gene could be detected until 12 days of gestation. Thus, the RAG-1$^{+}\lambda_5^{-}$ DJH$^{+}$ cells may not be B cell lineage restricted. B cells do develop in mice lacking $\lambda_5$ due to targeted deletion, although development is delayed (24). Thus, the RAG-1$^{+}\lambda_5^{-}$ DJH$^{+}$ cells may be representative of a $\lambda_5$-independent B lineage pathway (23).

Uncommitted B cell precursors from day 12 of gestation that do not yet express the high molecular weight isoform of CD45, B220, can be isolated based on AA4.1 expression (25). CD45 is a transmembrane protein tyrosine phosphatase (PTP) expressed on all nucleated
Figure 1-1. Ontogeny of hematopoiesis and B lymphopoiesis.

**Hematopoietic ontogeny**

- Hematopoietic progenitors (HP) in P-Sp
- HP in yolk sac
- Multipotent progenitors in AGM
- Circulation established
- Colonization of liver
- Colonization of thymus
- Bipotent B/macrophage cells in liver
- Precursors detected in BM

**Gestation**

<table>
<thead>
<tr>
<th>Days</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
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<th>18</th>
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<td>VDJ in liver</td>
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<td>VJK in liver</td>
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<tr>
<td>B lymphopoiesis begins in BM</td>
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</table>

**B lymphopoiesis**
hematopoietic cells (26). A more detailed description of CD45 and its function will be discussed later in this chapter. Committed B progenitors containing DJ rearrangements can first be detected in FL at day 12 of gestation (21). Although B lineage-committed progenitors have not been detected prior to day 12 of gestation in either yolk sac or embryo body, hematopoietic progenitors in these sites can develop into B lymphocytes in vitro and in vivo (17, 27-30).

Other sites appear to be transient locations of B generative potential, such as placenta and peripheral blood (day 10-13 of gestation) (31, 32), and fetal omentum (day 13-14 of gestation) (33). In placenta, however, development does not proceed to the μ+ stage (34). In fetal thymus, B cell precursors are detected at day 12 of gestation but rapidly decline in frequency afterwards, although at day 15-16 of gestation some cells are still present that are capable of transformation by a B-lymphotrophic virus (35). In FL, the first VDJ rearrangements can be detected at day 13 of gestation (36), Ig κ L chain rearrangements at day 14 of gestation (25, 36, 37), and the first sIgM+ cells can be detected by day 17 of gestation (34, 38). FL is therefore a rich source of B cell progenitors during the early period of development, between days 12-16 of gestation, since it is essentially free of mature sIgM+ B cells. Active B lymphopoiesis begins in the embryonic BM at day 17 of gestation, with the BM remaining the primary site of hematopoiesis throughout adult life (7).

c) Stages of B cell Development

After commitment to the B lineage, a cell transits through multiple stages of differentiation that are characterized by phenotypic and functional changes, such as sequential rearrangement of the Ig H and L chain loci and expression of cell surface molecules. Different nomenclatures have been used to classify the various B cell developmental stages (Table 1-1). The nomenclature varies among laboratories, including those of Hardy, Osmond and Melchers (Table 1-1). The Hardy nomenclature will be used throughout this thesis for defining the various B cell fractions.
In normal mice, the pre-B cell compartment can be divided into two populations. The first is large, cycling B-progenitor cells that express the CD43 molecule (Table 1-1; fractions A-C'). These develop into the second population, consisting mainly of small resting pre-B cells (Table 1-1; fraction D), which are CD43− (39). These two populations can be further subdivided based on the expression of other surface markers (Table 1-1). Cells entering the B lineage compartment are classified as Fraction A (B220+, CD43+, HSA−, BP-1−, CD19−). Some of the cells in this fraction contain DJ_H rearrangements, while VDJ_H and VJ_k rearrangements are undetectable (39, 40). Fraction A can be separated further into two AA4.1+ subfractions, designated as A1 and A2. Cells in these subfractions express high levels of germline μ transcripts, and very low levels of RAG-1 and RAG-2 transcripts, indicative of early B lineage commitment. These cells can differentiate into cells belonging to fractions B or C when placed in short term cultures with stromal cells, a characteristic property of B lineage precursors. The A1 fraction differs from fraction A2 by expressing low levels of CD4. Fraction A1 likely develops into fraction A2, based on the pattern of expression of a set of B lineage-restricted genes and of a series of transcription factors involved in regulating B cell development (18).

The cells then proceed to fraction B (B220+, CD43+, HSA+, BP-1−, CD19+). In this fraction, most IgH loci are in DJ_H configuration. However, some VDJ_H rearrangements are observed at this stage (40). At the next stage (Fraction C; B220+, CD43+, HSA+, BP-1+, CD19+), most cells contain VDJ_H rearrangements, and a small percentage contains VJ_k rearrangements (40). Cells with non-productive VDJ rearrangements remain in fraction C. Approximately 70% of cells in fraction C are rapidly eliminated by apoptosis (41). Cells in fraction C' are distinguished from those in fraction C by a higher expression of HSA (Table 1-1). Fraction C' is also enriched for cells containing productive VDJ_H rearrangements (42). Upon completion of a productive VDJ_H rearrangement, cells lose expression of CD43 and c-kit, and decrease RAG-1 and RAG-2 expression, while other cell surface markers are increased, such as CD25 and CD22 (Table 1-1). At this stage (fraction C'-D), cells express the pre-B cell receptor (BCR) on their surfaces. The pre-BCR consists of an Ig μ HC paired with the λ5 and VpreB
**Table 1-1. Stages of B cell development.**

<table>
<thead>
<tr>
<th>Melchers:</th>
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<th>preB1</th>
<th>preB1</th>
<th>large preBll</th>
<th>small preBll</th>
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<td>C</td>
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<tr>
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<td>+++</td>
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<td>+++</td>
<td>+++</td>
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</tr>
</tbody>
</table>

*a* Fraction C is enriched for cells with two nonproductive rearrangements.  
*b* Tdt is expressed in BM-derived B cell precursors only.  
This table is based on (18, 21, 39, 43-45), and references therein.
surrogate light (SL) chains (46). Fraction D consists mainly of small resting pre-B cells. The immature B cells observed in fraction E are defined by expression of sIgM. They are generated from fraction D upon completion of a productive rearrangement at one of the LC gene loci. These newly produced immature B cells leave the marrow and migrate to the spleen and other secondary lymphoid tissues where the majority normally die within a week (47, 48). A small minority of immature B cells are induced to mature into long-lived recirculating B cells (fraction F) (47-49). Mature B cells express both IgM and IgD and migrate among the follicles of lymphoid tissues for several weeks (49-52).

d) Transcriptional regulation of B cell development

Transcription factors that are essential for B cell development appear to be necessary for differentiation primarily at two stages: prior to the commitment to the B lineage, and at the fraction A-fraction B cell transition, when VDJ recombination is beginning. PU.1 and Ikaros transcription factors appear to be required prior to commitment to the B lineage, whereas E2A, EBF (early B cell factor), and BSAP (B cell specific activator protein) appear to act at the second developmental checkpoint (53-55). It has been suggested that PU.1 is important for the development of multiple hematopoietic lineages (55, 56). Targeted disruption of PU.1 is embryonically lethal. Analysis of FL derived from PU.1-/- mice demonstrated the lack of B and granulocyte/macrophage precursors. In addition no T cell development was observed in fetal thymic cell suspensions. Therefore PU.1 is required for development of B, T, and multiple myeloid lineages (57, 58). Further, the lack of both T and B cells in Ikaros-deficient animals indicates that this factor is acting in a lymphoid-restricted progenitor (59).

The differentiation of the earliest committed B cell appears to require E2A genes. E2A-/- mice are specifically blocked in B lymphopoiesis, fail to initiate DJH rearrangements, and lack B220+CD43+ progenitor B cells (60, 61). EBF is also required very early in B cell development, and its expression pattern appears to overlap with that of E2A, although EBF-deficient mice appear
to have more mature progenitor B cell populations. B cells from EBF-deficient mice do not progress beyond the B220+CD43+ stage, lack DJH rearrangements, CD79α/β, λ5, RAG-1 and RAG-2 (62). BSAP is required in the transition from fraction B to fraction C B cell stage in the BM, when cells are actively rearranging IgH genes (63, 64). B cells from BSAP-deficient mice express c-kit, B220, CD43, and IL-7R and have undergone DJH rearrangements, but cells expressing CD19 are not detected, and VDJH rearrangements are significantly reduced (64, 65). In contrast to the BM, the FL of BSAP-deficient animals does not contain any B220+ B cell progenitors. Adoptively-transferred mutant FL cells could, however, progress to fraction B, suggesting that BSAP functions early in the development of FL to allow cells to respond to a stimulus specific to the FL microenvironment (64).

e) Cell surface antigens expressed in B cell development

i) B220 (CD45)

B220, the 220 kDa isoform of CD45, is a type I integral membrane protein, whose cytoplasmic domain has PTP activity (26, 66, 67). Different isoforms of CD45 are generated by alternative splicing of variable exons 4-7 (26, 66, 68). Whereas CD45 is expressed on all nucleated hematopoietic cells, isoform usage changes depending on the developmental stage and activation state. The B220 isoform of CD45 is expressed on uncommitted lymphoid progenitors, NK cells, and on all B lineage cells except terminally differentiated plasma cells (18, 69-72). The first committed B cells express B220 (Fraction A; Table 1-1) (39). The expression of B220 on B cell precursors occurs at the time of D-JH rearrangement (25). In the absence of B220, B cell development proceeds unimpaired until the immature B cell stage (73). The critical role of B220 in B cell activation has been elucidated by studies using CD45 antibodies or CD45-deficient B cell lines (74-79). The function of B220 on B cells will be addressed in a later section of this chapter.
ii) Heat Stable Antigen (HSA)

Heat stable antigen (HSA/CD24) is a 31-50 kDa type III membrane protein that is expressed on progenitors of erythroid, myeloid, T and B lineages, as well as neuronal progenitors (80). The first committed B cell progenitors do not express HSA (B220+ CD43+). Expression of HSA and CD19 occurs concomitantly during development, and characterizes the cells in Fraction B in the developmental scheme (table 1-1) (39). HSA expression increases during B cell development and reaches highest levels at the Fraction C' stage, when CD43 is downregulated (39). Immature B cells also express high levels of HSA, which distinguishes newly generated BM and splenic transitional B cells from mature HSAlo long-lived peripheral B cells (81-83). HSA functions as a costimulatory molecule on antigen presenting cells and as an adhesion molecule on activated B cells (84-87). Mice deficient for HSA develop normal numbers of mature B cells but exhibit an incomplete block in development at the transition from fraction C to fraction D ((88)). Forced expression of HSA in transgenic mice results in reduced numbers of IL7-responsive B cell progenitors as well as a reduction in peripheral B cell populations (89). Antibody mediated aggregation of HSA induces apoptosis in B cell precursors (90). These results are consistent with a role for HSA as a negative regulator of B cell development.

iii) BP-1

BP-1 is a 110-150 kDa type II integral membrane protein, and the extracellular domain exhibits aminopeptidase A activity (91, 92). It was originally identified by its high expression on pre-B cells transformed by Abelson murine leukemic virus (A-MuLV) and on B cell progenitors cultured in Whitlock-Witte cultures (93, 94). BP-1 is expressed on a subset of B cell precursors as well as on thymic cortical epithelial cells, endothelial cells, enterocytes, epithelial cells of the intestine and proximal renal tubules, placenta splanchnopleura, and stromal cells of the BM and
thymus (95, 96). In B cell development, BP-1 expression is restricted to a brief period of
differentiation, from fraction C to the fraction D stage of development (39).

BP-1 expression is increased on B cell precursors by the stromal cell-derived cytokine, IL7
(94, 97). Further, B cell progenitors upregulate BP-1 expression in response to IFN-α or IFN-β
(98). The expression of BP-1 on stromal cells correlates with their ability to support the
proliferation of B cell progenitors (99). These results are consistent with the involvement of BP-1
in the growth and differentiation of B cell progenitors at the IL7 responsive stage of development.

iv) CD43 (leukosialin)

CD43/leukosialin, a 90-120 kDa type I integral membrane protein, is a major cell surface
sialoglycoprotein (100). The extracellular domain of CD43 has an extended rigid protein structure
caused by the extensive O-glycan linkages. In addition, the high negative charge, which is a
consequence of extensive sialic acid residues on the protein's surface, also prevents compact
folding of the protein (101). Because of the extensive O-glycosylation, CD43 has been classified
as a cell surface mucin (102). Characteristic isoforms result from differential O-glycosylation. T
cells express 2 specific isoforms of CD43, a 115 kDa form on resting T cells and 140 kDa form on
activated T cells (103). The 140 kDa form consists mainly of a hexasaccharide core whereas the O-
glycans of the 115 form consist largely of a tetrasaccharide core (104-106). Altered expression of
CD43 has been associated with Wiskott-Aldrich syndrome, an X-linked immunodeficiency
disease. In this disease, the hexasaccharide form of the molecule seems to be overexpressed
instead of the usual tetrasaccharide form (107-109).

CD43 is expressed on HSC, T lymphocytes, granulocytes, macrophages, platelets,
erthroid progenitors and B lymphocytes at specific stages of development (100). In B cell
development, CD43 is expressed from the early pro-B cell stage of fraction A, and is
downregulated at fraction C', prior to the expression of Ig light chain (LC) (39). CD43 is
expressed on splenic B-1 cells as well as on activated and terminally differentiated B cells (110, 111).

The function of CD43 remains unknown. Antibody mediated aggregation of CD43 on human cells has been reported to enhance monocyte activation (112, 113), increase NK activity (114), induce apoptosis in hematopoietic progenitor cells (115), and to stimulate T cell proliferation and aggregation (116, 117). CD43 has been shown to co-stimulate T cells independently of CD28, suggesting its involvement in an alternate co-stimulatory pathway (118). In Jurkat cells, ligation with a specific CD43 antibody can induce calcium (Ca\textsuperscript{2+}) signaling in the absence of TCR signaling, by the hydrolysis of phosphoinositides. This results in diacylglycerol (DAG) and inositol trisphosphate (IP\textsubscript{3}) production, which leads to increased concentrations of intracellular Ca\textsuperscript{2+} (113).

CD43 has been demonstrated to act as an adhesion molecule, as B cells bind specifically to immobilized purified CD43 (117). Paradoxically, CD43 has also been shown to have anti-adhesion properties. In some cell lines it was found that expression of CD43 inhibited intercellular adhesion, while ablation of CD43 expression facilitated adhesion (119, 120). CD43 transgenic mice display splenomegaly due in large part to increased numbers of B cells. These B cells exhibit increased survival in vitro, which correlates with their decreased susceptibility to apoptosis (121), and a decreased ability to homotypically aggregate (122, 123). Further, CD43 transgenic mice have a humoral immunodeficiency characterized by decreased serum Ig levels, and decreased antibody responses to both T-dependent and T-independent antigens. Mice carrying a targeted disruption of the CD43 locus display enhanced in vitro proliferative responses and adhesive properties (124). These results suggest that the extensive O-glycosylation and presence of sialic acid residues on CD43 prevent critical cell-cell interactions. The anti-adhesive effect of CD43 can be overcome by the binding of CD43 to several putative ligands, including ICAM-1(125), serum albumin (126), E-selectin (127), and galectin-1 (128).
v) AA4.1

The study of B cell differentiation has relied heavily on the expression of surface markers that characterize different stages of B cell development. In 1984, the identification of a new surface marker on developing B cells was reported along with a mAb specific for its detection, termed 'AA4.1'(13). AA4.1 was isolated from a fusion between spleen cells derived from the immunization of a rat with the pre-B leukemia line, 70Z/3, and the mouse myeloma SP2/O-Ag14. For ease of discussion I will hereafter also refer to the molecule detected by mAb AA4.1 as AA4.1. AA4.1 expression was originally thought to be confined to more immature B cell precursors. AA4.1 bound to all pre-B lymphomas, but none of the plasmacytomas tested. The highest proportion of AA4.1+ cells was found in adult BM. In the BM, all cμ+ cells were AA4.1+, whereas half of the sIgM+ cells in the BM were AA4.1+. Some AA4.1+ cells were also observed in adult spleen and FL (13). However, the phenotype of such cells was not examined. Thus it was unknown whether the AA4.1-expressing cells in adult spleen were of the B lineage. These results suggested that either AA4.1 recognizes a subset of B cells, or alternatively, AA4.1 expression is lost upon B cell maturation. Approximately half of AA4.1+ BM cells are sIgM- cμ-, suggesting that either they are not B lineage, or they recently became committed to the B lineage (13).

The expression of AA4.1 is not just confined to cells restricted to the B lineage. The AA4.1 antigen (Ag) is expressed on HSC, multipotent progenitors, and restricted erythroid/myeloid precursors as well as precursors that differentiate into B cells (12). As a result, AA4.1 has been an extremely valuable marker for the isolation of such cells, and their subsequent characterization. AA4.1 Ag expression can be used to isolate and enrich for the fetal PHSC (129). However, isolation of AA4.1+ cells does not enrich for the PHSC in adult BM. This discrepancy is thought to be due to differences in cell cycle. FL PHSC are likely to be in a state of exponential growth whereas BM-derived PHSC are expected to be in a resting Go phase. Thus AA4.1 is thought to be a marker of PHSC in cell cycle (130).
Multipotent cells capable of differentiating into both myeloid and lymphoid lineages can be isolated from the para-aortic splanchnopleura (P-Sp) at day 8-9 of gestation based on the expression of AA4.1 (131). Multilineage precursors from day 13 FL that contained erythroid, myeloid, and B lymphoid precursors can be isolated based on the expression of AA4.1 (12). Bipotent cells capable of generating both B cells and macrophages can be isolated from day 12 FL based on AA4.1 and Ly6 expression (11). The expression of AA4.1 has been successfully used to isolate and enrich for uncommitted B220- B cell precursors from day 12 of gestation (25). In addition, B cell precursors that have the capacity to differentiate in vitro into Ig-secreting cells can be isolated based on AA4.1 expression from as early as day 8.5 of gestation from yolk sac and embryonic tissue (28).

The expression of AA4.1 also identifies multi-lineage and committed progenitors from adult BM (130, 132). In adult BM, AA4.1 expression characterizes the earliest stage of commitment of HSC to the B lineage. B cell progenitors from this early developmental stage belong to fractions A1 and A2. A description of these fractions was mentioned previously in Section 1-c. A subset of AA4.1+B220- cells, termed A0, has been identified, and is characterized by the expression of low levels of CD4, and thus may be the immediate precursors of cells within fraction A1 (18). Low CD4 expression in mouse BM characterizes more differentiated cells than the long term reconstituting HSC, but these CD4lo cells do contain multilineage capacity (19). Thus cells in fraction A0 are likely not yet committed to the B lineage.

Chapter 3 of this thesis characterizes the expression of AA4.1 not only on B cell precursors but also on mature B cells. In addition, the structure and size of the molecule recognized by AA4.1 is also presented. To date, no definitive function for AA4.1 has been demonstrated.

f) The role of stromal cells in B cell differentiation.

In vivo, developing B cells are found in close physical association with microenvironmental cells known as stromal cells, which are responsible for the production of
extracellular matrix (ECM) and cytokines that promote lymphopoiesis. Stromal cells are a heterogenous population of cells which include fibroblasts, adventitial reticular cells, pre-adipocytes and macrophages (133-135). The molecular interactions between stromal cells and B cell progenitors are essential for B cell development, and involve a variety of adhesion molecules and growth factors (135). Stromal cells also provide supporting matrices that allow migration to blood vessels. The most immature B cell progenitors are found in the subendosteal region of the marrow in close association with reticular cells. As differentiation proceeds there is a migration from the endosteal region to the centrally located sinuses along a network of stromal cell processes. Immature B cells, expressing surface IgM but not IgD can be seen to traverse the endothelial lining of the sinusoids. These immature cells are held in an intravascular compartment, possibly for a final stage of differentiation, before being released into the circulation (136).

The establishment of in vitro systems which support hematopoiesis provided a tool for the identification of microenvironmental interactions which regulate B cell development (69, 137-141). Stromal cell clones were established from these cultures which could either support or inhibit B cell development (99, 142-145). In addition, clones were identified which could support B cell progenitors at different stages of development (146-150). The stromal cells which support lymphopoiesis are generally fibroblasts or pre-adipocytes, whereas macrophages are frequently inhibitory. Over 20 stromal cell-derived growth factors have been identified which can mediate many of the effects attributed to the microenvironment.

i) adhesion molecules

Adhesion molecules are involved in mediating the association of B cell progenitors with stromal cells. These include, NCAM-1, VCAM-1 and components of extracellular matrix such as fibronectin, laminin, hyaluronate and collagen (135).

The α4β1 integrin, VLA-4 (very late activation antigen-4) is expressed on most hematopoietic precursors in addition to lymphocytes, NK cells, monocytes, eosinophils and
basophils (151, 152). VLA-4 binds to several ligands, including VCAM-1 on stromal cells (153-155), and fibronectin on ECM (156, 157). Mice deficient for either the α4 or β1 integrin chains, or the VLA-4 ligands, fibronectin or VCAM-1, have all resulted in early embryonic death indicating their importance in murine development (158-164).

CD44 is a lectin involved in cell-cell and cell-matrix adhesion and the migration of lymphocytes between blood and lymphoid organs. It is expressed on virtually all hematopoietic cells as well as a variety of tissues including stromal cells. CD44 has multiple ligands, including the ECM glycosaminoglycan, hyaluronic acid (HA), collagens, mucosal vascular addressin, and fibronectin (135). Monoclonal antibodies directed against CD44 completely inhibited the production of lymphoid cells in long-term BM cultures suggesting that CD44 is involved in lymphopoiesis (165). Similarly, mAbs which inhibit the interaction of VCAM-1 with its ligand, VLA-4, abrogate B lymphopoiesis in vitro (166, 167).

ii) IL7

The first cytokine to be cloned from a stromal cell line was IL7 (168). IL-7 is a 25 kDa secreted glycoprotein produced by stromal cells from many hematopoietic tissues including FL, BM, spleen and thymus (169, 170). In addition, IL-7 is also constitutively produced by keratinocytes in the skin, intestinal epithelial cells, and by follicular dendritic cells, endothelial cells and stromal cells in human tonsils (171-174). The majority of cells from FL or BM that proliferate in response to IL-7 are B220+ IgM- B cell progenitors. IL-7 does not induce the proliferation or differentiation of cells from the fraction D cell stage of B cell development (146, 175, 176).

The essential role of IL-7 in B cell development has been demonstrated using several experimental systems (175, 177-179). The administration of neutralizing anti-IL7R mAb into adult mice completely abrogates B cell development at the fraction D cell stage, demonstrating the essential role of IL-7 in B lineage progression (178). IL-7-deficient mice have a severe reduction of mature B cells in the periphery caused by a block in B cell development at the fraction C cell stage.
These mice contain a small population of functionally mature B220+ IgM+ B cells in the spleen indicating that B cell development can occur in the absence of IL-7, although at significantly reduced levels. In addition, the presence of a population of immature B220+ IgM- cells in the spleen raises the possibility that B cells may develop in the peripheral lymphoid tissues in the absence of IL-7 (177). The number of mature B and T lymphocytes is elevated in IL-7 transgenic mice, and in mice administered IL-7 through subcutaneous injection (180-182).

The receptor for IL-7 is a heterodimer composed of a 75 kDa ligand binding α chain (CDw127) and a 64 kDa common γ chain (γc) (183-185). The γc chain is also a component of receptors for other cytokines such as IL2 (183), IL4 (186, 187), IL9 (188), and IL15 (189, 190). Mice deficient for the γc chain have similar lymphoid deficiencies as IL-7 or IL-7Rα deficient mice (191, 192).

iii) Mast cell growth factor (Steel factor, Stem cell factor or c-kit ligand)

Mast cell growth factor (MGF) also plays an important role in regulating B cell development. MGF is a type 1 transmembrane glycoprotein, which can also exist as a soluble 25 kDa dimer (193). The receptor for MGF, c-kit, is a tyrosine kinase receptor belonging to the class III family of receptor tyrosine kinases (PDGF-R, CSF-1R, Flt3) (194, 195). Mutations at either the dominant White spotting locus (W, c-kit) or Steel locus (Sl) results in mice with similar phenotypic abnormalities including loss of hair pigmentation, severe macrocytic anemia, mast cell deficiency and sterility (196). Studies of mutant Sl and W mice demonstrated that MGF and its receptor were critical for the normal development of hematopoietic progenitors, melanocytes, germ cells and mast cells (197).

The MGF receptor is expressed on uncommitted progenitors and B lineage progenitors until the fraction B cell stage, prior to cμ expression (198, 199). On its own, MGF does not stimulate the growth of B lineage cells. However, it is synergistic with IL-7 in inducing the proliferation of B220+ B cell progenitor in vitro (200). This synergistic effect is due to an increase
in the frequency of progenitors which can respond to IL-7 in the presence of MGF (201). However, MGF is not obligatory for the development of B lineage cells. Mice (W/W) that lack c-kit on their surface die perinatally due to severe anemia. A comparison of the FL, BM and spleen from W/W and wild type mice revealed that B cell development was not affected in the mutant mice. Furthermore, c-kit⁻/⁻ FL cells transferred into RAG⁻/⁻ mice developed into mature B cells as efficiently as wild type cells, whereas progenitors of the erythroid, myeloid or T lineages did not develop (202). These studies demonstrated that MGF signaling is essential for the development of erythroid, myeloid and T lymphoid lineages, but not for the development of B lineage cells. Additional stromal cell factors may exist which can substitute for MGF in supporting B lymphopoiesis. Flt-3/flk-2, a factor sharing many structural features with MGF, may fulfill this function (203).

**g) Selection in B cell Development**

In the adult mouse, 3-5x10⁷ newly formed B cells are generated per day in the BM by proliferation and differentiation of early B cell progenitors (21). However, only 2-3x10⁶ IgM⁺IgD⁺ mature B cells enter the peripheral pool of mature sIg⁺ B cells each day (21, 204, 205). In addition, up to 75% of developing B cells are eliminated at the transition from fraction C to D (40, 206). Thus, the extensive loss of B lineage cells during development and maturation reflects powerful selection processes that occur prior to the inclusion of newly generated B cells into the peripheral B cell pool. Upon BCR generation, it is possible that a given recombination event will lead to a protein incapable of constructing a functional BCR, or a receptor reactive to endogenous or self-antigen. For these reasons, selection processes have evolved that mediate the elimination of B cells expressing inappropriate BCR. The selection processes not only serve to encourage development of B cells with functional antigen receptors (positive selection), but also provide a mechanism for elimination of clones able to respond to endogenous or self-antigens (negative selection). Thus, signaling through the antigen receptor at defined stages of B cell development
provides checkpoints for ordered B cell maturation, and shaping of the repertoire that will be represented among the mature, immunocompetent B cells. This section will examine the selection processes that occur during B cell development.

