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FCRL5 Exerts Binary and Compartment-Specific Influence on Innate-Like B Cell Receptor Signaling

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FCRL5 EXERTS BINARY AND COMPARTMENT-SPECIFIC INFLUENCE ON
INNATE-LIKE B CELL RECEPTOR SIGNALING

by

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A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2012

FCRL5 EXERTS BINARY AND COMPARTMENT-SPECIFIC INFLUENCE ON
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ZILU ZHU

BIOCHEMISTRY AND MOLECULAR GENETICS

ABSTRACT

Innate-like splenic marginal zone (MZ) and peritoneal cavity (PEC)-derived B1 B lymphocytes are the major contributors to primary humoral responses and play an important role in connecting innate and adaptive immunity, but are known to significantly differ in their B cell receptor (BCR) signaling characteristics. In mice, FCRL5 is discretely expressed by innate-like MZ and B1 B cells and is furthermore equipped with cytoplasmic ITAM-like and ITIM elements, suggesting diverse signaling potential for it. Our results showed that FCRL5 markedly inhibited BCR-mediated activation in MZ B cells, but not in PEC B1 B cells. To characterize its signaling function, a series of A20-IIA1.6 B cell line transductants engineered with chimeric receptors bearing cytoplasmic FCRL5 Y>F variants in frame with the extracellular portion of FcγRIIb were generated. Co-ligation of the WT FcγRIIb/FCRL5 chimeric protein with the BCR inhibited Ca²⁺ flux, whole-cell protein tyrosine phosphorylation, and Erk kinase activation via the ITIM-dependent (Y566) recruitment of the SHP-1 phosphatase. Conversely, co-ligation of a chimeric mutant bearing the intact ITAM-like (Y543/Y556) sequence with the BCR resulted in enhanced Ca²⁺ mobilization and Erk kinase activation. Intriguingly, this activation effect correlated with the Y543-dependent recruitment of the Lyn Src-family kinase (SFK). To further validate its bi-functional potential, viable SHP-1

deficient (me^v/me^v) and $Lyn^{-/-}$ mice were employed. These respective models revealed that both the SHP-1 phosphatase and Lyn kinase provide FCRL5 with counter-regulatory potential in modulating BCR signaling. Moreover, crosslinking FCRL5 with the BCR protected MZ B cells from BCR-mediated apoptosis, but promoted the death of B1 B cells. Finally, intracellular staining showed an excessive SHP-1 expression in MZ B cells that could confer FCRL5's inhibitory function in this particular subset. Taken together, these data indicate that FCRL5 has both inhibitory and activating signaling potential as well as a compartment-specific role in innate-like BCR signaling.

KEYWORDS: FCRL5, Inhibitory receptor, B cell signaling, Marginal zone, B1 B cells

DEDICATION

I would like to dedicate this dissertation to my parents who have been the driving force behind me, always there to support me, pick me up when I fall, push me when I want to give up, and congratulate me when I finish; To my wife, for her understanding and love; To my friends, for their kind assistance.

ACKNOWLEDGMENTS

First and foremost, I want to express my gratitude to my mentor, Dr. Randall S. Davis. He is a great advisor to both my research and my life. His constant encouragement and guidance has been accompanying me throughout my whole graduate work at UAB. With his help, I am able to not only do the research work and solve questions but also learn how to think and find solutions for my research. What I learned from him will benefit me all my life after.

I would also like to thank my fellow lab members, both past and present, for helpful comments and discussions, committee members for offering their time and advice, and neighbors and colleagues for generously sharing reagents.

Special thanks go to Ran Li for her excellent organization of our lab, Goetz R. A. Ehrhardt for teaching me how to do calcium flux experiments and providing retroviral vectors, John F. Kearney for Flow Cytometer expertise and Marion Spell for sorting primary B cells.

I also thank the Department of Biochemistry and Molecular Genetics (BMG), NIH-NIAID/NCI, and American Cancer Society for funding.

Finally, I would like to thank my family for their unwavering love and support.

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LIST OF ABBREVIATIONS

Ab	Antibody
Ag	Antigen
ASC	Antibody-secreting cell
APC	Antigen presenting cell
BAFF	B cell activating factor
BCR	B cell antigen receptor
BLNK	B cell linker
Btk	Bruton's tyrosine kinase
CBP	Phosphorylated CSK-binding protein
CDR	Complementarity determining regions
CSK	C-terminal Src tyrosine kinase
DAG	Diacylglycerol
ERK	Extracellular signal-related kinase
FACS	Florescence activated cell sorting
FDC	Follicular dendritic cell
GC	Germinal center
GEF	Guanine exchange factor
Grb2	Growth factor receptor-bound protein 2
Ig	Immunoglobulin
I(1,4,5)P ₃	Inositol 1,4,5-trisphosphate
ILT	Immunoglobulin-like transcript
ITAM	Immunoreceptor tyrosine-based activation motif

ITIM	Immunoreceptor tyrosine-based inhibitory motif
JNK	c-Jun N-terminal kinase
LAIR-1	Leukocyte-associated Ig-like receptor-1
Leu-13	Interferon-induced transmembrane protein 1
LFA-1	Lymphocyte function-associated antigen-1
Lyn	Lck/yes-related novel tyrosine kinase
MAPK	Mitogen activated protein (MAP) kinase
MHC	Major histocompatibility complex
mIg	Membrane-bound immunoglobulin
MZ	Marginal zone
NF-AT	Nuclear factor of activated T-cells
NF- κ B	Nuclear factor- κ B
NLR	Nod-like receptors
OMCP	Orthopoxvirus MHC class I-like protein
PAMP	Pattern associated molecular patterns
PC-PLC	Phosphatidylcholine specific phospholipase C
PD-1	Programmed death-1
PH	Pleckstrin homology
PI(3,4)P ₂	Phosphatidylinositol 3,4-bisphosphate
PI(3,4,5)P ₃	Phosphatidylinositol 3,4,5-triphosphate
PI3-K	Phosphatidylinositol 3-kinase
PIR-B	Paired immunoglobulin-like receptor B
PKB	Protein kinase B
PKC	Protein kinase C

PLC γ 2	Phospholipase C gamma 2
PRR	Pattern recognition receptor
PTB	Phospho-tyrosine binding
pTyr	Phosphotyrosine
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase
RasGRP	Ras-guanine nucleotide releasing protein
RIG-I	Retinoic acid-inducible gene-I
RA	Rheumatoid arthritis
SFKs	Src-family kinases
SH2	Src-homology 2
SH3	Src-homology 3
SHIP	SH2-containing inositol 5'-phosphatase
SHP-1	SH2-domain containing protein tyrosine phosphatase-1
SNP	Single nucleotide polymorphisms
SOS	Son of sevenless protein
SRCR	Scavenger receptor cysteine-rich
Syk	Spleen-associated tyrosine kinase
TCR	T cell receptor
TD	T cell-dependent
TI	T cell-independent
TLR	Toll-like receptor

INTRODUCTION

Overview of B Lymphocytes

In vertebrates, the immune system consists of both innate and adaptive components that cooperate to eliminate invading pathogens. Innate immunity is the first line of defense in immune responses. Although it is immediate, it is rather non-specific and does not establish memory after the response. Innate immunity involves several mechanisms including physical, chemical and microbiological barriers as well as immune components such as neutrophils, monocytes, macrophages, complement, cytokines and acute phase proteins. For instance, the epithelial surfaces of the body provide a good physical barrier while tears and saliva make a good chemical barrier against various invading organisms. Immune cells such as neutrophils and macrophages are able to engulf and destroy invading microbes by phagocytosis. Phagocytes recognize pathogens containing pattern associated molecular patterns (PAMPS) via germline-encoded pattern recognition receptor (PRRs) such as toll-like receptors (TLR), Nod-like receptors (NLR), and RIG-I (retinoic acid-inducible gene-I)-like receptors (RLR) (1). Adaptive immune responses ensue if an infectious organism breaks the early defense by the innate arm of immunity.

In contrast to innate immunity, adaptive immunity comprises a more sophisticated immune reaction against invading microbes. Adaptive immunity is a hallmark of higher order vertebrates and comprises antigen-specific reactions. The major players in adaptive

immunity are T and B lymphocytes, also known as T and B cells. Adaptive immunity does not become fully activated until several days after infection. It develops during the lifetime of an individual as an adaptation to infections and in many cases it confers lifelong immunity to re-infection from the same pathogen. Humoral adaptive immunity involves generating soluble antibodies against a specific pathogen, whereas the cell-mediated immunity consists of the destruction of infected cells by T cells. B and T cells recognize antigens by very different mechanisms. B cells recognize antigens through membrane-bound immunoglobulin (Ig) proteins, termed the B cell receptor (BCR), or through secreted Igs which bind to antigens. In contrast, T cells recognize peptide fragments of antigen presented by major histocompatibility complex (MHC) molecules expressed on antigen presenting cells (APCs) through the T cell receptor (TCR) positioned on the cell surface. The BCR consists of two identical heavy (H) and two identical light (L) chains joined by disulfide bonds, giving each antibody two identical antigen-binding sites. The antigen-binding region varies extensively between different antibodies and it is therefore known as the variable (V) region. The three hypervariable loops determine antigen specificity by forming a surface complementary to the antigen, and are more commonly termed complementarity determining regions (CDRs) (2). The region responsible for its effector function does not vary as much and is thus known as the constant (C) region. There are five main forms or so called Ig isotypes; IgA, IgD, IgG, IgE and IgM, each possessing a specialized effector mechanism (3). Each developing B lymphocyte generates a unique antigen receptor, which ensures that one cell will only

recognize one epitope. The enormous diversity in receptor specificity is generated by somatic rearrangements of a large number of gene segments encoding the V region.

The development of B cells occurs in the bone marrow and is independent of foreign antigen. The process of B cell development can be divided into several stages based on changes in Ig gene rearrangement status and expression of specific protein markers (4). Progenitor B cells (pro-B cells) develop from the common lymphoid progenitor, a multipotent cell capable of differentiating into lymphocytes and DC cells. Expression of the recombinase genes RAG-1 and RAG-2 results in BCR rearrangements at the pro-B cell stage where Ig heavy chain (IgH) is recombined first (5-7). After a functional V_HDJ_H rearrangement has been made, pro-B cells differentiate into precursor B cells (pre-B cells). Pre-B cells express the pre-B cell receptor (pBCR) which is composed of the μ heavy chain and the surrogate light chain (SLC) (8). In association with $Ig\alpha$ and $Ig\beta$, the pBCR mediates a signal to halt further rearrangements at the heavy chain locus (9). The pBCR also signals to upregulate light chain rearrangement (10), induce silencing of SLC transcription (11) and to continue differentiation to the immature B cell stage. Immature B cells express complete BCRs that consist of a light chain and a heavy chain in association with the $Ig\alpha$ and $Ig\beta$ signaling adaptor proteins and exit the bone marrow compartment as naïve B cells which have yet to encounter antigen. Upon encounter with foreign antigen in the periphery, the antigen-dependent phase of B cell differentiation begins. This involves proliferation and differentiation into either antibody secreting plasma cells or long-lived memory cells.

B Cell Receptor Signaling

BCR signaling plays a very dynamic role in B cell development, proliferation and differentiation (12-15). The BCR signaling process has three steps: signal initiation, signal propagation, and signal integration (16) (**Figure 1**).

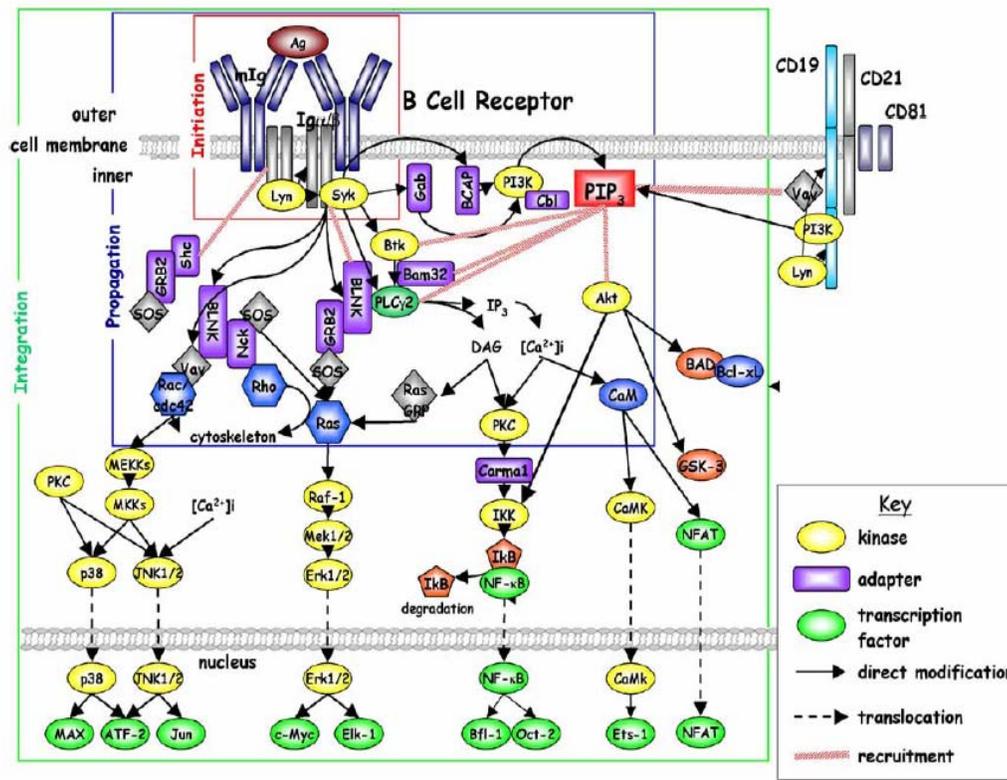
Signal initiation is an early and receptor proximal activation event which requires and involves the BCR complex itself. The BCR was first identified in 1970 (17), and is a multimeric complex consisting of an antigen-recognition subunit, the membrane-bound Ig (mIg), and a heterodimer of Ig α (CD79a) and Ig β (CD79b) that are non-covalently associated (18). Ig α and Ig β each carry in their intracellular domain an immunoreceptor tyrosine-based activation motif (ITAM) that is responsible for the recruitment and activation of protein tyrosine kinases (PTKs) to initiate BCR signaling (19-20). A canonical ITAM, YXXI/LX_{6,9}YXXI/L, is a conserved motif consisting of two precisely spaced tyrosine residues with an adjacent consensus sequence (21). Aggregation of the BCR results in the phosphorylation of the ITAM tyrosine residues on Ig α and Ig β primarily by PTK Lyn or Syk (22-23). The Src-family kinase (SFK) Lyn predominantly phosphorylates the first ITAM tyrosine, whereas Syk phosphorylates and binds to both ITAM tyrosines (24). Syk is a 72 kD kinase that contains two SRC-homology 2 (SH2) domains in tandem followed by a kinase domain. The phosphorylation of both ITAM tyrosines creates a specific and spatially-defined binding site for the tandem SH2 domains of Syk. Recruitment of Syk to doubly phosphorylated Ig α and/or Ig β proteins results in activation of the downstream kinases and adaptor proteins which facilitate the

initiation of several different signaling pathways. One way it does this is via phosphorylation and interaction with the adaptor molecule, B cell linker protein (BLNK).

Signal propagation is a downstream step of early activation events and is characterized by amplification and diversification of BCR signals via the association of adaptor proteins with key effectors and the production of second messenger molecules. BLNK, the major substrate of Syk, is a multidomain adaptor protein required for the formation of the “signalosome” which is crucial for initiating BCR-proximal events into several divergent signaling pathways (25-28). Phospho-BLNK has been shown to associate with the SH2 domain of Btk and PLC γ 2 (29-31). PLC γ 2, activated by BTK, cleaves membrane-associated phosphoinositide PI(4,5)P $_2$ into the second messengers I(1,4,5)P $_3$ and diacylglycerol (DAG). I(1,4,5)P $_3$ generation causes the mobilization of Ca $^{2+}$ from intra and extracellular stores which is required for the activation of transcription factors such as NF- κ B and NF-AT (32-33). DAG recruits and activates several signal effectors like protein kinase C (PKC)- β and the Ras guanine nucleotide release protein (RasGRP) which activate the classical NF- κ B and the mitogen activated protein kinase (MAPK) pathways separately (14-15, 34). Other BCR signaling components such as the guanine exchange factor (GEF), Vav, and adaptor complex of Grb2/SOS can also bind to phosphorylated BLNK and activate the Rac and Ras MAPK family pathways, respectively (35-36) .

Signal integration is a process in which the downstream effectors/second messengers regulate transcription factor activation and gene expression. As reviewed in

Figure 1, BCR signaling activates four major downstream pathways: MAPK, Ras/Raf/ERK, NF- κ B, and Ca²⁺-Calmodulin. Each pathway triggers very specific sets of transcription factors that regulate gene expression. For instance, the MAPK family consists of three members: the extracellular signal-related kinase (ERK), c-Jun NH₂-terminal kinase (JNK/SAPK) and p38 MAPK (37). Following activation, these kinases phosphorylate different sets of transcription factors including Elk-1 and c-Myc by ERK, c-Jun and ATF-2 by JNK, and ATF-2 and MAX by p38 kinase (38). Generation of a productive humoral response is possible only when these kinases are stimulated.



----- Mol. Immunol. 2004; 41: 599-613

Figure 1. The B cell antigen receptor signal transduction cascade

Signal transduction initiates at the cell membrane following ligand-induced aggregation of the membrane immunoglobulin and associated signal transducing elements Ig α and Ig β . Signals are then propagated by means of protein phosphorylation, modification, and interaction. The integration of the signaling cascade results in the regulation of transcription factor activation and gene expression. Co-receptor(s), such as the CD19/CD21/CD81 complex, can modulate BCR signaling pathway.

Modulation of B Cell Receptor Signaling

BCR signaling leads to a wide range of biological outputs, but the signal quality and quantity can be significantly influenced by multiple co-receptors and adaptor proteins.

1. Co-receptors on B cells

These receptors are distinct from the BCR and cytokine receptors and are able to modulate BCR signals positively or negatively.

1.1 Activating co-receptors

B cell activating coreceptors, such as CD19, Fc γ RI, and Fc γ RIII, may have their own cytoplasmic ITAM or they may associate with a transmembrane protein containing one or more ITAMs. CD19 is a B-cell-specific transmembrane glycoprotein that is expressed from the pro-B-cell to the plasma-cell stage. On mature B cells, CD19 associates with CD21, CD81, and Leu-13 to form a tetrameric complex involved in signal transduction (39-40). Upon BCR activation, the ITAM in the cytoplasmic tail of CD19 is phosphorylated by SFKs, and provides binding sites for the SH2 domains of Lyn, PI3-K, Btk, and Vav (41-44).

1.2 Inhibitory co-receptors

B cell inhibitory co-receptors include CD5, CD22, CD32, CD72, CD66a, ILT, PIR-B, PD-1, LAIR-1 and others, which downmodulate BCR signaling by setting a signaling threshold that prevents B cell overstimulation (45). Activation of these inhibitory co-receptors occurs by phosphorylation on their cytoplasmic immunoreceptor

tyrosine-based inhibition motifs (ITIMs), followed by recruitment of the tyrosine phosphatases SHP-1 or SHP-2, the lipid phosphatase SHIP, or the phosphatidylinositol 3-phosphatase PTEN (46).

1.2.1 Fc γ RIIB

Fc γ RIIB, a low affinity IgG receptor, is one of the most well characterized modulators of BCR-mediated signaling. Fc γ RIIB is the only IgG Fc receptor expressed on B cells and its inhibitory signal transduction is mediated primarily by its ITIM which is phosphorylated in a Lyn-dependent fashion (47). Fc γ RIIB tyrosyl-phosphorylation primarily recruits the SH2-containing inositol 5'-phosphatase SHIP (48). SHIP hydrolyzes PI(3,4,5)P₃ to PI(3,4)P₂, resulting in the disassociation of pleckstrin homology (PH) domain containing molecules PLC γ and Btk from the membrane and preventing their participation in activating downstream signaling pathways (49-50). Fc γ RIIB-associated SHIP also indirectly dephosphorylates CD19 leading to abrogated PI3-K recruitment and activation (51). Furthermore, Fc γ RIIB-associated SHIP can also serve to recruit the adaptor molecule downstream of kinase (DOK) which acts to inhibit other downstream signaling pathways (i.e. Ras/ERK activation) (52).

1.2.2 CD5

The CD5 co-receptor is a cysteine-rich scavenger receptor family glycoprotein that is expressed constitutively on all T cells and a subset of B cells (B1a B cells) (53-54). Several cell surface molecules, including CD72, gp40-80, and Ig-framework sequences, have been reported to be CD5 ligands (55). However, none of these has been shown to be

a functional physiological ligand. It is now generally accepted that CD5 is a negative regulator of T cell receptor (TCR) signaling in thymocytes and of BCR signaling in B1 cells (56-57). Although peritoneal B1a cells normally undergo apoptosis after engagement of surface IgM receptors (58), the same treatment of CD5-deficient B1a cells results in enhanced calcium mobilization and resistance to apoptosis (59). In wild-type B1a cells, prevention of the association between CD5 and mIgM rescues their growth response to mIgM crosslinking (59). In a CD5^{-/-} HEL-Ig/sHEL mouse model, “anergic” B cells lacking CD5 show enhanced proliferative responses in vitro and elevated intracellular Ca²⁺ levels at rest and after IgM cross-linking (59). These data support the inhibitory role of CD5 in regulating BCR signaling. Interestingly, signal transduction pathways activated by CD5 have also been reported in human T cells. CD5 can be tyrosine phosphorylated upon TCR ligation (60-61) and associate with p56^{lck}, p59^{fyn}, and Zap-70 (61-62). Some mitogenic anti-CD5 mAbs can also activate PKC resulting from the DAG released after phosphatidylcholine specific phospholipase C (PC-PLC) activation and de novo phospholipid synthesis (63).