Mice with targeted or naturally disrupted genes have been useful in characterizing these selection processes. SCID mice are naturally occurring mutant mice that are unable to produce T or B cells due to absence of the enzyme, DNA-PK, which is an integral part of the double-stranded break repair machinery necessary for the rearrangement of both Ig and TCR genes (207-211). RAG-1 and RAG-2 are proteins also necessary for the rearrangement of Ig and TCR genes, and hence mice with targeted disruption of either gene are unable to generate mature T or B cells (212-214).

In B cell development, cells belonging to fraction C may only progress to the developmental stage of fraction D if they express a membrane-bound μ-chain. Evidence for this is apparent by the lack of fraction D-expressing cells in SCID (215), RAG-2-deficient (214), and JHT mice (40). JHT mice have a targeted deletion of the entire JH locus such that B cell precursors cannot assemble DJH and VDJH complexes (40). All three mice strains mentioned above are unable to produce functionally rearranged μ chain genes. The expression of a μ chain transgene allows the developmentally arrested B cells in RAG-deficient mice to progress from the fraction C to fraction D cell stage (43, 46, 216).

The μ protein must be expressed on the cell surface in order to effectively select B cells into fraction D. The μMT mice are a B cell deficient mouse strain, in which the membrane exon of the μ chain was disrupted by gene targeting (217). Their B cells are able to produce functionally rearranged μ protein, but are unable to express the protein on their surfaces, and do not progress to the fraction D cell stage. Thus the presence of the membrane-bound μ-chain is necessary for the progression of cells to fraction D.

The presence of a membrane-bound μ-chain is necessary but not sufficient for the transition to the fraction D cell stage. Surrogate light chains (SL), λ5 and VpreB, are associated with μ HC on the cell surface of pre-B lines (218-221). The μH-SL complex expressed on in vitro pre-B cell
lines can transduce signals that induce an increase of intracellular free Ca\(^{2+}\) and tyrosine phosphorylation of intracellular proteins, indicating that the complex could function as a surface receptor (222-224). Targeted disruption of the \(\lambda 5\) gene results in depletion of cells of fraction D (B220\(^+\) CD43\(^-\) IgM\(^-\)) (24, 40, 46). Thus, the presence of the \(\mu\) chain in its membrane form together with the SL complex positively selects the cells within the CD43\(^+\) compartment to progress to fraction D (CD43\(^-\)).

Positive selection occurs upon signaling through the \(\mu\)-SL pre-BCR. Evidence for the role of signaling in this positive selection process can be demonstrated by examining mice or humans containing genetic deficiencies in signaling molecules. X-linked agammaglobulinemia (XLA) patients, that have a mutation in the protein tyrosine kinase (PTK), btk, have a severe deficit of circulating Ig due to a block at the C to D transition (225-227). However, the same mutation in the murine system reveals a milder phenotype. Neither the naturally occurring mutant, \(xid\), or the genetically targeted mutant, btk\(^{-/-}\), exhibit a severe impairment in B cell development, although the B cells that do develop are of an immature phenotype (228-231). The difference between the human and murine phenotype may be due to compensatory mechanisms present in the murine system only.

Another essential kinase in pre-BCR signaling and selection is the \(p72^{syk}\). B cells deficient in syk by gene-targeted disruption are blocked at the C to D transition (232, 233). The heterodimer Ig\(\alpha\)/Ig\(\beta\) (CD79\(\alpha\)/CD79\(\beta\)) is essential for cell surface expression of and signal transduction through, the BCR (234-237). The heterodimer is also noncovalently associated with the pre-BCR in murine and human pre-B lines (238-242). Cross-linking of \(\mu\) or Ig\(\alpha\) on pre-B cell lines induced the elevation of intracellular Ca\(^{2+}\), indicating that the pre-BCR/CD79 complex has the potential to transduce signals (222, 243, 244). Signaling through CD79 is accomplished by the tyrosine phosphorylation of a motif in the cytoplasmic tail of CD79, termed the ITAM motif (245, 246). A more detailed explanation of this motif and signal transduction through CD79 in B cells is discussed later in this chapter.
Interactions between CD79 and \( \mu \) are necessary for progression in development. B cells from mice expressing \( \mu \) chains mutated in the transmembrane domain (YS-VV) such that they could no longer associate with CD79 were unable to rescue progression to fraction D cell stage in RAG-1 deficient mice. Introduction of a fusion protein between the extracellular and transmembrane domains of \( \mu \) and the cytoplasmic tail of CD79\( \beta \) rescue the progression in RAG-1 deficient mice to the fraction D cell stage, while the same chimeric transgene with mutations in both tyrosines in the ITAM motif of CD79\( \beta \) were unable to rescue further development (247). In addition, signaling through CD79\( \beta \) by treatment of RAG-2 deficient mice with anti-CD79\( \beta \) mAb induced differentiation that phenotypically mimics the differentiation observed in RAG-2 deficient mice carrying a \( \mu \) transgene. The phenotypic differences included increased CD2, CD25, and BP-1 expression, together with decreased CD43, c-kit, and \( \lambda 5 \) expression, expansion of the B lineage compartment in the BM, and accumulation of smaller B220\(^+\) cells expressing CD25 (248). All of these are characteristic changes observed at fraction C to fraction D transition in normal B cell development (39, 43, 46). Thus positive selection at the fraction C cell stage occurs through signaling through the pre-BCR complex, which is necessary and sufficient for the progression in B cell development.

Negative selection can occur when a truncated form of \( \mu \) is expressed on the cell membrane at an earlier developmental stage, i.e. the stage of D-J\( _H \) rearrangement. B cells normally express DH elements in one particular reading frame (RF), namely RF1, although the DH elements can in principle be translated in at least two RFs. RF1 preferential usage is a result of selective processes. While RF3 translation results in stop codons, RF2 translation results in a truncated D\( \mu \) protein (249-251). The expression of a membrane-bound D\( \mu \) protein from DJ\( _H \) joints is counter-selected in development. This is suggested by the absence of RF2 counter-selection in B cell progenitors from \( \mu \)MT mice and bcl-xL transgenic mice (252, 253), and its effective counter-selection in nonproductive VDJ joints (254, 255). The negative selection by RF2-translated D\( \mu \) proteins on the B cell surface occurs through a signaling process. This is illustrated by mutations in signaling
molecules such as CD79β and the PTK, syk, which result in an increased frequency of RF2-expressing cells (233, 256).

Improper Ig rearrangements are another means by which a large proportion of B cell precursors are eliminated. Since the joining mechanism that mediates IgH and IgL chain gene recombination is imprecise and error prone, a large fraction of rearranging B cell progenitors fail to express functional IgM molecules. As a consequence, these non-functional B cell precursors die by apoptosis (41). Evidence for this comes from mice transgenic for the anti-apoptotic molecule, bcl-xL (253). These mice contain large expansions of B220+CD43+ cells (fraction C) containing mostly aberrant, non-productive Ig gene rearrangements.

2. B cell signaling

General overview

Engagement of the BCR by antigen or anti-Ig Abs induces an ordered series of biochemical signals that culminates in B cell activation (257, 258). This section describes some of the critical biochemical signals involved in this process.

The BCR mediates the internalization of antigen for its subsequent processing and presentation to T cells in conjunction with MHC encoded antigens. The BCR also initiates transmembrane signaling, which ultimately culminates in B-cell growth and differentiation.

The first detectable signaling events stimulated by BCR aggregation are the activation of PTKs, and a rapid increase in the tyrosine phosphorylation of a number of proteins (245, 259, 260). Protein tyrosine phosphorylation is strongly correlated with receptor signaling and with control of cell growth and differentiation in a number of cell types.

The BCR for antigen exists as a protein complex on the surface of the resting B cell. It is comprised of either mIgM or mIgD noncovalently associated with one or more heterodimers. Each heterodimer is comprised of one CD79α chain (IgM-α or IgD-α)(234, 261) and one CD79β chain
(Igβ or Igγ) (235). Specifically, one of the earliest signals that initiates the activation of a B cell is the immediate phosphorylation of the tyrosine residues in a 26 amino acid signaling motif termed an immunoreceptor tyrosine activation motif (ITAM) motif, in the cytoplasmic tail of CD79α and CD79β (245). This process is initiated by the activation of PTKs associated with the BCR. PTK activation is an essential component of signaling by the BCR (262). Anti-Ig-induced proliferation of mature resting B cells is blocked by PTK inhibitors (263). The BCR is associated with two classes of PTKs: the Src family PTKs, p53/56lyn, p59fyn, p56lck, p55blk, (260, 264-266) and the 72 kDa non-Src kinase, syk (267, 268). Although these PTKs are constitutively associated with the CD79 heterodimers of the BCR in resting B cells (267, 269), the activity of lyn, blk, fyn and syk is increased following BCR aggregation (260, 267, 268). A time course analysis of tyrosine phosphorylation of activated B cells suggests that syk is activated after activation of src PTKs. Another PTK that is activated following BCR aggregation which plays a major role in BCR signaling is btk, a Tec family PTK (270-272). Activation of btk occurs following the rapid activation of the src-family PTKs, blk and lyn, and prior to activation of syk (270).

The activation of PTKs results in the recruitment of the next level of second messenger-generating systems, which propagates this initial signal. One of these pathways is the phospholipase C (PLC) pathway. The tyrosine phosphorylation of PLCγ1 and PLCγ2 activates the enzymes, which results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate (IP3) and DAG (273-275). DAG has been shown to activate protein kinase C (PKC) enzymes, while IP3 mediates the release of Ca2+ from intracellular stores, which leads to entry of Ca2+ from the extracellular milieu (276). Induction of both PKC activity and Ca2+ flux are necessary for stimulating B cell entry into cell cycle (277, 278).

Another network of regulatory proteins activated by BCR aggregation are the proteins of the ras pathway (279-281). In its active state, p21ras binds GTP, and can activate raf-1, a serine/threonine kinase. Activated raf-1 is able to phosphorylate and activate MEK, which can then activate mitogen activated protein (MAPK) kinases. MAPK can in turn phosphorylate and activate transcription factors, thereby causing their localization to the nucleus, ultimately leading to the
transcription of specific genes, such as c-fos and c-jun (282-284). Activation of ras is mediated by the recruitment of SH2 and SH3-containing proteins to the phosphorylated CD79α ITAM. The activation of ras will be described further in a following section.

Another enzymatic pathway activated by BCR aggregation is the phosphatidylinositol-3-kinase (PI3-K) pathway, an enzyme that phosphorylates the D-3 position of inositol phospholipids (285, 286) (fig. 1-6). Three products can potentially result from this activity. One is phosphatidylinositol (3) phosphate, another is phosphatidylinositol (3,4) bisphosphate, and the third is phosphatidylinositol (3,4,5) trisphosphate (PIP3) (287, 288). One consequence of PI3-K activity is the ability of PIP3 product to activate a unique isoform of PKC, PKC-ζ (289). PIP3 has also been shown to be necessary for the btk-dependent activation of PLCγ2 (290). PI3-K may also be important for the activation of another kinase, S6K, which has been shown to be important for the progression of stimulated cells through the cell cycle (291, 292). PI3-K has also been shown to be required for cell survival. This is thought to occur through the PI3-K-mediated activation of the serine/threonine protein kinase, Akt or protein kinase beta (PKB), by binding of Akt via its PH domain to PIP3 (293, 294). Active Akt then functions by phosphorylating and inactivating the proapoptotic Bcl-2 family member, BAD, thereby suppressing apoptosis and promoting cell survival (295). Another substrate of Akt is glycogen synthase kinase-3 (GSK-3). The activity of GSK-3, which is necessary for apoptosis, is inhibited upon phosphorylation by Akt (296), providing another mechanism by which Akt activation by PI3-K leads to cell survival.

Other cytoplasmic proteins are also phosphorylated upon BCR aggregation by PTKs, and serve as adapter proteins linking different second messenger pathways. One such protein is the 95 kDa proto-oncogene product, vav, that possesses GDP/GTP exchange activity towards ras (297). BCR aggregation also leads to tyrosine phosphorylation of the SH2 domain-containing protein, Shc, and subsequent formation of a phospho-Shc-Grb2/SOS complex, involved in ras activation (281, 298). In addition, BCR aggregation also results in the tyrosine phosphorylation of the proline-rich cytosolic protein, cbl. Cbl was shown to associate with the p85 subunit of PI3-kinase
following BCR aggregation, and is thought to be one of the mechanisms responsible for the recruitment of PI3-K into B cell signaling pathways (299).

The ultimate consequence of signals converging from the BCR is the induction of de novo gene transcription and translation. Transcription factors activated by BCR aggregation can be divided into 2 categories: those that pre-exist in the resting B cell and are activated by BCR aggregation, and those encoded by immediate early genes whose expression is induced. An example of the former factor that becomes activated upon BCR aggregation is nuclear factor of activated T cells (NFAT) (300-302). The NFAT family consists of several members to date, including NFAT1 (NFATp, NFATc), NFAT2, NFAT3, and NFAT4. NFAT pre-exists in the resting B cell cytoplasm in an inactive form that is dephosphorylated as a consequence of BCR signaling, resulting in its translocation to the nucleus (303). Several other transcription factors are expressed in resting B cells that have their activity modified by BCR aggregation. These include, ets-l (304), NF-kB (305), and CREB (306). NFAT does not bind DNA with high affinity unless it is associated with another transcription factor, AP-1 (307). AP-1 is a protein complex composed of homo and heterodimers of protein products of the Fas and Jun gene families of transcription factors, that are activated upon BCR aggregation (308). Induction of c-fos in B cells is associated with activation of ERK, a specific member of the MAP kinase family. Phosphorylation and activation of Jun family members is mediated by specific members of the MAP kinase family (282, 284), c-Jun N-terminal kinase (JNK)(283). Besides the fos and jun family members, other immediate early transcription factors induced by BCR aggregation include egr-1 and c-myc (309-311). Induction of egr-1 is coupled to BCR-signaling via the p21ras pathway (312). Egr-1 expression has been associated with the proliferation of primary B cells (313).

a) CD79α and CD79β

CD79α (Ig-α) and CD79β (Ig-β), the gene products of mb-1 and B29, respectively, constitute the signaling components of the BCR (237). The physical association of CD79α/CD79β
heterodimers with μ HC is necessary for the efficient transport of μ to the cell surface (314). Both CD79α and CD79β are disulphide-linked heterodimers which associate non-covalently with mIg of all 5 isotypes (234, 315, 316). CD79α exists as a glycoprotein of 32-33 kDa, whereas CD79β exists as glycoproteins of 37 and 34 kDa which differ due to differences in glycosylation (315, 317, 318).

The initiation of the signal transduction cascade following aggregation of the BCR requires the phosphorylation of a pair of uniformly spaced tyrosines in the cytoplasmic domains of CD79 (319). These tyrosine residues are within an 18-20 amino acid sequence conserved among many signal-coupling proteins of different receptors. These regions are referred to as ITAMs, and have the consensus sequence (D/E)XXYXX(I/L)X6-8YXX(I/L), where D=aspartic acid, E=glutamic acid, Y=tyrosine, I=isoleucine, L=leucine, and X=any amino acid (320, 321). This motif is also found in the cytoplasmic tail of components of the T cell antigen receptor, of certain Fc receptors, and of CD22 (321-323).

In resting B cells, the ITAM of CD79 couples the BCR to transducer elements, initiating the signaling cascade from the receptor (324). After BCR aggregation and the activation of PTKs, the 2 tyrosines of the ITAMs of CD79α, and to a lesser extent, of CD79β become phosphorylated (245). Phosphorylated ITAM becomes a binding target for proteins with SH2 domains, thus initiating several signaling routes from the BCR (269, 325). The importance of CD79 in B-cell development has been illustrated by creating mice with only one CD79α gene, carrying mutations in both ITAM tyrosine residues (326). The resulting mice exhibit a severe block in B cell development in the transition from fraction C to fraction D. Decreased numbers of pre-B and immature B cells are observed in the BM, with only 1-3% of normal levels of peripheral B cells. In addition, these mice are unable to respond to T-independent antigens (326). B cells deficient in CD79β are arrested prior to V-DJ rearrangement, at fraction A-B developmental stage. This suggests that the CD79 heterodimer also has a signaling function in early B cell development, prior to the expression of μ chain (256).
b) Protein Tyrosine Kinases

B cell signaling is associated with the activation of 3 classes of PTKs, the Src family PTKs, the syk PTK, and the Tec family member, btk. The activation of Src PTKs initiates a cascade of signaling events that then amplify and propagate the signal through the cytoplasm and nucleus. Src family PTKs contain a conserved arrangement of protein/protein interaction domains, as well as a kinase domain and sites for autoregulation by phosphorylation (fig. 1-2). They contain a myristylation sequence at the N-terminus, which facilitates the association of the kinases with the plasma membrane. Adjacent to the myristylation sequence is a stretch of unique sequence, which differs for all the Src family PTKs (327). At the C-terminal of the unique sequence is a single SH3 domain, which is known to mediate protein/protein interactions by recognition of sequences rich in proline residues (328). C-terminal to the SH3 domain is the SH2 domain. SH2 domains are known to interact with phosphotyrosines containing the appropriate flanking residues (329, 330). The enzymatically active kinase domain is towards the C-terminus of the protein. Each of these family members also shares common regulatory mechanisms. One tyrosine residue, at the C-terminal of the protein, provides negative regulation. The phosphorylation of the C-terminal tyrosine by the PTK, Csk, inactivates the Src family PTK, thereby providing a mechanism to turn off the response (331-333).

Tyrosine phosphorylation of CD79 is accomplished by members of the Src family kinases (245, 260). In resting B cells, about 1-3% of total CD79 heterodimers are physically associated with Src PTKs blk, lyn, fyn and lck (265). Distinct regions in both lyn and fyn are reported to associate with non-phosphorylated and phosphorylated CD79α. When the BCR is in its basal conformation, the ITAM of CD79α is non-phosphorylated. In this conformation, both lyn and fyn bind to the ITAM through their N-terminal first 27 amino acids. Receptor activation leading to tyrosine phosphorylation of CD79α triggers a change in the orientation of the kinases such that the N-terminus of the kinase is released, and the interaction with CD79α is maintained through an SH2-mediated binding to the phosphorylated ITAM (266).
Figure 1-2. Molecular structures of proteins involved in B cell transmembrane signaling. Src homology domains (SH2, SH3), pleckstrin homology domain (PH), Tec homology domain (TH), proline-rich sequences (pro rich), guanine nucleotide exchange factor domain (GNEF), phosphotyrosine-binding domain (PTB), glycine and proline rich domain (gly:pro), phospholipase C domain (PLC), and protein tyrosine phosphatase domain (PTPase) are indicated. Dbl is a region of homology to Dbl protein, which contains guanine nucleotide exchange activity. Illustration is taken from review (334).
Another family of PTKs expressed and activated in B cells is represented by the syk PTK. Syk contains homology to the ZAP-70 PTK, which is found associated with the ζ chain in the T-cell receptor complex (335). ZAP-70 and syk contain tandem SH2 domains at the N-terminal, and contain a kinase domain in the C-terminal region (331) (fig. 1-2). Syk is also tyrosine phosphorylated and activated upon BCR aggregation, although with much slower kinetics than Src PTKs (270). Initial studies suggested that syk was associated with sIg from resting or activated B cells (267). But recent evidence suggests that syk is recruited to the BCR upon BCR aggregation (336). Syk activation is thought to occur by binding of both SH2 domains to tyrosine phosphorylated ITAMs. Syk requires phosphorylation of both ITAM tyrosine residues in order to bind. This binding elevates the rate of syk autophosphorylation resulting in an increase in syk specific activity and promotes the tyrosine phosphorylation of syk substrates (337). The BCR-associated Src-PTK, lyn, can also phosphorylate the tyrosine residue of syk upon BCR aggregation, enhancing the activity of syk (338).

The importance of syk in B cell development has been demonstrated through the creation of mice deficient in syk by gene targeting. Since such mice die shortly after birth from severe systemic haemorrhaging, radiation chimeras were generated by reconstituting irradiated RAG-2 deficient mice. Results demonstrated a severe block in B cell development at the fraction C to D transition. A small proportion of sIgM+ cells can be found in the BM, although no mature cells are found in the periphery. Thus, syk may be involved in the transition of B cells to the mature peripheral B cell pool, in addition to its role at the C-D transition (232, 233, 339).

A third PTK family that is necessary for B cell signaling is Bruton's tyrosine kinase (btk). Btk is an enzyme belonging to a subgroup within the Src family kinases that includes Tec kinases and itk as well as btk. These enzymes are similar to the rest of the Src family PTKs, in that they contain a single SH3, SH2, and SH1 (kinase) domain. They differ from other Src kinases in several ways. They contain a pleckstrin homology (PH) domain at the N terminal, and a proline-rich region in between the PH domain and the beginning of the SH3 domain (fig. 1-2) (340, 341). PH domains are found in a variety of proteins and their function is not completely defined,
although they are thought to mediate protein/protein interactions, or to mediate interactions with inositol phosphates (342-344). Unlike the Src-family kinases, Tec/btk kinases have no myristylation site, and are localized to the cytoplasm. They also lack the C-terminal negative regulatory phosphorylation site of Src PTKs. In btk, the proline-rich Tec homology domain has been shown to interact with SH3 domains of fyn, lyn and hck (345). The PH domain of btk has also been shown to interact with PKC (343). Btk has also been shown to be autophosphorylated and activated by BCR aggregation (271), although this activation is later than that of the Src PTKs (270). The role of btk in PLCγ2 activation will be addressed in a later section. The significance of btk in B cell development and activation has been demonstrated in mice with defective btk protein (xid), or in mice lacking btk generated by targeted disruption of the btk locus. In such mice, peripheral B cells are present, although somewhat reduced in number, and are skewed towards an immature phenotype. Survival of peripheral B cells is diminished. In addition, the humoral response to a subset of T cell-independent antigens is absent. B cells from such mice do not proliferate upon BCR aggregation, CD38 aggregation or CD40 aggregation (228-231, 346-348).

It is not known which PTK initially phosphorylates the CD79 ITAM motifs. However, by reducing the kinetics of the BCR signaling cascade using low temperatures, it was found that upon BCR aggregation, active lyn rapidly associates with CD79α. CD79α tyrosine phosphorylation occurs subsequent to the tyrosine phosphorylation of lyn. Syk auto/trans-phosphorylating activity also appears associated with CD79α, although with much slower kinetics, suggesting that lyn activation occurs first. CD45 has been shown to be physically associated with CD79α. It has been reported that CD45 is physically associated with lyn, but not with other Src family PTKs such as fyn or blk. (325, 349). CD45 activates lyn by dephosphorylating the C-terminal negative regulatory tyrosine of the kinase. The dephosphorylation of this tyrosine is obligatory for the participation of lyn in BCR signaling (350). Thus, these results support the following sequence of events. CD45 initially activates lyn, which subsequently phosphorylates CD79α, allowing syk to bind, leading to the propagation of downstream signaling events. However, recent studies have demonstrated that the tyrosine phosphorylation of CD79 can occur in the absence of lyn. Primary
B cells from lyn⁻/⁻ mice, expressing a transgenic BCR specific for hen egg lysozyme (HEL), underwent CD79 and syk tyrosine phosphorylation upon stimulation with HEL (351). By analysis using cell lines deficient in CD45 expression, CD79 heterodimers were inducibly phosphorylated upon BCR aggregation, and syk was recruited despite the apparent catalytic inactivity of lyn, fyn and blk. Therefore, syk may be able to phosphorylate the BCR complex upon receptor aggregation in the absence of Src family kinase activation. Thus, other PTKs may inducibly activate CD79 in the absence of CD45 expression (352, 353). In accordance with this, a Src family PTK other than lyn has been shown to phosphorylate the CD79 ITAM. Reconstitution of BCR signaling components, CD79 cytoplasmic tails together with different Src PTKs, in COS cells was performed. Upon aggregation of CD79, only the Src PTK, blk, was able to tyrosine phosphorylate and associate with CD79α/β (354). Thus, redundancy appears to exist with respect to different Src PTKs in their ability to phosphorylate CD79 ITAM residues.

The Src tyrosine kinase, lyn, was originally thought to be a positive regulator of B cell signaling. Recently, lyn has also been found to be a negative regulator of B cell signaling. Mice deficient in lyn have hyperactive B cells, and contain autoantibodies, similar to mice with disrupted CD22, and SHP-1. CD22 and SHP-1 are also negative regulators of B cell signaling (355-357). Lyn negatively regulates B cell signaling by phosphorylating the tyrosine based inhibitory motif (ITIM) of CD22, which then provides a docking site for the PTP SHP-1 (351). Binding of SHP-1 through its SH2 domain to the phosphorylated ITIM of CD22 activates the phosphatase (358). In lyn-deficient mice, the induced tyrosine phosphorylation of CD22 is extremely impaired upon BCR aggregation. Furthermore, the tyrosine phosphorylation of SHP-1 is low, and the association of CD22 with SHP-1 does not occur (359). Thus, it appears that positive signaling is less affected than negative signaling in lyn⁻/⁻ B cells. This could be due to the compensatory effects of other Src-like kinases or syk kinase on positive signaling but not negative signaling by lyn.
c) Ras Activation.

Ras is a membrane-associated guanine nucleotide-binding protein that can exist in an active GTP-bound state and an inactive GDP-bound state. The amount of active GTP-bound ras in a cell is controlled by two types of proteins. The ras GTPase-activating protein (GAP) stimulates the GTPase activity of ras, favoring the inactive GDP-bound form. The guanine nucleotide exchange factors (GNEFs) stimulate the release of GDP from ras, allowing GTP to bind (360). Some GNEFs include the homologues of the Drosophila son of sevenless protein (mSOS1 and mSOS2 in mouse) (361).

The SH2-containing adapter protein shc may link the activated BCR to the ras/raf-1/MAP-kinase pathway (362) (Fig. 1-3). In activated B cells, the 46 kDa and 52 kDa isoforms of shc are tyrosine-phosphorylated by activated lyn and/or syk (363), resulting in the SH2-mediated recruitment of the grb-2 adapter into the complex (281, 364). The 26 kDa grb-2 protein contains one SH2 domain flanked on either side by an SH3 domain. The two SH3 domains associate with the ras GDP/GTP exchange factor, SOS, which positively regulates the activity of Ras (365, 366). Tyrosine phosphorylation of shc is thought to promote the assembly of grb-2 with SOS in lymphocytes (367). The SH2 domain of shc preferentially binds to the tyrosine-phosphorylated ITAM motifs of the CD79 heterodimer in activated B cells (298). This targets the shc/grb-2/SOS complex to the membrane fraction where ras is localized (281). The lack of CD45 expression in the J558Lμm3 plasmacytoma resulted in abrogated shc tyrosine phosphorylation upon receptor ligation. As a result, grb-2 was not able to bind shc and recruit SOS to the membrane (353). The lack of p21ras activation in the CD45 deficient cell line is likely a consequence of the inability to interact with SOS (77).
Figure 1-3. Activation of p21ras upon anti-IgM stimulation. (●) represents phosphorylated tyrosine residues.
d) Mitogen activated protein kinases (MAPKs)

The MAP kinases are serine/threonine kinases that contain at least 3 subfamilies in mammalian cells. These are (i) the extracellular signal regulated kinases (ERKs) which were initially known just as MAPKs, (ii) the c-Jun N-terminal kinases (JNKs) or stress-activated protein kinases (SAPKs), and (iii) the p38 MAPKs. The ERK cascade is connected with regulation of cell growth and differentiation. In contrast, the JNKs/SAPKs and p38 MAPKs are involved in the response to environmental stresses such as UV light, hyperosmotic shock, TNFα and IL1, or LPS. Each family is activated by a cascade involving low molecular weight GTP-binding proteins (i.e., Ras, Rac-1, Cdc42) followed by a cascade of ser/thr kinases (Fig. 1-4).

ERK activation begins by Ras-mediated recruitment of Raf-1 to the plasma membrane. This results in the activation of Raf-1, a ser/thr kinase which phosphorylates and activates the dual specific kinases, MEK1 and MEK2 (MKK1/2). The latter phosphorylate and activate ERK 1/2. Although activated ras leads to the activation of all 3 MAPKs, JNK and p38 can also be activated by ras-independent signals. Activation of JNKs occurs through the activation of members of the low molecular weight GTP-binding protein family, rho, (i.e., Rac and Cdc42) (282-284). The GNEF, vav, has been shown to activate Rac in a tyrosine phosphorylation-dependent manner (368, 369). Rac activation leads to the activation of Raf isoforms, MEKK1/2, which phosphorylate M KK4, which in turn phosphorylates and activates the JNKs. p38 activation undergoes a similar cascade with TAK in place of MEKK1/2 and M KK3/6 in place of M KK4.

The activation of the MAPKs results in the phosphorylation and activation of transcription factors, upon subsequent nuclear translocation. ERKs phosphorylate ternary complex factors (TCF) on c-fos promoters, such as Elk-1, resulting in increased c-fos induction. ERK activation also leads to c-myc activation. JNK phosphorylates and activates c-jun and ATF2, which reside on the promoter of c-jun, resulting in its activation. JNK can also activate TCFs on the c-fos promoter. p38 activates ATF2 activity, promoting the transcription of c-jun. However, p38 also mediates the activation of MAPKAP kinase-2, which can phosphorylate CREB and ATF1.
Figure 1-4. The mitogen-activated protein kinase (MAPK) signaling family. From reviews (282-284, 370).
transcription factors (282-284). MAPKAP kinase-2 can also phosphorylate the low molecular weight heat shock protein, HSP27 in humans and HSP25 in mice (371). Another major substrate for MAPKAP-kinase 2 is LSP1. Both LSP1 and small heat shock proteins are F-actin binding proteins, suggesting a role for MAPKAP kinase-2 in the regulation of cytoskeletal structure or function (372).

e) PLCγ2 Activation.

Aggregation of the BCR results in the tyrosine phosphorylation and activation of the PLCγ2 isoform of PLCγ (274). PLCγ hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP2) to generate IP3 and DAG, resulting in increased Ca2+ and PKC activation, respectively (276) (Fig. 1-5). Structurally, PLCγ has a putative PH domain at its amino-terminus, followed by the two conserved parts of the catalytic domain, separated by two tandem SH2 domains, an SH3 domain, and a putative split PH domain (373) (Fig. 1-2).

The activation of PLCγ2 has been shown to occur through the actions of the syk PTK. BCR aggregation of the syk-negative DT40 cell line does not induce tyrosine phosphorylation of PLCγ2, suggesting that syk couples BCR to the PI pathway (374). In activated B cells, the C-terminal SH2 domain of PLCγ binds to a conserved phosphotyrosine residue in the linker region between the SH2 domains and the kinase domain of syk (375, 376).

PLCγ2 activation has also been shown to occur via the src PTK, btk. BCR aggregation of btk-deficient DT40 B cell lines results in significantly reduced PLCγ2 tyrosine phosphorylation, impaired IP3 production, and decreased Ca2+ mobilization. PLCγ2 activation is dependent upon the PH, SH2 and kinase domains of btk (179). A separate study found that the tyrosine phosphorylation of PLCγ2 did occur upon BCR aggregation in EBV-transformed B cell lines from XLA patients (i.e. btk-deficient). Further, IP3 production in these cells was abrogated, suggesting that PLCγ2 is not activated in the absence of btk. This suggests that the increased phosphotyrosyl content of PLCγ2 is necessary but not sufficient for its activation (379). In addition, splenic B cells
Figure 1-5. Hydrolysis of phosphatidylinositol (4,5)-bisphosphate by phospholipase C (PLCγ). Resulting products are diacylglycerol (DAG) and inositol (1,4,5)-trisphosphate (IP₃). From review (377).
Figure 1-6. Inositol lipid metabolism. The substrates and products of phosphatidylinositol 3-kinase (PI3K). From review (378).
from *xid* mice exhibit reduced PI hydrolysis compared with normal B cells upon BCR aggregation (380). One potential mechanism that explains how btk activates PLCγ2 suggests that PI-3,4,5-P₃ produced by active PI3K (fig. 1-6) interacts with the PH domain of btk. This results in the activation and auto/transphosphorylation of btk. This activation is further enhanced by Src family kinases, at least in part by their ability to phosphorylate btk within its activation loop (290, 381). Phosphorylation of btk has been shown to be mediated by lyn and syk, and this phosphorylation is necessary for BCR-induced Ca²⁺ mobilization (382). Another study found that lyn or fyn phosphorylate btk on Y551 in the kinase domain, leading to the activation of btk and ensuing phosphorylation at Y223, within the SH3 domain (383). Once btk is activated, its SH2 interacts with a tyrosine-phosphorylated ligand induced by BCR aggregation, which is necessary for co-localization of btk and PLCγ2, in order to phosphorylate the latter. A candidate tyrosine-phosphorylated protein could be syk, which is required for BCR-mediated Ca²⁺ mobilization, and is capable of binding both PLCγ2 and btk *in vitro* (290). The PH domain of PLCγ can also bind PtdIns-3,4,5-P₃, suggesting a further means of localizing PLCγ2 to membrane phospholipids (373).

f) Calcium Mobilization

Another signaling event that occurs upon BCR aggregation is the increase of intracellular Ca²⁺ levels. This is thought to occur by the PLCγ2-mediated hydrolysis of phosphatidylinositol (PI), resulting in IP₃ production (384-386). Once IP₃ has been released from the membrane, it diffuses into the cytosol where it binds to specific IP₃ receptors in the membrane of the endoplasmic reticulum (ER). The IP₃ receptor is a very large protein embedded in the membrane of the ER through distinctive membrane spanning regions located in the C-terminal domain. The IP₃-binding site is located at the N-terminal end that projects out into the cytoplasm in the form of a large bulbous head. Four of these subunits come together to form a functional IP₃-regulated Ca²⁺ channel (387). Upon IP₃ binding, the release of intracellular Ca²⁺ stores ensues. This Ca²⁺
release is thought to act as a trigger to cause the influx of extracellular Ca\(^{2+}\) (257). The Ca\(^{2+}\) response in anti-Ig stimulated B cells occurs in a 2 step process: a rapid transient rise due to release of Ca\(^{2+}\) from intracellular stores, followed by a sustained increase due to influx of extracellular Ca\(^{2+}\) (388). Sustained increases in [Ca\(^{2+}\)]\(_{i}\) are required for the proliferation and differentiation of resting B cells. Failure to reach a threshold intracellular Ca\(^{2+}\) level, as seen in anergic B cells, leads to cell death (389-391).

The mechanism and regulation of Ca\(^{2+}\) influx have not been fully defined. One mechanism by which Ca\(^{2+}\) is thought to enter through the plasma membrane is through the I\(_{CRAC/DAC}\) (Ca\(^{2+}\) release-activated or depletion-activated current). I\(_{CRAC}\) is highly Ca\(^{2+}\) selective and is inactivated by high intracellular Ca\(^{2+}\) levels. Depletion of intracellular stores will activate I\(_{CRAC}\) (392). Opening of the I\(_{CRAC}\) occurs only upon generation of a high amount of IP\(_{3}\) (393). The mechanism by which this is thought to occur is through the IP\(_{3}\) receptor in the ER. The receptor would 'sense' the intracellular Ca\(^{2+}\) levels and transmit the information to the I\(_{CRAC}\) channel by means of its large bulbous head structure (394, 395).

The generation of high levels of IP\(_{3}\), considered necessary for sustained Ca\(^{2+}\) influx, may be regulated by btk. Btk activation results in enhanced IP\(_{3}\) levels. This leads to the release of Ca\(^{2+}\) from a thapsigargin-sensitive Ca\(^{2+}\) store, which in turn controls Ca\(^{2+}\) influx in B cells. Thapsigargin is a drug that selectively inhibits Ca\(^{2+}\) re-uptake via sarco/endoplasmic reticulum Ca\(^{2+}\) ATPases (SERCAs) (379, 396). B cells from X-linked immunodeficient (xid) mice (containing mutated btk) exhibit a blunted increase in [Ca\(^{2+}\)]\(_{i}\), and fail to proliferate upon BCR aggregation (380). Btk-deficient DT40 B lymphoma cells also exhibit reduced PLC\(\gamma\) activation, and abrogated Ca\(^{2+}\) mobilization and IP\(_{3}\) production in response to BCR aggregation. (397).

There are some instances whereby Ca\(^{2+}\) influx can be dissociated from IP\(_{3}\) production and mobilization of intracellular Ca\(^{2+}\). Ca\(^{2+}\) influx can occur in the absence of IP\(_{3}\) generation and mobilization of intracellular Ca\(^{2+}\), such as upon mAb-mediated aggregation of CD38. CD38 is a 42 kDa transmembrane glycoprotein. It is an ectoenzyme with both ADP-ribosyl cyclase and cyclic ADP-ribosyl hydrolase activities. Tyrosine kinase activity is associated with CD38 aggregation.
B cells from xid mice do not proliferate in response to mitogenic anti-CD3 (347). This suggest that btk may be necessary for the Ca²⁺ influx response induced upon CD38 aggregation. B cells from xid mice are able to undergo extracellular Ca²⁺ influx upon BCR aggregation, but undergo decreased intracellular Ca²⁺ release and virtually no IP₃ production (380). Another example of the dissociation between BCR-mediated IP₃ production and Ca²⁺ influx is observed in immature B cells. Immature B cells from neonatal spleen undergo BCR-induced release of intracellular Ca²⁺ but no IP₃ production. They also do not proliferate in response to BCR aggregation (81, 399). The dissociation of BCR-mediated PI hydrolysis and Ca²⁺ mobilization is often associated with the lack of proliferation to BCR aggregation. Thus, BCR-mediated proliferation of B cells may require PI hydrolysis-mediated Ca²⁺ mobilization, including both Ca²⁺ influx and intracellular release.

Kinases are not the only proteins involved in Ca²⁺ influx. Phosphatases have been implicated in Ca²⁺ influx as well. Phosphatase inhibitors suppress Ca²⁺ influx induced by receptor-mediated intracellular Ca²⁺ store depletion in platelets (400). Since CD45 is also a phosphatase, these results may implicate CD45 in BCR-mediated Ca²⁺ influx.

More than one type of Ca²⁺ entry channel probably exists in B cells. One Ca²⁺ entry pathway is modulated by Ins (1,3,4,5) P₄ (401). Ins (1,3,4,5) P₄ was shown to induce Ca²⁺ influx by acinar cells, and enhanced the activity of the Ca²⁺ permeable channel (401). In lymphocytes, IP₃ receptors may be responsible for Ca²⁺ entry. In the T cell tumor line, Jurkat, IP₃ receptors were found in the plasma membrane (402). The IP₃ receptors in the plasma membrane may differ structurally from IP₃ receptors on the ER, as suggested by their different inositol phosphate specificities and sugar content (403). Capping of the T cell receptor-CD3 complex, which is associated with signal transduction, was accompanied by capping of IP₃ receptors in the plasma membrane, suggesting that the IP₃ receptor on T cells is responsible for the entry of Ca²⁺ that initiates proliferative responses (402). Thus IP₃ receptors may function in a similar manner in B cell proliferative responses.
Another type of Ca\(^{2+}\) entry pathway in B cells is through the protein, CD20. CD20 is a membrane-embedded protein with hydrophobic regions of sufficient length to pass through the membrane 4 times. Three different forms of CD20 (33, 35, and 37 kDa) result from differential phosphorylation of a single protein. The structure of CD20 suggests a function as a membrane transporter or ion channel. Thus, CD20 itself may be the ion channel for Ca\(^{2+}\) entry (404). Antibodies to CD20 increase transmembrane Ca\(^{2+}\) current in B cells (405). In combination with anti-CD40 or suboptimal amounts of anti-\(\mu\), CD20 antibodies also result in proliferation of resting B cells (406-408).

CD20 is linked to signal transduction molecules. Upon antibody-mediated CD20 aggregation, increased tyrosine phosphorylation of cellular substrates occurs, which is likely due to the association of CD20 with serine and tyrosine kinases (409). Specifically, lyn, fyn, and lck were found associated with CD20 (410). The expression of c-myc occurs upon mAb-mediated aggregation of CD20. CD20 aggregation also induces homotypic B cell adhesion by activation of PTKs through CD11a/CD18 independent adhesion pathways, which can be inhibited by CD45 ligation (404). CD20 can be phosphorylated by PKC, casein kinase II, and Ca\(^{2+}\)/calmodulin-dependent protein kinase II. PKC-mediated phosphorylation of CD20 inactivates CD20-associated Ca\(^{2+}\) flux, which may be one mechanism by which transmembrane Ca\(^{2+}\) conductance is regulated in B cells (404). Both PLC\(\gamma\)\(_1\) and PLC\(\gamma\)\(_2\) are tyrosine phosphorylated upon CD20 aggregation, and this correlates with increased intracellular Ca\(^{2+}\) concentrations that is due partially to Ca\(^{2+}\) influx (409).

g) Downstream effects of Ca\(^{2+}\) release

NFAT (nuclear factor of activated T cells) are a family of transcription factors of 120-140 kDa that play a central role in inducible gene transcription during the immune response. They are activated by stimulation of receptors coupled to Ca\(^{2+}\) mobilization. The isoform of NFAT expressed in B cells is NFAT1 (NFAT\(\text{p}\), NFAT\(\text{c}\)). In resting cells, NFAT is retained in the
cytoplasm in its phosphorylated state by a complex of proteins including the phosphatase, calcineurin. Upon receptor-induced activation, the binding of calmodulin triggered by increases in \([\text{Ca}^{2+}]_i\) leads to a conformational change in calcineurin that results in its activation. Activated calcineurin dephosphorylates NFAT, which causes a conformational change in NFAT that exposes its nuclear translocation signal. Prolonged elevation of \([\text{Ca}^{2+}]_i\) levels achieved by \text{Ca}^{2+} \text{ influx is required to maintain calcineurin and NFAT proteins in an activated state. Once in the nucleus, NFAT proteins interact together with AP-1 transcription factors in DNA binding and transactivation. NFAT function can also be modulated independently of \text{Ca}^{2+} \text{ through lck, ZAP 70, and vav in T cells (303).}

In addition to activating calcineurin, increased \([\text{Ca}^{2+}]_i\) leads to the activation of other calmodulin-dependent enzymes such as \text{Ca}^{2+}/calmodulin-dependent protein kinases (CaMK). CaMK have been implicated in c-fos induction. The mechanism of c-fos induction by \text{Ca}^{2+} \text{ occurs through phosphorylation and activation of the transcription factor CREB (cAMP response element binding protein) (Fig. 1-4). Some other genes that are also induced by \text{Ca}^{2+} \text{ include c-jun, zif 268, nur 77, junB and c-myc (411).}

3. B Cell Co-receptors

BCR stimulation is modified by co-receptor molecules. These co-receptors may both enhance and inhibit BCR signals. In B cells, the co-receptors CD5, CD19-CD21, CD22 and FcγRIIB (CD32) modulate the response to antigen. Two co-receptors, CD19 and CD22, will be described briefly in the next sections.

a) CD19

CD19 is a B cell restricted, single transmembrane, 95 kDa glycoprotein expressed throughout the B cell lineage with the exception of plasma cells. Co-aggregation of CD19 with
BCR decreases the threshold for BCR stimulation by at least two orders of magnitude (412). In addition, synergy of Ca²⁺ responses and PTK phosphorylation also occurs (412). CD19 has a large intracellular domain, which contains 9 tyrosine residues. It is rapidly tyrosine phosphorylated following BCR aggregation (413). Upon BCR aggregation, CD19 can associate with the signaling proteins PI3-K, lyn, fyn and vav (414-417). The PI3-K p85 regulatory subunit and the Src PTK, fyn, can bind directly to the CD19 tail. Lyn has also been shown to bind the CD19 cytoplasmic tail (416). The activation of PI3-K upon binding to CD19 results in PI hydrolysis, since a variant of the J558Lμm3 plasmacytoma expressing CD45 but lacking CD19 contains reduced IP₃ production and abrogated Ca²⁺ influx upon BCR aggregation (418).

CD19 is part of a membrane complex containing CD21 (complement receptor 2 (CR2)), CD81 (target of anti-proliferative antibody (TAPA-1)), and in some cells, the membrane protein Leu-13 (419-421). CD21 can link CD19 to the BCR by virtue of its ability to bind complement-opsonized antigen (421, 422). This association allows important signaling molecules such as PI3-K to be relocated to the BCR complex, allowing further amplification of signal transduction pathways.

Mice deficient for CD19 have significantly reduced numbers of peripheral B cells and exhibit severe defects in their responses to thymus dependent (TD) antigens and B cell mitogens (423, 424). In contrast, mice that overexpress CD19 have augmented mitogenic responses and higher level of spontaneous proliferation (423, 425). Together, these results demonstrate the positive regulatory role of CD19 in BCR signaling.

b) CD22

CD22, a 135 kDa B cell restricted glycoprotein, is a member of the sialo-adhesion family of adhesion molecules, and a co-receptor for mIg (426, 427). The CD22 extracellular domain is able to bind oligosaccharides with α-2,6-linked sialic acid residues on a variety of proteins (428, 429). The cytoplasmic domain of CD22 contains 6 tyrosine residues. Two regions of the CD22
cytoplasmic domain are homologous to ITAM residues. In addition, there are 3 regions homologous to the tyrosine-based inhibition motif (ITIM), which provides docking sites for SH2 domains of the SHP-1 PTP (320, 430).

CD22 associates with mIg in the plasma membrane and is rapidly tyrosine phosphorylated after mIg or CD22 aggregation (431-433). Tyrosine-phosphorylated CD22 recruits a number of intracellular signaling molecules, including lyn, syk, PI3-K, PLCγ1 (434-436), and a tyrosine-specific phosphatase, SHP-1 (PTP-1C) (358, 437). The phosphorylated ITIM reside of CD22 can also associate with the SH2-containing inositol polyphosphate 5-phosphatase, SHIP (438). In murine B cells lacking SHP-1, the threshold for mIg signaling is lowered, demonstrating that SHP-1 negatively regulates the mIg signaling complex (357, 439). The interaction of CD22 together with lyn and SHP-1, mentioned in the previous section on PTKs, demonstrates the negative regulatory role of CD22 in BCR signaling (355, 356).

CD22-deficient mice have normal numbers of B cell progenitors in the BM, indicating that early B cell development is normal (356, 440, 441). Their mature peripheral B cells have reduced levels of sIgM. They also have reduced numbers of recirculating B cells in the BM. The phenotype of CD22 deficient B cells is similar to anergic B cells that have been chronically exposed to soluble autoantigens in vivo (357). Despite this anergized surface phenotype, stimulation through the BCR in CD22−/− mice results in markedly enhanced Ca2+ signaling (356, 440, 441). This characteristic is also found in SHP-1−/− B cells transgenic for anti-HEL mIg (357), suggesting that the CD22−/− B cell phenotype is largely due to the lack of recruitment of SHP-1 to the BCR. The phenotypes of the CD22−/− mice differed with respect to the mIg induced in vitro proliferative and in vivo immune responses between the various studies (356, 440, 441). In one study, the phenotype of CD22−/− mice was consistent with a loss of a negative regulatory effect, resulting in enhanced proliferative responses to BCR aggregation, and the presence of raised levels of serum IgM and autoantibodies (440). In contrast, results from two other studies of CD22−/− B cells showed impaired in vitro proliferative responses to BCR aggregation, which correlated with decreased cell cycle entry and increased apoptosis (356, 441). The reasons for these discrepancies is not clear. The impaired
proliferative responses observed in two of the three CD22−/− mice studies may have been caused by a downmodulation of the signaling pathways distal to the BCR as part of the anergic phenotype (441).

4. CD45

a) Structure

CD45 is a transmembrane PTP consisting of a family of heavily glycosylated high molecular weight cell surface proteins expressed on all nucleated cells of hematopoietic origin. CD45 is an abundant protein, comprising 5-10% of surface protein (67, 442, 443). CD45 is comprised of a large extracellular domain approximately 400-500 amino acids, a single transmembrane domain and a cytoplasmic domain of 700 amino acids (Fig. 1-7). At least 9 different isoforms can arise from alternative mRNA splicing of 4 exons (exons 4-7) located near the amino terminal. Different isoforms result in structurally related proteins between ~180-220 kDa in size (26, 66, 68). All isoforms of CD45 share a large, highly conserved cytoplasmic domain containing tyrosine phosphatase activity (444). The cytoplasmic region of CD45 consists of 2 tandem PTP domains of ~240 residues separated by a 56 residue spacer region (444, 445). Mutational analysis has revealed that the catalytic activity of the more N-terminal domain I is both necessary and sufficient to restore T cell receptor-mediated signaling in a CD45-deficient cell line (446). Domain II of CD45 is not catalytically active, nor is it inducibly activated in response to ligand binding. However, domain II plays an important role in regulating the catalytic activity of domain I and potentially its substrate specificity as well (447, 448).

Different cell lineages express different isoforms of CD45, and isoform usage often varies as cells differentiate. For example, the majority of developing thymocytes and activated T cells express low molecular weight isoforms, while B cells express the high molecular weight isoform (220 kDa, B220) (66). Mature CD4+ and CD8+ T cells normally express multiple CD45 isoforms.
Figure 1-7. Schematic representation of the domain structure of murine CD45. The high-molecular weight isoform of CD45 containing all three alternatively spliced exons is shown.
on the cell surface. In T cells, CD45 isoform expression changes upon activation. Naive peripheral T cells switch from high molecular weight CD45 isoforms to low molecular weight isoform expression after stimulation (449, 450). In contrast, late stages of thymocyte development correlate with the expression of high molecular weight CD45 isoforms on mature thymocytes (451-453). B cells can also switch isoforms upon activation with specific stimuli. Stimulation with IL5 results in a CD44<sup>hi</sup> hyaluronate-adherent subpopulation of activated B cells that express predominantly lower molecular weight isoforms of CD45 (454, 455). Another report found that stimulation with anti-IgM antibody or phorbol myristate acetate (PMA) in combination with ionomycin resulted in a switch in isoform usage from B220 to an isoform lacking all variable exons (CD45RO) (456).

b) Analysis of CD45 function in B cells

i) Monoclonal Antibodies

CD45 specific mAbs have been shown to alter both B cell activation and differentiation, indicating that CD45 is involved in regulation of signal transduction through the BCR. These studies varied depending on the mAb used and the specific B cell population examined. Simultaneous addition of anti-CD45 mAbs abrogates BCR-mediated signal transduction and proliferation (457). Several events associated with B cell activation, including Ca<sup>2+</sup> mobilization, PI hydrolysis and increased gene transcription were inhibited by simultaneous ligation of CD45 and the BCR (78, 79, 458-460). Small resting B cells were found to be most susceptible to the inhibitory effects of anti-CD45. Isolation of B cells based on size or density revealed that as cells transit from a resting Go population, to one that contains a higher proportion of cells that have entered G1 and exhibit increased RNA synthesis, they become more resistant to the inhibitory effects elicited by aggregation of CD45 (78, 79). Although proliferation induced by the addition of IgM specific mAbs is suppressed in all B cell populations by simultaneous addition of CD45
specific mAb, the degree of inhibition was much less in cells that appear to be partially activated than that observed for resting B cells. This suggests that the functional role of CD45 in regulating signal transduction via the BCR changes with progressive activation such that the involvement of CD45 in BCR signaling is not as crucial in cells that have received initial activating signals.

Certain studies have shown that the addition of anti-CD45 can enhance B cell proliferation. Thus, in vitro culture of B cells with mAbs that recognize the high molecular weight isoform of CD45 has been shown to increase responses induced by anti-Ig (459, 460). Additionally, mAbs that are specific for carbohydrate determinants on CD45 appear to increase proliferative responses upon BCR aggregation. These results demonstrate that binding of mAbs to discrete epitopes in the extracellular domain of CD45 may elicit qualitatively different results in terms of their effects on CD45 function. This suggests that structural heterogeneity in the extracellular domain of CD45 is functionally relevant (461).