1.2.3 CD22

CD22 is a type 1 membrane protein and a member of the Siglec family of adhesion receptors which binds α 2, 6-linked sialic acid modified proteins (64). CD22 is expressed in a B-cell lineage specific fashion, starting with low levels at the pre-B cell stage, peaks on mature B cells (65), and is ultimately downregulated by plasma cells (66). CD22 has seven Ig-like extracellular domains and a cytoplasmic tail containing six

tyrosines, three of which belong to the ITIM sequences (67). After mIgM crosslinking, the CD22 cytoplasmic tail is quickly tyrosine phosphorylated via a Lyn-dependent pathway, since CD22 tyrosine phosphorylation is reduced in *Lyn*^{-/-} mice (47, 68). The phosphorylation of the two CD22 carboxyl terminal ITIMs leads to recruitment of the SHP-1 tyrosine phosphatase, which plays an important role in its inhibitory function (46). CD22-deficient B cells show significantly enhanced calcium mobilization (69-70) and increased tyrosine phosphorylation of CD19, Vav-1 and BLNK after BCR crosslinking (71-73). Thus, CD22, Lyn, and SHP-1 appear to be limiting elements in a common pathway modulating the BCR signaling threshold. Indeed, heterozygous CD22/*Lyn*/*SHP-1* knockouts alter the balance between tolerance and immunity, resulting in the development of autoimmunity (74-75). CD22 also physically associates with SHIP via Grb2/Shc interactions following BCR ligation and the SHIP/Grb2/Shc ternary complex reduces BCR-mediated calcium mobilization, which is essential for preventing B cell hyperresponsiveness (76). A positive signaling role for CD22 in B cell function is also suggested by the presence of two potential ITAM-like sequences that could serve as docking sites for the SH2 domains of Syk kinase and SFK Lyn (77-78). Some other positive effector molecules of B-cell activation, such as PI3-K and PLC γ 2, also bind tyrosine-phosphorylated CD22 through their SH2 domains (77-79).

1.2.4 CD72

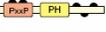
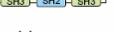
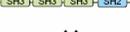
CD72 is a type II transmembrane protein of the C-type lectin family that is primarily expressed on B cells (80-81). The cytoplasmic tail of CD72 contains an ITIM

and an ITIM-like sequence. It has been reported that crosslinking BCR with CD72 induces phosphorylation of CD72 tyrosines, resulting in SHP-1 association (82-84). CD72-deficient B cells have a mildly increased Ca^{2+} response and hyperproliferation after BCR stimulation, suggesting that CD72 has an inhibitory role in B cell signaling (85). However, CD72 ligation alone plays a positive role in activation of B cells via the recruitment of adaptor protein Grb2 or indirectly by inducing phosphorylation of CD19, which in turn activates MAPK (86-89). The ligand for CD72 is CD100, a member of the semaphorin family expressed abundantly on T cells, but weakly on resting B and APCs (90). Binding of CD100 to CD72 reduces CD72 phosphorylation and triggers SHP-1 disassociation. This suggests that CD100 positively regulates B-cell activation by abrogating CD72 signal inhibition (91).

2. Adaptor proteins

Adaptor proteins are broadly defined as proteins that lack enzymatic or transcriptional activities but express various modular binding domains such as SH2, SH3, PH domains and phospho-tyrosine binding (PTB) or tyrosine phosphorylation sites (36, 92). These domains or motifs are known to be involved in modulating signal transduction by mediating protein-protein or protein-lipid interactions, which are important for signal transduction and integration (93). Recently the criteria for what defines an adaptor protein has been broadened to include some proteins that have enzymatic activity since they additionally contain protein- or lipid-binding domains. For instance, Lyn, a SFK, which has both SH2 and SH3 domains that can facilitate protein-protein interactions, is

now considered as an adaptor protein as well (36). A list of adaptor proteins identified in lymphocytes signaling is shown in **Figure 2**. In B cells, several adaptor proteins such as BLNK play important roles in B cell activation or development (94-95). Here we focus on the role of adaptor proteins on Lyn kinase activation events and SH2 domain-containing phosphatase SHP-1 activity.

Name	Structure	MW (kDa)	Potential interaction partners	Cellular expression
Transmembrane adapter proteins				
LAT		36–38	Grb2, Gads, SLP-76 (via Gads), PLCγ1/2, c-Cbl, PI3K	T cells, NK cells, mast cells, platelets
PAG		75–85	Csk, Fyn	Ubiquitous
SIT		30–40	SHP-2, Csk	B cells, T cells
TRIM		29–30	PI3K, CD3ζ	T cells, NK cells
Cytosolic adapter proteins				
Bam32		32	PLCγ2	B cells
3BP2		80	LAT, ZAP-70, Syk, Cbl, Grb2, PLCγ1	B cells, T cells
BRDG1		37	Tec	B cells, myeloid cells
Cbl		120	Grb2, Crk, Syk, ZAP-70, Src-PTKs, Btk, PI3K, Vav	Ubiquitous
Clnk		54	p92	T cells, NK cells, mast cells
CrkL		28, 40, 42	c-Cbl, C3G, Cas	Ubiquitous
Dok family		56, 62	SHIP, RasGAP, SLP-76, LAT, SAP	Ubiquitous
Gab family		97, 115	Grb2, SHP-2, Crkl, p85	Ubiquitous
Gads		40	LAT, SLP-76, Shc, HPK1	T cells, NK cells, mast cells, macrophages, platelets
Grap		28	SOS, LAT, Shc, Sam68	B cells, T cells
Grb2		28	SOS, LAT, c-Cbl, SLP-76, Shc, Vav, SHP-2, WASP, HPK1	Ubiquitous
Lnk		75	Lck, CD3ζ	B cells, T cells
Nck		47	SLP-76, SLP-65, SOS, PAK, WASP, Cbl	Ubiquitous
RIBP		45	Itk, Lck, Txk	T cells, NK cells
SAP		15	SLAM, 2B4, Dok	T cells, NK cells
Shb		55, 66	Grb2, LAT, PI3K, Eps8, PLCγ1, CD3ζ, Src	Ubiquitous
Shc		46, 52, 66	Grb2, CD3ζ, Igα/β, SHIP, ZAP-70, RasGAP	Ubiquitous
SKAP55		55	SLAP-130, Fyn	T cells, platelets
SKAPHOM		55	SLAP-130, Fyn	Ubiquitous
SLAP		34	ZAP-70, Cbl, SLP-76, Vav, CD3ζ	T cells, NK cells
SLAP-130		130	SKAP55, SKAPHOM, Fyn, SLP-76	T cells, myeloid cells
SLP-65		65	Btk, PLCγ2, Vav, Nck, Grb2	B cells, macrophages
SLP-76		76	Gads, Itk, SLAP-130, Nck, Vav, c-Cbl	T cells, NK cells, mast cells, macrophages, platelets
SOCS family		Variable length of amino-terminus	JAKs, STATs, Tec, elongin B/C	Leucocytes
TSAd		52	?	T cells

	SH2 domain		Proline rich region		SOCS box		Disulfide bond
	pTyr sites		SH3 domain		Coiled coil		Glycosylation site
	PTB domain		PH domain		RING domain		Nuclear location site

----- Curr. Opin. Immunol. 2001, 13:307-316

Figure 2. Adaptor proteins in lymphocytes

Note the different domains and motifs important for mediating protein-protein as well as protein-lipid interactions.

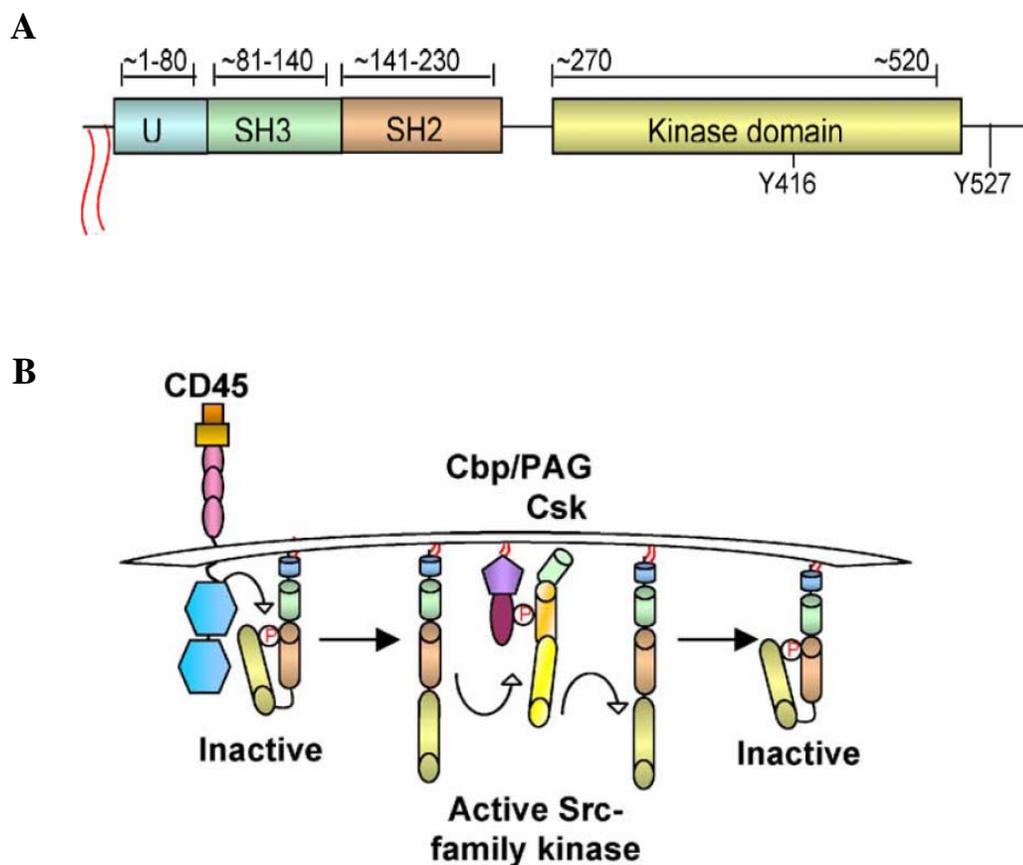
2.1 Control of Lyn activity

Src family nonreceptor tyrosine kinases (SFK) are found in both immune cells and non-hematopoietic cells where they are implicated in multiple intracellular signaling pathways. They can be activated by diverse growth factor, cytokine, adhesion, or antigen receptors. SFK members consist of Src, Fyn, Yes, Lck, Hck, Fgr, Lyn, Blk, and Yrk (96-97). Each of these proteins is ~ 60 kD and shares a conserved domain organization consisting of a unique N-terminal domain, followed by SH3, SH2 and tyrosine kinases domains (98-99) (**Figure 3A**). The unique N-terminal domain is known to play an important role in SFK membrane localization due to possessing of acylation sites for both myristoylation and palmitoylation (100). The SH3 domain mediates binding to proline rich regions (PXXP) of intracellular substrates (101-103). The SH2 domain mediates protein-protein interaction to tyrosine phosphorylated (especially YEEI motif) proteins (104-105). At the carboxyl (C)-terminus there is a protein kinase domain important for SFK catalytic kinase activity, which tyrosine phosphorylates downstream substrates (103). SFKs have two important tyrosine phosphorylation sites: Y416, an autophosphorylation site that is required for kinase activation and a C-terminal tyrosine residue Y527 that negatively regulates kinase activity (106).

Lyn is a SFK that, like two other SFKs Blk and Fyn, is predominantly expressed by B cells (99). Lyn is constitutively present in lipid rafts but is maintained inactive by tyrosine phosphorylation at its C-terminal Y527 site, which results in intramolecular binding to its own SH2 domain and hence keeps the kinase in a closed or inactive

conformation. The C-terminal Src tyrosine kinase (CSK) is a kinase that phosphorylates the C-terminal Lyn Y527 residue and thereby inhibits Lyn function in resting B cells (107). CSK is maintained in lipid rafts by binding to the phosphorylated CSK-binding protein (CBP), termed phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG), which resides in lipid rafts together with SFKs in the resting state (108-109). CD45, a receptor tyrosine phosphatase, is expressed by all hematopoietic cells except erythrocytes and platelets (110). BCR ligation first activates CD45, which dephosphorylates the Lyn C-terminal inhibitory Y527 (111-112). Meanwhile, BCR stimulation also dephosphorylates CBP and once dephosphorylated CBP no longer binds CSK and hence releases CSK to the cytosol, removing Lyn inhibition (108). This leads to the autophosphorylation of the Lyn Y416 residue thereby triggering its enzymatic activation. The Lyn C-terminal tyrosine is hyperphosphorylated in CD45-deficient B cells and hypophosphorylated in CSK-deficient B cells (107, 113), thus further indicating the opposing role of CD45 and CSK in regulating Lyn activation (**Figure 3B**).

Other than the well established role of SFKs in BCR signal initiation, it is now known that SFKs, in particular Lyn can play both a positive and negative regulatory role in BCR signaling (114-115). Lyn^{-/-} mice are susceptible to autoimmune disease and Lyn-deficient B cells are found to be hyperresponsive to BCR ligation despite defects in B cell development (116-118).



----- Mol. Immunol. 2004 41: 631-643

Figure 3. The domain structure and activation cycle of Src-family kinases

A. The Src kinase consists of four domains: the unique domain directs membrane targeting; the SH3 domain binds to proline-rich containing proteins; the SH2 domain interacts with tyrosine-phosphorylated proteins; and the C-terminal domain has catalytic kinase activity. Two important tyrosine sites: Tyr 416 enhances kinase activity while Tyr 527 mediates intramolecular binding to its own SH2 domain, which keeps SFKs in a closed and hence inactive conformation.

B. The activation cycle of SFKs mediated by the opposing effects of CD45 and Cbp/PAG associated CSK.

2.1 Regulation of SHP-1 activity

SH2 domain-containing protein tyrosine phosphatases (SHP) consist of two members, namely SHP-1 and SHP-2. Unlike the ubiquitously expressed SHP-2, SHP-1 is preferentially distributed in hemopoietic and epithelial cells (119). SHP-1 contains two tandem N-terminal SH2 domains that are important for binding to tyrosine phosphorylated proteins; a central phosphatase domain that orchestrates for phosphatase activities, and a C-terminal extension with unknown function (120). Signaling through the BCR is negatively regulated by SHP-1, which requires association with a tyrosine-phosphorylated peptide for activation (121). Inhibitory co-receptors, such as CD5, CD22, CD72, and PIR-B, contain ITIMs in their cytoplasmic regions and, upon phosphorylation, recruit SHP-1, suggesting that these molecules can activate SHP-1 in B cells (122). Thus, these co-receptors can also be viewed as adaptor molecules. Apart from ITIM-containing inhibitory receptors, SHP-1 is also activated by various tyrosine-phosphorylated signaling molecules including the erythropoietin receptor and the IL-3 receptor (123-124). In B cells, as a phosphatase when activated, SHP-1 can bind and deactivate $Ig\alpha$, $Ig\beta$, Syk, Vav, BLNK and CD22 by dephosphorylation (125). Mutation of SHP-1 in mice leads to enhanced BCR signaling with an increase in BCR-induced proliferation, MAPK activation, as well as general protein tyrosine phosphorylation (126).

Innate-like B cells

Overview of innate-like B cells

The mammalian immune system consists of innate and adaptive branches that cooperate to mount sophisticated responses against invading pathogens. B cells play a central role at the interface between the adaptive and innate immunity because they express both antigen-specific BCRs and various TLRs. Upon encounter with pathogens, experienced antigen-specific follicular B cells (FO B cells) produce antibodies. Development of these cells is a T cell-dependent (TD) process which includes commitment to germinal center (GC) reactions. In GCs, B cells undergo somatic hypermutation, affinity maturation and class-switching, and ultimately become antibody-secreting cells (ASCs) and memory B cells capable of producing high affinity antibodies. These lymphocytes help eliminate infections, and provide robust, long-lasting immunity (127). However, the peak of GC formation can be ten days or more after the initial pathogen exposure. To overcome this lag phase, it is now recognized that, distinct and specialized subsets of B cells exist, termed innate-like B cells, which can generate rapid responses to invading pathogens (128-129).

The innate-like B cells responsible for this vital responsibility include marginal zone (MZ) B cells in the spleen and B1 B cells positioned in the peritoneal and pleural cavities. MZ and B1 B cells share many similarities in terms of their unique anatomic position, function, Ab repertoire, and preactivated status (130). For example, both of these distinct B cell subsets can be triggered by TLRs ligands that lead to their quick

differentiation into ASCs (131-132). The antibodies secreted by these cells are polyreactive IgM, termed “natural” antibodies, which are important in protection against microbial infections and represent an immune mechanism of first-line defense against a vast array of pathogens (133-134).

MZ B Cells

Overview of MZ B cells

The marginal zone is a splenic structure that separates the white pulp from the red pulp and was firstly designated by MacNeal in 1929. This anatomic site is primarily enriched in MZ B cells, specialized macrophages, and reticular cells (135). The unique location of MZ B cells permits them to maximize encounters with blood-borne pathogens. Functionally, MZ B cells are primarily important for induction of IgM production in response to T cell-independent (TI) antigens rather than the TD-responses (129, 136). In addition to the well-characterized role of MZ B cells in TI-immune responses, MZ B cells also contribute to high levels of IgM production against protein-antigens and rapidly become extrafollicular plasma cells (137). MZ B cells are also able to initiate germinal center formation and undergo somatic hypermutation. They are also more important during the first week following antigenic challenge as IgG derived from MZ B cell ASCs has higher affinity for antigen than IgG produced by FO B cells (138). Taken together, MZ B cells participate in both TI and TD antibody responses, but are most important in the early phase of an immune response until high-affinity IgG antibodies derived from FO B cells undergoing GC-reactions are generated.

The B cell receptor repertoire of MZ B cells

MZ B cells express a more restricted, multi-reactive BCR repertoire than FO B cells, presumably a consequence of positive selection by self-antigens (139). Similar to multi-reactive B1 BCR specificities, the MZ B cell BCR repertoire is highly enriched for

IgH chains that lack non-template nucleotide insertions which are suggested to confer multi-reactive antibody-specificity (140-141). Furthermore, the CDR3 IgH complementarity determining region that makes contact with antigen is on average two amino acids shorter among MZ B cells than among FO B or NF B cells and this feature may also be linked to multi-reactivity (140).

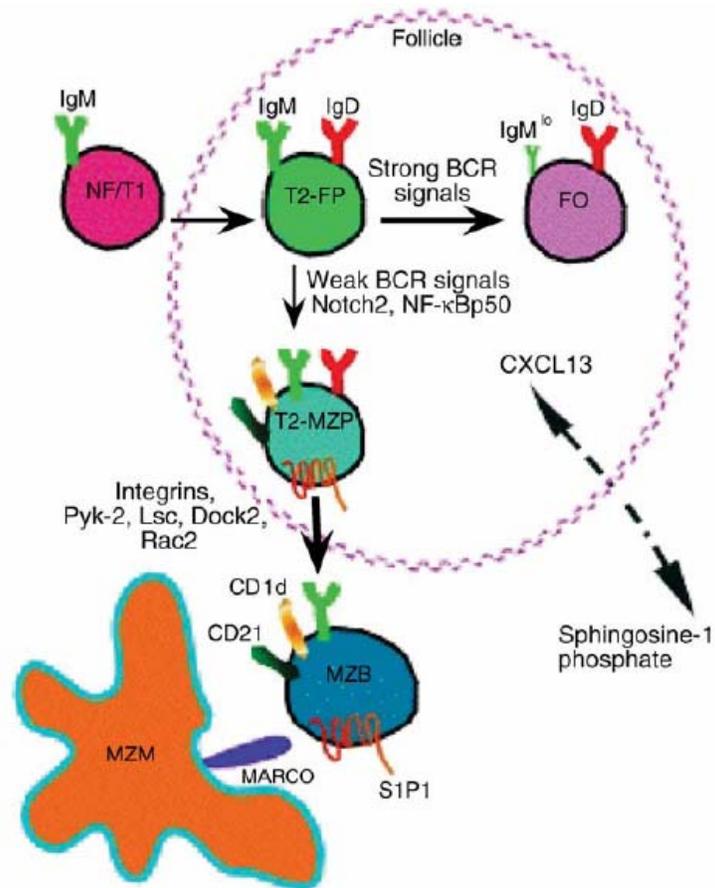
The phenotype of MZ B cells

In mice, MZ B cells are larger than FO B cells and have a unique phenotype. Unlike FO B cells that express high levels of IgD and CD23, but low levels of IgM and CD21, MZ B cells express high levels of IgM and CD21 and very low levels of IgD and CD23. They also express higher levels of CD1d, CD80, CD86, CD44, CD40, and CD25 than those on FO B cells (142-144). The $\alpha 4\beta 1$ integrin, LFA-1, and $\beta 2$ integrin are also expressed at higher levels on MZ B cells than on FO B cells (145). In addition, MZ B cells express higher levels of B7 proteins than FO cells and are overall described as having a “pre-activated” phenotype (142).

BCR signaling and MZ B cell development

During development, immature B cells migrate from the bone marrow to the spleen, where they further mature. Immature B cells in the spleen are called transitional 1 (T1) or newly formed (NF) B cells. T1 B cells that are not anergized will progress to the T2 stage. The maturation of T2 B cells into MZ or FO B cells is not very clear. However, indications show that two major driving forces are crucial for MZ B cell development (146). The primary determining factor is the specificity of the BCR, which allows a

developing cell to choose between FO and MZ B cell fates. The second major force is Notch signaling together with NF- κ Bp50, which is an additional factor that drives B cells to mature into the MZ B cell lineage (**Figure 4**). “Weak” signals delivered by the BCR appear to suffice for MZ B cell maturation. In contrast, “strong” BCR signaling is required for FO B cell development (146). A number of studies support this signal-strength hypothesis. For example, mutations of positive regulators of BCR signaling, such as Ig α , CD45, BTK, BCAP, PI3-K, and PLC γ 2 favor MZ B cell development. By contrast, deficiency of negative regulators of BCR signaling, such as CD22, Lyn, and SHP-1 causes a loss of MZ B cells (146). Notch, an ancient single-pass transmembrane receptor, consists of four different members and its signaling regulates cell fate ‘choice’ for a variety of cells (147). Ligands for Notch receptors include Delta-like 1, -3, and -4 and Jagged 1 and -2. Conditional knockout studies show that Notch2, its ligand Delta-like1, and the Notch down-stream signaling component RBP-J are specifically required for MZ B cell development (148-151).