The addition of mAbs to CD45 can affect other proteins, specifically, the Ig-associated signaling complex, CD79α and CD79β. Treatment of B cells with anti-CD45 mAb together with a secondary aggregating antibody resulted in increased tyrosine phosphorylation of Ig-α/Ig-β. This effect was not observed in the absence of CD45 aggregation. The hypothesis is that aggregation of CD45 results in sequestration of CD45 from the BCR complex, and is consistent with the role of CD45 in the maintenance of the observed basal level of tyrosine phosphorylation (75).

Studies utilizing an isoform specific anti-CD45 mAb have demonstrated the role of CD45 in regulating B cell activation in vivo during T-dependent B cell activation. Injection of mice with a CD45 specific mAb, RA3-6B2, which recognizes a B-cell-restricted epitope of CD45 (B220), abrogated the T-cell dependent antibody response against the hapten fluorescein isothiocyanate (FITC) (462). Both plaque-forming cells and reduction of anti-FITC serum antibodies are inhibited by a single dose of CD45 mAb. Therefore, CD45 was exerting its effect at the level of B cell activation, blocking early events prerequisite for B cell differentiation into plasma cells. Injection of anti-CD45 did not affect the secondary response following rechallenge with the antigen, which may be a result of the loss of the specific epitope recognized by the anti-CD45 on memory B cells.
Alternatively, memory B cell stimulation may utilize alternative signaling pathways that do not require the action of CD45(462).

CD45 specific mAbs have also been shown to inhibit postactivation events associated with B cell differentiation such as Ig class switching (463). Thus addition of anti-CD45 to LPS-stimulated cultures abrogates class switching but does not affect IgM production (464, 465), whereas both IgM and IgG production in response to stimulation of B cells with *Staphylococcus aureus* Cowan (SAC) were inhibited with anti-CD45 (466). In contrast, aggregation of CD45 was observed to enhance T cell-dependent isotype switching (467). The reason for this discrepancy is unclear. However, the physical localization of CD45 within a discrete cap in the plasma membrane (75), may prevent it or associated proteins from interacting with one or more substrates in the cell, thereby altering their function. Alternatively, since CD45 extends from the plasma membrane as a rigid extended rod-like structure, it is one of the largest molecules on the lymphocyte surface. Another consequence of CD45 aggregation and capping could be enhanced interaction between B and T cells via surface molecules involved in antigen recognition due to the reorganization of CD45 within the membrane.

ii) CD45-Deficient B cell lines.

CD45-deficient cell lines have been extremely valuable in elucidating the role of CD45 in lymphocyte activation, however, there is still heterogeneity among different CD45-deficient B cell lines in terms of the signals transduced upon BCR aggregation.

The most extensively studied CD45-deficient B cell line is the B cell plasmacytoma, J558Lum3, which expresses surface IgM specific for the hapten nitrophenyl (NP). Signal transduction in response to BCR aggregation, mediated by either antigen or anti-Ig, was abnormal, as indicated by the absence of a detectable Ca$^{2+}$ mobilization response. Upon transfection of a cDNA encoding the high-molecular-weight isoform of CD45, the Ca$^{2+}$ response was restored (74). This suggested that CD45 expression is required for B cell activation and exerts a positive
effect on signaling. However, not all B cell activation events seem to be governed by CD45, since aggregation of BCR on J558Lμm3 cells leads to the inducible tyrosine phosphorylation of numerous substrates (76, 352, 353). This suggests that there must be additional PTKs that do not require CD45 for activation.

Several downstream proteins are affected by the lack of CD45. Recruitment to the BCR complex and activation of the src family kinases p53/56 lyn, p59 fyn and p55blk upon BCR aggregation is attenuated in the absence of CD45, although the PTK syk is inducibly activated and recruited to the BCR upon BCR aggregation (350, 352, 353, 468). The activation of btk is observed regardless of CD45 expression, although it is greatly reduced in CD45 deficient cells upon BCR aggregation. This is consistent with the findings that btk phosphorylation and activation are dependent in part on Src PTKs (381, 469). While the tyrosine phosphorylation of PLCγ is normal (presumably due to syk activation), IP3 production is significantly decreased in the absence of CD45 (353), suggesting that the lack of IP3 production is a result of the lack of PLCγ activation, which ultimately is responsible for the lack of Ca2+ mobilization observed in the J558Lμm3 cell line upon BCR aggregation (74). Tyrosine phosphorylation of shc was abrogated, as was the association between shc and grb-2 (353). The inducible association of shc with grb-2 is necessary for p21ras activation (281), as described in a previous section on Ras activation. Ras activation does not occur in the absence of CD45 in J558Lμm3 cells upon BCR aggregation (77). A downstream effect of ras activation is the activation of the MAP kinase, ERK. ERK activation also does not occur upon BCR aggregation in the J558Lμm3 cell line (76). Thus, activation of the ras pathway appears to be dependent on CD45.

Another study involved a variant of the B lymphoma cell line, K46. K46 cells express the IgG2a-BCR on the surface, representing a mature B cell stage (470). K46-17μmλ variants were obtained by transfection of membrane μ HC and λ LC, encoding specificity for the hapten NP (471). Studies with the CD45-deficient mutant of the K46-17μmλ B lymphoma cell line support some of the observations observed in the J558Lμm3 cell line. BCR aggregation in K46-17μmλ results in the induction of tyrosine phosphorylation of several substrates, although the pattern of
phosphorylation is qualitatively and quantitatively different from that seen in cells expressing CD45. The tyrosine phosphorylation of p72^syk is again unaffected by the lack of CD45. However, the activation of p53/56^lyn is inhibited in these cells. Tyrosine phosphorylation of PLC\gamma is observed, although no information has been obtained regarding IP\_3 production. BCR aggregation results in mobilization of Ca^{2+} from intracellular stores, whereas extracellular Ca^{2+} influx does not occur (461).

A further study involved the avian leukosis virus-induced bursal lymphoma B cell line, DT40 (472). Studies with a variant of DT40, in which CD45 has been "knocked out" by homologous recombination, demonstrate that BCR aggregation induces diminished but detectable tyrosine phosphorylation. As was observed in other CD45-deficient B cell lines, the activation of p72^syk is unaffected by the absence of CD45, however, the phosphorylation and activation of p53/56^lyn is disregulated. Lyn tyrosine phosphorylation and activation does not occur upon BCR aggregation. Lyn is constitutively phosphorylated at both the autophosphorylation and negative regulatory C-terminal tyrosine site, providing evidence that these sites are targets of CD45 in B cells (350). The tyrosine phosphorylation of cbl upon BCR aggregation is thought to be dependent on lyn kinase activity (299, 473). In this study, BCR aggregation of CD45-deficient DT40 cells resulted in a significantly reduced induction of tyrosine phosphorylation of cbl, consistent with the involvement of lyn in this process (350). One function of the adaptor protein, cbl, is the recruitment of PI3-K into B cell signaling pathways (299). In addition to the lyn activation deficiency, the Ca^{2+} mobilization response is also abnormal in CD45-deficient DT40 cells, consisting of a delayed and gradual increase in intracellular Ca^{2+} levels (350). Although this suggests the presence of only Ca^{2+} influx and absence of Ca^{2+} release from intracellular stores, both responses were not formally tested separately.

Another study involved CD45-deficient clones which were generated from the sIgM and sIgD-positive mature B cell line, BAL-17. CD45-deficient clones underwent normal tyrosine phosphorylation and Ca^{2+} mobilization upon BCR aggregation, however, BCR-mediated growth inhibition was abrogated (474).
The WEHI-231 cell line is phenotypically similar to immature B cells in that it expresses sIgM and low levels of sIgD and MHC class II antigen, and is susceptible to growth arrest in G1 phase of cell cycle and cell death by apoptosis upon stimulation with anti-IgM (475-478). The CD45-deficient variant of WEHI-231 cell line contained many substrates that were constitutively hyperphosphorylated on tyrosine, and aggregation of the BCR did not induce any additional phosphorylation. Specifically, the PTK p53/56lyn was hyperphosphorylated and activated in the absence of CD45 (468). There was also a delayed and prolonged Ca2+ mobilization response to BCR aggregation, similar to CD45 deficient DT40 cells (350, 461). In addition, there was an increase in growth inhibition and induction of apoptosis upon BCR aggregation relative to the parental lines expressing CD45 (479). This suggests that the presence of CD45 on immature B cells may provide some protection from BCR-induced apoptosis.

In conclusion, studies with CD45-deficient B cell lines demonstrate an important role of CD45 in signal transduction via the BCR. The variation among cell lines may be accounted for by the differentiation state of the cell line, and/or the method of transformation unique to each cell line.

iii) CD45 Knockout Mice

The function of CD45 in early lymphocyte development cannot be tested very effectively in cell lines. Knockout mice have been extremely useful in elucidating the function of many proteins in lymphocyte development as well as in lymphocyte activation.

CD45 knockout mice were created by disrupting exon 6 of the CD45 gene with a neomycin gene (73). As a result, CD45 was absent on B cells. A limited number of peripheral T cells expressed an isoform of CD45, generated by alternative splicing of the mutated exon. However, there was a substantial reduction in peripheral T cells and mature CD4+ and CD8+ single positive thymocytes, whereas the number of immature CD4+CD8+ double positive thymocytes was comparable to that observed in normal mice. Thus, there was a block in T cell development between the transition of double positive to single positive thymocytes. Clonal deletion of T cells
expressing superantigen (Mls\(^a\)) reactive T cell receptors proceeded unimpaired in the CD45\(^{-/-}\) animals. Cytotoxic T cell responses against LCMV infection were absent (73). CD45-deficient mice were able to mount type 1 and type 2 T-cell-independent B cell responses to vesicular stomatitis virus (VSV) (480). Further, the lack of CD45 affected T cell activation as the proliferation of T cells to Con-A or mAb-mediated TCR-CD3 aggregation was significantly reduced.

In terms of B cell development, precursor frequencies of pre-B cells and B cells from BM, spleen or FL were normal in the absence of CD45. In contrast, B cell activation was affected by the lack of CD45. While CD45\(^{-/-}\) B cells were able to proliferate in response to LPS stimulation, aggregation of sIgM did not result in proliferation (73). These results demonstrate the important role of CD45 in B cell activation as well as T cell development and activation. The question that remains is how does the lack of CD45 result in defective B cell proliferation in response to anti-IgM stimulation. This question is examined more thoroughly in Chapter 2 of this thesis.

Subsequent to our study, a second CD45 knockout was generated by gene targeting of an exon common to all isoforms (exon 9) (481). This resulted in a complete CD45 knockout, with no CD45 expression on any cells. T cell development was blocked at 2 points: the double negative stage to double positive stage, and the double positive to single positive stage. In contrast to CD45 exon 6\(^{-/-}\) mice, CD45 exon 9\(^{-/-}\) mice exhibited a block in both positive and negative thymocyte selection, probably due to the complete loss of CD45 expression. Peripheral T cells were reduced dramatically as seen in CD45 exon 6\(^{-/-}\) mice. Twice the number of B cells were found in the spleens of CD45 exon 9\(^{-/-}\) mice compared to controls. Upon stimulation of such B cells with either polyclonal F(ab')2 goat anti-mouse IgM or anti-IgD, no proliferation was observed. Proliferation to anti-CD40 was normal. However, proliferation to anti-CD38 stimulation was significantly reduced (481).

Thus CD45 knockout mice demonstrate an important function of CD45 in T cell development and activation, as well as B cell activation. The role of CD45 in B cell maturation will be examined and discussed further in Chapter 2.
c) Regulation of CD45 function.

The importance of CD45 in lymphocyte biology, and its high level of expression in hematopoietic cells suggests that its function must be tightly regulated. The activity of CD45 could be regulated through mechanisms that affect its inherent catalytic activity and/or its ability to access relevant substrates in the cell. It has been demonstrated that ligand binding to CD45 may regulate its function through an allosteric mechanism involving dimerization and functional inactivation. Specifically, transfection of chimeric CD45 molecules composed of the extracellular/transmembrane region of epidermal growth factor receptor (EGF) fused to the cytoplasmic domain of CD45 into CD45-negative cells restores TCR-mediated signal transduction (446, 482). However, addition of EGF to cells expressing the EGF-CD45 chimera inhibits signal transduction in response to TCR aggregation. The inhibitory effect of EGF could be reversed by cotransfecting cells with either a truncated EGF receptor lacking a cytoplasmic domain or wild-type CD45 (482). These results demonstrate that dimerization of the cytoplasmic portion of CD45 could regulate its function, possibly through regulating its access to substrates. While it remains unclear whether natural ligands for CD45 function in an analogous fashion, support for this mechanism has been provided by analyzing the crystal structure of the membrane-proximal catalytic domain of murine RPTPα (483). Examination of a dimeric form of this domain indicates that the aminoterminal segment of each monomer forms a helix-turn-helix structure wedge that fits into the active site of the opposing monomer. Therefore, based on the conservation of primary sequence of the dimer interface between CD45 and RPTPα (483), the results with the EGF-CD45 chimera study (482), and the fact that CD45 can be recovered from cells as a homodimer (484), it is possible that ligand-mediated dimerization of CD45 restricts its interaction with substrate, thereby regulating its function.
5. a) Immature B cells

Immature B cells can be derived from a number of sources: neonatal spleen (≤ 4 days), where they constitute 95% of IgM+ B cells (399), and adult BM or adult spleen (82, 83). In adult BM, immature-stage cells represent less than 10% of mononuclear cells (485). Immature B cells can also be derived from the spleens of sublethally irradiated recipients reconstituted with autologous BM (82, 83).

Immature B cells differ phenotypically from their mature counterparts in that they contain high expression of sIgM and low or no expression of sIgD (399). In addition, they contain high levels of HSA expression (82, 83), a molecule discussed previously (Section 1d(ii)). Immature B cells comprise 10-15% of adult spleen, and are B220lo sIgMhi HSAhi, and 50% sIgD+ (82).

b) Transitional B Cells.

In order that mature (HSAloB220hi) B cells develop from immature (HSAhiB220lo) B cells, they must pass through an intermediate developmental stage in which they are sensitive to tolerance induction. They are considered "transitional" B cells at this stage of development (81). Transitional B cells are characterized by changes in cell surface markers that distinguish them from immature and mature B cells (Table 1-2). Early markers of differentiation reach the highest level of expression on transitional B cells, such as Pgp1 (CD44) and ThB. Late markers associated with progressive maturation increase, such as MHC class II, CD23 and sIgD. IgM is expressed at modest levels on immature B cells, reaches the highest level of expression on transitional B cells, and is downregulated on mature resting B cells, where it is co-expressed with high levels of mIgD. Transitional B cells are targets of negative selection. Injection of anti-IgM Abs results in deletion by apoptosis of transitional B cells, while most of the more immature HSAhi B220lo IgMlo precursors survive (81).
Table 1-2. Late stages of B cell maturation from immature to mature B cell.

<table>
<thead>
<tr>
<th>pro-B/pre-B</th>
<th>immature</th>
<th>transitional</th>
<th>mature</th>
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<td>fas-</td>
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Transitional B cells comprise 10-15% of adult splenic B cells and ~12-20% of B220+ cells in adult BM. They are unable to proliferate in response to anti-IgM (82). Transitional B cells are rare in the BM of newborn mice before 6 days of age. However, they are already present in the spleen 2 days after birth (81). The neonatal spleen is a transient site for lymphopoiesis from day 18 of gestation until 4 weeks after birth (10), while active B lymphopoiesis begins at day 17 of gestation in embryonic BM, and it remains the primary site of hematopoiesis throughout adult life (7, 17). The spleen may have more ongoing hematopoiesis right after birth, while hematopoiesis in the BM may predominate later. This may account for the higher proportions of transitional B cells found in neonatal spleen compared to neonatal BM. Transitional cells can develop from IgM-depleted BM precursors upon in vitro culture. Based on surface expression levels of HSA, IgM and B220, after 24 hours in culture, most sIgM+ cells that develop were immature. After 36 hours, 15% of sIgM+ B cells were in transition from the immature to the mature B cell stage (81).

c) Signaling

Immature B cells differ functionally from mature B cells in that they are refractory to anti-IgM-mediated proliferation (83, 399, 486). Ligation of sIgM on immature B cells from neonatal spleen, adult BM or day 14 auto-reconstituted spleen results in death by apoptosis (487). PTK activation is critical for initiation of B cell activation responses (262, 263). Both neonatal splenic and adult BM-derived immature B cells are deficient in the expression of the Src family PTKs, fyn and fgr, which may account for the lack of proliferation in response to BCR aggregation (488). In contrast, immature B cells from the spleen of 14 day auto-reconstituted mice express levels of fyn and fgr comparable to that seen in mature splenic B cells. This may reflect the fact that these cells are more mature than those isolated from either BM or neonatal spleen since they have proportionately more IgD-expressing cells. Lyn, blk and lck expression levels are comparable in all immature and mature populations (485).
BCR aggregation on mature B cells induces tyrosine phosphorylation and activation of PLCγ1, which hydrolyzes phosphatidylinositol 4,5-bisphosphate into second messengers, DAG and IP3 (273, 274, 276, 489). Immature B cells do not undergo PI hydrolysis and subsequent IP3 production (399). DAG, one product of PI hydrolysis, activates the enzyme PKC (276). Expression of genes egr-1 and c-fos are a consequence of PKC activation (311, 490, 491). Neither gene was induced in immature B cells following BCR aggregation (399), which is consistent with the inability to activate the PI pathway leading to PKC activation. BCR aggregation does stimulate Ca2+ mobilization in both immature and mature B cells (399). These results demonstrate uncoupling of BCR-mediated signals from the PI pathway-linked signal transduction machinery. In T cells, Ca2+ increases in the absence of PKC activation (due to lack of PI hydrolysis) have been observed to elicit a tolerogenic response (492). Since immature B cells also undergo Ca2+ increases in the absence of PI hydrolysis, this may explain their lack of proliferation in response to BCR aggregation.

Entry into and progression through cell cycle are regulated by the activity of a series of related serine/threonine protein kinases known as cyclin-dependent kinases (cdk) (493). Inhibition of either cyclin E expression or cdk2 activity in early G1 induces a G1 arrest (494, 495). BCR aggregation on immature B cells from neonatal mice does not induce their progression through the G1 phase of cell cycle. This abortive transition into cell cycle in immature B cells may be due to the lack of induction of cyclin E/cdk2 expression upon BCR aggregation (496). A signal transduction intermediary that has been implicated in the regulation of cyclin E expression is the proto-oncogene, c-myc. Mitogen stimulation of a variety of cells increases c-myc expression (497). Forced expression of c-myc induces expression of cyclins E and A (498). Immature B cells fail to induce c-myc expression following BCR aggregation (496), suggesting that the lack of c-myc induction may be responsible for the lack of cyclin E expression, and thus the inability to complete cell cycle. A further connection between immature B cell growth arrest and the lack of c-myc induction is seen in the immature B cell line, WEHI-231. In this study, BCR aggregation lead to a temporary increase in c-myc followed by a drastic drop in c-myc expression below detectable basal
levels. This correlated with induction of growth arrest and apoptosis. However, a revertant of WEHI-231 which was resistant to BCR-mediated apoptosis retained strong c-myc expression levels, which were not lost upon stimulation. A separate study demonstrated that overexpression of c-myc in WEHI-231 cells prevented apoptosis induced by BCR aggregation.

**d) Selection**

Previously, selection checkpoints in B cell development were discussed as an important means of ensuring that only appropriately rearranged Ig receptor-bearing B cells were allowed to mature. Another major selective checkpoint occurs at the immature B cell stage. At this stage, self-reactive B cells are eliminated or anergized upon exposure to auto-antigens. One of the transgenic models that has been useful in demonstrating this effect uses transgenic mice carrying rearranged HC and LC genes encoding antibody specific for H-2K$^k$ or H-2K$^b$. When these mice were mated with H-2K$^k$ or H-2K$^b$-expressing mice, the auto-reactive B cells were eliminated in the BM. B cells expressing the transgenic BCR were not detected in the periphery, but were present in the BM where they had downmodulated IgM expression. A proportion of cells succeeded in replacing the κ LC encoded transgene with a λ LC arising from the expression of a newly assembled endogenous V$\lambda$ gene. Such cells now displayed a different specificity, no longer anti-self, and escaped negative selection. This process is referred to as 'receptor editing'.

Another model of negative selection at the immature B cell stage involves transgenic mice carrying rearranged HC and LC genes encoding IgM and IgD that bind to hen egg lysozyme (HEL). When these mice were mated with mice carrying transgenes for a membrane-form of HEL (mHEL), no anti-HEL B cells were present in the periphery, but large numbers of self-reactive immature HEL-specific B cells were found in the BM. The immature self-reactive B cells were eliminated by a two step process, arrested development followed by apoptosis. When these
HEL/anti-HEL mice were mated with mice bearing the anti-apoptotic gene, bcl-2, as a transgene. The death of the immature self-reactive B cells between the BM and the periphery was prevented, although the cells still remained immature. Thus, bcl-2 reversed the apoptosis but not the maturational arrest (506).

In contrast to the developmental arrest that occurs in the presence of mHEL, no developmental arrest or elimination occurred when mice expressing HEL-specific BCR were mated to mice expressing soluble HEL (sHEL) (501). It is likely that the binding of sHEL triggered intracellular signaling in the immature B cells, since IgM receptors were down-modulated (357, 508, 509). The binding of sHEL rendered the immature B cells anergic (509). In contrast to the membrane-bound form, auto-antigen was unable to induce deletion of self-reactive B cells. These differences are due to the differential valency of each antigen, and capacity to cluster antigen receptors together (505). The 'Signaling Threshold' model predicts that immature B cells are eliminated in the BM if they bind self-antigens that are abundant, highly multivalent, and induce strong signals. Less multivalent antigens, such as sHEL, trigger weaker signaling, and result in anergy (510). The concept of signaling strength in the selection of immature B cells into the mature B cell pool will be discussed further in the Discussion chapter of this thesis.

Selection of immature or transitional B cells into the mature B cell pool is thought to occur by signals generated through the BCR. Mouse models that are deficient in proteins involved in signal transduction through the BCR have been useful in identifying which proteins are necessary for the transition to the mature B cell stage. Mice deficient in btk either by gene targeting, or by a naturally mutated btk protein, contain a block in maturation at the immature to mature B cell stage. The majority of their splenic B cells are sIgM\(^{hi}\) sIgD\(^{lo}\), consistent with a block at the transitional B cell stage (231, 346, 511). In addition to a block in B cell development at fraction C to fraction D cell stage, syk deficient mice contain a total block in the recruitment of immature B cells into the recirculating mature B cell pool (339). Mice lacking the cytoplasmic domain of CD79\(\alpha\) show a modest reduction in immature BM B cells but an almost complete lack of peripheral B cells (326), demonstrating another important signaling molecule required for maturation. Lyn-deficient mice
contain more IgM\textsubscript{hi} IgD\textsubscript{lo} immature cells and fewer IgM\textsubscript{lo} IgD\textsubscript{hi} mature cells, demonstrating a lack of maturation from immature to the mature B cell stage (512). Finally, the results presented in Chapter 2 of this thesis demonstrate that CD45 deficient mice also contain a block in maturation. Most B cells in these animals are transitional in phenotype, and they are deficient in mature B cells (513).

6. T-dependent B cell activation

B cell activation to Ig secretion is a sequential process. Ab responses to soluble antigens usually require the collaboration of B cells with helper T cells. In contrast to T-independent (TI) antigens, which are typically polysaccharides possessing highly repeated or polymeric epitopes, thymus-dependent (TD) antigens are monovalent or paucivalent proteins. The binding of TD antigens to specific IgM and IgD on the B cell surface does not result in extensive aggregation of the BCR (514). Therefore, unlike TI antigen binding, the signals transduced through mIg by TD antigens are insufficient to induce B lymphocyte cell cycle entry, proliferation or subsequent Ab production without the delivery of additional signals to B cells from helper T cells. These additional signals include signals delivered via direct cell-cell contact as well as signals that result from the binding of soluble cytokines (515).

B cells bind antigen with their antigen receptor membrane Ig, internalize and degrade the antigen. Then the peptides from the antigen are presented on the B cell surface bound to class II MHC molecules. Finally, the T cell recognizes the processed antigen in the context of MHC class II on the B cell surface through a cognate interaction, and mutual activation results (515, 516).

Besides antigen, the second signal that is required for B cell activation occurs through the interaction between CD40 on B cells, and its ligand, gp39 (CD154), on activated T cells (517, 518).

The signaling pathways that are involved in CD40 signaling will be discussed in a later section of this chapter. Signaling through CD40 triggers B cell activation and progression through the G1-G2
stage of cell cycle (519). Activated B cells upregulate the expression of the IL-2, IL-4, and IL-5 interleukin receptors, and become highly responsive to the growth and differentiative effects of these lymphokines (520, 521). In response to CD40-gp39 interactions and IL4, B cells become fully competent antigen-presenting cells (APCs) by increasing expression of B7.1 and B7.2 cell surface molecules (522, 523), which are critical for the costimulation of T cells through the counter-receptors, CD28 and CTLA-4 (524, 525). The CD28/B7 interaction can determine whether antigen presentation results in T cell anergy or proliferation (526). B7 is upregulated on activated B cells, and may determine in part whether B cells induce tolerance or T cell clonal expansion (527, 528). At present, B7 has not yet been shown to deliver a signal to the B cell. At this stage, the fully activated B cells are competent to progress through G2 and mitosis, producing soluble antibody, and undergo terminal differentiation (529).

a) **CD40 Signaling**

CD40, a 45-50 kDa transmembrane glycoprotein, is a member of the tumor necrosis factor receptor (TNFR) superfamily of surface molecules. It is constitutively expressed on a variety of cell types, including B cells (530), interdigitating cells (IDC) (531), monocytes (532), vascular endothelial cells (533), follicular dendritic cells (FDC) (534), thymic epithelial cells (535), hematopoietic progenitor cells (536), and some carcinomas (537, 538).

As previously mentioned, signals through CD40 are crucial for T-dependent B cell activation. This section will describe the important role of CD40 in B cell activation, differentiation and survival. Heterologous Abs or mAbs to CD40 induce B cell proliferation (79, 408, 539-541), and the engagement of CD40 by mAb provides a stimulatory signal that synergizes with signals delivered by IL-4 or Ab to either sIgM or CD20 (540, 542, 543). Anti-CD40 in the presence of IL-4 promotes long-term B cell growth (519). CD40 also induces homotypic B cell adhesion among resting B cells (544). This adhesion is due in part to interactions between the leukocyte function-associated antigen-1 (LFA-1) (CD18/CD11a) and intercellular adhesion molecule-1 (ICAM-1)
CD40 also plays an important role in B cell survival. B cells undergo affinity maturation of their Ig receptors in germinal centers. Only cells with a high affinity receptor will survive, whereas low affinity Ig receptor-expressing B cells die by apoptosis. One mechanism by which B cells survive in germinal centers is through ligation of CD40. Anti-CD40 prevented apoptosis of germinal center B cells in vitro by the induction of the anti-apoptotic gene, bcl-2 (547). The anti-apoptotic effects of CD40 are also due to the induction of bcl-xL expression (548). Both bcl-2 and bcl-xL are members of the bcl-2 family of proteins that regulate cell death in eukaryotic cells in positive or negative ways (549). CD40 ligation rescued the immature B cell lymphoma, WEHI-231, from anti-IgM-mediated apoptosis, by inducing bcl-xL RNA and protein expression (550). CD40 ligation on B cells can also have negative effects on cell survival by the induction of Fas expression, rendering cells susceptible to Fas-induced apoptosis (551, 552). Fas is also a member of the TNFR family, that mediates apoptosis (553, 554). Treatment of B cells with anti-CD40 and anti-IgM also induces Fas expression, but such cells are no longer sensitive to Fas-mediated apoptosis (555, 556). IL4 can also reverse the susceptibility of B cells to Fas-mediated apoptosis induced by anti-CD40 (557).

CD40 also mediates the induction of Ig class switching. Anti-CD40 induces IgE synthesis in the presence of IL-4 (558, 559), and together with other cytokines, CD40 supports isotype switching (560, 561). For example, culture of purified tonsillar B cells with anti-CD40 and IL-10 resulted in secretion of IgM, IgG and IgA (562).

In order to understand how CD40 mediates the above mentioned effects, CD40 structure and signaling pathways will be described. The CD40 protein consists of 277 amino acids (530). CD40 contains tandem cysteine-rich pseudo motifs in the extracellular domain that classify it as a member of the TNFR superfamily. The cytoplasmic domain of murine CD40 contains 4 threonines, 3 serines and 1 tyrosine (563), whereas the tyrosine is absent in human CD40 (530).
The ligand for CD40, gp39, is a 33-39 kDa type II transmembrane protein expressed on activated CD4+ T cells (564, 565).

CD40-mediated signal transduction pathways are distinct from those triggered by BCR aggregation. Since CD40 lacks intrinsic enzymatic activity, signal transduction is mediated through the association of CD40 binding proteins together with the cytoplasmic domain of CD40. It is also mediated by the downstream activation of nonreceptor tyrosine kinases (566). CD40 ligation results in the rapid tyrosine phosphorylation and activation of PTKs, lyn and syk, and induces the tyrosine phosphorylation of multiple substrates, including PI3-kinase, and PLCγ2 (567, 568). The Tec family PTK, btk, is also required for CD40 signaling, as B cells from xid mice, which have a point mutation in btk, are unable to proliferate in response to mAb-mediated aggregation of CD40 (348). The tyrosine protein kinase regulated guanine nucleotide exchange factor, vav, is also phosphorylated upon CD40 ligation (218). The activation of PLCγ2 by phosphorylation results in IP3 production, as mentioned previously. Consistent with this, increased IP3 production is also observed following anti-CD40 treatment (569). Increases in intracellular Ca2+ upon CD40 ligation remain controversial. Nevertheless, one study observed increased levels of intracellular Ca2+, which was due to Ca2+ influx. The Ca2+ response was physiologically relevant, since an inhibitor of calcineurin, FK506, inhibited anti-CD40-mediated B cell proliferation (570). CD40 ligation also activates the serine/threonine protein kinases, such as the SAPKs, JNK, and other members of the MAP kinase family (i.e. ERK and p38) (571-575). Ultimately, the above different activation pathways result in the activation of various transcription factors, including NF-kB, NF-kB-like molecules (e.g. p50, p65, cRel), c-fos and c-jun, and NF-AT (576, 577).

The mechanisms by which signals are transduced through CD40 ligation are through CD40-binding proteins. Four members of the TNF receptor-associated factor (TRAF) family of proteins were found to directly associate with the cytoplasmic domain of CD40, TRAF2, TRAF3, TRAF5, and TRAF6 (578-582). TRAF2, TRAF5 and TRAF6 mediate NF-kB activation (579, 580, 583, 584). In addition, the Janus kinase (Jak), Jak3, is constitutively associated with the cytoplasmic domain of CD40 (585). Jak kinases are constitutively associated with the membrane-
proximal regions of cytokine receptor cytoplasmic domains, and become activated upon ligand-induced receptor homodimerization or heterodimerization. The activated Jaks phosphorylate both themselves and other substrates, including members of a family of signal transducers and activators of transcription (STATs), which then dimerize and translocate to the nucleus where they transactivate the promoters of cytokine-responsive genes containing the interferon γ activation site (GAS), related sequences, or both (586-589)(Fig. 1-7). CD40 aggregation leads to tyrosine phosphorylation and activation of Jak3 and STAT3, which leads to the induction of expression of CD23, ICAM-1, and lymphotoxin-α (585).

The essential role of CD40 in T cell-dependent Ig class switching and germinal center formation is demonstrated in mice deficient in CD40. Such mice were able to mount IgM responses but no IgG, IgA or IgE responses to TD antigens. However, responses to TI antigens were normal. Furthermore, germinal center formation was defective in CD40-deficient mice. The impaired antibody responses to TD antigens were reflected by serum Ig levels of unimmunized mutant mice. Serum Ig levels of IgG1, IgG2a, IgG2b, and IgA were significantly reduced, while serum IgM and IgG3, the major isotypes of TI antibody responses, were normal or enhanced (590). Mice deficient in CD40 ligand exhibited a similar phenotype as CD40-deficient mice, except that serum IgE was undetectable, whereas all other isotypes were normal. Serum levels of all isotypes except IgM decreased, however, with increasing age (591). The phenotype of CD40 ligand-deficient mice is similar to that found in Hyper-IgM Syndrome (HIM) in humans. HIM is an X-linked immunodeficiency containing deleterious mutations of CD40L, gp39 (592-595). However, HIM is characterized by elevated serum IgM levels, a phenotype not observed in CD40L-deficient mice (591).

b) IL4 Signaling

Interleukin 4 (IL4) is a cytokine secreted by activated T cells, mast cells and basophils (596-598). It was originally described as a factor that enhances the growth of resting B cells
Figure 1-8. IL4 signal transduction pathways. (Y) represents a tyrosine residue. (●) represents phosphorylated tyrosine residues.
stimulated with anti-IgM antibody (599). IL4 also increases the surface expression of MHC class II and CD23 on B cells. In addition, IL4 plays an essential role in Ig class switching, by stimulating the transcription of germ line γ1 and ε, promoting the switch to IgG1 and IgE (600-602).

IL4 exerts its effects by binding to the IL4 receptor (IL4R) complex (Fig. 1-8), which consists of 2 subunits. One subunit is the IL4Rα chain, and the other is the γ common (γc) chain (186, 603). γc is a component for other cytokine receptors such as IL2, IL7, IL9, and IL15 (184, 186, 190). The IL4Rα and γc chain are associated with the tyrosine kinases, Jak1 and Jak3, respectively (604, 605). Ligand binding induces heterodimerization of the receptor components, which triggers phosphorylation of the Jak kinases. The phosphorylation of Jak1 and Jak3 results in the phosphorylation of cytoplasmic signaling molecules. Specifically, IL4 signals through the phosphorylation of 2 distinct signaling proteins (606). One protein is a 170 kDa substrate, IL4-induced phosphotyrosine substrate (4PS) or insulin receptor substrate (IRS)2. 4PS/IRS2 is related to IRS1 and contains a pleckstrin homology domain and phosphotyrosine-binding domain at its amino terminus. Activated 4PS/IRS2 associates via phosphotyrosine residues with proteins containing an SH2 domain, such as PI3-kinase, grb-2, and SHP-2 (607). 4PS/IRS2 has been shown to be essential for IL4-induced mitogenesis, such that a myeloid progenitor cell line, 32D, deficient for 4PS/IRS2 does not proliferate in response to IL4. Forced expression of 4PS/IRS2 or IRS1 restores the IL4-induced proliferative response in this cell line (608-610).

The second major signaling protein activated by IL4 signaling is Stat6. Upon IL4 receptor signaling, Jak1 and Jak3 become activated and phosphorylate the tyrosine residues of Stat6 (Fig. 1-7). This activates Stat6, which translocates to the nucleus and binds DNA sequences located in promoters of IL4-responsive genes, including CD23, MHC class II, and germline ε transcripts (607, 611). Stat6-deficient mice demonstrate severely impaired proliferative responses of B cells costimulated with anti-IgM and IL4, suggesting that the Jak-Stat pathway is involved in the IL4-mediated proliferative signal (612, 613).
IL4-deficient mice exhibited reduced serum levels of IgG1 and IgE. The IgG1 dominance in a T cell-dependent immune response was lost, and IgE was not detectable upon nematode infection (614). The mild phenotype of IL4 deficient mice may be attributed to the compensatory role of IL13, which shares many similar functions and signaling components with IL4 (615, 616). Since both IL4 and IL13 signal through Stat6, a more severe phenotype is observed in Stat6-deficient mice. Augmentation of CD23, MHC class II, and IL4Rα on B cells in response to IL4 was not observed in Stat6-deficient mice (612, 617, 618). When stimulated, naive CD4+ T helper cells can develop into two cell types, designated as Th1 and Th2 cells. Th1 cells produce IFN-γ, tumor necrosis factor β, and IL2, whereas Th2 cells produce IL4, IL5 and IL10. Th2 cells become dominant during helminthic infections and in response to common environmental allergens (619). *N. brasiliensis* is known to generate a selective activation of Th2-type immune response. In Stat6-deficient mice, Th2 cytokine production was almost completely abolished after infection with this parasite (612). This blockage is even more severe than that observed in IL4-deficient mice (620). In addition, the production of IgG1 and IgE after *N. brasiliensis* infection was severely reduced (612). Thus, almost all IL4-mediated functions are impaired in Stat6-deficient mice, indicating the essential role of Stat6 in IL4 signaling.

**Thesis Outline**

B cell development occurs through a regulated series of stages, that involves the acquisition of a number of cell surface markers. Certain markers are expressed very early in development while others are expressed during later stages of B cell development. This thesis examines two such surface markers expressed very early in B cell development, AA4.1 and CD45.

In chapter 2, the function of CD45 in B cell activation using CD45 exon 6 knockout mice is examined. Previously, it was demonstrated that B cells from CD45−/− mice were unable to proliferate upon BCR aggregation (73). As described in chapter 2, I extend these findings, and through the characterization of the molecular basis of the signaling lesion associated with BCR
activation of CD45-/- B cells, examine the cause of the lack of proliferation to BCR aggregation. This is achieved by examining signaling pathways that may be affected by the lack of CD45, as well as the phenotype of B cells that develop in these mice. Phenotypic analysis of B cells from CD45-/- mice reveal the presence of a developmental block at the transition from the immature to mature B cell stage. I demonstrate that some element of the activation sequelae induced via BCR aggregation are unaffected in CD45-deficient B cells. Specifically, tyrosine phosphorylation of CD79α, PLCγ2, and ERK2 activation are all evident. Although Ca2+ release from intracellular stores does occur upon BCR aggregation, the induction of Ca2+ influx is impaired in CD45-/- B cells. The defect in proliferation can be partially rescued by addition of anti-CD40 or IL4, but Ca2+ influx remains impaired. These results suggest that although CD45 may be dispensable for the activation of some B cell signaling pathways, it may be crucial for the induction of Ca2+ influx, and for the transition from immature to mature B cell.

Results presented in chapter 3 characterize another cell surface molecule expressed early in B cell development. Previous results had suggested that AA4.1 expression is lost upon maturation. I demonstrate that this is not the case, rather, AA4.1 expression decreases during B cell development. The loss of high levels of AA4.1 correlates with the acquisition of sIgM expression, and occurs prior to the acquisition of high levels of CD22 expression. AA4.1 expression remains at low levels on mature B cells. Once a mature B cell becomes activated to enter into cell cycle, the level of AA4.1 expression changes depending on the source of stimulation. Finally, upon immunoprecipitation of AA4.1, I demonstrate that mAb AA4.1 recognizes a highly glycosylated protein of 115-130 kDa in size.

The discussion presented in Chapter 4 presents an integration of results presented in the data chapters and focuses on the resolution of unanswered questions.
CHAPTER 2

Immunoglobulin-mediated Signal Transduction in B cells from CD45-deficient Mice

Contents of this chapter appear in the Journal of Experimental Medicine (1996). Volume 183: 329-334. Immunoglobulin-mediated signal transduction in B cells from CD45-deficient mice. Tania Benatar, Rita Carsetti, Caren Furlonger, Nilupa Kamalia, Tak Mak, and Christopher J. Paige. I would like to acknowledge Caren Furlonger for her initial experiments with IL4 and anti-IgM in the CD45-knockout mice. I would also like to acknowledge Nilupa Kamalia for her help with the analysis of the mixed bone marrow chimeras.
INTRODUCTION

Evidence from a variety of sources clearly demonstrates that the expression of CD45 is essential for activation of B lymphocytes after cross-linking of the B cell receptor (74-79). Simultaneous addition of anti-CD45 mAbs abrogates B cell AgR-mediated signal transduction as well as proliferation (457). *In vitro* activation studies using primary cells treated simultaneously with anti-CD45 and anti-Ig resulted in the inhibition of B cell activation events such as Ca\(^{2+}\) mobilization (79), phosphoinositol hydrolysis, and increased gene transcription of genes such as c-myc (458), suggesting that CD45 facilitates early B cell signaling events.

Cell lines have also been useful in examining the role of CD45. In some cases, conflicting results have emerged from such studies. The plasmacytoma J558Lμm3, which lacks CD45, failed to mobilize Ca\(^{2+}\) after BCR cross-linking. This effect was restored in J558μm3 cells by transfection with a cDNA encoding the B220 isoform of CD45 (74). In a separate study, CD45 deficient mutants of the K46-17μm λ B lymphoma cell line failed to undergo extracellular Ca\(^{2+}\) influx, although they did mobilize Ca\(^{2+}\) from intracellular stores in response to BCR aggregation (461). In contrast, CD45- variants of the CD45+ immature cell line, WEHI-231, exhibited a delayed but prolonged Ca\(^{2+}\) mobilization response upon BCR cross-linking (479), very similar to what is seen with the CD45-deficient YAC-1 T cell line (621). The CD45-deficient BAL-17 cells had reduced Ca\(^{2+}\) mobilization in response to BCR aggregation (474). Whether these discrepancies accurately reflect the different maturation states of these cell lines, or are due to abnormalities peculiar to transformed cells, remains to be determined.

To examine the role of CD45 more extensively, CD45-deficient mice were generated by targeted disruption of exon 6 (73). Mice homozygous for the CD45-exon 6 mutation lacked CD45 expression on B cells and on the majority of thymocytes and peripheral T cells. A block in T cell development occurred at the transition from immature CD4+CD8+ double-positive to mature single-positive thymocytes resulting in a significant reduction in peripheral T cells. Residual peripheral T cells exhibited impaired anti-CD3 and anti-TCR signaling. In contrast to the paucity of
peripheral T cells, approximately normal numbers of B cells were found in peripheral lymphoid tissues. Furthermore, the B cells detected were responsive to the B cell mitogen, lipopolysaccharide, and serum levels of both IgM and IgG were near normal. However, thymidine incorporation assays revealed that CD45-deficient cells failed to proliferate after cross-linking with IgM-specific antibodies. These results confirmed that CD45 plays a critical role in B cell activation as previously demonstrated in other experimental systems.
MATERIALS AND METHODS

Mice. Mice used were CD45 exon 6 -/-, originally derived by Kenji Kishihara, after fifth backcross with C57BL/6. Control mice were either age-matched C57BL/6 or CD45 exon 6 +/+ mice of the fifth backcross to C57BL/6.

B cell purification. BM was prepared by flushing tibias and femurs with a 26-gauge syringe. Single cell suspensions of splenic B cells were prepared by mechanical disruption through a metal screen with a syringe plunger, followed by a 5-min incubation on ice to remove large aggregates. Erythrocytes were lysed with ACK (0.155 M ammonium chloride, 0.1 mM disodium EDTA, 0.01 M potassium bicarbonate, pH 7.3) for 5 min on ice. T cells were lysed with anti-CD4 (RL172), anti-CD8 (3.168), and anti-thy1.2 (H013.4) antibody supernatants (1:20 dilution) together with low tox guinea pig complement (1:12 dilution) (Cedarlane Laboratories Ltd., Hornby, Canada) for 1 h at 37°C. The remaining cells were separated on a Percoll gradient (Sigma Chemical Co., St. Louis, MO) as described (622).

[3H] Thymidine Incorporation Assay. Splenic B cells were placed in flat-bottomed 96-well plates (Costar Corp., Cambridge, MA) in Opti-MEM (Gibco BRL, Gaithersburg, MD) medium supplemented with 2.4 g/liter NaHCO3, 5 mg/liter streptomycin, 5 x 10^3 U/liter penicillin, 5 x 10^-5 M 2-mercaptoethanol, 0.5% FCS (Gibco BRL, Gaithersburg, MD) at 8-10 x 10^4 cells/well with either medium alone, LPS (20 μg/ml), B76 (rat IgG1 anti-mouse Igμ; 30 μg/ml), or goat anti-mouse Igμ (25-30 μg/ml; Jackson Immunoresearch Laboratories, West Grove, PA) alone or in combination with anti-CD40 (Pharmingen) (10 μg/ml) or IL4 (Genzyme Corporation, Cambridge, MA) (5 ng/ml). B cells were harvested at various intervals after a 6-h pulse with 1 μCi of [3H] thymidine per well.
**Flow cytometry.** Cells (1-5 x 10^5/sample) were washed and exposed to appropriate antibodies for 15 min on ice. Before analysis, cells were fixed in 1% paraformaldehyde. 10^4 cells/sample were analyzed on a FACScan® flow cytometer (Becton Dickinson and Co., San Jose, CA) using Lysys II software. The following mAbs were used: RA3-6B2 (B220, PE-labeled; Pharmingen, San Diego, CA); 33-60 (anti-sIgM; FITC and biotin-labeled; the hybridoma, 33-60 ((623)); anti-sIgD (PE-labeled; Southern Biotechnology Associates, Birmingham, AL); anti-CD23 (PE-labeled; Pharmingen); M5-114 (anti-H-2^bd-FTTC-labeled). Biotinylated antibodies were visualized with streptavidin-PerCP (Becton Dickinson) or streptavidin-Quantum red conjugate (Sigma Chemical Co.).

**BM reconstitution experiments.** Donor BM was separated by panning on plates coated with anti-IgM, 33-60, for 1 h at 4°C to remove mature B cells. Nonadherent cells were harvested and resuspended in PBS at 5 x 10^6/ml. Recipients were irradiated with 900 rads and subsequently reconstituted with 2 x 10^6 BM cells via tail vein injection. Mice were analyzed 9-11 wk after reconstitution by flow cytometric analysis of spleen and BM.

**Immunoprecipitation and Western Blotting.** Anti-phosphotyrosine blots of whole cell lysates were performed by stimulation of 3x10^6 high buoyant density splenic B cells with the anti-μ antibody, B76 (20 μg/ml) for indicated time periods, followed by lysis in 50 μl of lysis buffer (50 mM Tris pH=8, 150 mM NaCl, 2 mM EDTA, 1 mM sodium orthovanadate) with protease inhibitors (10 μg/ml aprotinin/leupeptin, 1 mM PMSF) for 30 min on ice. Lysates were boiled for 5 min in 2x reducing sample buffer before separation of proteins on 7.5% polyacrylamide gels, followed by transfer to nitrocellulose by electroblotting for 3 h at 50 V. The membrane was blocked with wash buffer (20 mM Tris, 0.5 M NaCl, 0.1% Tween, pH 7.6) containing 3% gelatin for 1 h at room temperature. Anti-phosphotyrosine blots were performed using a 1/1000 dilution of anti-phosphotyrosine mAb, 4G10 (Upstate Biotechnology Inc., Lake Placid, NY), followed by a 1/2000 dilution of horseradish peroxidase (HRP)-coupled goat anti-mouse IgG (Sigma Chemical Co.).
Bands were visualized by enhanced chemiluminescence detection system (NEN Dupont, Boston, MA).

MB-1 immunoprecipitation was performed as described (245). A 1:50 dilution of rabbit antisera against mb-1 was used. Samples were boiled 5 min before separation of proteins on 10% polyacrylamide gels, followed by transfer to nitrocellulose by electroblotting for 2 h at 50 V. The membrane was blocked with wash buffer (20 mM Tris, 0.5 M NaCl, 0.1% Tween, pH 7.6) containing 3% gelatin for 1 h at room temperature. Antibody incubations were done in wash buffer containing 1% gelatin for 1 h at room temperature, followed by 5 x 5 min washes. Anti-phosphotyrosine blots were performed as described above. Blots were stripped by incubation for 15 min in 10 mM Tris-Cl pH 2.3; 150 mM NaCl, followed by washing 5 times in the same buffer at pH 8. For anti-phospholipase C (PLC)γ2 immunoprecipitation, lysis was performed in 0.5 ml of RIPA modified buffer (1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 50 mM Tris, pH 7.5, containing protease inhibitors: 1 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml aprotinin/leupeptin) for 30 min on ice. Lysates were incubated with 15 μl rabbit antisera towards mouse PLCγ2 (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at 4°C, followed by addition of protein A-Sepharose (Sigma Chemical Co) for 1 h at 4°C. Immune complexes were washed three times with lysis buffer containing protease inhibitors followed by one wash in TN buffer (50 mM TrisCl, pH 7.5, 150 mM NaCl) containing protease inhibitors. Proteins were separated on 8% polyacrylamide gels. Anti-phosphotyrosine blots were performed as described above. Anti-PLCγ2 blots were visualized by stripping and probing blots with anti-PLCγ2, followed by protein A-HRP.

**In Vitro Kinase Assay.** Cells were stimulated for various time periods, and then washed once with ice cold 50 mM TrisHCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 100 μM Na3VO4. Cells were lysed in 50 mM TrisHCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 100 μM Na3VO4, 1 mM EDTA, 1 mM PMSF, and 10 μg/ml aprotinin/leupeptin, for 10 min. Immunoprecipitations were performed using 1 μg/5x10⁶ cells of ERK2 (Santa Cruz Biotechnology) for 1 h at 4°C,
followed by addition of protein A sepharose for 1 h at 4°C. Samples were washed twice in 50 mM TrisHCl (pH 8.0), 150 mM NaCl, 0.1% Triton X-100, 100 μM Na3VO4, followed by an additional wash in 5 mM Heps, 10 mM MgCl2, 100 μM Na3VO4. Kinase reactions were performed in 50 μl of kinase buffer (5 mM Heps, 10 mM MgCl2, 100 μM Na3VO4, 5 μg per sample of myelin basic protein (MBP) (UBI, Lake Placid, NY), 1 μM cold ATP, 10 μCi of 32P-γATP/ reaction. Kinase reactions were performed for 15 min at 30°C. Reactions were stopped by boiling in 2x sample buffer. Proteins were separated on 12.5% SDS-PAGE.

**Determination of [Ca2+]i.** Cells at 5 x 10⁶/ml in HBSS (pH 7.0) received the acetoxyethyl ester of indo-1 (Molecular Probes, Eugene, OR) to a final concentration of 1 μM. After 30 min at 37°C, an equal volume of HBSS containing 5% FCS (pH 7.4) was added to cells for an additional 30 min at 37°C. Cells were washed once in HBSS/FCS (pH 7.4) or HBSS containing 0.5% BSA and 10 mM Heps (pH 7.3), and finally resuspended in the latter buffer at 5 x 10⁶/ml. Before fluorescence measurements, aliquots of 5 x 10⁵ cells were preincubated at 37°C for 1-2 min, and were maintained at this temperature during measurement of the [Ca2+]i. Flow cytometric analysis of [Ca2+]i was carried out using a FACStar® Plus (Becton Dickinson) using an ion laser (Innova Enterprise; Coherent, Santa Clara, CA) optimized for UV argon ions, set for 355-nm excitation at a power setting of 50 mW. Differential analysis of intracellular mobilization of Ca2+ vs. extracellular influx was carried out by assessing the responsiveness of cells resuspended in Ca2+-free HBSS (buffered with 1.8 mM EGTA) followed by repletion of the external Ca2+ by addition of 4 mM CaCl2. The response that occurs in Ca2+-free medium reflects intracellular release.
RESULTS

Cell surface phenotype of CD45-deficient cells. We have previously reported that normal numbers of B cells arise in CD45-deficient mice (73). We examined cell surface antigens that are associated with the progression of cells from the immature to the mature stage of B cell differentiation. Results revealed that high buoyant density splenic B cells obtained from CD45-/- mice differ significantly from their normal littermate controls (fig. 2-1a). The majority of splenic B cells from normal mice were B220+, δ+, μlo, and CD23+. As expected, the B cells obtained from the CD45-/- mice failed to express the B220 antigen. However, we also found a significant decline in the frequency of the δ+, μlo population. In addition, there was a significant decline in B cells expressing CD23 in CD45-/- mice. There was also a decline in the number of MHC class II+ cells. Similar results were obtained for low buoyant density (1.079/1.066) B cells (fig. 2-1b), whereas BM B cells were phenotypically normal (fig. 2-1c). These results raise the possibility that B cells in CD45-/- mice may have undergone a developmental arrest characterized by the transition from the immature to mature B cell stage (81).