----- Annu. Rev. Immunol. 2005. 23:161-96

Figure 4. The BCR signal strength model of peripheral MZ and FO B cell development

BCR signaling differences between MZ and FO B cells

BCR signaling not only regulates the lineage commitment of peripheral MZ and FO B cells, but also significantly differs between these two subsets. In vitro, MZ cells exhibit greater calcium mobilization and whole-cell protein tyrosine phosphorylation than FO B cells upon BCR ligation (152). In vivo, MZ cells differentiate into plasma cells in response to low doses of antigens much faster than FO cells (139, 153). The molecular mechanism underlying this difference has been explained by enhanced tyrosine phosphorylation and activity of PLC γ 2 and Syk protein tyrosine kinases in MZ B cells, but not in FO B cells (152). Interestingly, the expression levels of negative regulators of BCR signaling, such as SHP-1 and SHIP, are also higher in MZ B (152). However, the expression and tyrosine phosphorylation of Btk, BLNK, Vav, or PI3-K and the abundance of Lyn, Fyn, and Blk are equivalent between these two subsets (152). Furthermore, MZ cells have reduced proliferative responses but increased apoptosis susceptibility after BCR crosslinking, but have a greater capacity to serve as APCs (142-144).

MZ B cells and autoimmune diseases

Autoimmune diseases are complex and depend on many factors that lead to the breakdown of tolerance to self-antigens. There are several features of MZ B cells suggesting that they are involved in autoimmunity such as multi-reactive IgM, their low threshold of activation, anatomical localization that provides easy accessibility to antigens, the capacity to transfer immune complexes to follicular dendritic cells (FDCs),

and also a potent ability to activate naïve CD4⁺ T cells. For example, an expanded MZ B cell population is observed in the SLE model (NZBxNZW) F1 mice (154-155). BAFF, a B cell activating factor of the tumor necrosis factor family, is important for normal mature B cell homeostasis (156). BAFF has been implicated in autoimmunity because its excess favors the maturation of autoreactive B cells into the MZ B or FO B subsets (157). BAFF-transgenic mice have an expanded MZ B cell population and develop SLE-like symptoms and also Sjögren's syndrome, induced by autoimmune destruction of the salivary glands (158-159). Moreover, MZ B cells produce pathogenic anti-dsDNA IgG autoantibodies in BAFF-transgenic mice lacking T cell help (160). Thus, BAFF also promotes MZ B cell-mediated autoimmunity.

B1 B Cells

Overview of B1 B cells

Mouse B1 cells were first described in 1983 by Herzenberg as a relatively small population of CD5⁺ splenic B cells that spontaneously produce IgM (161). Later studies showed that B1 cells are predominant in the peritoneal and pleural cavities, but are rare in lymph nodes. Based on CD5 surface expression, B1 cells can be further subdivided into B1a (CD5⁺) and B1b (CD5⁻) subtypes (162). The 'B2 cells' are a phenotypically distinct mature FO B cell population because they develop later in ontogeny than B1 cells and originate from a common bone marrow precursor (163). Unlike B2 cells, B1 cells are involved mainly in TI and innate-like immune responses. By virtue of expressing a specific BCR repertoire that reacts to both self antigens and microbial antigens, B1 cells produce a large amount of natural antibodies and participate in maintaining tissue homeostasis (164-165). B1 cells also have the capacity to secrete IgA antibodies which are important in immune defenses against mucosal pathogens (166).

The role of BCR signaling in B1 B cell development

The origin of B1 cells is controversial. Two models have been proposed to explain their development (167). The 'lineage model' suggests that B1 cells are derived from a discrete B cell precursor. B1a cell-restricted precursors have been found in the splanchnopleura region of the developing embryo and fetal liver. However, unlike B1a cells, B1b cell-restricted precursors have been identified in fetal liver and adult bone marrow, but not in the splanchnopleura region. The 'induced differentiation model'

postulates that the B1 phenotype can be induced according to specific signals received by B cells. Wortis and coworkers reported that B cell activation with certain anti-IgM Abs could result in CD5 induction on splenic B2 cells (168). Furthermore, Lam and Rajewsky demonstrated the alteration of the B2 cell surface phenotype to a B1 type by replacing a “B2 type” BCR with a “B1 type” using an inducible system in splenic B cells (169).

BCR signaling strength is essential for B1 cell selection, survival, and expansion. B1 cells have a distinct antibody repertoire distinguished by self- and polyreactive specificities which is generated in the absence of foreign antigen exposure (130, 153). The relatively low-affinity BCR repertoire for self antigens allows B1 cells to be positively selected, which is justified by the observation that natural autoreactive B cells recognizing membrane-bound Thy-1 (CD90) enrich the B1 compartment (170). Evidence in support of BCR-mediated positive selection during B1 cell development includes, mutations that disrupt BCR signaling result in a decreased number of B1 cells or their complete elimination, but a largely expanded B2 compartment (162). Conversely, mutations or transgenes that enhance BCR signaling increase the frequency of B1 cells (162).

BCR signaling differences between B1 and B2 B cells

Although the BCRs on both B1 and B2 cells are composed similarly of the Ig heavy and light chains in complex with the signaling subunits $Ig\alpha$ and $Ig\beta$ (171), signaling differences in response to BCR activation have been documented between these two subsets. Whereas BCR engagement on B2 B cells leads to robust intracellular

calcium mobilization, NF- κ B activation and proliferation, the peritoneal B1 B cell response is characterized by reduced Ca^{2+} mobilization, impaired proliferation, and increased apoptosis (59, 172-174). This indolence may be a sign of previous BCR activation, or Ag experience, and may represent a form of tolerance (175-177). It is suggested that the impaired BCR signaling in B1 B cells requires the co-expression of CD5 and the T cell SFK Lck (178-179).

B1 cells and autoimmune diseases

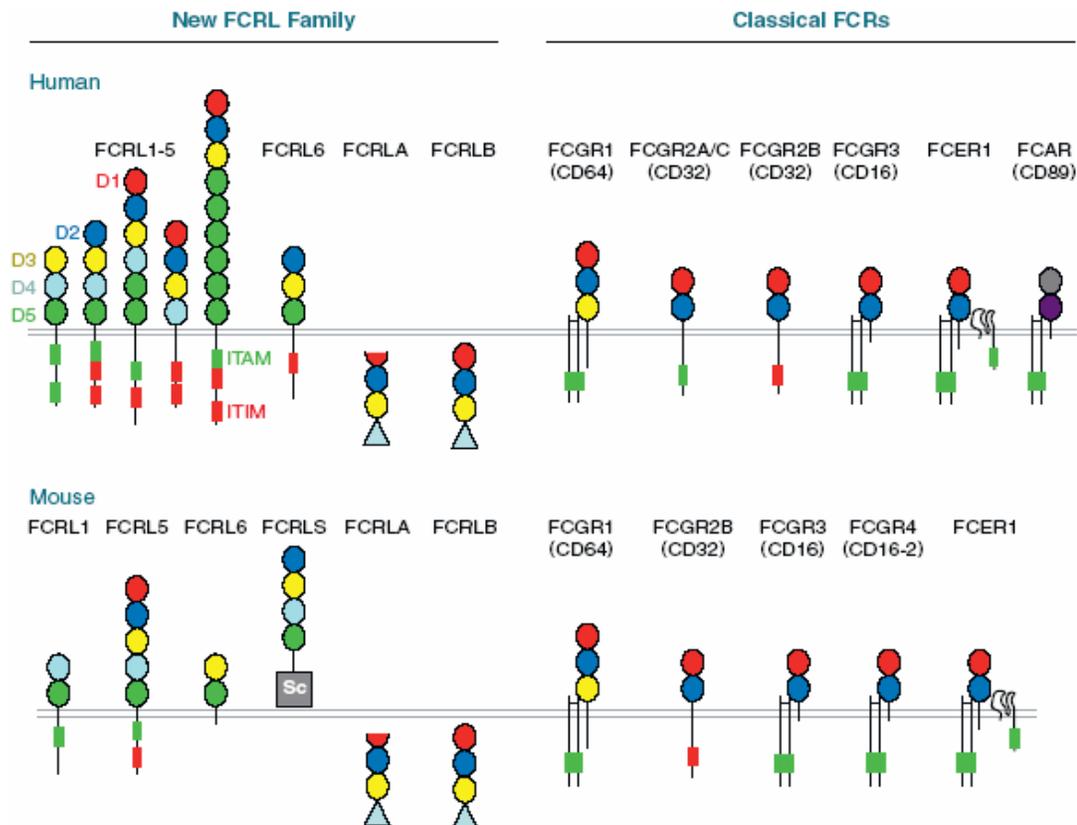
B1 cell dysregulation is associated with some autoimmune diseases. In humans, increased numbers of B1 cells have been reported in patients with rheumatoid arthritis (RA) (180) and Sjorgen's syndrome (181). In mice, elevated numbers of B1 cells have been found in a number of naturally occurring and genetically manipulated strains that develop autoimmune manifestations, such as SLE (NZBxNZW) F1 mice and NZB mice that develop autoimmune hemolytic anemia (AHA) (161). A number of gene knockout and transgenic mice also have high levels of B1 cells and manifestations of autoimmune disease (115). For example, CD22-deficient B cells are hyperresponsive to BCR crosslinking (69). CD22^{-/-} mice have increased number of peritoneal B1 cells and develop high titers of IgG antibody specific for dsDNA at 20 months of age (182). The mechanisms by which B1 cells contribute to autoimmune diseases are not very clear. They might play a role by producing pathogenic autoantibodies, presenting self-antigen to autoreactive T cells, or via IL-10 secretion (183-184).

Fc Receptor-Like Molecules (FCRLs)

Overview of FCRLs

Fc receptor-like molecules (FCRLs) are related to the well-known receptors for the Fc-portion of immunoglobulin (FCR). They were initially identified through a database search using a 32 amino acid consensus sequence derived from the Fc-binding portions of CD16, CD32, and CD64 as well as by sequencing a translocation breakpoint (1;14)(q21;32) in a multiple myeloma cell line by the Dalla-Favera group (185-186). Until now, eight members in human and six members in mice have been found (187) (**Figure 5**). FCRLs have similar gene sequence and protein structure, as well as linked genomic localization with the classical FCRs, indicating that they share a common ancestor. The human *Fcrl* genes are located at three distinct loci spanning a 4 Mb region of chromosome 1q21-23 within the *FCR* cluster. In mice, the *Fcrl* genes are split between two chromosomes (187). *Fcrl1* and *Fcrl5* are located on chromosome 3 approximately ~8.5 Mb centromeric of the *Fcgr1* gene. The atypical *Fcrls* gene is also situated on chromosome 3 in the opposite orientation relative to *Fcrl1* and *Fcrl5* and encodes an Ig domain-scavenger receptor cysteine rich (SRCR) fusion protein. *Fcrl6*, *Fcrla*, and *Fcrlb* are located on chromosome 1 and feature high organizational conservation relative to their human counterparts. FCRLs vary in their numbers of extracellular Ig-like domains, and contain ITAMs, ITIMs, or both in their intracellular regions. The presence of multiple tyrosine-based activation and inhibitory motifs indicates that FCRL family members may have varied and complex signaling potential. In contrast to the

conventional FCR, FCRL molecules possess diverse extracellular frameworks, dual signaling properties, and preferential B lineage expression, suggesting that they are important immunomodulatory molecules in B cells.



----- Annu. Rev. Immunol. 2007. 25:525-60

Figure 5. A comparison of protein characteristics between human and mouse FCRL and FCR molecules

Extracellular Ig domains are color-coded according to phylogenetic relationships. Cytoplasmic ITIM and ITAM are represented by red and green boxes, respectively. FCRLS contains a Group B Scavenger Receptor Cysteine-Rich (SRCR) domain indicated by a gray rectangle. Both FCRLA and FCRLB contain C-terminal mucin-like regions (blue triangles) and are expressed intracellularly.

Human FCRLs

Overview of human FCRLs

Human *FCRL1-6* encode type I transmembrane glycoproteins with 3-9 extracellular Ig domains varying in sequence identity from 45% to 83% (186, 188-189). FCRL immunoregulatory potential is implicated by the presence of ITAM and/or ITIM sequences in their cytoplasmic tails except for FCRL6 and FCRLA/B. All of them are expressed by B cells at different stages in differentiation with the exception of FCRL6 (187). FCRL2 and 4 are predominantly expressed in memory B cells, whereas FCRL1 is a pan B cell marker. FCRL3 and 5 are expressed in bone marrow in pre-B cells, and in most mature B cells with the highest levels on naïve and memory B cells (187). Although FCRL molecules have homology with the classical FCRs, none of the FCRL family members have been shown to bind Igs (187), except for FCRLA, which binds Igs in the endoplasmic reticulum (190). Recently, Schreeder et al. showed that human FCRL6 which is selectively expressed on cytotoxic T and NK cells, directly binds to the HLA-DR MHC class II molecule (191-192).

The role of human FCRLs in BCR signaling

The tyrosine-based influence of FCRL molecules on BCR signaling has now been studied by several groups. FCRL1 has two ITAMs in its cytoplasmic tail, suggesting that it functions as an activating receptor. Indeed, co-ligation of FCRL1 with the BCR results in its tyrosine phosphorylation and enhanced calcium mobilization and cell proliferation (193). FCRL2-5, which contain one or more ITIM as well as ITAM-like sequences, have

all been found to dominantly inhibit BCR-induced cellular activation which is mediated by their capacity to recruit SHP-1 and/or SHP-2 (194-197). Interestingly, Sohn et al. have recently shown that FCRL4 ligation alone enhances innate TLR9-dependent signaling despite its inhibition on the antigen-induced BCR signaling via the SHP1 and SHP-2 recruitment to the three tyrosine residues in its cytoplasmic domain (198). These findings suggest that FCRL4 may act as a molecular switch to dampen adaptive immune signaling and enhance innate signaling in response to chronic antigenic stimulation.

Human FCRLs in autoimmune diseases and malignancies

The pathogenic roles of FCRLs have been widely identified in malignancies and autoimmune disorders. FCRL1 is expressed by several B cell malignancies including chronic lymphocytic leukemia (CLL), follicular lymphoma (FL), hairy cell leukemia (HCL), and mantle cell lymphoma (MCL) (199). FCRL2 is expressed in higher levels in B-CLL than in AML (acute myelogenous leukemia), ALL (acute lymphoblastic leukemia), CML (chronic myelogenous leukemia), MM (multiple myeloma), and NHL (non-Hodgkin lymphoma) (200). Polymorphisms in the FCRL3 gene have been associated with RA and autoimmune thyroid disease in Asians; SLE in Asians and Caucasians; MS in Caucasians; Grave's disease and Addison's disease (201-202). In the peripheral blood of HIV⁺ viremic patients, FCRL4⁺ cells have impaired proliferation and differentiation into ASCs in response to polyclonal stimuli, suggesting that FCRL4 could promote B cell exhaustion (203). Upregulated FCRL5 expression is also found in the

blood or on the malignant cells of patients with multiple myeloma, CLL, and mantle cell lymphoma (204).

Mouse FCRL5

Overview of mouse FCRL5

The *Fcrl5* gene encodes a type I transmembrane glycoprotein with five extracellular Ig-like domains, an uncharged transmembrane region, and a cytoplasmic tail with four tyrosines (187, 205). The *Fcrl5* gene consists of 13 exons. Through the use of differential mRNA splicing, *Fcrl5* generates two different splice variants including a 507 amino acid short isoform and a 595 amino acid long isoform (205). The short isoform, lacks the sequence encoding the first Ig domain, but the rest of the sequence is identical to long counterpart which includes all five Ig domains. Both isoforms have a 23 amino acid transmembrane domain and a relatively short 79 amino acid cytoplasmic domain. Analysis of full-length cDNAs from five different mouse strains defines two FCRL5 alleles. BALB/c, 129, NZB, and CBA strains share a common FCRL5 allele, whereas the C57BL/6 allele is distinct (206). The variations present in the C57BL/6 allele are located throughout the extracellular and cytoplasmic coding sequence and consist of a single amino acid (+164S) insertion in the D2 domain and 15 single nucleotide polymorphisms (SNP). Among these SNPs, five are synonymous and ten are nonsynonymous. One of these nonsynonymous C57BL/6 SNPs (T376N) generates an additional N-linked glycosylation site in the D4 Ig domain which may alter ligand binding (206).

The expression of mouse FCRL5

In mice, *Fcrl5* transcripts are highly enriched in splenic MZ B cells and PEC-derived B1 cells (187, 205). In bone marrow B cells, *Fcrl5* is transcribed at very

low levels, but transcripts can be detected beginning at the pre-B cell stage and increase with differentiation. Expression outside the B lineage has not been observed for this gene. By using a panel of specific monoclonal anti-FCRL5 Abs, Won et al. confirmed that FCRL5 expression is confined primarily to splenic MZ and peritoneal B1a and B1b B cells (206). Interestingly, FCRL5 is also identified on MZ precursor B cells. Thus, mouse FCRL5 appears to be a discrete marker of innate-like MZ and B1 B cells.

The ligand for mouse FCRL5

Despite the many similarities between the FCR and FCRL families, there is no evidence that FCRL5 can bind Igs. Recent work by Campbell et al. identified a viral-encoded immunoevasin as a mouse FCRL5 ligand (207). The orthopoxvirus MHC class I-like protein (OMCP) was identified using a Hidden Markov model (HMM) and was initially found to bind to NK cells via NKG2D, but also demonstrated reactivity with innate-like B cells (208). Using an expression cloning approach, FCRL5 was characterized as the interacting OMCP partner. The three N-terminal Ig domains of FCRL5 are required for OMCP binding (207). However, the FCRL5 endogenous ligands and their physiological role remain enigmatic.

Tyrosine-based signaling potential of mouse FCRL5

The FCRL5 cytoplasmic domain contains 4 tyrosines and three of these residues have typical spacing and surrounding amino acids that conform to a noncanonical ITAM-like sequence (Y543/Y556) and a consensus ITIM (Y566), but the fourth (Y544) does not fit any known tyrosine-based motif (187, 205). Moreover, the FCRL5

ITAM-like sequence differs from the traditional motif (D/EXXYXXL/IX₆₋₉YXXL/I) by possessing a glutamic acid residue at the second Y+3 position instead of an aliphatic leucine or isoleucine residue. Our previous studies have shown that FCRL5 co-ligation with the BCR in primary MZ B cells can inhibit Ca²⁺ mobilization, suggesting its dominant inhibitory role (206). However, the possession of both tyrosine-based activation and inhibitory sequences in FCRL5 suggests that this immunoregulatory receptor may have dual signaling properties.

In this work, we present a bi-functional role for FCRL5 in the context of the BCR complex signaling. By generating FcγRIIb/FCRL5 chimeric receptors, we are able to find that the FCRL5 cytoplasmic ITAM-like sequence and ITIM motif are capable of enhancing or inhibiting BCR-mediated activation. The activating effect was mediated by ITAM-like-dependent interaction with the Lyn SFK, while the inhibitory effect was mediated by ITIM-dependent recruitment of the SHP-1 tyrosine phosphatase. FCRL5 significantly inhibits BCR signaling in primary MZ B cells, but it had no obvious influence in PEC B1 B cells. Its disparate function in these innate-like B cells correlated with significant differences in SHP-1 expression. Further dissection of FCRL5 dual signaling properties in the PEC B1 B cells derived from homozygous viable motheaten mice (me^v/me^v) and Lyn^{-/-} mice confirmed its counter-regulatory role in BCR-mediated signaling.

FCRL5 EXERTS BINARY AND COMPARTMENT-SPECIFIC INFLUENCE ON
INNATE-LIKE B CELL RECEPTOR SIGNALING

by

ZILU ZHU, RAN LI, TONG ZHOU, AND RANDALL S. DAVIS

In preparation for publication

Format adapted for dissertation

ABSTRACT

Innate-like splenic marginal zone (MZ) and peritoneal cavity (PEC) B1 B lymphocytes share critical responsibilities in primary humoral responses, but have divergent B cell receptor (BCR) signaling characteristics. A discrete marker of these subsets with tyrosine-based dual regulatory potential termed FCRL5 was investigated to explore this discrepancy. Although FCRL5 repressed MZ cell BCR activation, it had no influence in PEC-derived B1 B lymphocytes. The molecular basis for its inhibitory function derived from SHP-1 phosphatase recruitment to a cytoplasmic ITIM. Surprisingly, mutagenesis of this docking site unearthed co-activation properties for FCRL5 that were orchestrated by independent association of the Lyn Src-family kinase with an FCRL5 ITAM-like sequence. The contributions of SHP-1 and Lyn to balancing FCRL5's unique binary-functionality directly correlated with the disparate antigen receptor biology evident between MZ and B1 B cells and imply a specialized counter-regulatory role for FCRL molecules at the intersection of innate and adaptive immunity.

INTRODUCTION

Innate-like B lineage cells positioned at strategic microanatomical sites generate the initial wave of primary effector defenses that bridge host protection until adaptive mechanisms emerge (1). The lymphocytes charged with these responsibilities include splenic marginal zone (MZ) B cells harbored in a location optimal for filtering blood-borne antigens and B1 lineage cells that guard the peritoneal and pleural body cavities (2-4). Their capacity for producing antibodies with broad neutralization capacities is associated with evolutionarily conserved immunoglobulin repertoires, distinct sensitivity to T-independent (TI) stimuli, and rapid differentiation into plasma cells that secrete “natural,” polyreactive antibodies (5-7). These features distinguish MZ and B1 B cells from their more abundant B2-lineage counterparts that recirculate and participate in T cell-dependent (TD) responses.