T cells are not responsible for the lack of mature B cells. CD45-/- mice lack functional T cells. To determine if the B cell phenotype was due to the lack of T cells, we analyzed the B cell phenotype in irradiated recipients of BM mixtures derived from CD45-/- and normal mice. One of the most reliable markers for the CD45-/- B cell phenotype is the level of δ expression. In control studies in which the median δ expression was compared in age-matched normal and CD45-/- splenic B cells (five separate experiments, each with at least two mice per group) the ratio of the median δ intensity of normal to CD45-/- μ+ B cells was 1.72±0.6. A similar comparison between two irradiated, reconstituted mice, one reconstituted with +/- BM and one with -/- BM resulted in a δ ratio of 1.6 (+/+ : -/-). This result indicates that the irradiation/reconstitution protocol does not alter the levels of δ subsequently obtained. The median δ level was then determined in recipients (either +/- or -/-) reconstituted (for 9-11 wk) with a 1:1 mixture of normal and CD45-/- B cell-
Figure 2-la. Flow cytometric analysis of high buoyant density (1.085/1.079) splenic B cells from CD45+/+ and CD45-/- mice. The percentage of cells within the boxed regions are based on the total number of IgM+ B cells. Mean fluorescent values determined for the following parameters were (+/+ vs -/-): $\mu$ (95 vs 129); $\delta$ (203 vs 71); CD23 (64 vs 21); and MHC class II (215 vs 101).
Figure 2-1b. Flow cytometric analysis of CD45+ cells from B220+/+ and CD45-/- mice. The percentage of cells within the boxed regions are based on the total number of Ia+ B cells. Mean fluorescent values determined for the following parameters were: (+/+) 72% vs. 28% for CD23 (72 vs. 28); MHC class II (266 vs. 119); T cell (105 vs. 119); B cell (104 vs. 119).
Figure 2.1c. Flow cytometric analysis of BM cells from CD45++ and CD45+ mice. The percentage of cells within the boxed regions are based on the total number of IgM+ B cells.
depleted BM. A comparison was made between μ+B220+ cells (derived from the normal BM and representing ~40% of all B cells) and μ+B220- cells (derived from CD45-/- BM and representing ~60% of all B cells). The ratio of the median δ intensity was found to be 1.7±0.2 (+/+-/-). This result demonstrates that even in the presence of normal T cells and B cells, the CD45-/- B cells retain the immature phenotype.

**BCR Cross-linking fails to induce B cell proliferation.** Density fractionation of splenic cells on Percoll gradients revealed that the CD45-/- cells differed considerably from their normal counterparts. In four separate experiments, using a total of at least 10 individual spleens, we found a two-to fourfold reduction in the number of high buoyant density (>1.079) B cells. In the same spleens, however, we found a two-to fivefold increase in the number of low buoyant density (1.079-1.066) B cells. Experiments using either monoclonal anti-μ or polyclonal reagents, revealed that neither the low nor the high buoyant density B cells incorporate [3H]TdT upon cross-linking of Igμ (fig. 2-2). It has been reported that immature cells fail to proliferate upon anti-Ig cross-linking (486). In this respect, they are similar to the CD45-/- B cells and might be used to support the conclusion that the B cells in CD45-/- mice are developmentally arrested. Immature B cells, however, do express CD45 on their surfaces, albeit at lower levels (39). Thus the failure of immature cells to proliferate cannot be based on exactly the same parameters as the failure of CD45-/- mice to proliferate. CD45-/- B cells were able, however, to proliferate upon exposure to the polyclonal B cell mitogen, LPS, suggesting that CD45 is not required for signaling pathways initiated by this stimulus.

**Ag stimulation leads to protein tyrosine phosphorylation in both CD45+/- and CD45-/- B cells.** Studies with B cell lines have shown that CD45 may not be required for receptor-mediated PTK activation (76, 352, 353). To determine if this is also true of nontransformed B cells, we examined the induction of tyrosine phosphorylation of cellular proteins in response to BCR aggregation from mice lacking CD45. As shown in figure 2-3, in
All assays were performed in triplicate.

Anti-mouse IgM or LPS (20 μg/ml), incorporation of [3H]thymidine was determined at 24, 48, or 72 h.

Figure 2-2. Lack of IgM-induced proliferation in CD45+/- splenic B cells. Cells were cultured in either media alone (NS) or in the presence of 50 μg/ml of purified anti-IgM (B76), 30 μg/ml of polyvalent goat anti-mouse IgM or LPS (20 μg/ml). Incorporation of [3H]thymidine was determined at 24, 48, or 72 h.
Figure 2-3. Induction of tyrosine phosphorylation of cellular proteins. High buoyant density splenic B cells from CD45+/+ and CD45-- mice stimulated for various time periods at 37°C with 20 μg/ml of anti-μ, B76. Proteins were separated on 7.5% SDS-PAGE. Blot was probed with antiphosphotyrosine, 4G10, followed by goat- anti-mouse IgG-HRP.
agreement with previous cell line studies, proteins were inducibly tyrosine phosphorylated in both CD45+/+ and CD45−/− B cells. These results confirm that CD45 is not required for receptor mediated PTK activation of some cellular proteins. Alternatively, other proteins may have replaced the role of CD45 in this function.

**Tyrosine phosphorylation of mb-1 occurs in response to Ig-μ cross-linking.** Previous experiments have shown that the Ig-associated protein, CD79α or Ig-α (mb-1), is hyperphosphorylated on tyrosine in a B cell line deficient in CD45 expression (74). Furthermore, CD79α was found to be hyperphosphorylated when CD45 was sequestered from the Ig receptor complex by cross-linking with anti-CD45 antibodies on resting splenic B cells (75). In addition, incubation of tyrosine-phosphorylated CD79α/CD79β with CD45, results in the rapid dephosphorylation of these proteins *in vitro* (74). These experiments suggested that CD45 may play a role in the maintenance of the basal level state of CD79α tyrosine phosphorylation. In contrast to these reports, CD79α was not hyperphosphorylated in unstimulated B cells from CD45−/− mice (fig. 2-4). Upon Igμ cross-linking, CD79α is inducibly phosphorylated on tyrosine in both CD45+/+ and CD45−/− B cells, suggesting that CD45 is not required for the tyrosine phosphorylation of CD79α.

**Anti-Ig induced PLCγ2 activation in CD45−/- B cells.** Another important signaling event that occurs upon Ig cross-linking is the tyrosine phosphorylation and activation of PLCγ2, which leads to the release of Ca2+ from intracellular stores (274, 489). The CD45 negative J558μm3 plasmacytoma did not undergo Ca2+ mobilization in response to anti-Ig crosslinking, which was restored upon transfection of a cDNA encoding the highest molecular weight isoform of CD45, suggesting a role for CD45 in Ca2+ mobilization (74). We therefore examined the induction of tyrosine phosphorylation of PLCγ2 in CD45−/- B cells. Results demonstrate that in response to Igμ cross-linking, a band of 140-150 kDa, corresponding to PLCγ2, is inducibly phosphorylated in both CD45+/+ and CD45−/− B cells (fig. 2-5). This suggests that upon Ig
Figure 2-4. Induction of tyrosine phosphorylation of Ig-α. High buoyant density splenic B cells from CD45+/+ and CD45/- mice stimulated for various time periods at 37°C with 30 μg/ml of anti-μ, B76. Ig-α was immunoprecipitated with anti-Ig-α followed by separation of proteins on 10% SDS-PAGE. (A) Antiphosphotyrosine blot of immunoprecipitated Ig-α. (B) Blots were stripped and reprobed with anti-Ig-α followed by anti-rabbit HRP. The migration position of molecular weight markers are indicated on the left. The position of Ig-α is indicated by the arrow on the right.
Figure 2-5. Induction of tyrosine phosphorylation of PLCγ2. Whole splenic B cells were stimulated using 30 μg/ml of goat anti-mouse IgM. (A) Antiphosphotyrosine blot c immunoprecipitated PLCγ2. (B) Blots were stripped and reprobed with anti-PLCγ2 followed by protein A-HRP. The arrow on the right represents the position of PLCγ2.
cross-linking, CD45 is not required for the induction of tyrosine phosphorylation of PLCγ2 in B cells.

**Activation of ERK2 in CD45−/− B cells.** Crosslinking of the BCR results in the activation of the ERK2 form of MAP kinase in B lymphoma lines and purified splenic B cells (624, 625). Neither the activation of p21ras nor of the downstream MAP kinase, ERK2, occurs upon BCR aggregation in the CD45-deficient plasmacytoma, J558Lμm3 (76, 77). This suggested a crucial role of CD45 in the activation of the MAP kinase pathway. We, therefore, examined ERK2 activation in CD45 exon 6−/− B cells. Results indicate that upon BCR aggregation on CD45−/− B cells, ERK2 was activated (fig. 2-6). This suggests that CD45 is not required for the activation of the MAP kinase, ERK2.

**Mobilization of intracellular Ca²⁺ is normal but influx from extracellular stores is abrogated in CD45-deficient B cells.** Results from studies with CD45-deficient cell lines has suggested that CD45 is necessary for induction of Ca²⁺ mobilization (74). We examined Ca²⁺ mobilization in response to anti-Igμ cross-linking in high buoyant density splenic B cells. Treatment with anti-μ resulted in an increase in intracellular Ca²⁺ concentration in both normal and CD45−/− B cells (fig. 2-7A). However, while the amplitude of the Ca²⁺ mobilization in CD45−/− B cells was similar to that observed in normal B cells, Ca²⁺ levels returned to baseline values more rapidly in the CD45−/− B cells. This result suggested that CD45−/− cells may be unable to use extracellular stores of Ca²⁺. This was directly tested by stimulating cells in the absence of extracellular Ca²⁺. As shown in figure 2-7B, in the absence of extracellular Ca²⁺, both normal and CD45−/− B cells responded to anti-Igμ with a rapid and short-lived increase in [Ca²⁺]_[i]. The addition of extracellular Ca²⁺ to normal cells (arrow b) resulted in a second wave of increased [Ca²⁺]_[i] reflecting the use of extracellular Ca²⁺. In contrast, the addition of extracellular Ca²⁺ does not affect [Ca²⁺]_[i] levels in CD45−/− B cells. These results suggest that CD45 is required for the induction of Ca²⁺ influx upon BCR ligation.
Fig. 2-6. Induction of Erk-2 activation. High buoyant density splenic B cells were stimulated using 30 μg/ml of goat anti-mouse IgM for indicated time periods. Erk-2 was immunoprecipitated, and activation was assessed by in vitro kinase assay using myelin basic protein (MBP) as a substrate (a). Erk-2 levels were discerned by silver staining of gels(b).
Figure 2-7. Intracellular release of Ca$^{2+}$ vs extracellular Ca$^{2+}$ influx in CD45$^{-/-}$ B cells. High buoyant density splenic B cells were loaded with indo-1, and stimulated with anti-μ, B76 (40 μg/ml) (A, arrow a), or B76 (40 μg/ml) + EGTA 1.8 mM (B, arrow a) followed 5 min. later by addition of 4 mM CaCl$_2$ (B,arrow b). Intracellular calcium increases were measured as the ratio of fluorescence of calcium-bound indo-1/calcium-free indo-1 (405:530 nm) by flow cytometry.
Partial rescue of proliferation using anti-CD40 and IL4. CD40 ligation has been shown to induce B cell proliferation (79, 408, 539-541), and engagement of CD40 by mAb synergizes with IL4 or anti-IgM (542, 543). Anti-CD40/anti-IgM costimulation was refractory to the inhibition of proliferation induced by anti-CD45 mAbs on anti-IgM stimulated B cells (79). We hypothesized that although CD45- B cells are not able to proliferate upon anti-IgM crosslinking, perhaps costimulation with anti-CD40 or IL4 could rescue their proliferative responses. Therefore, high buoyant density splenic B cells were stimulated with the various combinations of stimuli (fig. 2-8). Results demonstrate that proliferation induced by CD40 ligation or anti-CD40+IL4 was similar to controls in CD45- B cells. This suggests that these pathways do not require CD45. Costimulation of CD45- B cells with anti-IgM+anti-CD40 resulted in a substantial increase in proliferation compared to anti-IgM alone. A similar although less robust increase in proliferation was observed with anti-IgM+IL4 costimulation. However, both costimulatory proliferative events were still significantly lower than that of wild type B cells under identical conditions. Therefore, costimulation with anti-IgM+anti-CD40 or anti-IgM+IL4 could only partially rescue the proliferative defect in CD45- B cells.

Partial rescue of proliferation does not rescue Ca2+ influx. Since costimulation of CD45- B cells with anti-IgM+anti-CD40 or anti-IgM+IL4 partially rescued the proliferative response, we wished to determine whether this coincided with the reinstatement of the Ca2+ influx response. We examined Ca2+ mobilization in splenic B cells in response to anti-IgM, anti-IgM+anti-CD40, and anti-IgM+IL4 (fig. 2-9). Responses were separated into intracellular Ca2+ release followed by extracellular Ca2+ influx. Results demonstrate that upon stimulation of CD45- B cells, none of the above mentioned conditions resulted in Ca2+ influx, in contrast to normal controls. This suggests that the partial rescue of proliferation in CD45- B cells using anti-CD40 or IL4 together with anti-IgM was not due to the restoration of Ca2+ influx.
Figure 2-8. Partial rescue of proliferation in CD45−/− B cells. Cells were cultured in either media alone (N.S.) or in the presence of the various conditions under the following final concentrations: goat anti-mouse IgM (gamIgM; 25 μg/ml); anti-CD40 (10 μg/ml); IL4 (5 ng/ml) for 48 hours.
Figure 2-9. Lack of calcium influx upon costimulation of CD45⁻/⁻ B cells. High buoyant density splenic B cells were stimulated with goat anti-mouse IgM (40 μg/ml) alone, or together with anti-CD40 (10 μg/ml) or IL4 (10 ng/ml). Cells were loaded with indo-1, and stimulated under the various conditions together with EGTA (1 mM)(a), followed 5 min. later by addition of 2 mM CaCl₂ (b).
DISCUSSION

Our results demonstrate that mice lacking CD45 contain B cells that differ from their normal counterparts in that the majority of B cells are $\mu^{hi}\delta^{lo}$, with significantly reduced mature $\mu^{lo}\delta^{hi}$ cells. In addition, in B cells from CD45-deficient mice there is a reduction of IgM+ cells expressing CD23 and MHC class II. The expression of ThB and pgp-1 were slightly elevated in both high and low density splenic B cells from CD45-/− mice as compared to controls (data not shown). These phenotypic characteristics resemble those of transitional B cells, an intermediate stage of differentiation between immature and mature B cells (81) (Table 1-2). The B cells from CD45-deficient mice are transitional rather than immature since immature B cells either express very low or no IgD on their surfaces, and do not express CD23 (81, 399), whereas B cells from CD45-deficient mice contain some cells that express CD23, and most cells express IgD. The decrease in mature B cells suggests that there is a maturational block in B cell development from CD45-/− mice. Therefore, CD45 may be necessary for the final maturational stage of B cells. Similar results were subsequently observed in CD45 exon-9 deficient mice, which lack all isoforms of CD45 (481).

We demonstrate that induction of tyrosine phosphorylation of some cellular proteins does occur normally in mice lacking CD45. Specifically CD45 is not required for induction of tyrosine phosphorylation of CD79α, PLCγ2, and activation of ERK2. The data also suggest that CD45 is not required for maintaining the basal level phosphorylation state of CD79α. However, in the absence of BCR stimulation, the CD45-deficient B cell plasmacytoma, J558L\(\mu\)m3, contains hyperphosphorylated CD79α, implicating CD45 in the maintenance of the basal level phosphorylation state of CD79α. Furthermore, upon BCR crosslinking, the MAP kinase, ERK2, was not activated in the CD45-deficient B cell plasmacytoma J558L\(\mu\)m3. (74, 76) In addition, J558L\(\mu\)m3 cells are unable to mobilize Ca\(^{2+}\) in response to receptor ligation, whereas B cells from mice lacking CD45 are able to mobilize Ca\(^{2+}\) from intracellular stores, but are not able to influx Ca\(^{2+}\) in response to receptor ligation. One explanation for these discrepancies is that the population
of B cells found in CD45<sup>-/-</sup> mice may have undergone selection for alternative pathways, which do not require CD45. According to this hypothesis, B cells from normal mice, having the luxury of using CD45 all their lives, may still depend on CD45 for the observed effects. Alternatively, the protein signaling differences observed in my studies compared to studies involving cell lines may be a reflection of the transformed state unique to each cell line, or of the maturational stage representative of each cell line. In agreement with my studies, tyrosine phosphorylation of PLCγ2 did occur in the CD45-deficient cell lines, J558Lμm3 and K46-17μmλ (461) (353). The tyrosine phosphorylation of PLCγ2 could have occurred by the actions of either p72<sup>tyk</sup> or btk, which are not affected by the lack of CD45 (76, 352, 353). A separate study also supported my findings by demonstrating a functional MAPK pathway in CD45-deficient B cells. CD45-deficient B cells from mice expressing a transgenic BCR specific for the HEL antigen did undergo ERK2 activation in response to polyclonal anti-IgM, although ERK2 was not activated in response to the less potent receptor crosslinking antigen, HEL<em> in vitro </em>(625).

This study demonstrates that B cells from CD45<sup>-/-</sup> mice are unable to proliferate in response to polyclonal anti-IgM. Similar results were obtained with CD45 exon 9<sup>-/-</sup> B cells, that are also unable to proliferate in response to anti-IgD crosslinking and to polyclonal anti-IgM stimulation (481). This is in contrast to the study by Kong et. al., (626) in which CD45 exon 6-deficient B cells proliferated with polyclonal anti-μ stimulation, but not with monoclonal anti-μ stimulation. A possible explanation for this difference may be due to the activation state of the starting population. In the Kong study, no zero time points were shown. Therefore, the starting cell population may already be in an activated state, which would affect the results.

We also demonstrate that the increase in the absolute number of low buoyant density B cells relative to high buoyant density B cells in CD45 exon-6-deficient mice does not account for the inability to proliferate upon BCR aggregation since both CD45<sup>-/-</sup> low and high buoyant density B cell populations were similarly unresponsive. Lower density splenic B cells may either represent cells that have become partially activated<em> in vivo</em>, or immature B cells that have not yet attained the resting stage of B cell differentiation, or a combination of both of these types of cells.
Recent evidence suggests that maturation from the immature to mature B cell stage requires signaling through the BCR (reviewed in Chapter 1, Section 5-d). Thus, the increase in the low buoyant density population of splenic B cells in CD45-deficient mice may reflect an arrest in maturation from the transitional to mature B cell, whereby a partial signal is sent through the BCR resulting in a blast-like phenotype, but is unable to complete the maturation to the small resting phenotype due to the lack of CD45. Some splenic B cells in CD45-deficient mice are able to attain a small dense size, but are still phenotypically immature with respect to cell surface molecules and the in vitro response to BCR aggregation.

The Kong study also found that CD45 exon 6-/- B cells could differentiate into antibody-producing cells upon stimulation with LPS+IL4, and undergo class switching. CD45-/- B cells were able to respond normally to type I or type II thymus(T)-independent antigens. However, IgG1 and IgE production upon stimulation with a T-dependent stimulus was drastically reduced, indicating that the lack of appropriate CD4+ T cell help was the cause. Adoptive transfer of CD4+ T cells into CD45 mutant mice now restored the IgG1 and IgE production by CD45-/- B cells in response to T-dependent stimulus. They infer from these results that CD45 is not required for B cell activation (626). However, it is not known whether the elimination of CD45 expression resulted in an adaptive developmental response leading to the preferential selection of B cells that have effectively compensated for the loss of CD45 through increased expression and/or function of PTKs or alternative PTPs.

We demonstrate here that in response to anti-IgM crosslinking, extracellular Ca²⁺ influx does not occur in CD45-/- B cells. Ca²⁺ response is rapid and attenuated, characteristic of release of Ca²⁺ from intracellular stores alone. Immature B cells do not undergo PI hydrolysis and subsequent IP₃ production (399). However, they do undergo Ca²⁺ mobilization upon BCR aggregation (399). Thus, BCR-mediated signals are uncoupled from the PI-pathway-linked signal transduction machinery in immature B cells. In T cells, Ca²⁺ increases in the absence of PKC activation (due to the lack of PI hydrolysis) have been observed to elicit a tolerogenic response (492). Since immature B cells also undergo Ca²⁺ increases in the absence of PI hydrolysis, this
may explain their lack of proliferation in response to BCR aggregation (83, 399, 486). In response to BCR aggregation, CD45-deficient B cells undergo tyrosine phosphorylation of PLCγ2, and increases in intracellular Ca²⁺, but do not undergo Ca²⁺ influx. It is unknown whether IP₃ is produced. However, intracellular Ca²⁺ release is due to IP₃ production (384, 386). Thus, IP₃ is likely produced in CD45-deficient B cells. Opening of the I_{CRAC} Ca²⁺ entry channel in the plasma membrane occurs upon depletion of intracellular stores. A high concentration of IP₃ is needed to open the I_{CRAC} channel (393). Thus, insufficient IP₃ generation in CD45-deficient B cells may be responsible for the lack of Ca²⁺ influx upon BCR aggregation. This hypothesis could be tested by measuring the amount of IP₃ generated upon BCR aggregation in CD45⁻/- B cells compared to wild type B cells.

Partial rescue of the proliferative response in CD45⁻/- B cells occurred by costimulation using anti-IgM+anti-CD40 or anti-IgM+IL4. The increase in the proliferative response was not due to restoration of Ca²⁺ influx since Ca²⁺ influx still did not occur. Therefore, while ligation of CD40 or the IL4 receptor-associated pathways conducted a proliferative signal that synergized with anti-IgM-induced aggregation, that signal was not dependent on Ca²⁺ influx or the presence of CD45. Signaling through CD40 alone or CD40 together with IL4 resulted in equivalent proliferative responses in both CD45⁺/+ and CD45⁻/- B cells. This may reflect the fact that Ca²⁺ influx mediated by CD45 is not a necessary component of those signaling pathways. Thus the results obtained in this study suggest that a signal that relies on CD45-mediated Ca²⁺ influx, that is mediated through signaling pathways activated upon BCR aggregation, must occur in order to restore proliferation to levels observed in BCR-stimulated CD45⁺/+ B cells. Indeed, although PKC activation alone is sufficient to synergize with IL4 in inducing B cells to enter cell cycle, the co-mitogenic effects of anti-Ig and IL4 are also dependent on Ca²⁺ signals generated through ligation of sIg (628). Immature B cells from neonatal spleen or adult BM do not proliferate upon BCR aggregation with anti-IgM antibodies (486, 487). Apoptosis induced by anti-IgM can be prevented by costimulation with IL4 in immature B cells derived from adult BM, but
not from neonatal B cells (486, 487). Based on this result, CD45-deficient B cells resemble adult BM-derived immature B cells rather than FL-derived immature B cells.

Further support for the role of CD45 in Ca\(^{2+}\) influx and proliferation is evident in CD45 exon 9\(-/-\) B cells, which were unable to proliferate in response to anti-CD38 stimulation (481). Triggering of CD38 with mAbs results in an influx of extracellular Ca\(^{2+}\) and an increase in tyrosine phosphorylation (398). It also induces proliferation of small resting B cells (629). Thus proliferation to CD38 may not have occurred in the absence of CD45 since this response depends in part on Ca\(^{2+}\) influx.

Tolerant HEL-specific B cells that develop in mice with a soluble HEL transgene undergo chronic activation of their BCR (390). This chronic stimulation results in asynchronous Ca\(^{2+}\) oscillations, which are a result of extracellular Ca\(^{2+}\) influx since the Ca\(^{2+}\) oscillations are abrogated by chelation of extracellular Ca\(^{2+}\). However, when these mice were crossed to CD45 exon 6\(-/-\) mice, the Ca\(^{2+}\) oscillations ceased (390), again further demonstrating that CD45 is necessary for Ca\(^{2+}\) influx.

Sustained increases in [Ca\(^{2+}\)]\(_i\) that are provided by Ca\(^{2+}\) influx are believed to be required for the proliferation and differentiation of resting B cells. Failure to reach a threshold intracellular Ca\(^{2+}\) concentration, as is seen in anergic B cells, leads to cell death (389-391). Thus, the defect in Ca\(^{2+}\) influx may be responsible for the lack of proliferation to anti-\(\mu\) and/or final maturation in CD45\(-/-\) B cells. The amplitude and duration of Ca\(^{2+}\) signals in B cells controls the differential activation of the transcriptional regulators NF-\(\kappa\)B, JNK and NFAT. NF-\(\kappa\)B and JNK are selectively activated by a large transient [Ca\(^{2+}\)]\(_i\) rise, whereas NFAT is activated by a low, sustained Ca\(^{2+}\) plateau. Differential activation results from differences in the Ca\(^{2+}\) sensitivities and kinetic behavior of the 3 pathways (389). Prolonged elevation of [Ca\(^{2+}\)]\(_i\) achieved by Ca\(^{2+}\) influx is required to maintain NFAT in an activated state in the nucleus (303). Ligation of the BCR on CD45\(-/-\) B cells does not induce Ca\(^{2+}\) influx, which likely does not lead to prolonged NFAT activation. The lack of prolonged NFAT activation may be responsible for the inability of CD45\(-/-\) B cells to proliferate upon BCR aggregation. Examination of NFAT activation in CD45\(-/-\) B cells
will help confirm this hypothesis. Another means of testing directly whether Ca\(^{2+}\) influx is necessary for BCR-mediated proliferation would be to use Ca\(^{2+}\) ionophore to artificially induce Ca\(^{2+}\) influx in CD45\(^{-/-}\) B cells in vitro together with anti-IgM antibodies. If CD45\(^{-/-}\) B cells are now able to proliferate, this would confirm that Ca\(^{2+}\) influx is required for BCR-mediated proliferation.
CHAPTER 3

Differential Expression and Characterization of AA4.1 on Murine B Cells.

Contents of this chapter have been submitted to the journal Blood. Differential Expression and Characterization of AA4.1 on Murine B lineage Cells. Tania Benatar, Jenny Jongstra-Bilen, and Christopher J. Paige.
INTRODUCTION

The hematopoietic system consists of distinct cell lineages that arise from a common multipotential stem cell by a developmentally regulated process. This developmental process takes place in the BM and thymus of adult animals, and, during embryonic development, predominantly in FL and thymus. Stem cells are undifferentiated cells that can maintain their numbers by self-renewal, while retaining their potential to differentiate into committed progenitor cells for all lymphoid and myeloid cells (66, 68) (630, 631). The study of stem cells and multilineage progenitors is difficult since the stem cells and multilineage cells are present in very low numbers in hematopoietic tissues (<1 in $10^4$-$10^5$ cells)(632, 633), and most studies rely on analysis of their clonal progeny. Another useful approach has been the characterization of the cell surface properties of these cells by coating with monoclonal antibodies that recognize determinants found on these cells. AA4.1 is a mAb that recognizes an as yet uncharacterized determinant present on the surface of cells of the hematopoietic lineage (13). The generation of AA4.1 has facilitated the enrichment of these populations and their subsequent characterization.