MZ and B1 B cell development is strongly influenced by B cell receptor (BCR) specificity in concert with the composite array of surface and intracellular regulatory proteins that help balance antigenic responses. Mutations in CD45, BTK, or PLC γ 2 that dampen BCR signaling favor MZ development and a loss of follicular (FO) B cells (8). However, defects in negative regulatory components, such as Lyn, SHP-1, or CD22 lead to a loss of MZ B cells, a relative expansion of the B1 compartment, and an increased

susceptibility to autoimmunity (9). While many other trophic, migratory, and retention factors instruct their development and positioning, these signals must be integrated in the context of BCR signaling which primarily drives B cell fate and survival (8, 10-12). Correspondingly, BCR triggering differs markedly between these subpopulations. MZ B cells exhibit more robust whole-cell protein tyrosine phosphorylation, calcium mobilization, and PLC γ 2 and Syk tyrosine kinase activation than FO B cells, but have greater apoptotic sensitivity (13-14). In contrast, B1 B cells have blunted calcium mobilization, NF- κ B activation and proliferation, but may also possess relatively increased rates of apoptosis compared to PEC B2 cells (15-17). Notably, these properties do not differ according to CD5 expression, as both the B1a (CD5⁺) and B1b (CD5⁻) subsets respond similarly to BCR ligation (18). Although they differ from anergic cells, it remains unclear whether the unique biology of B1 B cells derives from chronic antigenic exposure, suppressive signals present in the coelomic cavity microenvironment, or other causes (2, 16).

An evolutionarily conserved gene family related to the Fc receptors (FCR) for IgG and IgE, termed FCR-like (FCRL), is preferentially expressed by B cells and encodes transmembrane proteins with tyrosine-based immunoregulatory motifs (19). FCRL proteins do not appear to bind Ig; however, two representatives have recently been found to interact with MHC-like molecules (20-21). Given their growing clinical relevance in infectious diseases, autoimmunity, malignancies, and immunodeficiencies, several groups have investigated FCRL signaling function (22-26). In humans, FCRL1

has two immunoreceptor tyrosine-based activation motif (ITAM)-like sequences and enhances BCR-induced calcium mobilization and cellular proliferation (27). In contrast, FCRL2-5, which feature one or more consensus immunoreceptor tyrosine-based inhibition motifs (ITIM) as well as ITAM-like sequences, all inhibit BCR activation via recruitment of the Src homology-2 (SH2) domain-containing SHP-1 and/or SHP-2 phosphatases (28-31). We have previously shown that the FCRL5 mouse ortholog distinctly marks innate-like B cells and possesses an ITIM as well as an ITAM-like sequence that differs from the canonical motif (D/EX₂₋₃YXXL/IX₆₋₈YXXL/I) due to a glutamic acid residue at the second Y+3 position rather than an aliphatic residue (19, 32). Although, FCRL5 inhibits BCR-mediated calcium mobilization in MZ B cells, the molecular basis for this activity and its function in B1 cells has remained unclear. Furthermore, the conservation of both activation and inhibitory sequences in FCRL5 and other FCRLs suggests they have dual-signaling features, but definitive functional evidence for this is lacking. Given its discrete distribution and regulatory potential, the biological role of FCRL5 in these specialized B lymphocytes with recognized differences in their adaptive signaling capacity was investigated.

Here we report that FCRL5 has dual-modulatory and compartmental subset-specific effects on antigen receptor signaling. Upon association with the BCR its ITAM-like and ITIM sequences are tyrosine phosphorylated and recruit the Lyn Src-family kinase (SFK) and SHP-1 protein tyrosine phosphatase. The non-redundant contributions of these elements to FCRL5's unique counter-regulatory function further

revealed that adaptive signaling differences between MZ and B1 B cells directly correlate with SHP-1 activity.

RESULTS

FCRL5 Attenuates MZ but Not B1 B Cell Receptor Signaling

Because MZ and B1 B cells have disparate BCR signaling capacity, we investigated whether FCRL5 regulation might also differ. To approach this question, calcium mobilization was examined in spleen and PEC-derived primary B lymphocytes isolated from wild-type (WT) C57BL/6 mice. Consistent with previous results, the strong calcium response observed in gated MZ B cells following BCR engagement alone was significantly diminished by FCRL5 co-crosslinking (Figure 1) (32). In contrast, FCRL5 did not alter this already blunted activation cascade in B1 B cells. An analysis of global tyrosine phosphorylation by intracellular phospho-specific flow yielded similar results. Although FCRL5 could also inhibit this outcome in MZ cells, it again had no influence in B1 B cells, which showed less robust whole-cell tyrosine phosphorylation. These data indicate that FCRL5 has a compartment-specific regulatory role in BCR signaling.

FCRL5 Immunoregulatory Function is Tyrosine-based

To define the molecular basis for its differential innate-like B cell activity, we next dissected the roles of the FCRL5 cytoplasmic tyrosine-based motifs. Fc γ RIIb/FCRL5 chimeric constructs encoding six different cytoplasmic mutants as well as intact Fc γ RIIb

and a vector control were stably transduced into the mouse B cell line A20-IIA1.6 (IgG2a, κ) that lacks endogenous Fc γ RIIb expression (Figure 2A) (31). After confirming equivalent levels of surface expression, responses following co-ligation of the various chimeric proteins were compared to BCR ligation alone (Figures S1A and S1B).

Since the FCRL5 cytoplasmic tail contains both ITAM-like and ITIM sequences, we initially examined whether both motifs could be phosphorylated. After treatment with the sodium pervanadate phosphatase inhibitor, the WT, Y543F, Y556F, Y566F, and FF (Y543F/Y556F) chimeras were all tyrosine phosphorylated, but the FFF (Y543F/Y556F/Y566F) mutant was not (Figure S2). This latter mutant confirmed the functional inactivity of a fourth cytoplasmic tyrosine at position 544, which does not correspond to any known tyrosine-based signaling motif. Thus, this mutational analysis indicates that FCRL5 tyrosine-based signaling is confined to its ITAM-like and ITIM units.

We then investigated the impact of the FCRL5 WT and FFF mutants on BCR-induced whole-cell tyrosine phosphorylation. BCR triggering alone induced rapid tyrosine phosphorylation of multiple intracellular proteins, including the ERK MAP kinase; however, co-ligation with the WT chimera greatly reduced these effects as a function of time (Figure 2B). In contrast, global tyrosine phosphorylation and ERK activation were unaffected by FFF crosslinking. Immunoprecipitation experiments indicated that the WT FCRL5 cytoplasmic tail was tyrosine phosphorylated following its association with the BCR, but not upon BCR or FCRL5 ligation alone (data not shown).

Results in this cell line were thus consistent with FCRL5 regulation in MZ B cells and demonstrated that its cytoplasmic domain can inhibit BCR activation in a tyrosine-dependent manner.

FCRL5 ITIM and ITAM-like Sequences Counter-regulate BCR Signaling

We next examined the functional contributions of the individual FCRL5 tyrosines in calcium mobilization assays. Whereas BCR ligation-alone induced a characteristic wave of calcium flux, WT chimeric receptor crosslinking significantly abrogated this effect (Figure 3A). In fact, the degree of inhibition was even more potent than that of the FcγRIIb positive control. The function of the ITIM was then examined by disrupting the ITAM-like 543 and 556 tyrosines. Co-ligation with this mutant completely shut down the calcium response. In turn, mutating the ITIM Y566 residue unearthed activation properties for FCRL5 by virtue of enhanced calcium flux. Finally, among the ITAM-like tyrosines, the Y543F mutant completely blocked calcium signaling, whereas the Y556F variant disclosed only partial inhibition similar to that of the WT tail. As expected, the FFF mutant had no effect on BCR-mediated Ca²⁺ flux. Because our results shown in Figure 2 indicated that the MAP kinase cascade could be restrained by FCRL5, we next deconstructed the roles of these tyrosines in ERK activation. The Y543F, Y556F, and FF variants, which all retain an intact ITIM, inhibited ERK phosphorylation (Figure 3B). In contrast, the Y566F mutant with an unmodified ITAM-like sequence moderately enhanced ERK activation. Thus, FCRL5's modulation of ERK activation correlated with

its effects on calcium mobilization. Similar observations were also made with regard to whole-cell tyrosine phosphorylation by phospho-specific flow (data not shown). These findings collectively indicate that FCRL5 has dual-regulatory tyrosine-based signaling properties that are balanced and graded. The ITIM (Y566) exerts strong inhibitory properties individually, whereas the ITAM-like (Y543/Y556) sequence has the inverse effect and can augment downstream BCR-mediated effects. Importantly, within the non-canonical ITAM, Y543 plays a critical role, whereas the Y556 residue appears to be dispensable. The opposing influence of these motifs therefore outfits FCRL5 with counter-regulatory function.

SHP-1 and Lyn Are Recruited to Independent FCRL5 Cytoplasmic Tyrosines

The cytosolic effector proteins that mediate FCRL5 functions were then examined. To define the elements responsible for its inhibitory function, chimeric protein immunoprecipitates were probed for the SHP-1, SHP-2, or SHIP SH2-domain containing tyrosine phosphatases. Except for the FFF mutant, all chimeric receptors were tyrosine phosphorylated following BCR co-engagement (Figure 4A). Among these candidates, SHP-1 associated with the WT, Y543F, and Y556F single mutants, as well as the FF double mutant, but not the Y566F or FFF chimeras. In contrast, neither SHP-2 nor SHIP co-precipitated with any of the chimeras. This ITIM-dependent association was also confirmed by SHP-1-specific immunoprecipitation which identified a low-level of constitutive FCRL5 binding that was enhanced by BCR co-ligation (Figure 4B).

Putative signaling components responsible for FCRL5 activating properties were then assessed. The Syk and PLC γ 2 kinases did not associate with the FCRL5 cytoplasmic tail (Figure 4C). Other kinases including Btk and PI3K, as well as the adaptor proteins Grb2 and BLNK, were also undetectable in immunoprecipitates (data not shown). Because Lyn is the predominant SFK expressed in B cells and is required for the activating function of Ig α /Ig β , CD19, and CD180/RP105 as well as the inhibitory properties of CD5, CD22, CD32, and CD72 (9), we explored whether it associated with FCRL5. Lyn indeed co-precipitated with the WT, Y556F and Y566F chimeras, but not with the Y543F, FF, or FFF mutants. We also examined whether the physiologic status of Lyn was active (pY397) or inactive (pY508) (33). Western blotting revealed that the SFK member associated at the Y543 position was in the active state. This newfound Lyn-dependent activation property was also supported by kinase inhibitor studies. FCRL5 calcium signaling enhancement was lost upon treatment with SFK- and PLC-, but not Syk- or Btk-specific inhibitors (Figure 4D). Thus, following BCR co-ligation, the FCRL5 Y566 ITIM residue is phosphorylated and coordinates the receptor's inhibitory activity by recruiting SHP-1. The ITAM-like tyrosines are also phosphorylated, but offset the receptor's potent ITIM-mediated repression by recruiting the active form of Lyn to the Y543 residue, which engenders a Syk and Btk-independent activation pathway.

SHP-1 and Lyn Mediate the Unique Dual-Functionality of FCRL5 in Innate-like B Cells

To enable uncoupling of its regulation, we next validated FCRL5 function in viable motheaten mice (me^v/me^v), in which SHP-1 phosphatase activity is disabled by a point mutation (~10–20% of normal), and Lyn deficient mice (34-36). Both of these mice develop severe autoimmunity and have a loss of MZ and FO B cells in the spleen as well as B2 cells in the PEC, but develop a relative expansion of B1a and B1b B cells (Figures S3A and S3B; (9, 37-38)). After confirming that FCRL5 is expressed at comparable levels in the three strains (Figure S3B), we examined its regulatory impact in these genetically modified B1a and B1b B cells. In remarkable contrast to WT B1 B cells, FCRL5 crosslinking in SHP-1 deficient cells strongly augmented BCR-mediated calcium mobilization and whole-cell tyrosine phosphorylation (Figures 5A and 5B). Intriguingly, the opposite effect was observed in $Lyn^{-/-}$ B1 B cells. Here, co-ligating FCRL5 with the IgM BCR resulted in marked repression of calcium flux and whole-cell tyrosine phosphorylation. Notably, this outcome resembled observations made for WT splenic MZ and A20-IIA1.6 B cells. Responses were similar between the B1a and B1b subsets. Furthermore, FCRL5 engagement alone again had no impact on calcium flux or tyrosine phosphorylation (data not shown). These results in mutant mice confirm the inverse contributions of SHP-1 and Lyn to FCRL5 function and demonstrate that their relative cellular abundance has a direct effect on driving FCRL5 biology. While these data validate the essential inhibitory role for SHP-1, an unsuspected non-redundant requirement for Lyn in mediating its activating function was additionally uncovered.

These data collectively indicate that FCRL5 may serve as a cellular sensor for SHP-1 and Lyn activity in innate-like B cells.

To determine whether dual-functionality was a feature unique to FCRL5, other well-characterized regulatory proteins including CD5, CD22, CD32, and CD72 were also analyzed. Cell surface staining demonstrated their similar expression levels on PEC B1a and B1b B cells derived from WT, me^v/me^v , and $Lyn^{-/-}$ mice (Figure S4 and data not shown). IgM expression was slightly lower on me^v/me^v B1 B cells, whereas IgD was uniformly dim. In contrast to FCRL5, all four receptors tested inhibited BCR-mediated calcium mobilization in WT B1 cells, albeit to different extents (Figure 5C). In SHP-1 deficient cells, CD22 and CD32 retained inhibitory function, likely through their association with the SHIP inositol phosphatase (39-40). However, the dampening capacity of CD5 and CD72 was abolished indicating that their inhibitory properties are indeed SHP-1 dependent (41-42). Furthermore, unlike FCRL5, none of these immunoregulatory molecules acquired enhancing function in SHP-1 deficient B1 cells, including CD5, CD22, and CD72 which have each been shown to play both positive and negative roles in B cells (43-45). Nevertheless, these receptors could all block BCR calcium signaling in $Lyn^{-/-}$ B1 B cells. These data indicate that ITIM tyrosine phosphorylation, which is required for SHP-1 and SHIP docking and the consequent inhibitory function of each of these proteins including FCRL5, could be compensated by SFKs other than Lyn that are co-expressed in these cells. However, the FCRL5 ITAM-like Y543 residue does not appear susceptible to promiscuous SFK activity in this

fashion and instead is strictly Lyn-dependent. Taken together, these results indicate that FCRL5 possesses unique counter-regulatory function in B cells that is directly mediated by SHP-1 and Lyn.

Divergent FCRL5 Function in Innate-like B Cells Reflects Differences in SHP-1 Activity

To determine the nature of its disparate activity in MZ and B1 B cells, we reassessed FCRL5 signaling properties in primary cells. Because innate-like B cells represent only a minority of the total B lymphocytes in normal adult mice, we employed a human soluble BAFF transgenic (Tg) mouse model as a source of larger quantities of MZ B cells. As a fundamental immature B cell survival factor, mice overexpressing BAFF markedly expand their total B cell compartment and MZ B cell frequency (46). The resulting human BAFF Tg mice demonstrated a phenotype similar to previous established models (Figures S5A and S5B). As expected, BCR co-ligation of FACS-sorted splenic MZ B cells resulted in FCRL5 tyrosine phosphorylation and the coincident association of SHP-1 and Lyn (Figure 6A). These interactions were also confirmed in PEC B1 B cells derived from TCL1 (T-cell leukemia/lymphoma 1) Tg mice that have a marked expansion of chronic lymphocytic leukemia (CLL)-like B1 B cells (data not shown, (47)).

Previous biochemical studies had found that MZ B cells express comparatively more SHP-1 than FO B cells, but similar levels of total Lyn (13). Furthermore, splenic MZ and PEC B1 cells show higher constitutive whole-cell tyrosine phosphorylation than

conventional B2 cells present in their respective compartments (13, 48). To directly examine compartment-specific differences in these variables, we employed a flow cytometry-based analysis for quantitative comparisons. Consistent with previous work, calcium signaling among WT splenic subsets was most intense in MZ B cells followed by newly formed (NF) and FO B cells, whereas PEC B2 B cells demonstrated a stronger response than B1 cells (Figure S6) (14, 16). Both innate-like B cell subpopulations had significantly higher constitutive tyrosine phosphorylation relative to their B2 lineage counterparts by phospho-flow; however, activation was greatest in MZ B cells (Figure 6B). Correspondingly, basal SHP-1 expression was significantly elevated in MZ and B1 cells relative to conventional B2 cells and was two-fold greater in MZ than B1a or B1b B cells (Figure 6C). Relative SHP-1 abundance also strongly correlated with global tyrosine phosphorylation potential in response to pervanadate treatment (Figure 6D). Although total Lyn expression was similar among these subsets, its active and inactive forms were slightly higher in PEC B1 compared to B2 cells (Figure 6E). Along with our findings in mutant mice defining the requisite contributions of Lyn and SHP-1 to its function, these data indicate that divergent FCRL5 biology in MZ and B1 B cells directly correlates with SHP-1 activity.

FCRL5 Binary Immunoregulation Discloses the Contributions of SHP-1 and Lyn to MZ and B1 B Cell Receptor-induced Apoptosis

Because BCR stimulation markedly induces apoptosis in MZ B cells, but does so to a lesser extent in B1 cells (14-15, 49), we next investigated the differential influence of Lyn and SHP-1 on antigen receptor-mediated survival. In the absence of exogenous stimulation, survival of WT MZ B cells was much shorter than that of B1 B cells purified from WT, me^v/me^v , or $Lyn^{-/-}$ mice. Although SHP-1 deficient B1 B cells were about twice as sensitive to spontaneous apoptosis as the WT, $Lyn^{-/-}$ B cell viability was higher overall (Figure 7). Thus, Lyn deficiency favored B1 B cell survival (38, 50), while SHP-1 deficiency promoted apoptosis (51). We next examined the impact of FCRL5 on BCR-mediated survival. Anti-IgM crosslinking markedly increased MZ B cell apoptosis; however, consistent with their elevated SHP-1 activity, FCRL5 co-engagement rescued a significant number of these cells. While BCR ligation also enhanced apoptosis in WT PEC B1 B cells, in contrast to its failure to modulate calcium or tyrosine-phosphorylation, here FCRL5 augmented apoptosis. This effect was in line with the comparatively lower SHP-1 levels in these cells and was magnified in me^v/me^v B1 B cells. These data are also consistent with studies by Luciano et al. disclosing that Lyn promotes BCR-induced apoptosis (52). Conversely, in the absence of Lyn, FCRL5 demonstrated regulation akin to that in MZ B cells and suppressed apoptosis. These findings collectively indicate that compartmental differences in SHP-1 and Lyn activity play a key role in influencing the differential adaptive function of innate-like MZ and B1 B cells.

DISCUSSION

In these studies a distinguishing marker of innate-like B cells was found to exert compartment-specific regulatory function. Although FCRL5-BCR co-ligation could inhibit the striking BCR activation typical of splenic MZ B cells, it had no obvious influence on this cascade which is intrinsically dampened in PEC B1 B cells. These findings led to broader questions concerning the molecular basis for FCRL5 function and its role in these specialized subsets. Although it failed to initiate downstream responses by itself, as a co-receptor, FCRL5 had unique dualistic influence on BCR signaling that derived from its tyrosine-based recruitment of Lyn and SHP-1. The incongruent biology evident for FCRL5 as well as the BCR in MZ and B1 B cells directly correlated with the intrinsic SHP-1 activity of these cells. Collectively these findings demonstrate that FCRL5 is a discrete counter-regulatory biomarker of innate-like B cells directly coupled to the Lyn-SHP-1 biochemical circuit (Figure S8).

Through their direct and indirect BCR association, Lyn and SHP-1 have profound effects on B cell selection and function (53-56). Deficiency of either of these proteins leads to a loss of MZ B cells, expansion of the B1 lineage, and a breakdown in peripheral tolerance (35, 38, 57). We found that basal calcium influx, whole-cell tyrosine phosphorylation, and SHP-1 levels were significantly higher in MZ B cells than in B1 B cells, but Lyn activity did not vary substantially. These differences indicate that splenic MZ B cells are more globally activated at homeostasis and are thus primed for stronger

signal transduction once triggered. Given its governing effects on BCR signaling strength (51), the effector roles of these cells, and their developmental loss in its absence, SHP-1 is likely upregulated to raise the triggering threshold and offset the pre-activation induced by tonic BCR and other environmental signals these lymphocytes experience in the splenic marginal zone niche (8, 58). Dampening their pre-amplified physiologic resting state by means of elevated phosphatase activity would not only suspend BCR activation, but would in turn heighten their overall potential for explosive responsiveness once this restriction is lifted. The relationship between SHP-1 expression, pre-activation status, and BCR responsivity was also apparent in the other splenic subpopulations and recent work by the DeFranco group has demonstrated that increased signaling sensitivity among transitional B2 cells declines in a maturation-dependent fashion as the Lyn-SHP-1 pathway becomes active (59). As a distinctive substrate in this circuit, FCRL5 inhibitory function in MZ B cells reflects more elevated SHP-1 than Lyn activity. This was also corroborated by findings that neither total Lyn abundance nor its activity substantially differed among splenic subsets. Thus, compensatory SHP-1 upregulation appears critical for buffering MZ B cell excitation and fate. Although what modulates its expression remains unclear, based on the extreme sensitivity of MZ cells to spontaneous apoptosis *ex vivo* and their marked SHP-1 induction, factors originating from the marginal zone milieu likely contribute.