The fetal totipotent HSC was isolated and characterized based on AA4.1 expression. AA4.1 expression resulted in the enrichment of the totipotent HSC by a factor of 100-200 fold from day 14 FL (129). The expression of AA4.1 was not found, however, to enrich for the PHSC from adult BM. Rather, the data from this study suggests that AA4.1 is expressed as a stage specific marker of PHSC in cell cycle(130). AA4.1 expression was used to select for multilineage precursors from day 13 FL that contained erythroid, myeloid and B lymphoid precursors (12). The earliest B cell precursors that have the capacity to differentiate in vitro into Ig-secreting cells can be isolated based on AA4.1 expression from yolk sac and embryonic tissue from fetuses at day 8.5 (10-12 somite stage)(28). AA4.1 has also been used effectively to enrich for B cell precursors from day 12 FL that are uncommitted for Ig HC expression (25). The earliest B lineage stage in mouse BM has also been identified based on AA4.1 expression (18).
B lymphocyte development involves several distinct developmental stages that can be distinguished by the expression of specific cell surface markers (18, 39). Early studies found high levels of AA4.1 expression on all pre-B lymphomas, on only a few B lymphomas tested, and no expression on plasmacytomas or T cell lines (13). Adult BM contained the highest percentage of AA4.1+ cells. All cytoplasmic μ+ (cμ+) cells in the BM and just less than half of the sIgM+ BM cells were AA4.1+. A few cells in the periphery expressed AA4.1, although their identity was not examined (13). Together, these results suggested that either AA4.1 recognizes a subset of B cells, or the expression of AA4.1 is lost upon B cell maturation.

In the present study, we examined AA4.1 expression levels on different B cell populations in adult BM in combination with different maturation markers. We also examined AA4.1 expression on B cells in the spleen. Stimulation of splenic B cells resulted in modulation of AA4.1 expression levels, depending on the specific stimuli used. In addition, the protein recognized by AA4.1 was characterized based on size, glycosylation, and disulphide-linkage.
MATERIALS AND METHODS

Mice. C56BL/6 mice, 6-8 week old, were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed at the animal facility at the Wellesley Hospital Research Institute.

Cell lines. Cell lines used were: 70Z/3, pre-B cell lymphoma (sμ+) (634), 18-81, A-MuLV transformed pre-B lymphoma (sμ+) (635), M1, myeloid leukemia cell line (American Type culture collection (ATCC), Rockville, MD), MC-9, mast cell line (ATCC), NFS-70, pro-B lymphoblast (sμ−) (ATCC), J558 plasmacytoma (ATCC), and VCD28 T cell thymoma (a kind gift from Dr. Dominique Couez, Angiers, France).

B cell purification. Cell suspensions from BM were prepared by flushing tibias and femurs with a 26-gauge syringe. Single cell suspensions of splenic B cells were prepared by mechanical disruption through a metal screen using a syringe plunger, followed by a 5 min incubation on ice to remove large aggregates. Erythrocytes were lysed with ACK (0.155 M ammonium chloride, 0.1 mM disodium EDTA, 0.01 M potassium bicarbonate, pH 7.3) for 3 min on ice. The separation of BM cells based on AA4.1 expression was performed by panning as described using Optilux 100 mm plastic Petri dishes (Falcon no. 1001; Becton Dickinson, Mountain View, CA) (69). Petri dishes were coated with mouse anti-rat IgG (5 μg/ml; Jackson Immunoresearch, Jackson, ME) in 0.05 M Tris-HCl, pH 9.5, 0.15 M NaCl) at room temperature (RT) for a minimum of 1 h or overnight at 4°C. After washing the plates 3 times in Balanced Salt Solution containing 5% fetal calf serum (BSS/5% FCS), 5 ml of AA4.1 hybridoma supernatant, diluted 1:2, was applied for 1 h at RT or overnight at 4°C. The dishes were washed 3 times in BSS/5%FCS, and cell suspensions were then incubated on the plates for 60 min at 4°C. Nonadherent AA4.1− cells were recovered by three gentle washes with BSS/5% FCS. Adherent B220+ or AA4.1+ cells were recovered by
scraping with a plastic scraper (no. 3010; Costar, Cambridge, MA) after carefully washing the plates in 5% FCS/BSS.

**Cell culture conditions.** Cells were maintained in OPTI-MEM (Gibco/BRL, Grand Island, NY) supplemented with 50 μM 2-mercaptoethanol, 2.4 g/l NaHCO₃, 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco/BRL), 100μg/ml kanamycin sulfate (Gibco/BRL). BM cultures contained 10% FCS. Splenic B cell cultures contained 5% FCS that was heat-inactivated for 30 min at 56°C. LPS (Salmonella typhosa W0901; Difco, Detroit, MI) was added to BM cultures at a final concentration of 15 μg/ml.

**Isolation of resting B cells and B cell activation.** Small resting B cells were obtained as described (622). Briefly, erythrocyte-free spleen cells were T cell-depleted by lysing with anti-CD4 (RL172), anti-CD8 (3.168) and anti-thy 1.2 (H013.4) antibody supernatants (1/20 dilution) together with low tox guinea pig complement (1/10 dilution) (Cedarlane Laboratories Ltd., Hornby, Canada) for 1 h at 37°C. The remaining cells were separated on a Percoll gradient (Sigma Chemical Co., St. Louis, MO) as described (622). Small resting B cells were obtained from interfaces ≥1.085. For analysis of AA4.1 expression following B cell activation, resting splenic B cells were plated in 24-well plates at 4×10⁵/well with either B76 (rat IgG1 anti-mouse Igμ: 10 μg/ml, LPS (20 μg/ml), anti-CD40 (Pharmingen, San Diego, CA; 10 μg/ml), B76 + anti-CD40, B76 + IL4 (Genzyme Corporation, Cambridge, MA; 5 ng/ml), or anti-CD40 + IL4. AA4.1 expression was analyzed by flow cytometry on day 2.

**Flow Cytometry.** Cells (1-5×10⁵/sample) were washed and exposed to appropriate FITC-conjugated, phycoerythrin (PE)-conjugated or biotinylated antibodies for 15 min on ice. Cells were washed with Phosphate Buffered Saline (PBS) containing 5% FCS. Cells stained with biotinylated antibodies were further incubated for 15 min with Quantum Red-avidin (Sigma Chemical Co.) to reveal the biotin reagent. Before analysis, cells were fixed in 1%
paraformaldehyde. 10^4 cells/sample were analyzed on a FACScan® flow cytometer (Becton Dickinson and Co., San Jose, CA) equipped with a 15 mW 488 nm, air-cooled Spectra Physics argon-ion laser, using Lysys II software. The following mAbs were used: RA3-6B2 (B220, PE-labeled: PharMingen); 33-60 (anti-IgM; biotin-labeled; the hybridoma, 33-60 (636)); anti-IgD (PE-labeled; Southern Biotechnology Associates, Birmingham, AL); anti-CD22 (Cy34.1-PE, PharMingen); anti-HSA (Biotin-labeled M1/69, PharMingen); anti-BPI (6C3-FITC, PharMingen); AA4.1 (FITC-labeled, supernatant, (13)).

**Surface Biotinylation.** Cells were washed three times with ice-cold PBS (pH=8), and then resuspended at 2.5x10^7 cells/ml in PBS (pH=8). Biotinylation was performed by adding 0.5 mg of sulfo-NHS-LC-biotin (Pierce, Rockford, IL) per ml reaction volume for 30 min at RT. Cells were then washed 3 times with ice-cold PBS (pH=8) and subsequently exposed to lysis buffer.

**Immunoprecipitation.** Cells were lysed (at 5x10^7/ml) in Lysis buffer (50 mM Tris, 150 mM NaCl, 2 mM EDTA) with protease inhibitors (10 μg/ml aprotinin and leupeptin, 1 mM PMSF) for 30 min on ice. Nuclei were removed by centrifugation at 12,000 rpm for 5 min at 4°C. Immunoprecipitation was performed by addition of 30 μg/ml of AA4.1 or MAC-1 mAb (from hybridoma, ATCC) for 1 h at 4°C, followed by addition of protein G-sepharose (Pharmacia Biotech, Baie d'Urfe, Quebec) for 1 h at 4°C. Immune complexes were washed 3 times with lysis buffer, and bound proteins were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto Immobilon PVDF membranes (Millipore, Bedford, MA). For reduced proteins, samples were boiled in 2x Laemmlli Sample Buffer (637) containing 10% 2-mercaptoethanol. Nonreduced samples did not contain 2-mercaptoethanol. Membranes were blocked for 1 h in PBS containing 0.1% tween 20 with 1% milk at RT, and then probed for 1 h at RT in 1% milk with Streptavidin-HRP (Amersham Corp., Arlington Heights, IL). Proteins were then visualized by exposure to enhanced chemiluminescence (ECL).
Deglycosylation of N and O-linked proteins. N-linked sugars were removed as follows: After immunoprecipitation, bound proteins were removed from beads by boiling for 5 min in PNGase buffer [0.15 M sodium phosphate buffer (pH 7.7), 0.06 M EDTA, 0.9% SDS, 1% 2-mercaptoethanol]. Eluted proteins were diluted with five volumes of 0.9% NP40 prior to enzyme digestion. N-linked proteins were then removed by addition of 8 U per ml of sample volume of N-glycosidase F (Boehringer Mannheim, Mannheim, Germany) at 37°C overnight. O-linked sugars were removed as follows: Immunoprecipitated proteins were removed from beads by boiling in 50 mM Na₂HPO₄ (pH 6.5) + 1% SDS at 50μl/10⁷ cells for 5 min, followed by dilution in 10 volumes of 1% NP40. Prior to cleavage of O-linked sugars, sialic acid residues were removed by addition of 120 mU/10⁷ cells of Neuraminidase (Boehringer Mannheim) for 30 min at 37°C. O-glycosidase (Boehringer Mannheim) was then added at 0.14 mU/10⁷ cells to remove O-linked sugars. Samples were incubated at 37°C overnight, prior to separation of deglycosylated proteins on SDS-PAGE.
RESULTS

**AA4.1 Expression in BM is downmodulated upon maturation.** Previously, it was thought that the expression of AA4.1 was lost upon B cell maturation (13). I examined the expression of AA4.1 on developing B cells in the BM together with cell surface markers involved in B cell maturation. Lymphocyte-gated BM was analyzed by 3 colour flow cytometry for AA4.1 expression. The B220⁺ IgM⁻ BM fraction contains the highest level of AA4.1 expression (fig. 3-la and b). This fraction consists of immature B cell progenitors. Upon maturation, cells acquire sIgM on their surfaces. The sIgM⁺ cells in the BM contain a population of cells with high AA4.1 expression levels and a smaller population with lower AA4.1 expression levels (fig. 3-lc and d).

CD22 has been shown to be expressed early in B cell development in the BM at low levels, and is increased upon sIgM expression (638). The surface phenotype of BM cells were examined by 3 colour flow cytometry for IgM, CD22 and AA4.1 expression (figs. 3-1c and d). The highest levels of AA4.1 are found on the more immature sIgM⁻ CD22lo BM population (fig. 3-1c and d; group 1). As cells mature further, expression of sIgM is attained. The sIgM⁺ CD22lo/med cells contain a small population of AA4.1lo-expressing cells (fig. 3-1c and d; group 2). Finally as cells reach maturity, they acquire high levels of CD22 (638, 639). The acquisition of high levels of CD22 is coincident with the majority of these cells expressing low levels of AA4.1 (fig. 3-1c and d; group 3). Thus AA4.1 decreases from high to low levels upon B cell maturation in the BM. This occurs upon sIgM expression and prior to acquisition of high CD22 levels. Similar results were obtained from 3 separate experiments.

**Expression of AA4.1 on splenic B cell subsets.** Early studies had suggested that mature B cells in the periphery do not express AA4.1 (13). In order to verify this point, I examined AA4.1 expression on B cells from adult spleen (fig. 3-2a). I found that all B220⁺ splenic B cells do in fact express low levels of AA4.1 on their surfaces (fig. 3-2a). Splenic B cells can be further separated based on levels of sIgM versus sIgD into μhiδlo immature B cells
Figure 3-1. AA4.1 expression is decreased upon maturation. Bone marrow cells falling within the viable lymphocyte gate were analyzed by three-colour flow cytometry. (a) Cells were stained with FITC-AA4.1, PE-anti-B220, and biotin-anti-μ followed by avidin-quantum red. (b) The expression of AA4.1 of gated B220+ μ- (1) and gated B220+μ+ (2) is presented. (c) Cells were stained with FITC-AA4.1, PE-anti-CD22, and biotin-anti-μ followed by avidin-quantum red. (d) The expression of AA4.1 of gated CD22loμ- (1), CD22medμ+ (2) and CD22hiμ+ (3) are depicted.
Figure 3-2. AA4.1 is expressed on splenic B cells at low levels. Total adult spleen cells were stained. (a) Cells were double stained with FITC-AA4.1 and PE-anti-B220. (b) Cells were 3-colour stained with FITC-AA4.1, PE-anti-IgD, and biotin-anti-μ followed by avidin-quantum red. (c) The expression of AA4.1 of gated μ^{hi}δ^{lo} (1) and μ^{lo}δ^{hi} (2) are shown.
(fig. 3-2b, region 1), and $\mu^{lo}B_{hi}$ mature B cells (fig. 3-2b, region 2). Results demonstrate that the immature $\mu^{hi}B_{lo}$ B cells in the spleen express higher levels of AA4.1 in comparison to the mature $\mu^{lo}B_{hi}$ population. Similar results were obtained from 4 separate experiments.

**Assessment of AA4.1 expression during B cell activation.** The activation of mature, unstimulated B cells requires either the interaction of specific antigen with the B cell Ig receptor alone (T-independent response), or additionally, T cell-derived signals mediated by CD40 and/or cytokine receptors (T-dependent response) (517, 518, 640). A variety of *in vitro* stimulation techniques have been developed to mimic these responses. We chose to examine whether the activation of mature resting B cells by various stimuli that result in B cell entry into cell cycle could modulate AA4.1 expression. The stimuli chosen consisted of anti-IgM (fig. 3-3a), anti-IgM+anti-CD40 (fig. 3-3b), anti-IgM+IL4 (fig. 3-3c), anti-CD40+IL4 (fig. 3-3d), and LPS (fig. 3-3e,f). These stimuli are known to induce B cell entry into cell cycle (476, 542, 543, 596, 641, 642). Results show that AA4.1 expression levels either decreased moderately (fig. 3-3, panel a, c and d), or remained unchanged (fig. 3-3, panel b) upon exposure to stimuli that mimic antigen encounter (anti-IgM) and/or T-dependent stimuli (anti-IgM+anti-CD40; anti-IgM+IL4; anti-CD40+IL4). In contrast, upon exposure to the T-independent type 1 stimulus, LPS, AA4.1 expression levels increased after 2 days (fig. 3-3e), and continued to rise to higher levels when assessed at 4 days post stimulation (data not shown). The rise in AA4.1 expression levels was not simply a consequence of the increase in cell size with stimulation since the levels of sIgM expression remained unchanged after LPS stimulation (fig. 3-3f). B cells increased cell size in response to all the stimuli described in figure 3-3, although AA4.1 expression levels increased only upon LPS stimulation. All stimuli shown in figure 3-3 resulted in B cell activation, as assessed by increased MHC class II expression after 2 days (data not shown).

**Immunoprecipitation of the AA4.1 protein.** The identity of the protein recognized by AA4.1 was previously unknown. To initiate characterization of the AA4.1 protein, I determined
Figure 3-3. AA4.1 expression is modulated following B cell activation. High buoyant density splenic B cells were cultured in 24 well plates with various stimuli for 48 h. Cells were harvested and stained with FITC-AA4.1 (solid line) or FITC-rat IgG2b isotype control (dashed line). They were compared against unstimulated splenic B cells stained with FITC-AA4.1 (bold line). Cells were stimulated with (a) anti-IgM, (b) anti-IgM + anti-CD40, (c) anti-IgM + IL4, (d) anti-CD40 + IL4, (e) LPS. Cells were stimulated with LPS and stained with FITC-anti-IgM (f).
Figure 3-4. AA4.1 recognizes a 115-130 kd protein on AA4.1+ cell lines. 6x10^6 biotin-surface-labeled cells were lysed in 1% NP40 and then subjected to immunoprecipitation with either mAb AA4.1 or MAC-1. The specifically bound material was eluted in sample buffer, separated on 8% SDS PAGE, and transferred to PVDF membrane. Membranes were probed with Streptavidin-HRP. The migration position of molecular weight markers are indicated on the left. The numbers on the right identify the lane numbers corresponding to the different cell lines. The status of AA4.1 expression in each cell line based on immunofluorescence (FACS) analysis is indicated.
Figure 3-5. AA4.1 also recognizes similar 115-130 kd protein in AA4.1-panned BM. Adult BM cells were separated by panning on AA4.1 coated plates. Nonadherent AA4.1- cells and adherent AA4.1+ cells were collected, and subjected to immunoprecipitation after prior surface biotinylation, as described in fig. 3-4. Membranes were probed with Streptavidin-HRP. AA4.1+ cell line, 70Z/3, and AA4.1- cell line, J558, were included as controls.
the molecular mass of proteins immunoprecipitated from lysates obtained from various cell lines (fig. 3-4). Preliminary experiments demonstrated that the AA4.1 mAb is not suitable for the Western Blotting technique. Therefore, the protein was visualized by biotinylating cell surface proteins and revealing the precipitated proteins with streptavidin coupled to HRP. AA4.1 surface expression was also verified on the cell lines by FACS, summarized on the right of figure 3-4. Results revealed a protein of approximately 115-130 kDa in cell lines that were positive by FACS for AA4.1 (fig. 3-4; lanes 1, 2, 4, 5). As an isotype control, MAC-1 mAb was used for immunoprecipitation in parallel, and as expected, the 170 kDa αm CD11b and 95 kDa CD18 β2 integrin chains were only visible in the M1, myeloid leukemic cell line. AA4.1 was also immunoprecipitated from normal BM cells isolated by panning on AA4.1-coated plates into the nonadherent AA4.1- fraction, and the AA4.1+ adherent fraction. Surfaces were biotinylated, and AA4.1 was immunoprecipitated from the lysates. As a comparison, identical treatments were applied to the AA4.1+ pre-B cell line, 70Z/3, and the AA4.1- plasmacytoma cell line, J558. Results shown in figure 3-5 reveal a band of approximately 115-122 kDa, which was specifically precipitated by the AA4.1+ mAb.

**AA4.1 protein is not part of a disulphide linked complex.** Many cell surface molecules exist as disulphide-linked proteins, such as cytokine receptors, B, and T cell receptors. In order to determine whether AA4.1 is part of a covalently-linked complex, biotinylated surface proteins immunoprecipitated by AA4.1 were separated under reducing or nonreducing conditions (fig. 3-6). Results demonstrate that the AA4.1 protein migrates at the same molecular weight under either reduced or nonreduced gel electrophoresis conditions. This indicates that AA4.1 does not exist as a covalent, disulphide-linked molecule.

**Glycosylation state of AA4.1 protein.** The broad nature of the 115-130 kDa band and slightly varying sizes in different cell lines suggested that AA4.1 may be glycosylated. To reveal N-linked glycosylation, AA4.1 was immunoprecipitated from cell surface biotinylated 18-81 cells
The figure shows the expression analysis by FACS for AA4.1. Strains of AA4.1 were analyzed by FACS. Cell lines were loaded per lane with 106 cells per lane. The cell lines correspond to the various cell lines indicated on the right. Cell equivalents were loaded per lane. Lane numbers correspond to the various cell lines, indicated on the right. Cell equivalents were loaded per lane. Lane numbers correspond to the various cell lines, indicated on the right. Cell equivalents were loaded per lane. Lane numbers correspond to the various cell lines, indicated on the right.

**Figure 3-6. AA4.1 Lacks covalent linkage with other proteins.** Cell lines were subjected to surface labeling and immunoprecipitation with AA4.1 as described in the figure. Proteins were separated under reducing conditions by electrophoresis in sample buffer containing beta-mercaptoethanol, or nonreducing conditions by electrophoresis in sample buffer containing beta-mercaptoethanol. Blots were probed with streptavidin-HRP. 7X106 cell equivalents were loaded per lane. Lane numbers correspond to the various cell lines, indicated on the right. Cell equivalents were loaded per lane. Lane numbers correspond to the various cell lines, indicated on the right. Cell equivalents were loaded per lane. Lane numbers correspond to the various cell lines, indicated on the right.
performed from 1.5x10^7 cell equivalents.

O/N at 37°C. Neuraminidase was removed as described in previously 2 samples, except enzymes were omitted. Immunoprecipitation was performed from 1.5x10^7 cell equivalents.

Figure 3-7. AAV1 is a glycoprotein rich in O-linked carbohydrates. N-linked sugars were analyzed from 70/37 cells (A). B: synapsin.
and subsequently treated with N-glycosidase F. This procedure removes high mannose and complex N-glycans. As shown in Figure 3-7A, there is a small shift in the mobility of AA4.1 upon enzyme addition, suggesting the existence of a few N-linked sugars, supporting the conclusion that AA4.1 is a glycoprotein. The presence of O-linked sugars was examined by treatment with neuraminidase and O-glycosidase (fig. 3-7B). The neuraminidase treatment resulted in a slight increase in mobility, suggesting the presence of some sialic acid residues. However, when O-glycosidase was added together with neuraminidase, a large increase in mobility was observed, resulting in a band of 86-90 kDa in size. This demonstrates that AA4.1 contains abundant O-linked sugars. The difference in the amount of O-linked sugars could account for the slightly different sized bands between various cell lines.
DISCUSSION

AA4.1 has been successfully used in the past as a marker to isolate early B lineage cells (7, 12, 25, 28, 129, 643). Early studies have shown that AA4.1 is expressed on all cd+ cells but on less than half of sIgM+ cells in adult BM, and at very low percentages in the periphery, suggesting that either AA4.1 is lost upon further maturation or that AA4.1 recognizes a subset of B cells (13). This report shows that AA4.1 is expressed at high levels on developing B cells in the BM, and is not lost upon maturation, but is rather downregulated to low levels. The decrease in AA4.1 expression occurs upon sIgM expression and prior to acquisition of high CD22 expression levels.

The majority of B cells in the spleen are of a mature sIgMlo sIgDhi phenotype, whereas a minority of splenic B cells are of an immature or transitional sIgMhi sIgDlo phenotype (352) (81). AA4.1 is expressed on all splenic B cells, and also decreases upon maturation in that site. Decreased AA4.1 expression in the spleen correlates with increased sIgD expression since sIgDhi sIgMlo splenic B cells express lower levels of AA4.1 than sIgDlo sIgMhi cells (fig. 3-2). Thus the progressive decrease in AA4.1 expression coincides with B cell development in the BM, and B cell maturation in the spleen. The decreased levels of AA4.1 expression may reflect an inhibitory effect of AA4.1 on later stages of B cell development in the BM, and/or selection into the mature, peripheral B cell pool.

The expression of AA4.1 on mature B cells can be modulated depending on the nature of the activation stimuli. AA4.1 expression levels remain either unchanged at low levels, or decrease to even lower levels upon stimulation under various conditions, such as anti-IgM, anti-IgM+IL4, anti-IgM+anti-CD40, or anti-CD40+IL4. Anti-CD40 and IL4 mimic stimuli provided by T cell help in an ongoing immune response. Low levels of AA4.1 expression may be beneficial in such circumstances. AA4.1 is a large protein, with extensive O-linked sugars. O-glycosylated regions of proteins are typically extended, rigid structures (644). Higher expression levels of AA4.1 on B cells may interfere with cell-cell interactions, which are necessary during T cell contact. In contrast, signals generated by the type 1 T-independent stimulus, LPS, result in increased AA4.1 surface
expression. This may reflect an additional function of AA4.1 in plasma cell formation and/or IgM secretion.

It has been suggested that AA4.1 expression is associated with HSC that are in cell cycle. FL HSC are AA4.1+ and are thought to be in a state of exponential growth in comparison to BM HSC which are thought to be in Go, and not dependent on AA4.1 expression (130). We demonstrate that AA4.1 expression is at highest levels on B220+IgM- BM cells, which would be expected to contain significant numbers of cells in cycle as compared to the more mature B220+IgM+ cells (39). Mature B cells in the spleen are believed to be in Go, which would correlate with low AA4.1 levels. If AA4.1 was simply a cell cycle associated marker, then stimulation of splenic B cells with each of the stimuli that were used in this study should result in increased AA4.1 levels. However, only LPS stimulation resulted in increased AA4.1 expression. Thus, AA4.1 does not appear to be a cell cycle associated marker, at least not on mature splenic B cells.

We demonstrated that AA4.1 is a 115-130 kDa glycoprotein that is not found in a covalently-linked form. This does not exclude possible noncovalent associations with other proteins. Our method of visualization of AA4.1 is based on surface expression. Although we did not detect any other surface proteins co-precipitating with AA4.1, AA4.1 might associate with intracellular signaling molecules through noncovalent interactions, which could not have been detected under our experimental conditions. The use of non-ionic detergents milder than NP40 did not reveal any AA4.1-associated proteins (data not shown). Therefore, the lack of any AA4.1-associated proteins in the NP40 lysates is not simply due to dissociation of these proteins under harsh conditions.

The fact that AA4.1 is expressed at the earliest stages of development, and AA4.1 expression is never extinguished throughout the lifespan of the B cell, and can be modulated by specific stimuli, suggests a crucial and perhaps conserved function during B cell development and activation. It will be important to discover the exact function, therefore, of AA4.1 on B cells and other hematopoietic cell lineages.
Chapter 4
DISCUSSION

In this thesis I have characterized 2 proteins expressed in early B cell development and maturation. AA4.1 is expressed at high levels at the earliest B cell committed stage, and its expression decreases upon maturation to sIgM+ B cells. Mature resting B cells express AA4.1 at low levels. Upon B cell stimulation, AA4.1 expression levels are modified such that stimuli that mimic T-dependent signals result in decreased AA4.1 expression, whereas the T-independent stimulus, LPS, resulted in increased AA4.1 expression.