B1 B cells were less constitutively activated than splenic MZ B cells; however, their global tyrosine phosphorylation, and SHP-1 and Lyn activity were slightly higher than PEC B2 cells. These features along with their impotent BCR responsiveness again highlight their unconventional biology. The inverse functionality of FCRL5 in me^v/me^v and Lyn deficient B1 B cells, but relative indolence in WT cells, indicates the SHP-1-Lyn circuit is more balanced in this subset at homeostasis. Under these conditions Lyn binding to the FCRL5 ITAM-like Y543 residue could be stoichiometrically compensated by SHP-1 recruitment to the FCRL5 Y566 ITIM. Importantly, lower SHP-1 activity would yield relatively higher Lyn kinase function. This was apparent by the augmentation of FCRL5-induced apoptosis in me^v/me^v compared to WT lymphocytes. Diminished SHP-1 activity might also be related to constitutively elevated levels of phosphorylated ERK (60-61). Despite its enigmatic source of stimulation in B1 B cells, ERK activation in response to agonistic T cell receptor ligation can modify SFK activity in a feedback loop that blocks SHP-1 function (62). Perhaps additional signals native to the coelomic cavities trigger Toll-like and/or cytokine receptors and commandeer B1 B cell function. Sustained BCR triggering in combination with these stimuli may downmodulate adaptive signaling as well as SHP-1 expression, leading to their exhaustion as found for T cells in chronic inflammatory states (63).

The possession of both functional ITAM-like and ITIM sequences distinguishes FCRL5 from other co-receptors. These experiments showed that CD5, CD22, CD32, and CD72 could each inhibit BCR-mediated calcium mobilization in WT and Lyn deficient

B1 B cells but, unlike FCRL5, did not acquire activation properties in the absence of SHP-1. Relative to its balanced function in WT cells, me^y/me^y B1 B cells not only validated the dualistic properties of FCRL5, but indirectly exposed Lyn's preferential affinity for the ITAM-like Y543 residue. The lack of SFK redundancy at this site is suggestive of sequence-specific properties that distinctly coordinate Lyn's recruitment there. Although the molecular features conferring SFK tyrosine phosphorylation at the Y566 ITIM appear more promiscuous, phosphatase docking was restricted to SHP-1. Though Lyn and SHP-1 can concomitantly associate with FCRL5, their relative cytosolic amounts balance its overall function. As a scaffold that directs their tandem positioning, it is possible that Lyn occupancy at Y543 could promote processive phosphorylation at the Y566 ITIM (64). Lyn-dependent transphosphorylation activity, which has been shown for CD19, would maintain FCRL5 binary function at balance unless quantitative differences or other intracellular factors compromised Lyn and/or SHP-1 recruitment to these respective sites (65). Conversely, SHP-1 could conceivably intra-molecularly interfere with Lyn's activity. Lyn also imbues FCRL5 with properties analogous to other activation receptors such as $Ig\alpha$, $Ig\beta$, CD19, and CD180/RP105 (9), and fosters a Syk and Btk-independent activation pathway. When untethered from SHP-1 counter-suppression, this Lyn-mediated function may play a role in FCRL5-mediated B cell pathogenesis.

Although the endogenous ligands for B cell expressed FCRLs have yet to be elucidated, two other representatives interact with MHC-like proteins. In humans FCRL6,

expressed by cytotoxic CD8⁺ T and NK cells, interacts with MHC class II, whereas in mice, an immunoevasin termed orthopoxvirus MHC class I-like protein (OMCP) was identified as an FCRL5 ligand (20-21). Although the consequences of these interactions are under investigation, their associations have intriguing implications for these receptors in mechanisms of tolerance induction. The signaling properties defined here for FCRL5 have additional relevance for other FCRL proteins. These receptors have thus far been found to generally inhibit BCR signaling, but their conserved cytoplasmic motifs along with prior mutagenesis studies revealed subtle hints of their potential bifunctionality (28, 30). Thus, unearthing the FCRL5-Lyn relationship raises the likelihood that other FCRLs also possess activation features.

One underlying question relates to the integration of these signaling properties with the discrete expression of FCRL5 by MZ and B1 B cells. Given their front-line access to potential pathogens, a hallmark feature of these lymphocytes is their preferential responsiveness to innate agonists and TI antigenic stimulation. Accordingly, recent work demonstrated intriguing signaling potential for FCRL4 in human B cells. Despite its possession of a tyrosine-based switch motif and two ITIMs that inhibit adaptive BCR function via SHP-1 and SHP-2 binding, Sohn et al. have shown that FCRL4 also enhances innate TLR9-dependent signaling (66). Its variable influence on these pathways was transmitted in the absence of direct FCRL4 engagement indicating that its expression alone may differentially modulate B cell responsivity to adaptive or innate stimuli. These features found for another FCRL, along with the ability of FCRL5

to recruit the active form of Lyn shown here, strongly indicate that these molecules are important modifiers of B cell adaptive and innate signaling. Importantly, Lyn is also required for CD180/RP-105 TLR activation in response to LPS binding (67). Thus, future studies in deficient mouse models should better clarify FCRL5's role in these two facets of B cell biology.

Finally our clearer understanding of FCRL5 function provides new insight into the possible pathogenic roles of other FCRL molecules in disorders with which they have already been widely associated. For example, FCRL2 and FCRL3 are expressed by a putative MZ effector memory population that circulates in human blood as well as by chronic lymphocytic leukemia (CLL) B cells that are believed to originate from this subset (25, 68-69). In addition to this lymphoproliferative disorder, FCRL3 has been linked to autoimmune diseases such as rheumatoid arthritis (24), and FCRL4 promotes B cell exhaustion, which is associated with dysfunctional humoral responses found in HIV and malaria infected patients (22-23). These observations underscore the probability that FCRL molecules participate in perturbed immunity. The current findings therefore lay essential groundwork for better informed exploration of the fundamental roles of the FCRL family in normal and pathogenic B cell function.

EXPERIMENTAL PROCEDURES

Mice

Wild-type C57BL/6 and viable motheaten C57BL/6J-Ptpn6^{me-v}/J (+/me^v) mice were purchased from the Jackson Laboratory. Lyn^{-/-} mice were kindly provided by Dr. Clifford A. Lowell (University of California, San Francisco, CA) (35). Generation of BAFF transgenic mice is described in the Supplemental Information. All studies and procedures were conducted with mice 6-10 wks of age and were approved by the UAB Institutional Animal Care and Use Committee.

Antibodies and Reagents

Table S1: Antibodies

Name	Manufacturer, Cat.#/Clone
Anti-HA	Roche 12CA5
Anti-SHP-1	Santa Cruz SC-287
Anti-SHP-2	Santa Cruz SC-280
Anti-Lyn	Santa Cruz SC-7274
Anti-Lyn	Santa Cruz SC-15
Anti-Syk	Santa Cruz SC-51703
Anti-PLC γ 2	Santa Cruz SC-5283
Ani-Btk	Santa Cruz SC-81736
Anti-PI3-K	Santa Cruz SC-71896
Anti-BLNK	Santa Cruz SC-8003
Anti-Grb2	Santa Cruz SC-503
Anti-SHIP	Upstate 06-932
HRP anti-phosphotyrosine 4G10	Upstate 16-105
Anti-Lyn (phospho Y396)	Abcam ab40660
Anti-Lyn (phospho Y507)	Abcam ab53122
Anti-phospho-p44/42 MAPK	Cell Signaling 9106
Anti-p44/42 MAPK	Cell Signaling 9102
Anti-phospho-Src (Y416)anti-HA	Cell Signaling 2101

Anti- β -actin	Sigma A5316
Rabbit anti-mouse IgG	Jackson ImmunoResearch 315-005-003
F(ab') ₂ rabbit anti-mouse IgG	Jackson ImmunoResearch 315-006-003
F(ab') ₂ goat anti-mouse IgM	Jackson ImmunoResearch 115-006-020
Biotin F(ab') ₂ goat anti-mouse IgM	Jackson ImmunoResearch 115-066-020
PE anti-phosphotyrosine PY20	BD Biosciences 558008
PE mouse IgG2bk	BD Biosciences 555058
Biotin anti-CD23	BD Biosciences 553137
Biotin anti-Gr-1	BD Biosciences 553125
PE anti-rabbit IgG	SouthernBiotech 4050-09S
Rabbit IgG	SouthernBiotech 0111-01
Biotin anti-CD5	SouthernBiotech 1546-08
Biotin anti-CD22	SouthernBiotech 1580-08
Biotin anti-CD72	SouthernBiotech 1725-08
PE anti-CD21	eBioscience 12-0211-81
FITC anti-CD23	eBioscience 1102-32
PE anti-CD23	eBioscience 1202-32
FITC anti-CD5	eBioscience 11-0051
PE anti-CD5	eBioscience 12-0051
FITC anti-CD11b	eBioscience 11-0112
APC anti-CD11b	eBioscience 17-0112
PerCP-Cy5.5 anti-CD19	eBioscience 45-0193
Biotin anti-Thy-1.1	eBioscience 13-0900
APC anti-CD21	Biolegend 123411
Biotin anti-CD16/32	Biolegend 101303
Biotin anti-F4/80	Biolegend 123105
Biotin anti-CD11c	Biolegend 17303
Biotin anti-NK1.1	Biolegend 108703
Anti-mouse FCRL5 mAb (C57BL/6 allotype-specific)	Our lab 3B7 (mo γ 1 κ) (32)
Anti-mouse FCRL5 mAb	Our lab 9D10 (rat γ 2 κ) (32)
Anti-mouse FCRL5 mAb	Our lab 5-3B2 (mo γ 1 κ) (70)

Anti-mouse FCRL5 (3B7) and an irrelevant isotype-matched mouse IgG1 control mAb were digested using F(ab')₂ Preparation Kit (Pierce, Rockford, IL) and purified fragments were biotinylated using the EZ-Link Sulfo-NHS-LC-Biotin Kit (Pierce). Anti-mouse FCRL5 (9D10) was labeled using the Alexa Fluor 647 Labeling Kit (Invitrogen, Eugene, OR).

Table S2: Protein Kinase Inhibitors

Name	Manufacturer, Cat.#/Clone
Syk Inhibitor IV, BAY 61-3606	Calbiochem 574714
BTK Inhibitor, LFM-A13	Sigma L8789
Src Inhibitor, SU6656	Sigma S9692
PLC Inhibitor U-73122	Cayman 70740

Cell lines and Generation of Chimeric Fc γ RIIb/FCRL5 Constructs

The cDNAs encoding the intracellular portion of FCRL5 were fused in-frame with the extracellular and transmembrane domain of mouse Fc γ RIIB and cloned into the pDisplay vector (Invitrogen) adding the Ig kappa chain leader sequence and a hemagglutinin (HA) tag to generate the FCRL5 chimeric protein. A *NotI* site was introduced between the transmembrane domain of Fc γ RIIB and the intracellular domain of FCRL5, translating into a 3-alanine spacer underlined in Figure 2A as previously described (31). Site directed mutagenesis was performed by PCR using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) converting cytoplasmic tyrosine (Y) residues to phenylalanines (F). For retroviral transduction, cDNAs encoding the chimeric proteins were excised from pDisplay and cloned into the pMX-PIE retroviral expression vector which expresses the gene of interest upstream of an internal ribosomal entry site (IRES) element and the enhanced green fluorescent protein (a kind gift from Goetz R. A. Ehrhardt, University of Toronto, Toronto, Canada) and transduced into the A20-IIA1.6 B cell line (71).

Calcium Mobilization

For A20-IIA1.6 cells, 1×10^6 cells were washed twice in Hanks' balanced salt solution (HBSS) without phenol red, supplemented with $1 \mu\text{M}$ Ca^{2+} and Mg^{2+} , resuspended in $250 \mu\text{l}$ of HBSS containing $2 \mu\text{M}$ Fluo-4 and $4 \mu\text{M}$ SNARF-1 (Invitrogen, Carlsbad, CA), and incubated at 37°C for 30 min before analyzed with an FACSCalibur flow cytometer (BD Biosciences) after adding intact ($25 \mu\text{g/ml}$) or $\text{F(ab}')_2$ fragments ($16.6 \mu\text{g/ml}$) of anti-IgG.

For primary mouse B cells, single cell suspensions of whole splenocytes or lavage-isolated PEC cells were labeled with Indo-1/AM (Molecular Probes, Eugene, OR) in HBSS with $1 \mu\text{M}$ Ca^{2+} and Mg^{2+} and incubated at 37°C for 40 min before staining splenic MZ B cells with anti-CD23-FITC, CD21-APC and CD19-PerCP-Cy5.5, or PEC B1 B cells with anti-CD5-FITC, CD11b-APC, and CD19-PerCP-Cy5.5 and counterstaining with biotinylated $\text{F(ab}')_2$ anti-mouse IgM and anti-FCRL5 (3B7) or an IgG1 κ isotype control. In parallel experiments, PEC cells were preloaded with Indo-1/AM followed by Fc-block using anti-CD16/32 (2.4G2), then stained with the above surface markers, and counterstained with biotinylated $\text{F(ab}')_2$ anti-mouse IgM and either anti-CD5, anti-CD32, anti-CD22, anti-CD72, or an isotype-matched control. All antibodies were used at a final concentration of $10 \mu\text{g/ml}$. Ca^{2+} mobilization was induced by adding $20 \mu\text{g/ml}$ of streptavidin (SA) (Jackson ImmunoResearch) before analysis with a LSRII flow cytometer equipped with a UV laser (BD Biosciences). Fluorescence intensities of bound or free Ca^{2+} in the gated splenic MZ or PEC B1 cell subsets were

recorded in the FL-14 and FL-15 channels for 300s and the FL-14/FL-15 ratio was analyzed using the Flowjo software package (Treestar, Ashland, OR).

Protein Immunoblotting Analysis

To monitor the effects of WT FCRL5 on BCR-induced whole-cell protein tyrosine phosphorylation, 1×10^7 WT or FFF Fc γ RIIb/FCRL5 A20-IIA1.6 cell aliquots were washed twice and incubated for 2 hr in medium lacking FBS and supplemented with 20 mM HEPES (pH 7.2), before stimulation with intact (25 μ g/ml) or F(ab')₂ fragments of anti-mouse IgG (16.6 μ g/ml). After stimulation for 0, 1, 3, 5, 10, or 30 min at 37°C, cells were spun and lysed in lysis buffer. Whole-cell lysates (WCLs) were quantitated using the BCA reagent (Pierce), resolved by SDS/PAGE, and transferred to PDVF membranes (Millipore) before immunoblotting with the anti-pTyr (4G10) (Millipore), visualization using the ECL reagent (GE Healthcare, Piscataway, NJ), and detection by Biomax XAR film (Kodak, Rochester, NY). Blots were reprobbed with anti-pERK and anti- β -actin Abs to analyze downstream MAP kinase activation and verify equal protein loading. For immunoprecipitation, whole-cell lysates were incubated at 4°C for 30 min with 4 μ g of anti-HA Abs, followed by the addition of 30 μ l of a 50% slurry of protein G beads (GE Healthcare), and incubated overnight at 4°C. Beads were washed five times with 1 ml of lysis buffer to reduce non-specific binding and resuspended in an equal volume of SDS sample buffer and boiled. Proteins were resolved by SDS-PAGE and

immunoblotted with 4G10 to assess receptor phosphorylation status and with anti-HA as a loading control.

To investigate the role of different tyrosines in the FCRL5 cytoplasmic tail on BCR-induced ERK activation, 5×10^6 A20-IIA1.6 cells expressing the various mutants were left untreated (-) or stimulated with intact anti-IgG Abs (I) or F(ab')₂ fragments of anti-IgG Abs (F) for 10 min. Lysates were probed with anti-pERK then stripped and reprobed with anti-ERK to verify equivalent protein loading. The densities of p-ERK were quantified, using transductants stimulated with anti-IgG F(ab')₂ fragments as a baseline of 1.00.

To examine the association of SHP-1 and Lyn with native FCRL5, 5×10^6 MZ B cells sorted from BAFF Tg mice (Figure S5B) were stained with biotinylated F(ab')₂ anti-mouse IgM and anti-FCRL5 (3B7 clone) or an IgG1 κ isotype control. Cells were stimulated by SA crosslinking of IgM with FCRL5 versus an isotype control at a final concentration of 20 μ g/ml. Cells were then lysed, immunoprecipitated with an anti-FCRL5 mAb (9D10), and IPs were subjected to immunoblotting with anti-pTyr (4G10), anti-SHP-1, and anti-Lyn Abs. Blots were stripped and reprobed with anti-FCRL5 mAb (5-3B2) as a loading control.

Intracellular-Staining and Phospho-Flow Analysis

For intracellular staining, primary splenocytes or PEC cells (1×10^6) isolated from WT C57BL/6 mice were stained for the indicated MZ or B1 B cell surface markers. Cells

were then fixed and permeabilized using the FIX & PERM kit (Invitrogen) before staining with rabbit anti-mouse SHP-1, rabbit anti-mouse Lyn, rabbit anti-human phospho-Lyn (Y396) (cross-reactive with active form of mouse Lyn (Y397)), rabbit anti-human phospho-Lyn (Y507) (cross-reactive with inactive form of mouse Lyn (Y508)) or a rabbit IgG serum control, and counterstaining with a secondary PE-conjugated goat anti-rabbit IgG.

Phospho-specific flow cytometry was performed by modification of previously reported protocol (72). After red blood cell lysis, primary lymphocytes were starved for 2 hr at 37°C before staining at a density of 1×10^6 cells/ml with discriminating MZ or B1 cell surface markers and counterstaining with biotinylated F(ab')₂ anti-mouse IgM, anti-FCRL5 (3B7) or an IgG1 κ isotype control on ice. Cells were stimulated at 37°C for 10 min by SA crosslinking of IgM with FCRL5 or an isotype control at a final concentration of 20 μ g/ml. In parallel experiments, cells are stimulated with 0.1 μ M Pervanadate. Cells were fixed with the Phosflow Fix Buffer I (BD Biosciences) for 10 min at 37°C and permeabilized with methanol at -20°C for 30 min. Cells were then stained with PE-conjugated anti-pTyr (PY20) at 37°C for 10 min, and analyzed with a FACSCalibur flow cytometer and FlowJo Software (Treestar).

Statistical Analysis

Results are expressed as mean \pm SEM and were determined with an unpaired two-tailed student's t-test.

ACKNOWLEDGEMENTS

We thank Dr. Goetz R. A. Ehrhardt at University of Toronto for providing materials and expert technical help, Dr. Larry Gartland and Marion Spell in the University of Alabama at Birmingham CFAR (NIH P30 AI027767) for their cell sorting assistance, Dr. Clifford Lowell at UCSF for Lyn KO mice, Dr. Carlo Croce at Ohio State University for TCL1 Tg mice, and Drs. John Kearney and Peter Burrows for critical reading of the manuscript. This work was supported in part by funding from the NIH (AI067467) and the ACS (RSG 08-232-01).

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FIGURE LEGENDS

Figure 1. FCRL5 Differentially Regulates Innate-Like B Cell Signaling

Spleen and PEC leukocytes from WT mice were stained to define the indicated B cell subsets and FCRL5 (black line) or control (shaded grey) surface expression (left two panels). Indo-1/AM loaded cells were stained for distinguishing markers as well as IgM and FCRL5 (3B7) or control biotinylated F(ab')₂ Abs (all 10 µg/ml), and Ca²⁺ mobilization was analyzed in gated subsets after adding 20 µg/ml streptavidin (SA). Intracellular whole-cell tyrosine phosphorylation (pTyr) was monitored after SA crosslinking for 10 min, fixation, permeabilization, and intracellular (IC) counterstaining with anti-pTyr (PY20) (right two panels). Results are representative of three independent experiments.

Figure 2. FCRL5 Immunoregulatory Function Is Tyrosine-based

(A) Schematic illustration of FCRL5 cytoplasmic tyrosine-based motifs and FcγRIIb/FCRL5 chimeric constructs as detailed in the Supplemental Information.

(B) WT or FFF transductants (10⁷) were stimulated with intact (25 µg/ml) or F(ab')₂ (16.6 µg/ml) anti-IgG over time, and WCL were immunoblotted with Abs to pTyr (4G10), pERK, or β-actin as a loading control. WCL were immunoprecipitated with anti-HA (12CA5) and analyzed for pTyr and HA to verify equal loading with anti-HA (bottom). The arrow indicates the position of the chimeric receptor. Results are representative of three independent experiments.

Figure 3. FCRL5 Counter-Regulates BCR Signaling

(A) Ca^{2+} mobilization in Fluo-4 NW and SNARF-1 loaded A20-IIA1.6 transductants (10^6) was analyzed after BCR stimulation alone (black) or receptor cross-linking (grey).

(B) Lysates from transductants (5×10^6) left untreated (-) or stimulated with intact (I) versus F(ab')_2 (F) anti-IgG for 10 min were probed for pERK and total ERK. Relative changes were quantified by densitometry. Results are representative of at least three independent experiments.

Figure 4. FCRL5 ITIM and ITAM-Like Sequences Independently Recruit SHP-1 and Lyn.

(A) WCLs from A20-IIA1.6 transductants (10^7) stimulated with intact anti-IgG for 10 min (+) or left untreated (-) were immunoprecipitated with anti-HA and blotted for pTyr, indicated phosphatases, or HA as a loading control.

(B) WCLs from the FF and FFF transductants (10^7) stimulated as in (A) were immunoprecipitated with anti-SHP-1 and probed for HA or SHP-1 as a loading control.

(C) Immunoprecipitates from the chimeric panel stimulated as in (A) were blotted for the indicated kinases.

(D) Ca^{2+} flux was monitored in control A20-IIA1.6 B cells incubated with or without kinase inhibitors at different concentrations for 30 min after stimulation with F(ab')_2 anti-IgG. Optimally determined concentrations were applied before ligating Y566F mutant cells with intact or F(ab')_2 anti-IgG.

Figure 5. FCRL5 Has Unique SHP-1 and Lyn-Dependent Dual Functionality in B1 B cells

(A and B) PEC leukocytes (10^6) isolated from me^v/me^v and $Lyn^{-/-}$ mice were stimulated as in Figure 1 and Ca^{2+} mobilization and IC pTyr were analyzed in the gated subsets. The data are representative of at least two independent experiments.