CD45 is also expressed at the earliest stage of B cell commitment, but the effects of CD45 on B cell development are not observed until the transition from the immature to the mature B cell stage. In the absence of CD45, B cells are blocked at this transition. Stimulation of B cells in the absence of CD45 does not affect early signaling pathways such as the tyrosine phosphorylation of CD79, an event critical in the initiation of B cell signaling. In addition, the lack of CD45 does not appear to affect the activation of the ERK2 MAP kinase, or the tyrosine phosphorylation of PLCγ2. However, while the downstream release of intracellular Ca^{2+} is unaffected, the influx of intracellular Ca^{2+} is abrogated. Thus CD45 appears to be necessary for the induction of Ca^{2+} influx upon BCR stimulation. CD45 is also necessary for the transition into the mature recirculating B cell pool. It is unclear whether Ca^{2+} influx is required for the transition to the mature resting B cell pool.

Possible function of AA4.1 in B cells.

My studies have demonstrated that AA4.1 is a heavily glycosylated protein, particularly rich in O-linked sugars. O-glycosylation renders some proteins resistant to proteases (644). For example, when the membrane protein, LDL receptor, is expressed in a mutant of the Chinese hamster ovary cell line that is defective in O-glycosylation, the major effect noted is rapid
proteolytic cleavage of the protein and release into the media (645). Most cells are completely covered with a carbohydrate layer termed the glycocalyx, which consists of glycoproteins and glycolipids inserted in the cell membrane, and proteoglycans, which may be more loosely associated with the cell surface (646). Abundant O-linked sugars on a protein allow it to adopt a rigid rodlike structure that can extend beyond the glycocalyx on the cell's surface. AA4.1 may function by extending above the surface of the B cell, protecting the surface proteins from protease degradation.

There is evidence that O-linked sugars participate in cell-cell interactions (646, 647). AA4.1 is 115-130 kDa in size, similar in size to 2 other heavily O-glycosylated surface proteins, CD43 and CD34, which are also expressed early in B cell development. CD43 is a 90-120 kDa protein that plays a role in cellular adhesion and cellular activation. CD43 has been described as containing both adhesive and anti-adhesive properties (112, 116, 119, 120) (refer to Chapter 1: Section 1d(iv)). Ligands have been described for CD43 such as ICAM-1, E-selectin, and galectin-1 (125, 127, 128). CD34 is a 105-120 kDa protein expressed on HSC, myeloid and lymphoid progenitor cells as well as on nonhematopoietic cell types (648). It maps to an area of the chromosome containing other adhesion molecules (649, 650). CD34 was shown to be a ligand for L-selectin, the lymphocyte homing receptor (651).

The early stages of B cell development have been found to depend on interactions with cells of the BM microenvironment called stroma. The molecular interactions between stromal cells and B cell progenitors are essential for B cell development and involve a variety of both adhesion molecules and growth factors (133, 135). The similarities in glycosylation and temporal expression between AA4.1, CD43 and CD34 raise the possibility that AA4.1 may also be involved in lymphocyte adhesion. However, AA4.1 contains relatively few sialic acid residues, whereas both CD43 and CD34 are extensively sialated, imparting them with a net negative charge (100, 652), which may also prevent cell-cell interactions at certain stages of development.

The earliest B cell precursors in the BM are found in the subendosteal region, peripherally adjacent to the surrounding bone. As the B cell progenitors mature, they migrate toward the central
region of the BM along a network of stromal cell processes. Immature B cells, expressing sIgM but not sIgD congregate within a sinusoidal compartment before exiting into the peripheral circulation via the central venous sinus (136). My studies demonstrate that AA4.1 is expressed at high levels on the earliest B cell progenitors. AA4.1 is decreased to low levels upon maturation after sIgM is expressed. Therefore, if AA4.1 was involved in adhesion, high levels of AA4.1 on the developing B cell surface would enhance the attachment to crucial stromal elements necessary for this development. Perhaps this interaction involves the binding of AA4.1 to a specific ligand on the stromal cell. As the cell matures to a sIgM+ immature B cell, it decreases AA4.1 expression, which would decrease interactions with stromal elements, thereby facilitating the release of B cells into the periphery.

Upon activation of the B cell, AA4.1 expression levels are modified. AA4.1 tends to decrease in expression upon activation with stimuli that mimic T-dependent responses. In such responses, direct contact between the T and B cell are crucial for the B cell to become activated. If a ligand for AA4.1 is not present on the T cell, then perhaps the presence of such a large rigid rodlike molecule on the B cell surface may prevent optimal contact. In contrast, upon B cell activation with the T-independent stimulus, LPS, AA4.1 increases in expression to high levels. Cell-cell contact is not required for B cell responses to LPS. The increase in AA4.1 expression may reflect a function of AA4.1 in plasma cell generation.

So far, experiments to test the function of AA4.1 in B cells have not been revealing. Experiments which involve the addition of mAb AA4.1 to in vitro B cell progenitor culture systems to try and block their development, as well as the addition of mAb AA4.1 to splenic B cells in vitro to try and block their responses to mitogens have all yielded negative results. Possible explanations for such consequences may be that mAb AA4.1 is not a blocking or stimulating antibody. Alternatively, blocking by mAb AA4.1 may occur, but the function that AA4.1 may serve in B cell development may not be necessary for in vitro maturation, and may only be required in vivo. For example, AA4.1 may bind to a ligand on a stromal cell in vivo, and modulate the maturation of the B cell, but the function of this ligand binding may not be necessary for maturation in vitro due to
the presence of factors in the media that substitute for its function. Alternatively, in vitro maturation may bypass the need for the binding of AA4.1 to its ligand, similar to the development of λ5-deficient B cells, which undergo λ5-independent B cell maturation in vitro (653). Injection of AA4.1 mAb into mice in vivo, and subsequent examination of B cell development, may help distinguish these possibilities, and may shed light on the function of AA4.1.

Future experiments to determine the function of AA4.1

Assuming that the gene for AA4.1 is cloned, experiments could be performed to assess the function of AA4.1 in early and late B cell differentiation. Since AA4.1 is expressed on early B cell progenitors at high levels, the ablation of the gene will address the function of AA4.1 in early B cell development. The absence of any B cells would suggest a critical function of AA4.1 in B cell commitment. However, the lack of AA4.1 expression may result in a block later in development, suggesting either functional redundancy at earlier timepoints, and/or a more crucial role of AA4.1 function at later stages of B cell development. The lack of AA4.1 expression may help address the function of AA4.1 in plasma cell generation, by examining whether B cells are able to secrete Ig in response to LPS stimulation in B cells derived from AA4.1 gene knockout mice.

Overexpressing AA4.1 particularly at later stages of B cell development and/or in the periphery would address the hypothesis that AA4.1 is inhibitory to later stages of B cell differentiation when expressed at high levels. If the immunization of mice that contain high levels of AA4.1 by genetic overexpression with T-dependent antigens results in deficient primary and/or secondary antibody responses, this would suggest that AA4.1 is indeed inhibitory to T-B cell contact.
Transition from immature to mature B cells.

The transition from immature to mature B cell is believed to depend upon signals generated through the BCR. The signaling threshold determines whether a B cell will be positively selected into the mature recirculating B cell pool. Too weak a signal leads to the lack of positive selection into the mature B cell pool, whereas too strong a signal leads to negative selection by deletion. Evidence to support a signaling threshold can be found in signaling deficient mice. B cells from mice lacking either CD79α, syk, btk, or lyn and CD45 show a partial or complete lack of recruitment of B cells into the mature B cell pool (231, 326, 339, 511-513).

In the HEL/anti-HEL double transgenic mice, CD45 seems to be required for inhibition of B cell survival by soluble self-antigens. The presence of circulating HEL auto-Ag results in negative selection of autoreactive CD45+ B cells, whereas loss of CD45 leads to a decrease in BCR-mediated signal intensity, potentiating the positive selection of autoreactive B cells (625). According to this study, in order for mature B cells to accumulate, signaling through the antigen receptor is required, and the amount of signaling determines positive or negative selection. In the absence of CD45, HEL/anti-HEL double transgenic B cells are positively selected into the mature B cell pool, since engagement of antigen receptors by soluble self-antigen induces a weak signal that was sufficient to promote the maturation of CD45−/− B cells. This suggests that a basal level of antigen-receptor signaling is necessary for normal accumulation of mature long-lived B cells, and this signaling is deficient in nontransgenic CD45−/− B cells. CD45 appears to positively regulate the B cell selection and promotes the elimination of autoreactive B cells by negative selection.

The cytosolic tyrosine phosphatase, SHP-1, acts as a counterbalance to CD45. Motheaten (Me or Mev) mice contain a disrupted SHP-1 (654, 655). These animals contain high levels of serum Ig and autoantibodies. B cells from Mev mice are hyperresponsive to BCR stimulation (439). HEL transgenic B cells deficient in SHP-1 show a greatly exaggerated rise in intracellular Ca2+ compared to wild type B cells, establishing that SHP-1 negatively regulates antigen receptor signaling in B cells. sHEL/anti-HEL double transgenic mice defective in SHP-1 undergo deletion
in the BM when exposed to self-antigen, in contrast to wild type B cells that are not deleted by sHEL (357). SHP-1 therefore appears to regulate signals from the BCR negatively, and in the absence of this negative signaling by SHP-1, the BCR is hyperactivated resulting in deletion due to negative selection. The removal of both CD45 and SHP-1 allows cells to accumulate in the mature B cell pool (656). Therefore, it is the balance of these 2 phosphatases, CD45 and SHP-1, that determines B cell fate and selection. In the absence of CD45, SHP-1 negative signaling predominates, lowering signaling through the BCR to levels below that of the activation threshold necessary for selection into the mature B cell pool. In the absence of SHP-1, CD45 positive signals predominate, resulting in hyperactive B cells, raising signaling through the BCR to levels that exceed the signaling threshold required for maturation, resulting in deletion. By removing CD45 and SHP-1, both positive and negative signals are removed and the signaling threshold returns to wild type levels to allow normal selection into the mature B cell pool.

**Does the lack of CD45 block maturation?**

We demonstrate that CD45−/− B cells are phenotypically immature, which suggests that the presence of CD45 is required for selection of B cells into the mature B cell population. An alternative possibility, as is always possible in any gene 'knockout' model, is that in the absence of CD45, the only B cells that are able to develop are those that resemble transitional B cells phenotypically, but are not actually equivalent to any identifiable B cell population in normal mice. In this situation, the lack of Ca²⁺ influx in response to BCR aggregation would likely be a consequence of the type of B cells that develop rather than the cause. However, most of the discussion in this thesis is based on the assumption that CD45-deficient B cells are in fact equivalent to their transitional B cell counterparts in normal mice.
Influence of Ca\textsuperscript{2+} influx on B cell maturation.

My studies demonstrate the importance of CD45 in the induction of Ca\textsuperscript{2+} influx in response to BCR ligation, as well as the importance of CD45 in selection of B cells into the mature B cell pool.

It is still unclear whether Ca\textsuperscript{2+} influx is required for the transition from immature to mature B cells. It was mentioned in Chapter 2 that in the double transgenic HEL/anti-HEL B cells, chronic stimulation by autoantigen results in asynchronous Ca\textsuperscript{2+} oscillations that were a result of Ca\textsuperscript{2+} influx. The HEL/anti-HEL B cells do not undergo the Ca\textsuperscript{2+} oscillations in the absence of CD45, although they are selected into the mature B cell pool (390). These results suggest that Ca\textsuperscript{2+} influx may not be required for positive selection into the mature B cell pool. Alternatively, chronic stimulation with autoantigen early in B cell development may select for cells that utilize signal pathways leading to maturation that bypass the requirement of Ca\textsuperscript{2+} influx that normally would be necessary for this maturational process in nontransgenic B cells. An examination of Ca\textsuperscript{2+} influx in mice deficient in both CD45 and SHP-1, in which mature B cell pool levels are restored, may help resolve this issue. If Ca\textsuperscript{2+} influx does not occur in these mice, it would suggest that Ca\textsuperscript{2+} influx is not required for the transition from immature to mature B cells. Normal immature B cells do undergo Ca\textsuperscript{2+} mobilization and influx upon BCR aggregation (399), and are able to mature. In contrast, CD45-deficient B cells do not undergo Ca\textsuperscript{2+} influx, and are blocked in maturation. This suggests that a necessary component of maturation may be the capacity to induce Ca\textsuperscript{2+} influx upon BCR aggregation.

How the lack of CD45 leads to abrogated Ca\textsuperscript{2+} influx.

The BCR exists in an equilibrium between phosphorylated and unphosphorylated states that is normally tuned by balancing BCR density, src family and syk PTKs, and signal-enhancing coreceptor docking sites (e.g., CD19/CD21) against phosphatases (e.g., SHP-1) and inhibitory
coreceptor docking sites (e.g., CD22 and FcγRIIB1). A basal level of tyrosine phosphorylation of CD79, syk PTK, CD22 and CD19 exists in the absence of antigen, and is rapidly increased when antigen receptors cluster upon activation (245, 270, 413, 431). In the absence of CD45, the balance is skewed towards negative regulators of BCR signaling. There are likely compensatory molecules that serve in place of CD45 in some of the signaling pathways, since the phosphorylation of CD79α, PLCγ2 and ERK2 are not affected when CD45 is absent. The lack of BCR-mediated Ca²⁺ influx observed in CD45⁻/⁻ B cells may be responsible for decreasing the positive signal that is required for the transition into the mature B cell pool. The mechanism by which this occurs is unclear. Mice deficient in lyn and CD22 have elevated Ca²⁺ with an increase in the peak intracellular Ca²⁺ rise as well as in the sustained Ca²⁺ rise, suggesting both intracellular Ca²⁺ and Ca²⁺ influx are affected (351, 356). In addition, cell lines deficient in CD22 expression are hyperresponsive to BCR aggregation, and contain enhanced Ca²⁺ influx (657). This suggests that CD22 has a negative role in Ca²⁺ signaling, particularly in Ca²⁺ influx. Therefore, the lack of CD45 may shift the balance towards the negative signaling pathways, so that instead of lyn interacting with CD45, it is free to interact with CD22. This results in the tyrosine phosphorylation of the ITIM of CD22, and subsequent recruitment of SHP-1, leading to the activation of SHP-1. Evidence to support this is derived from lyn-deficient B cells, as the induced tyrosine phosphorylation of CD22 and the recruitment and activation of SHP-1 are impaired (359). The end result is that although some signaling pathways are activated normally, as was also seen in lyn⁻/⁻ B cells, Ca²⁺ influx is abrogated in CD45⁻/⁻ B cells, possibly due to CD22 and the actions of the associated negative regulator, SHP-1.

BCR-induced proliferation can be inhibited by the co-aggregation of BCR and FcγRIIB1 (658-660). Specifically, this co-aggregation inhibits the influx of extracellular Ca²⁺(661, 662). Additionally, co-aggregation of BCR and Fc receptors subsequent to the opening of the plasma membrane Ca²⁺ channel(s), stimulates the closure of the channel (662). This co-aggregation results in the tyrosine phosphorylation of the ITIM residue in the cytoplasmic tail of FcγRIIB1, which subsequently recruits SHIP (663, 664). SHIP catalyzes the hydrolysis of the membrane
phospholipid PI \( (3,4,5)P_3 \) to PI \( (3,4)P_2 \) and the soluble inositol polyphosphate Ins \( (1,3,4,5)P_4 \) to Ins \( (1,3,4)P_3 \) (665, 666) (see fig. 1-6). SHIP recruitment is thought to be responsible for the block in Ca\(^{2+}\) influx (664, 667), and is supported by the demonstration that SHIP\(^{-/-}\) DT40 cells exhibit enhanced Ca\(^{2+}\) influx upon BCR aggregation (668). By reducing the level of PIP\(_3\), SHIP regulates the association of btk with the membrane through the btk PH domain. SHIP\(^{-/-}\) DT40 cells contained enhanced membrane-associated btk, which was further increased upon BCR aggregation (668). The essential role of btk in the activation of Ca\(^{2+}\) responses is detailed in the section on PLC\(\gamma_2\) activation in Chapter 1 (Section 2e).

Not only does SHIP associate with the ITIM of F\(\gamma RIIB\)1, but it also associates with the phosphorylated ITIM of CD22 in vitro (438). Another mechanism by which CD22 could abrogate Ca\(^{2+}\) influx may be by the recruitment of SHIP to its phosphorylated ITIM residue. This recruitment localizes SHIP to the vicinity of the plasma membrane. In the absence of lyn, SHIP is not recruited from the cytoplasm to the plasma membrane (359). This lends further support to the negative regulatory role of lyn in B cell activation through tyrosine phosphorylation of CD22. Upon recruitment to the membrane, SHIP may negatively affect Ca\(^{2+}\) influx by reducing PIP\(_3\) levels, which are necessary for recruitment of btk and PLC\(\gamma_2\) to the plasma membrane. Since high levels of IP\(_3\) are necessary for Ca\(^{2+}\) influx, levels of IP\(_3\) may be reduced such that there is sufficient IP\(_3\) for intracellular Ca\(^{2+}\) release but not for Ca\(^{2+}\) influx. Alternatively, another proposed mechanism of Ca\(^{2+}\) entry through the plasma membrane is modulated by IP\(_4\), a molecule that also has been proposed to interact with btk (401, 669). SHIP is also able to catalyze the hydrolysis of IP\(_4\). (665, 666) Thus, SHIP could abrogate Ca\(^{2+}\) influx by removing IP\(_4\), affecting IP\(_4\)-regulated Ca\(^{2+}\) channels.

Btk may be necessary for Ca\(^{2+}\) influx. Btk-deficient B cells fail to proliferate upon BCR aggregation (380, 670). In early studies, upon analysis of the defect in proliferation, no inositol phospholipid hydrolysis and IP\(_3\) production occurred. However, Ca\(^{2+}\) mobilization did occur, although the peak Ca\(^{2+}\) response was reduced (380). This reduction was due to IP\(_3\)-mediated Ca\(^{2+}\) release from intracellular stores. However, this study only examined Ca\(^{2+}\) responses up to 10
minutes post-stimulation. A subsequent study examined Ca\(^{2+}\) responses by Digital Image analysis up to 90 minutes post-stimulation (391). Digital image analysis allows the analysis of some early activation events at the level of the single B cell that may be masked when they are studied in cell populations (671). In this study (391), btk-deficient cells were shown not to sustain Ca\(^{2+}\) responses due to Ca\(^{2+}\) influx at 90 minutes post-stimulation, whereas wild type cells did. This suggests that btk-deficient B cells are also defective in Ca\(^{2+}\) influx. Therefore, another mechanism by which the lack of CD45 may lead to the abrogation of Ca\(^{2+}\) influx may be through the lack of btk activation. Btk activation is partly dependent on phosphorylation by a Src PTK (381). Transphosphorylation of btk has been shown to be mediated by lyn, fyn and syk, and this phosphorylation is necessary for BCR-induced Ca\(^{2+}\) mobilization (382, 383). CD45 selectively associates with lyn, and is responsible for the activation of lyn, since cell lines deficient in CD45 contained hyperphosphorylated lyn involving both the autophosphorylation and C-terminal negative regulatory tyrosines. Interestingly, Ag-induced btk activation in these CD45-deficient cell lines was greatly reduced relative to their CD45\(^{+}\) counterparts (350, 352). Therefore, the lack of CD45 may lead to either the inactivation of lyn or lack of recruitment of lyn to the BCR complex, resulting in abrogated btk activation. An examination of the function of lyn and btk in CD45\(^{-/-}\) B cells will help corroborate this proposed mechanism.

Another molecule that may be responsible for the lack Ca\(^{2+}\) influx in CD45-deficient B cells is CD19. CD19 is a co-receptor that positively regulates signal transduction through the BCR (412). A more detailed description of CD19 can be found in Chapter 1. B cells from CD45 exon 6\(^{-/-}\) mice do not undergo tyrosine phosphorylation of CD19 upon BCR aggregation (656). The removal of SHP-1 as well as CD45 rescues B cell maturation to the \(\mu^{lo8hi}\) mature phenotype, and the proliferative responses to BCR aggregation are restored. In addition, BCR aggregation-induced tyrosine phosphorylation of CD19 is also restored (656). The inability to phosphorylate CD19 upon BCR aggregation in CD45\(^{-/-}\) B cells may be a consequence of the lack of lyn or fyn recruitment to CD19, since both lyn and fyn have been shown to associate with CD19 (415, 416). The removal of SHP-1 and CD45 restores the inducible tyrosine phosphorylation of CD19 (656).
Therefore, SHP-1 may be responsible for the lack of inducible tyrosine phosphorylation of CD19 in the absence of CD45. SHP-1 has been shown to associate with CD19 (Siminovitch, K., unpublished observations). One explanation for the negative effect of SHP-1 on CD19 phosphorylation in the absence of CD45 may be that another PTP, the activity of which is dependent on CD45, might prevent SHP-1 from binding to CD19. This may occur by the removal of key phosphorylated tyrosines required for SHP-1 binding.

In the absence of CD45, SHP-1 binding to CD19 may also be responsible for dephosphorylating critical tyrosine residues necessary for PI3-K activation. The phosphorylation of Y484 and Y515 of CD19 mediates the association and subsequent activation of PI3-K with CD19 (414) (418). A variant of the J558Lμm3 plasmacytoma expressing CD45 but lacking CD19 contains very little active PI3-K upon BCR aggregation. Although PLCγ2 and PLCγ1 tyrosine phosphorylation still occurs, IP3 production is significantly reduced. Furthermore, Ca2+ influx is abrogated (418). The inhibitory effect on Ca2+ mobilization and IP3 generation were both due to the lack of PI3-K activity (418). B cells from CD19−/− mice do not proliferate upon mAb-mediated BCR aggregation (423). PI3-K activation is severely diminished in CD19−/− B cells. Furthermore, a decrease in Ca2+ mobilization is also observed in CD19−/− B cells in response to aggregation of the BCR (418). Although Ca2+ influx was not tested separately, the decrease appears to be a result of both Ca2+ influx and intracellular release. Therefore, another mechanism by which the lack of CD45 may be affecting Ca2+ influx is through the lack of signals emanating from the BCR co-receptor, CD19, specifically, through the inability to activate PI3-K. Other compensatory mechanisms resulting in PI3-K activation may operate in CD45−/− B cells, although they may not be as efficient as those mediated by CD19. Thus, sufficient IP3 might be produced to mediate intracellular Ca2+ release, but not Ca2+ influx. Examination of PI3-K activation as well as IP3 production in CD45-deficient B cells may help clarify this issue. The mechanism through which PI3-K is activated by CD19 cannot be solely responsible for the block in maturation observed in CD45−/− B cells, since CD19−/− B cells, although reduced in numbers in the periphery, do not
exhibit an immature phenotype. Therefore, this may be another circumstance whereby Ca^{2+} influx is dissociated from B cell maturation, and may not be a necessary component for maturation.

Is the lack of proliferation physiologically relevant?

CD45-deficient B cells do not proliferate upon BCR aggregation. One question that remains is whether the *in vitro* response to BCR aggregation is physiologically relevant. The original study demonstrated that CD45^-/- mice did not mount a biologically relevant cytotoxic T cell response against LCMV (73). It was suggested that this was due to the paucity of peripheral T cells in CD45^-/- mice. Immunization of CD45^-/- mice with type I (TNP-LPS) or type II (TNP-Ficoll) TI antigens resulted in an intact TNP-specific IgM and IgG response to both antigens (626). This was unexpected since CD45^-/- mice are unable to respond *in vitro* to anti-IgM stimulation (73). Upon adoptive transfer of CD4+ T cells into CD45^-/- recipients, immunization with TD antigen (KLH-DNP) resulted in specific IgG1 and IgE production comparable to controls (626). This suggested that CD45 is not required for B cell activation and differentiation. Another study demonstrated that immature neonatal B cells could function as efficient antigen presenting cells for stimulating mature T cells, and could respond to stimuli generated by cognate interactions with T helper cells (486). Therefore, based on these results, it appears that *in vivo*, immature B cells may be able to function as effectively as mature B cells in maintaining the humoral immune response to foreign antigen. However, CD45-deficient B cells transgenic for HEL are selected into the peripheral mature B cell pool when exposed chronically to HEL (390). Based on this result, autoreactive B cells in CD45-deficient mice may be selected into the periphery, rather than be deleted, which may eventually lead to autoimmune disease. The examination of the B cell repertoire in older CD45-deficient mice may help substantiate this theory.
How do CD40 or IL4 signaling pathways rescue proliferation?

I have demonstrated that the proliferative response to anti-μ can be partially rescued in CD45⁻/⁻ B cells by co-aggregation with either anti-CD40 or IL4. Both CD40 and IL4 signaling pathways activate PI3-kinase (568, 672). One suggestion mentioned previously is that the failure to induce Ca²⁺ influx may be due to the inability to activate PI3-K as a consequence of the lack of CD19 phosphorylation. Signaling through CD40 or IL4 together with anti-μ may rescue PI3-K activation, however, since Ca²⁺ influx was not rescued by either of these stimuli, PI3-K activation may not be responsible for the lack of Ca²⁺ influx. Alternatively, the defect in Ca²⁺ influx may lie downstream of PI3-K activation, such as that observed as a consequence of deficient btk activation. PI3-K may perform a separate function upon CD40 or IL4 stimulation, that enables entry of B cells into cell cycle, by recruitment into a separate BCR-independent pathway. Evidence for this is seen by the inducible association of PI3-K with 4PS/IRS2 upon IL4 stimulation (672).

Another protein that is activated in response to CD40 or IL4 stimulation is the PTK, JAK3 (585, 606, 673). Thus JAK3 may also be involved in a PI3-K independent downstream pathway leading to B cell cycle entry.

Proliferative responses in CD45-deficient B cells were not restored to wild type levels upon co-aggregation of anti-μ together with CD40 or IL4, and Ca²⁺ influx was also not restored. Therefore, Ca²⁺ influx responses induced through BCR aggregation may be necessary for restoring proliferation upon co-aggregation to wild type levels.

Based on the studies described in this thesis, the role of CD45 in B cell maturation and signaling pathways that govern this process has been further characterized. Specifically, the connection between CD45 and Ca²⁺ influx responses in B cells is a novel finding. However, details of the signaling pathways that are intermediate between CD45 and Ca²⁺ influx, as well as between Ca²⁺ influx and proliferation, still need to be elucidated.
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