(C) PEC leukocytes (10^6) from the indicated mice were preloaded with Indo-1/AM and blocked with anti-CD16/32 (2.4G2) before staining with discriminating surface markers, followed by biotinylated $F(ab')_2$ anti-IgM and CD5, CD22, CD32, CD72, or isotype-matched control mAbs (all at $10\mu\text{g/ml}$). Ca^{2+} mobilization was monitored after SA addition ($20\mu\text{g/ml}$).

Figure 6. FCRL5 Function in Innate-Like B cells Directly Correlates with SHP-1 Activity

(A) MZ B cells (5×10^6) sorted from BAFF Tg mice (Figure S5) were stained with IgM and FCRL5 or control biotinylated $F(ab')_2$ Abs. After SA crosslinking ($20\mu\text{g/ml}$) for 10 min at 37°C , WCLs were immunoprecipitated with anti-FCRL5 (9D10), and immunoblotted for pTyr, SHP-1, or Lyn. Blots were stripped and reprobbed with anti-FCRL5 (5-3B2) as a control.

(B) WT splenocytes or PEC leukocytes (10^6) were rested in serum free medium for 2 hr, surface stained, fixed, permeabilized, and stained IC with anti-pTyr.

(C) WT splenocytes or PEC leukocytes (10^6) prepared as in (B) were IC stained with rabbit anti-mouse SHP-1 or a rabbit IgG serum control, and counterstained with a secondary PE-conjugated goat anti-rabbit IgG.

(D) WT splenocytes or PEC leukocytes (10^6) were rested, surface stained, and stimulated with 0.1 mM Na_3VO_4 for 10 min at 37°C prior to fixation, permeabilization, and IC staining for pTyr.

(E) WT splenocytes or PEC leukocytes (10^6) prepared as in (B) were IC stained with rabbit anti-mouse Lyn, rabbit anti-human phospho-Lyn (Y396), rabbit anti-human phospho-Lyn (Y507), or a rabbit IgG serum control before counterstaining with a secondary PE-conjugated goat anti-rabbit IgG Ab.

Data are shown as mean \pm SEM, * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$, and representative of at least three independent experiments per panel.

Figure 7. FCRL5 Reveals Opposing Roles for SHP-1 and Lyn in MZ and B1 B Cell Receptor-Mediated Survival

Purified splenic MZ or PEC B1 B cells (10^5) from the indicated mice (Figure S7) were directly cultured or stained with IgM and FCRL5 or control biotinylated F(ab')_2 Abs. After SA crosslinking, apoptosis was determined for MZ cells at 12 hr or 24 hr for B1 B cells by annexin V and propidium iodide (PI) staining. Total apoptotic cells include both early (annexin V positive, PI negative) and late fractions (annexin V positive, PI positive).

Data are shown as mean \pm SEM, **p < 0.01 and ***p < 0.001, and are representative of at least three independent experiments.

FIGURES

Figure 1

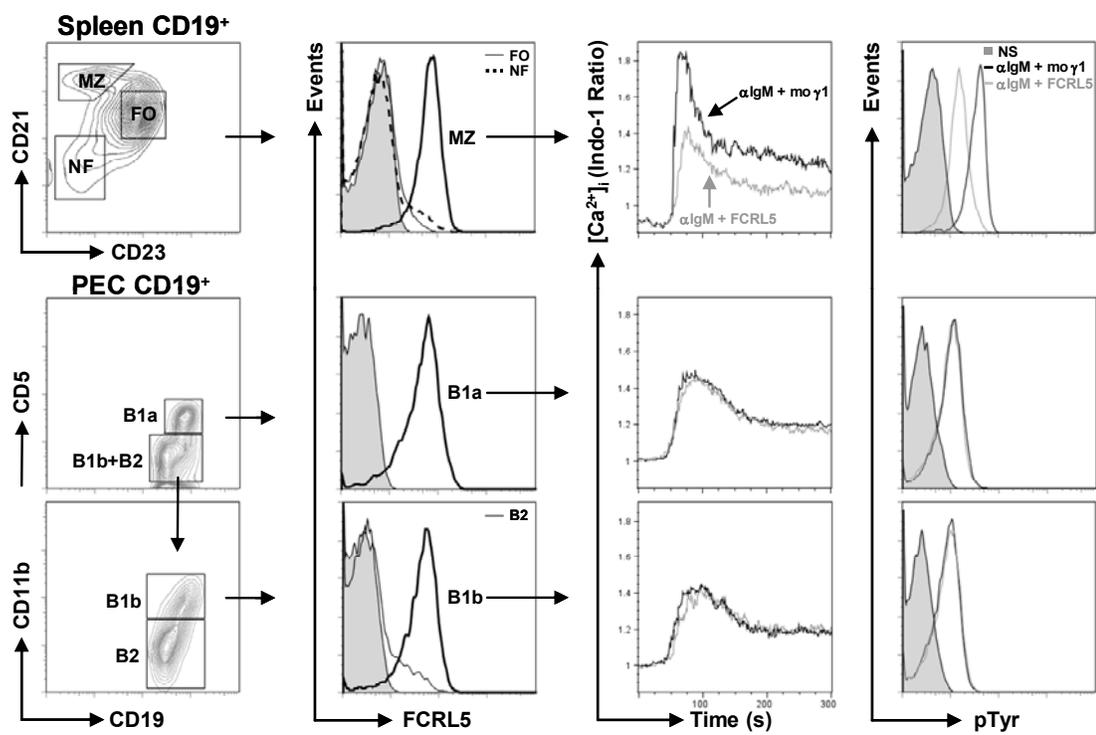
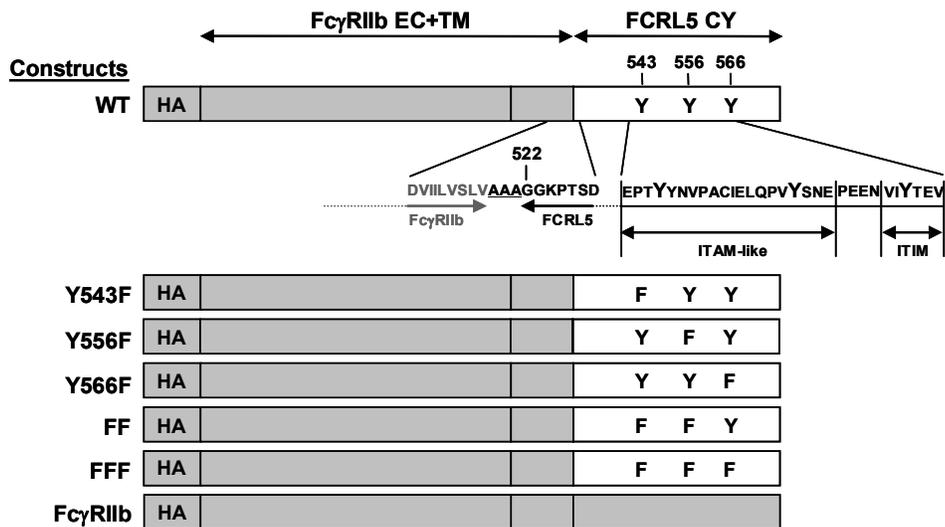


Figure 2

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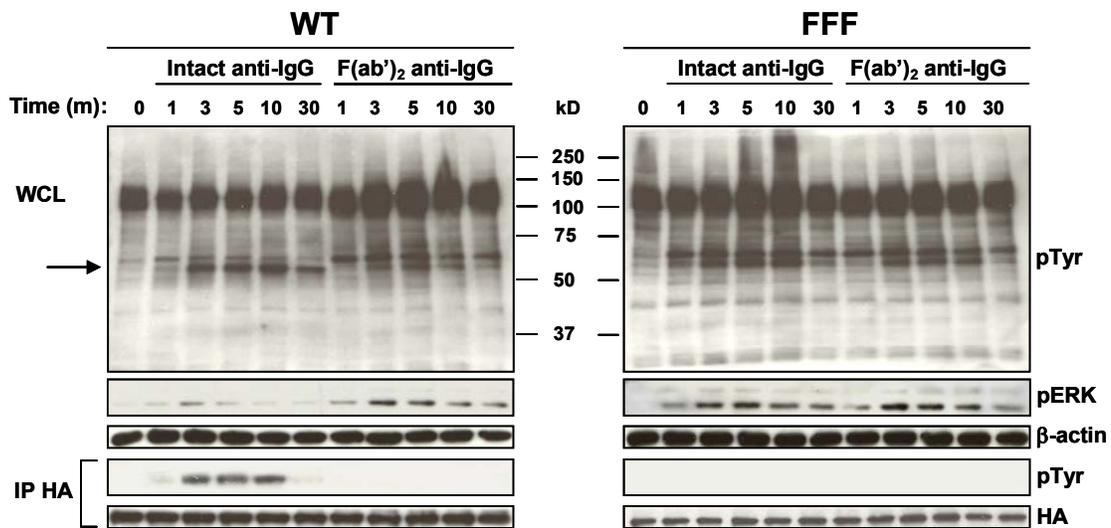
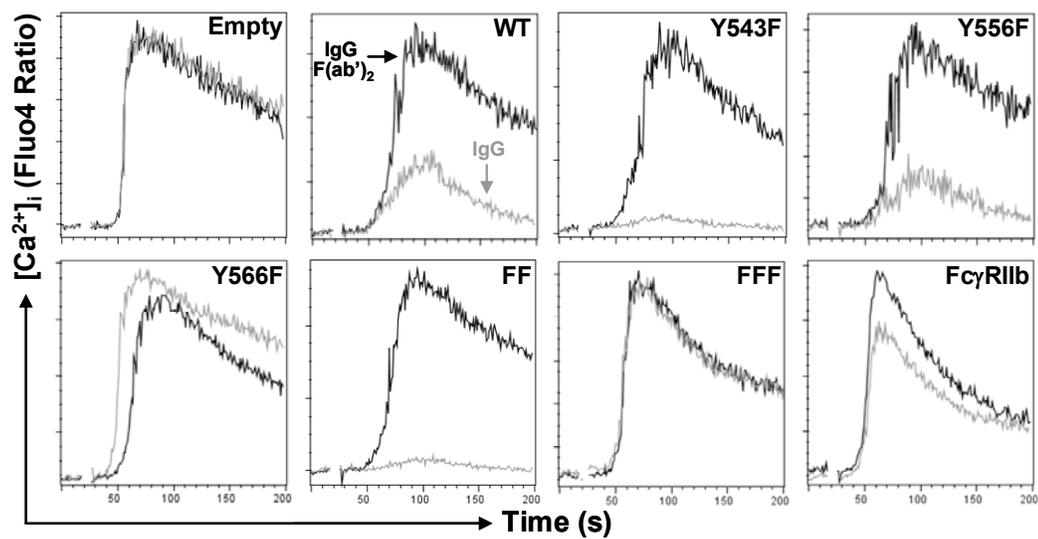


Figure 3

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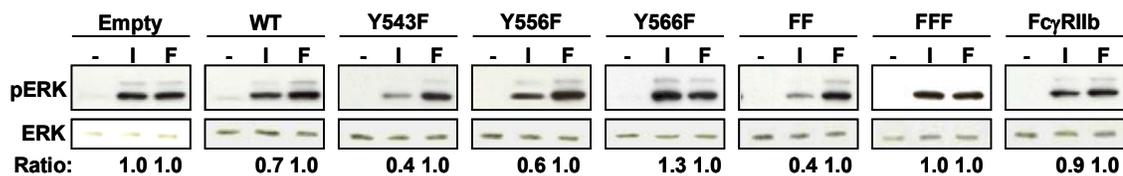


Figure 4

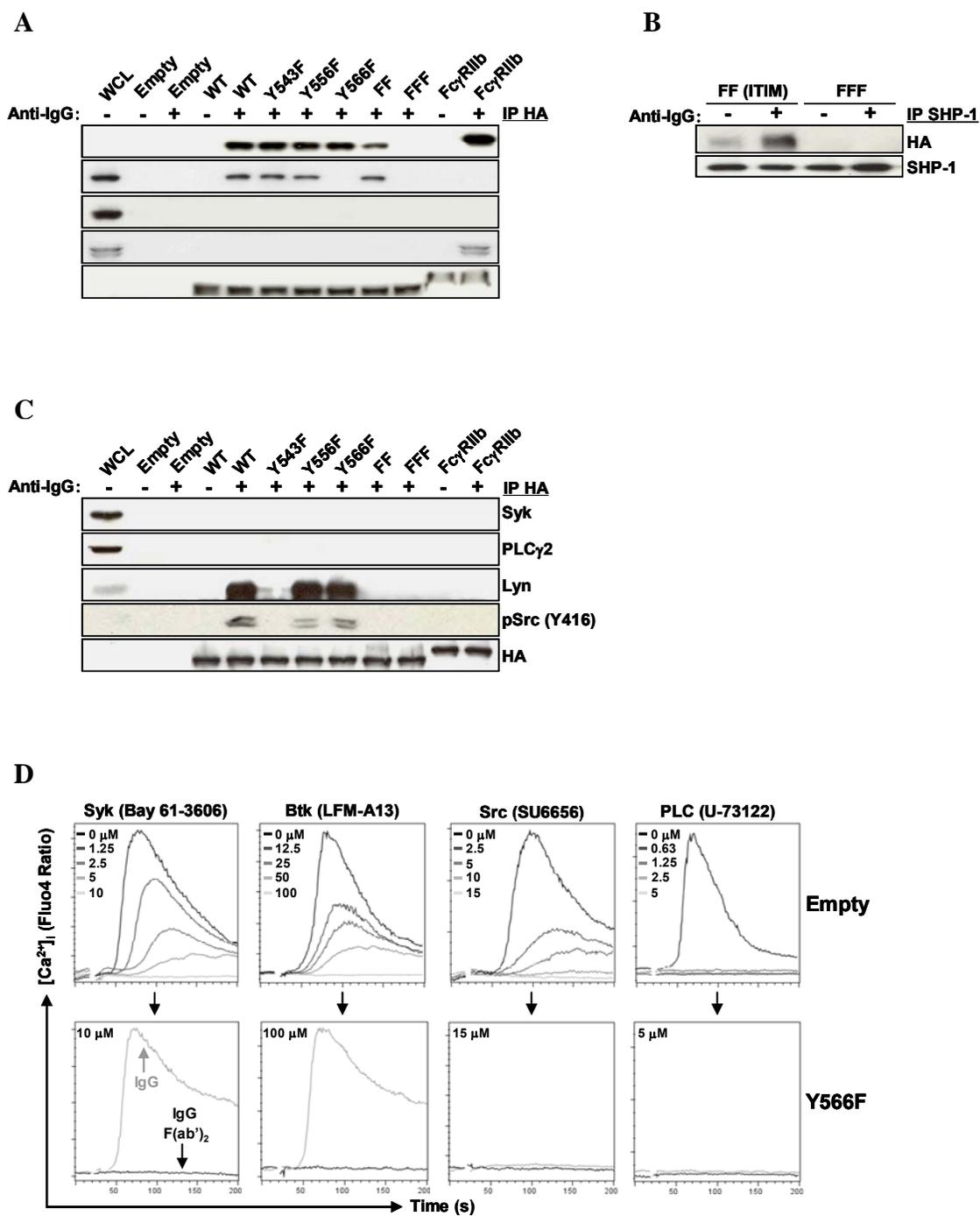


Figure 5

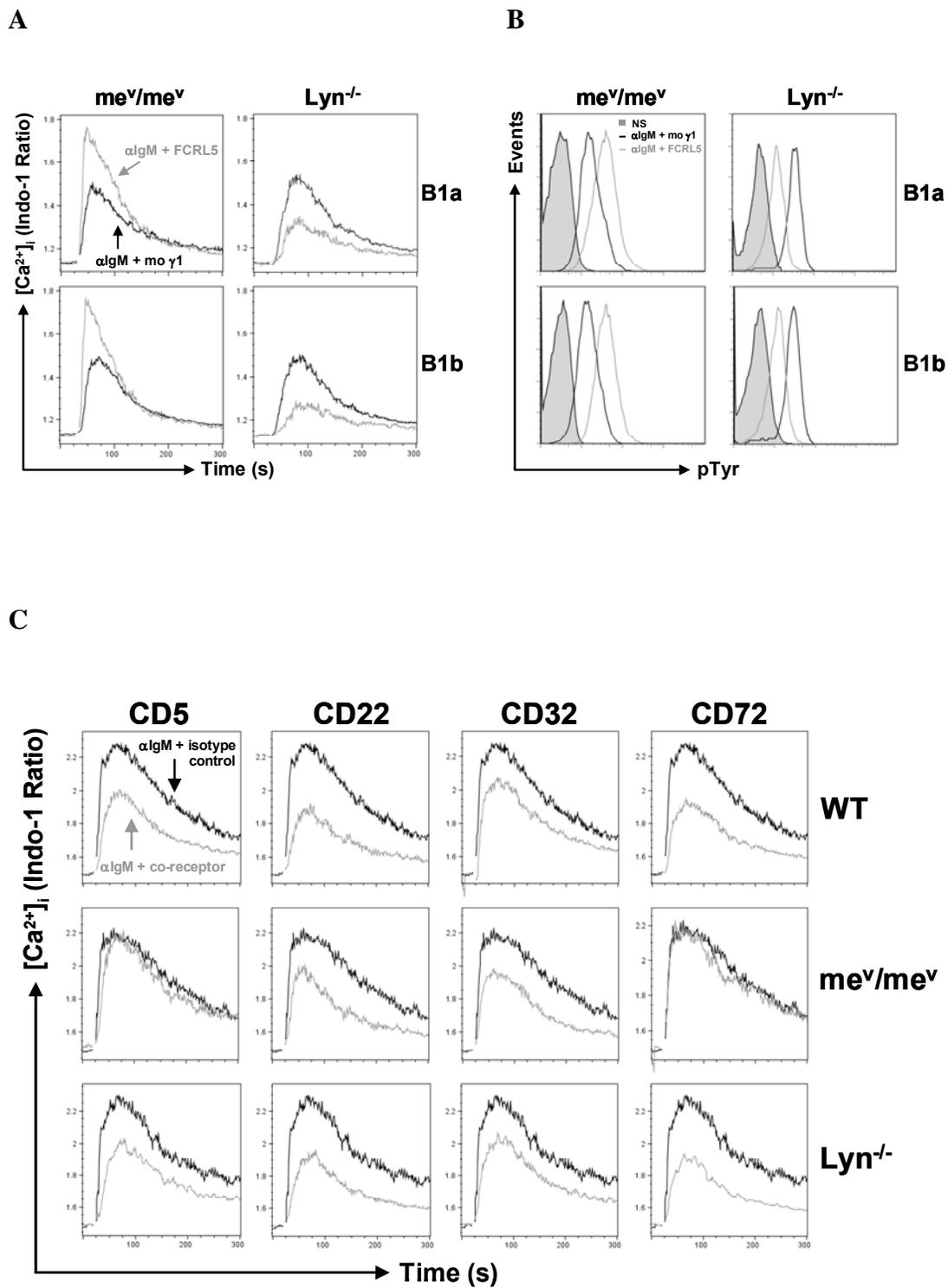


Figure 6

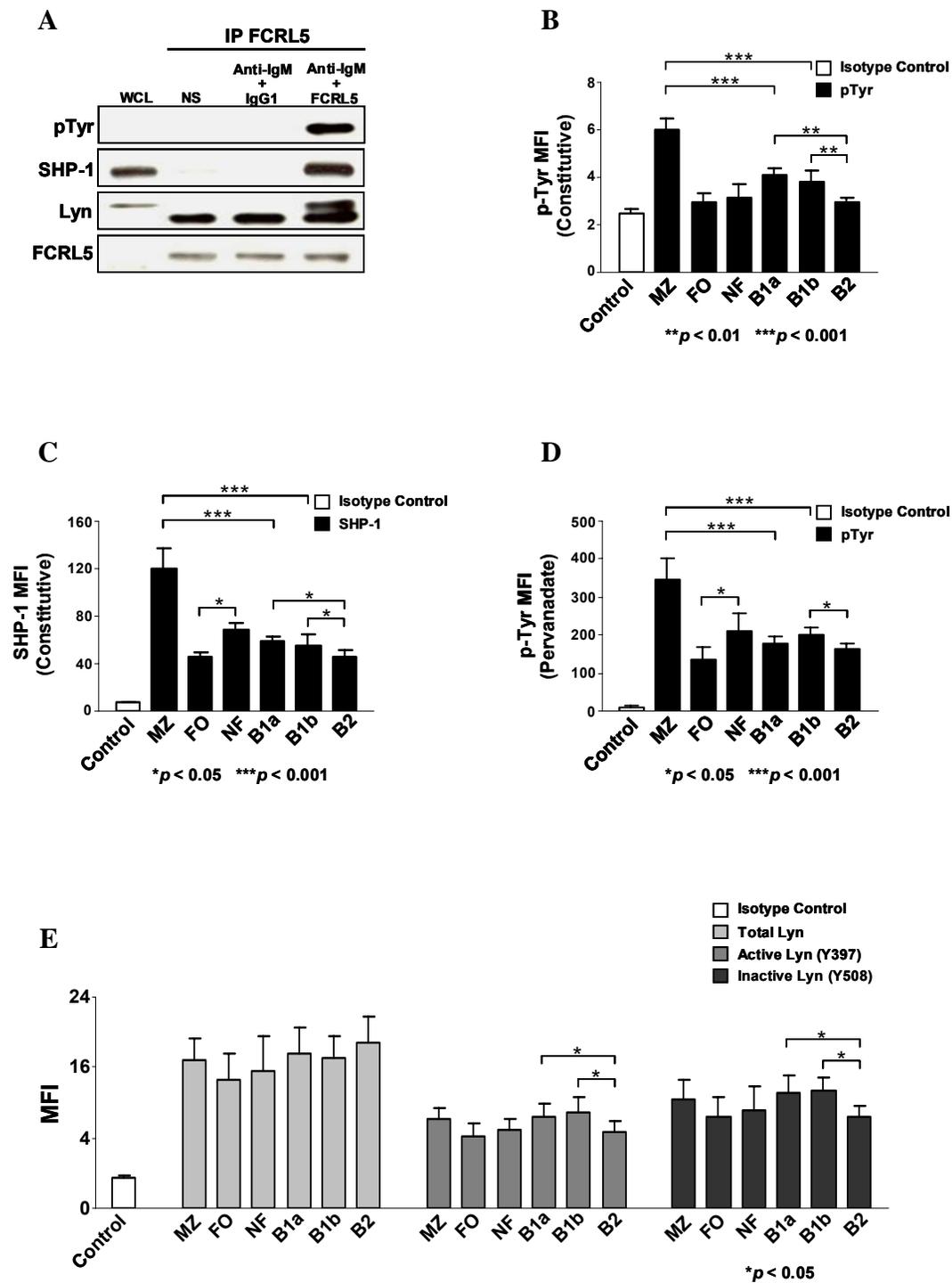
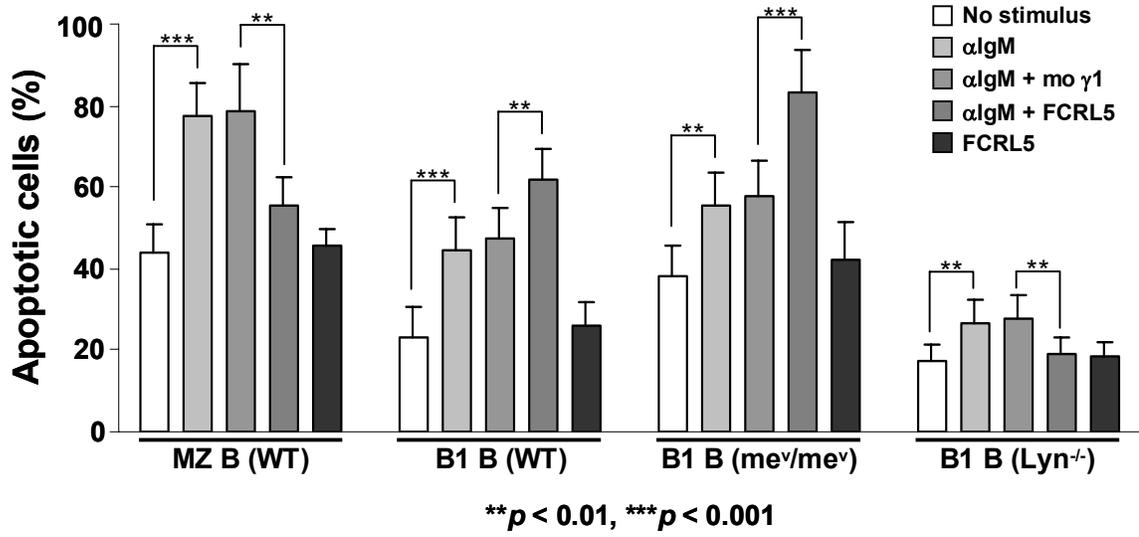


Figure 7



SUPPLEMENTAL DATA

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Equivalent Fc γ RIIb/FCRL5 Chimera Expression and Ligation Strategy in A20-IIA1.6 B Cells

(A) A20-IIA1.6 cells transduced with either the empty vector or indicated constructs were stained with anti-HA and examined by flow cytometry to assess EGFP and cell surface chimeric receptor expression.

(B) F(ab')₂ anti-IgG fragments stimulate the BCR only (left), while intact anti-IgG co-ligates the BCR with the Fc γ RIIb extracellular portions of various chimeric proteins (right). The ITAM-like sequence is represented in green and ITIM sequence in red.

Figure S2. The FCRL5 ITAM-like and ITIM Sequences Can Be Tyrosine-phosphorylated

A20-IIA1.6 B cell transductants (1×10^7) expressing wild type (WT) or mutant forms of FCRL5 were washed and rested in serum free RPMI 1640 medium for 2 hr, and then stimulated with 0.1 mM Na₃VO₄ (Sigma, St. Louis, MO) at 37°C for 10 min. Whole-cell lysates were immunoprecipitated with anti-HA and blotted with anti-pTyr (4G10). Blots were then stripped and reprobed with anti-HA as a loading control. The arrow indicates the expected position of the chimeric proteins.

Figure S3. B Both me^v/me^v and $Lyn^{-/-}$ Mice Have A Loss of Splenic MZ B Cells, but Their PEC B1 B Cells Express FCRL5 at Levels Similar to WT C57BL/6 Mice

(A) Splenocytes from WT C57BL/6, me^v/me^v , and $Lyn^{-/-}$ mice were stained with anti-CD21-PE, CD23-FITC, and CD19-PerCP-Cy5.5 and analyzed by flow cytometry. The percentage of total splenic B cells (upper panel) and the MZ subset frequency (lower panel) are shown.

(B) PEC cells isolated by lavage from WT C57BL/6, me^v/me^v , and $Lyn^{-/-}$ mice were stained with anti-CD5-PE, CD11b-FITC, and CD19-PerCP-Cy5.5, then counterstained with Alexa 647-conjugated anti-FCRL5 (9D10) (black line) or a rat $\gamma 2a\kappa$ isotype control (shaded grey) and assessed by flow cytometry.

Figure S4. Immunoreceptor Surface Expression by PEC B1a B Cells Derived from C57BL/6, me^v/me^v , and $Lyn^{-/-}$ Mice

PEC cells isolated by lavage were stained with anti-CD19-PerCP-Cy5.5 and CD5-PE to gate the B1a ($CD19^{high}CD5^{high}$) subset and then counterstained with biotinylated anti-CD5, CD22, CD32, CD72, IgM, or IgD (black line), or an isotype-matched control (shaded grey) followed by streptavidin-PE.

Figure S5: Generation of Human Soluble BAFF Tg Mice and Demonstration of Their Expanded Total Splenic and MZ B Cell Fractions

(A) Full-length human BAFF (285aa) is type II transmembrane protein possessing extracellular (EC), transmembrane (TM) and cytoplasmic (CY) portions, but soluble

hBAFF (152aa, grey box) can be released from the membrane by proteolytic cleavage (upper panel). The soluble hBAFF cDNA sequence was cloned into the human CD2 promoter/enhancer cassette, p29Δ2 (Sal-) via *EcoRI-SalI* restriction digest sites (low panel). After *NotI* digestion to remove the vector backbone, the construct was microinjected into C57BL/6 fertilized oocytes using standard procedures (UAB Transgenic Mouse Facility, Birmingham, AL).

(B) Splenocytes from WT C57BL/6 or BAFF Tg mice were stained with anti-CD21-PE, CD23-FITC, and CD19-PerCP-Cy5.5, then counterstained with Alexa 647-conjugated anti-FCRL5 (9D10) (black line) or a rat γ 2 κ isotype control (shaded grey). The percentage of total B cells in the spleen (left panel, top), the MZ frequency (left panel, middle) and FCRL5 expression (left panel, bottom) are indicated. MZ B cells (black dots) from BAFF Tg mice were sorted by FACS Aria to 95% purity relative to unsorted splenic B cells (grey dots) (right panel).

Figure S6. Splenic MZ B Cells Are More Preactivated and Possess Stronger BCR Signaling Potential than PEC B1 Cells

Single cell suspensions of whole splenocytes or lavage-isolated PEC cells from WT C57BL/6 mice were Indo-1/AM labeled before staining splenocytes with anti-CD23-FITC, CD21-APC and CD19-PerCP-Cy5.5, or PEC B cells with anti-CD5-FITC, CD11b-APC, and CD19-PerCP-Cy5.5 followed by stimulation with 10 μ g/ml F(ab')₂ anti-mouse IgM. Calcium mobilization in the gated (MZ:

CD19^{high}CD21^{high}CD23^{low}, newly formed (NF): CD19^{high}CD21^{low}CD23^{low}, FO: CD19^{high}CD21^{low}CD23^{high}, B1a: CD19^{high}CD5^{high}, B1b: CD19^{high}CD5^{low}CD11b^{high} and B2: CD19^{high}CD5^{low}CD11b^{low}) subsets was analyzed with a UV laser equipped LSRII flow cytometer (BD Biosciences) (left panel). Intracellular whole-cell protein tyrosine phosphorylation in stained WT splenic MZ and PEC B1 B cells left unstimulated or stimulated with 10 µg/ml F(ab')₂ anti-mouse IgM or 0.1 mM Na₃VO₄ for 10 min was determined after fixation and permeabilization by PE-conjugated anti-pTyr (PY20) via phospho-specific flow cytometry (right panel).

Figure S7. Splenic MZ and PEC B1 B Cell Purification Strategies

(A) Single cell suspensions of whole splenocytes from WT C57BL/6 mice were labeled with anti-mouse CD43 (Ly-48) microbeads and sorted by magnetic-activated cell sorting (MACS). Purity was verified by surface staining with anti-CD19-PerCP-Cy5.5 and analysis by FACS (left). Splenic B cells were then labeled with anti-CD23-PE and CD21-APC (middle) and FACS-sorted (right).

(B) After lavage, PEC leukocytes from WT mice were labeled with biotinylated anti-mouse F4/80, Gr-1, CD11c, NK-1.1, Thy-1.1 and CD23. Flow cytometry analysis of a small fraction of these cells counterstained with SA-FITC indicated the frequency of FITC-negative fraction containing PEC B1 cells (left). The remaining untouched subset was incubated with SA microbeads and sorted by MACS. Using this dumping strategy, FACS staining with anti-CD19-PerCP-Cy5.5, CD5-PE and CD11b-APC verified the

B1-cell purity at >90% (middle and right). The SHP-1 and Lyn-deficient PEC B1 cells were similarly isolated by this method with purities of ~90% (data not shown).

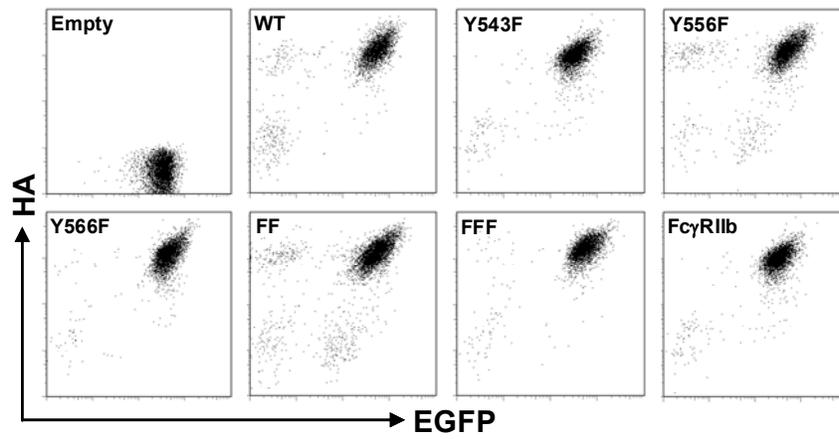
Figure S8. A Proposed Model for the Role of FCRL5 in Modulating Innate-like B Cell Antigen Receptor Signaling

FCRL5 extracellular Ig domains (color coded) and its cytoplasmic ITAM-like (green box) and ITIM (red box) subunits are shown. FCRL5 inhibitory function is conferred by SHP-1, which is recruited to other ITIM-bearing receptors (shaded red on left). FCRL5 enhancing function is Lyn-dependent and Lyn also equips other activation molecules (shaded green on right). Note the relative balance of Lyn-SHP-1 signaling differentially modulates the role of FCRL5 in innate-like B cell receptor signaling.

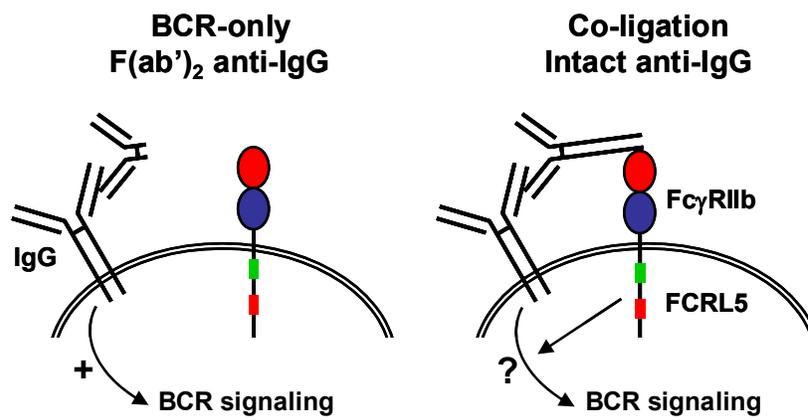
SUPPLEMENTAL FIGURES

Supplemental figure 1

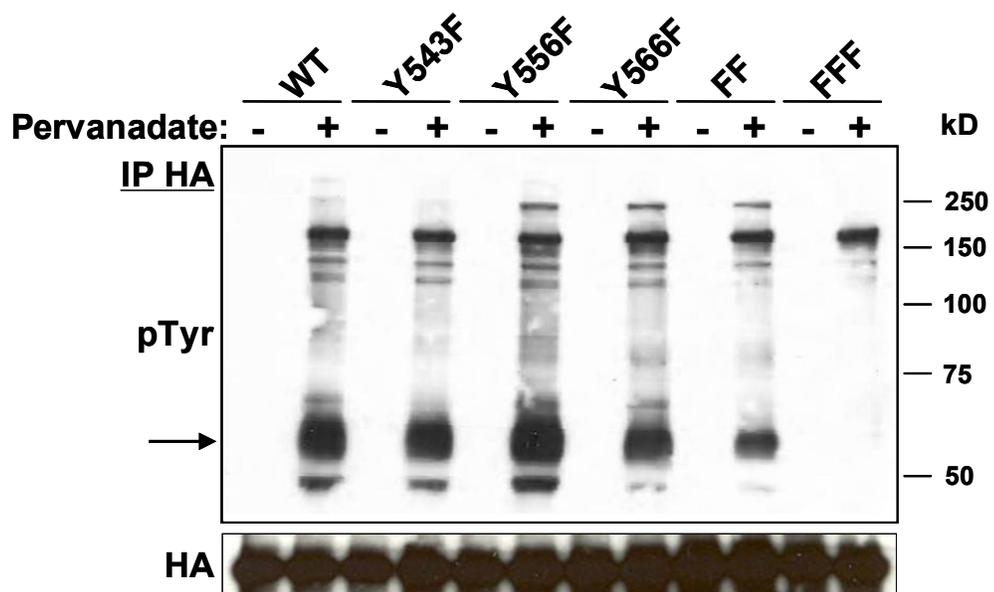
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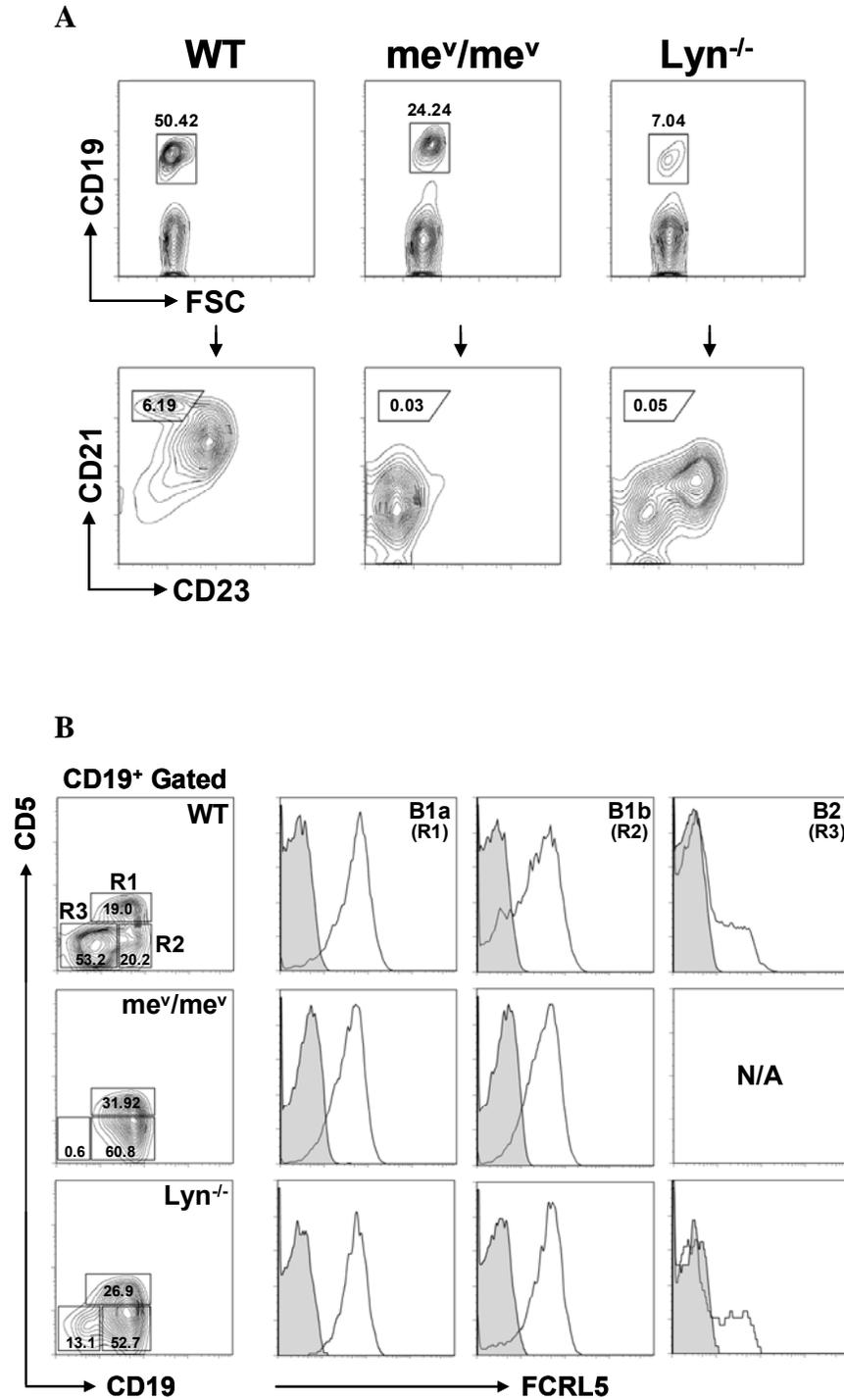
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Supplemental figure 2

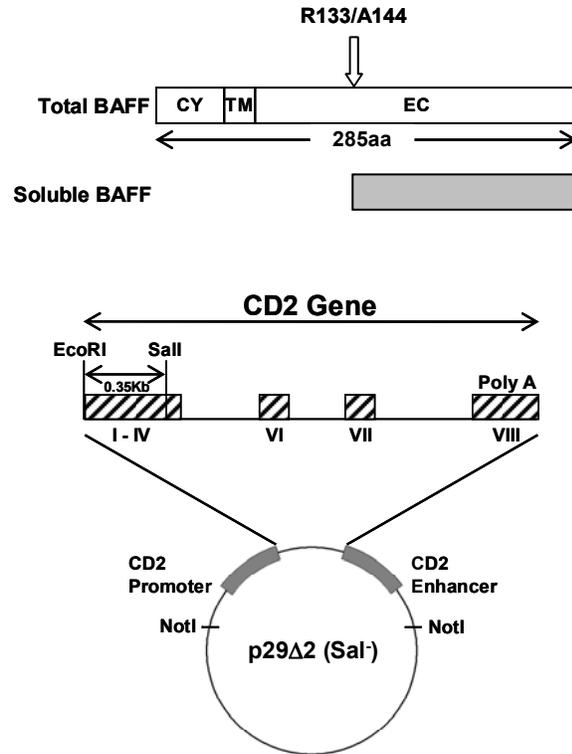


Supplemental figure 3

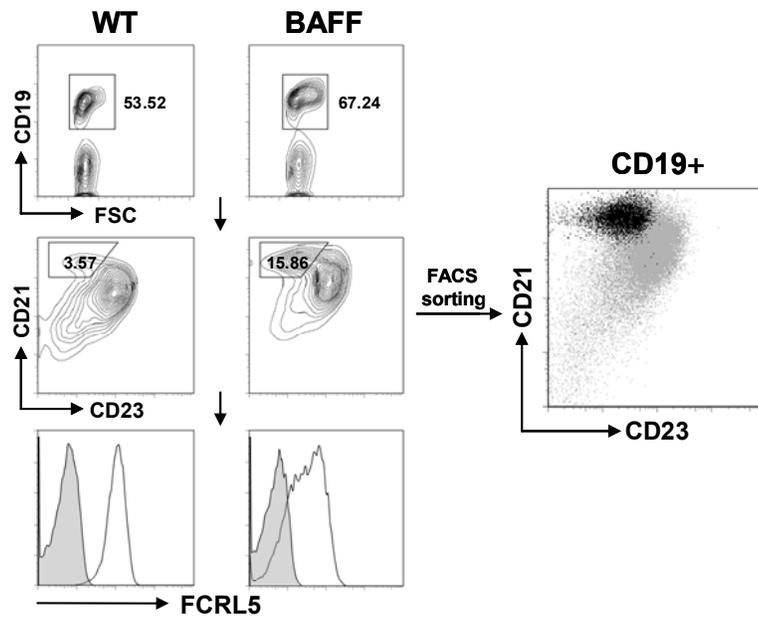


Supplemental figure 5

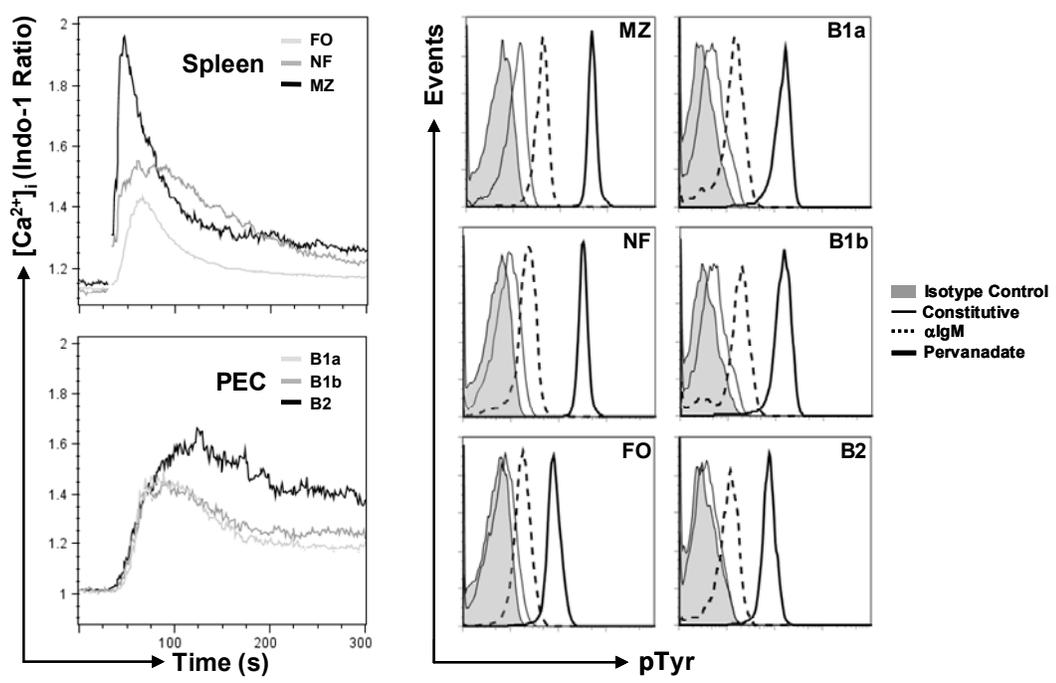
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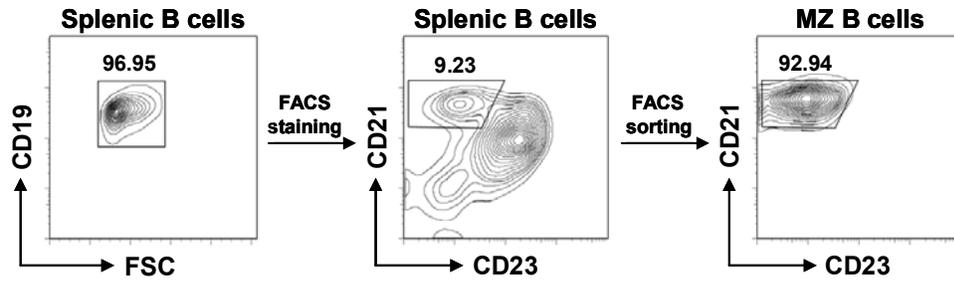


Supplemental figure 6

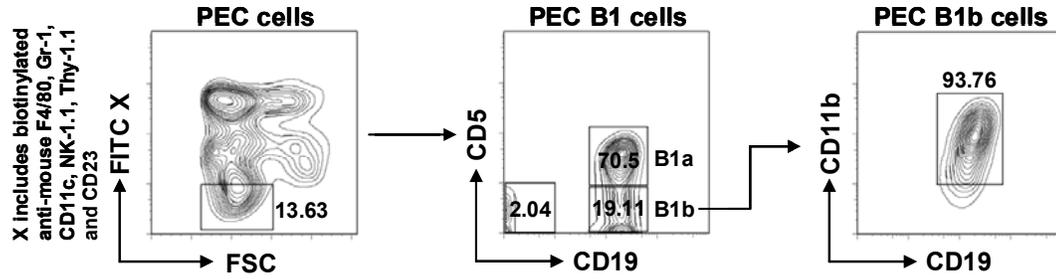


Supplemental figure 7

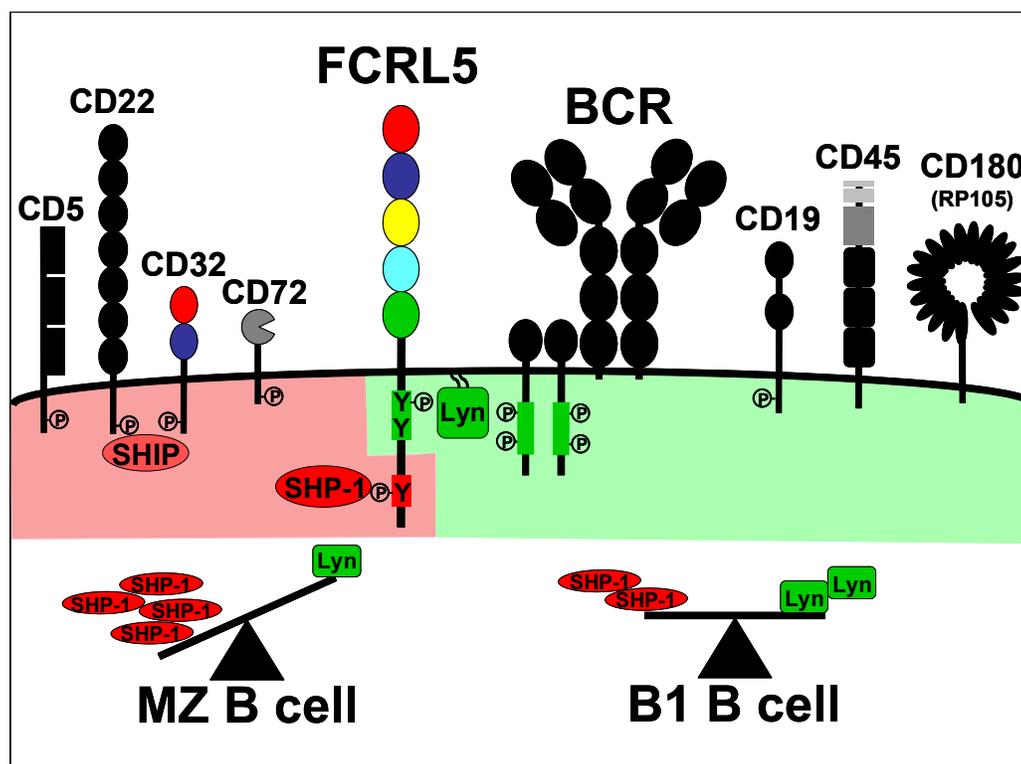
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Supplemental figure 8



CONCLUSIONS AND FUTURE DIRECTIONS

Innate-like B cells consist of splenic MZ B cells and PEC-derived B1 B cells. Their unique anatomical locations allow them to generate rapid immune responses to invading pathogens that bridges host protection until adaptive mechanisms are established. In the past several decades, these subsets have been extensively studied to elucidate their developmental, phenotypic, and functional properties. It is now known that their capacity for broad and prompt responses is associated with evolutionarily conserved immunoglobulin repertoires, distinct sensitivity to TI stimuli, and the potential to rapidly differentiate into cells that secrete “natural,” polyreactive antibodies.

FCRL5 is distinctly expressed on mouse MZ and B1 B cells and possesses cytoplasmic ITAM-like and ITIM sequences. In this study, we molecularly dissected its regulatory properties in cell line-based studies and validated them in primary B cells. By engineering a series of FcγRIIB/FCRL5 chimeric proteins possessing cytoplasmic Y>F mutants transfected into A20-IIA1.6 B cells, we first found that the three tyrosines comprising its ITAM-like and ITIM sequences could all be phosphorylated by pervanadate treatment and were potentially functional. Although it had no impact in A20 B cells when ligated alone (data not shown), FCRL5 could exert opposite effects on downstream BCR responses. The ITAM-like Y543 and ITIM Y566 residues were critical to its function, whereas the C-terminal Y556 site was dispensable. With both modules intact, BCR crosslinking disclosed its dominant inhibitory influence on Ca²⁺ flux, ERK

activation, and whole-cell tyrosine phosphorylation. Importantly, its activating and inhibitory properties offset each other. Disruption of the Y543 ITAM-like site completely abrogated Ca^{2+} mobilization, while mutation of the Y566 ITIM residue revealed its ability to enhance BCR-mediated activation. In contrast to previous findings in the field, these data indicate that FCRL5 can both inhibit and enhance B cell responses. The proteins responsible for its bi-functionality were then defined. Among SH2 domain containing tyrosine phosphatase candidates, SHP-1 associated in an ITIM-dependent fashion, whereas SHP-2 and SHIP failed to co-precipitate with any A20 variant. Putative signaling components responsible for its activating properties were also assessed, neither the Syk, PLC γ 2, Btk, or PI3-K kinases nor the Grb2 or BLNK adaptor proteins associated with FCRL5. Because Lyn is the predominant SFK expressed in B cells and is required for the activating and inhibitory function of many regulatory proteins, its association with FCRL5 was also explored. Lyn indeed co-precipitated with the WT, Y556F and Y566F chimeric receptors, but not with the Y543F, FF, or FFF mutants. Furthermore, Western blotting revealed that the SFK at the Y543 position was in the active state. To validate these studies in primary cells a F(ab')₂ anti-FCRL5 C57BL/6 allotype mAb (3B7) was employed. Crosslinking native FCRL5 with mIgM confirmed these effector/recruitment relationships in MZ B cells sorted from BAFF Tg mice that expand this subset. These data demonstrate that FCRL5 counter-regulates B cell responses by recruiting SHP-1 to its ITIM, while the ITAM-like Y543 residue fosters a Lyn-dependent activation pathway.

We additionally examined the significance of Lyn-SHP-1 signaling circuit in WT C57BL/6, variable motheaten (me^v/me^v , SHP-1 mutant) as well as Lyn KO mice. Both these genetically modified models develop a loss of splenic MZ and FO B cells and PEC B2 cells, but a relative expansion of B1a and B1b B cells. FCRL5 expression by PEC B1 B cells in these mutant mice was similar to WT. We were able to demonstrate divergent compartment-specific roles for FCRL5 that were Lyn and SHP-1 dependent. While it could suppress MZ BCR signaling, FCRL5 had no impact in WT B1 cells. By contrast, crosslinking FCRL5 in me^v/me^v B1 cells augmented BCR-mediated Ca^{2+} mobilization and whole-cell pTyr. Intriguingly, the opposite effect was observed in $Lyn^{-/-}$ B1 B cells which resembled observations made for WT splenic MZ and A20 B cells. These results in mutant mice confirm the inverse contributions of SHP-1 and Lyn to FCRL5 function. These features were also unique compared to other receptors (e.g. CD5, CD22, CD32, and CD72) that failed to acquire activating function in me^v/me^v B1 B cells. These findings further demonstrate that the relative activity of Lyn and SHP-1 has a direct effect on driving FCRL5 biology in innate-like B cells. While these data validate the critical contribution of SHP-1 to its inhibitory function, a non-redundant requirement for Lyn in mediating its positive regulation was uncovered. This was underscored by the inability of other SFKs in Lyn KO mice to redundantly target the ITAM-like Y543 site and offset SHP-1 inhibition. Taken together, our results indicate that FCRL5 may serve as a direct cellular sensor of SHP-1 and Lyn activity in innate-like B cells.

MZ and B1 B cells possess distinct preimmune antibody repertoires and innate sensing potential that facilitate their rapid responsiveness to TI stimuli. Although many other trophic, migratory, retention, and environmental factors modulate their development and activation status, our knowledge of the basic signaling properties of these cells and why they respond so quickly to stimuli still remains limited. The discrete expression of FCRL5 by these cells at homeostasis indicates its genetic regulation is complementarily distinct. Previously in our lab, we found that *Fcrl5* promoter region contains NF- κ B consensus binding sites capable of recruiting the p50 and p65 (RELA) elements that can transactivate *Fcrl5* in luciferase reporter assays (**Figure 1**). Furthermore, our colleague Wong-jai Won in the laboratory found that FCRL5 was upregulated in MZ B cells upon LPS exposure (206). He further examined a larger TLR ligand panel and found that Pam3, FSL-1, and LPS could moderately upregulate FCRL5 on most splenic CD19⁺ B cells at 24 hrs; however, the TLR9/CpG pathway induced strong and sustained FCRL5 expression by the total CD19⁺ population (unpublished data). We further examined FCRL5 regulation in *Myd88*^{-/-} and *Myd88*^{-/-}*Trif*^{-/-} DKO mice. Basal FCRL5 expression in MZ B cells from both Tg strains was similar to WT; however, LPS-dependent induction was absent in MyD88 KO splenic B cells, whereas CpG failed to upregulate FCRL5 in splenocytes lacking both MyD88 and TRIF adaptors (unpublished data). Collectively these findings strongly suggest a role for FCRL5 in the innate arm of immunity.

Recently, the Pierce group found that human FCRL4 expression alone can heighten B cell responsiveness to innate stimuli in the absence of receptor ligation (198). These data, along with its discrete expression pattern and the normal frequencies and numbers of MZ and B1 B cells in FCRL5 KO mice, suggest that FCRL5 may not directly influence innate-like B cell development, but rather play a regulatory role in their effector function. We therefore examined the influence of FCRL5 on innate stimulation. A20 B cells that lack endogenous FCRL5 expression were transduced with either the intact FCRL5 (WT) cytoplasmic tail or a variant with all ITAM-like and ITIM tyrosines mutated (FFF). After confirming similar surface expression, both transductants were examined for whole-cell pTyr by Phospho-specific Flow before or after treatment with TLR ligands. **Figure 2** demonstrates that similar to the human FCRL4 protein, the introduction of FCRL5 into the A20 B cell line confers responsiveness to LPS as well as CPG (data not shown) in a tyrosine-based fashion. These data indicate that FCRL5 possesses tyrosine-based properties that may catalyze innate stimulatory pathways in B cells. Based on these findings, we hypothesize that FCRL5 promotes innate B cell stimulation via the recruitment of Lyn kinase to its cytoplasmic ITAM-like sequence. However, how Lyn specifically integrates with TLR signaling in B cells remains unclear. Next, we may carry out following experiments to verify this hypothesis: (1) Investigate if FCRL5 is capable of being tyrosine-phosphorylated after stimulation with LPS or CpG DNA. (2) Determine if FCRL5 ITAM-like sequence can recruit Lyn kinase upon innate stimulation; (3) Examine if FCRL5 engagement further augments whole-cell activation

and other downstream signaling events after innate stimulation. (4) Assess the functional influence of FCRL5 on innate activation in MZ and B1 B cells.

Innate-like MZ and B1 B lymphocytes are the major effector cells involved in primary humoral responses. Given their unique developmental and biological characteristics, there is growing appreciation for their specialized roles at homeostasis and in complex immune-disease states in multiple species. The identification of a large family of immuno-regulatory FCRL molecules with preferential B cell expression has opened a new field of investigation. Their evolutionary conservation, associations with human lymphoproliferative, infectious, immunodeficiency, and autoimmune disorders, compellingly indicate their immunologic importance. Deconstructing FCRL5 function in mice will promote definition of the extended FCRL family, provide novel insight into their roles in pathogenesis, and stimulate innovative approaches to treating FCRL-associated human diseases.

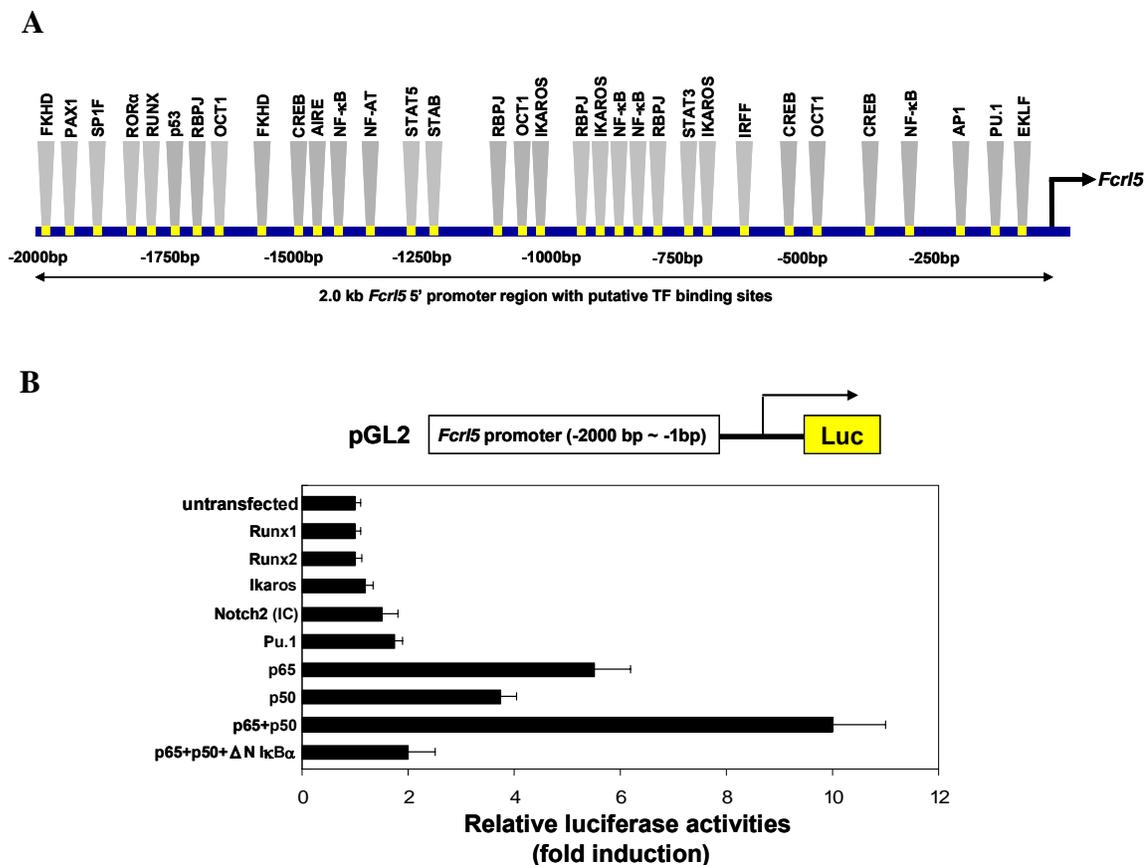


Figure 1. *Fcrl5* promoter activity is induced by NF- κ B in 293T cells.

(A) The predicted transcription factor (TF) binding sites in the 2.0 kb promoter region of the *Fcrl5* gene were analyzed using Genomatrix program.

(B) A 2.0 kb DNA fragment of the 5' flanking region of the *Fcrl5* gene was inserted just upstream of the firefly luciferase (Luc) gene positioned in the pGL2 vector. This PGL2 recombinant construct and the retroviral constructs for the indicated transcription factors and retina Luc gene (internal control) were then co-transfected into 293T cells. 48 hours after transfection, the Luc activity in lysates was assayed with the Dual-Luciferase Reporter Assay System (Promega).

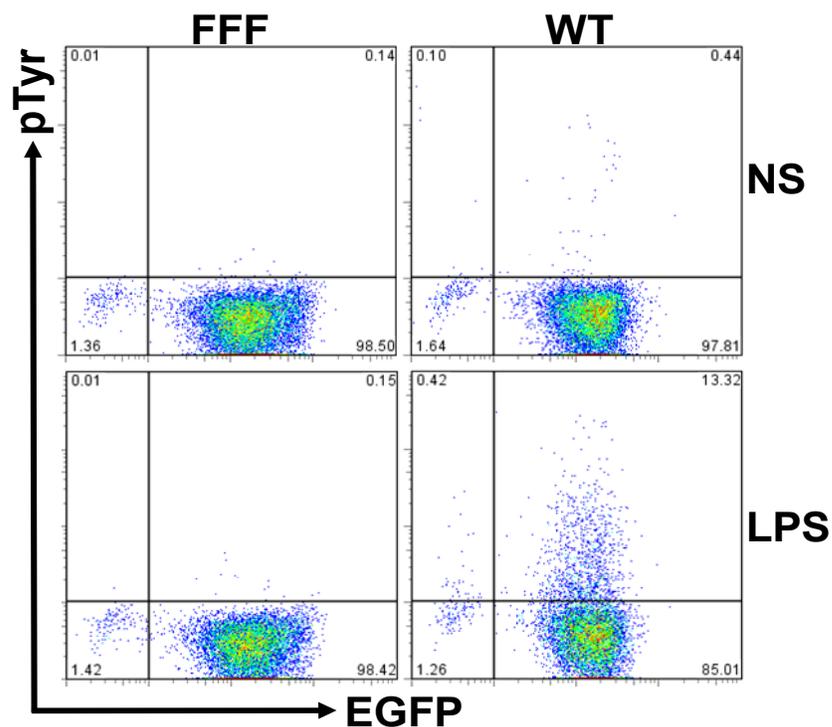


Figure 2. FCRL5 expression confers B cell responsivity to innate stimuli. A20-IIA1.6 B cell transductants bearing intact (WT) or FFF cytoplasmic FCRL5 mutants were cultured in the absence (NS) or presence of LPS (10 μ g/ml) for 60 mins, fixed, permeabilized, and stained with PE-anti-pTyr (PY20) or a control before analysis by flow cytometry. Results represent one of three experiments.

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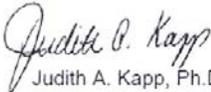
THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF RENEWAL

DATE: October 7, 2011

TO: RANDALL SCOTT DAVIS, M.D.
SHEL-402 2182
FAX: (205) 975-7218

FROM: 
Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: Functional Role of Fc Receptor Homologs on B Lineage Cells
Sponsor: NIH
Animal Project Number: 111107947

As of November 16, 2011, the animal use proposed in the above referenced application is renewed. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Mice	A	800
Mice	B	250
Mice	C	25

Animal use must be renewed by November 15, 2012. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 111107947 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.

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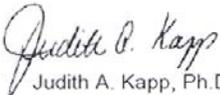
THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: October 7, 2011

TO: RANDALL SCOTT DAVIS, M.D.
SHEL-402 2182
FAX: (205) 975-7218

FROM: 
Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.

The following application was renewed by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on October 7, 2011.

Title of Application: Functional Role of Fc Receptor Homologs on B Lineage Cells

Fund Source: NIH

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

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