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Analysis Of The Role Of Fcrl5 And Figlers In B Cell Development, Signaling, And Malignancy

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ANALYSIS OF THE ROLE OF FCRL5 AND FIGLERS IN B CELL DEVELOPMENT, SIGNALING, AND MALIGNANCY

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MICROBIOLOGY

ABSTRACT

The regulation of signaling and migration is critical to B lymphocyte development, activation, and proliferation. This dissertation reports a functional role for Fc receptor-like molecule 5 (FCRL5), a B cell specific member of the Fc receptor-like family of molecules, and the discovery of a novel family of conserved transmembrane proteins with fibronectin, immunoglobulin, and leucine-rich repeat domains (FIGLER) expressed in bone marrow and B lineage cells. Until now, the signaling potential of FCRL5 was unknown. A panel of FcγRIIB/FCRL5 chimeric receptors were created and expressed in a B cell line lacking endogenous FcγRIIB. Coligation of the chimeric receptor with the B cell antigen receptor (BCR) resulted in the marked inhibition of whole cell tyrosine phosphorylation, calcium mobilization, Erk activation, and Igα/Igβ heterodimer phosphorylation. The inhibitory effect was shown to be mediated by dual ITIM recruitment of SHP-1, a SH2-containing protein tyrosine phosphatase. Although all 3 intracellular FCRL5 tyrosines are capable of being phosphorylated, only the ITIM tyrosines appear to contribute to the signaling potential of FCRL5 in the context of BCR signaling. Furthermore, the inhibitory effect of FCRL5 on BCR signaling was demonstrated in primary tonsillar memory B cells. The evidence presented represents the

first evidence of the potent inhibitory potential of FCRL5 and the first signaling potential analysis of an Fc receptor-like family member containing both ITAM-like and ITIM signaling motifs. Secondly, using bioinformatic searches for molecules resembling the IL-7α receptor, a novel family of 9 human *FIGLER* genes was identified. The FIGLER family proteins encode type I transmembrane glycoproteins, with 6–12 leucine-rich repeats (LRRs), a single C2 Ig and FNIII domain, and cytoplasmic domain containing at least one tyrosine. In addition to the nine human FIGLER molecules, homologs were identified in macaque, orangutan, chimpanzee, mouse, rat, dog, chicken, toad, and puffer fish databases. *FIGLER 1*, *2*, *3*, and *5* mRNA transcripts were detected in primary B cell lines. The structure and lack of evident intracellular signaling motif indicate the FIGLER molecules may serve as trophic or cellular adhesion molecules.

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A CONSERVED GENE FAMILY ENCODES TRANSMEMBRANE PROTEINS WITH FIBRONECTIN, IMMUNOGLOBULIN AND LEUCINE-RICH REPEAT DOMAINS (FIGLER)

LIST OF ABBREVIATIONS

BCR	B cell receptor
BLAST	Basic Local Alignment Search Tool
FCRL5	Fc receptor-like protein 5
FIGLER	Fibronectin Immunoglobulin Leucine Rich Repeat
IL-7	Interleukin-7
ITAM	Immunoreceptor tyrosine-based inhibitory motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
SHP	SH2 domain-containing tyrosine phosphatase

INTRODUCTION

Overview of B Lymphocytes

Vertebrates have evolved a complex combination of innate and adaptive immune defenses of or use in protecting the body from invading pathogens. The primary defense mechanism involves the epidermis and the mucosal membranes which serve as physical barriers between the internal and external environments. A secondary defense mechanism is provided by the innate or nonspecific immune system which consists of the soluble proteins such as lysozyme, lactoferrin, transferrin, interferon, and complement; innate leukocytes such as natural killer cells, mast cells, eosinophils, and basophils; and the phagocytic cells including macrophages, neutrophils, and dendritic cells (DCs). Cells of the innate immune system recognize potential pathogens via pattern-recognition receptors (PRRs) and lack the ability to generate immunological memory. The best understood PRRs are members of the Toll-Like Receptor (TLR) family which have the ability to recognize molecular features on the surface of pathogens. Each TLR family member can bind to an array of pathogen-associated molecular patterns (PAMPs) to initiate signaling events leading to the activation of innate immune responses. TLRs respond to interactions with pathogens in either a Myeloid Differentiation factor 88 (MyD88) dependent or independent manner (1, 2). Microbial induced signaling by TLRs initiates acute inflammatory responses such as the expression of anti-microbial genes, the release of inflammatory cytokines and chemokines, expression of costimulatory molecules such as CD80 and CD86, and the maturation of dendritic cells (3). TLR-induced DC

maturation is essential for naïve T cell activation and differentiation into T helper (T_H) cells. DC secreted cytokines such as IL-4 and IL-12 assist in activating T cells and directing T cell responses toward infection (4, 5). B lymphocytes, the principle effector cell of the humoral immune response to infection, also express TLR receptors, thereby providing a bridge between the innate and adaptive immune responses.

The adaptive immune system is composed of T and B lymphocytes that act in coordination to produce a directed immune response. B cells respond to antigen through different mechanisms, depending on the nature of the antigen challenge. In thymus-independent antigen responses, B cells specific for antigenic repetitive epitopes can be activated without the help of T_H cells. In thymus-dependent antigen responses, antigen first interacts with the BCR initiating the B cell to up-regulate MHC class II and B7 costimulatory molecules. The B cell then serves as an antigen presenting cell (APC) by presenting its antigen to antigen specific T_H cells with secondary signals provided by CD40/CD40L and B7/CD28 interactions to achieve the activation of both B and T cells (6, 7). Ligation of CD40, a member of the tumor necrosis family of receptors, regulates B cell proliferation by recruiting the tyrosine kinases Lyn and Syk with the subsequent activation of phospholipase C leading to the B cell proliferation and differentiation and the upregulation of cytokine receptors (8, 9). Cytokine-cytokine receptor interactions may direct the differentiation of B cells into memory B cells or antibody secreting plasma cells.

B cell precursors are generated from hematopoietic stem cells in the fetal liver and in bone marrow. Progenitor B cells (pro-B cells) develop from the common lymphoid progenitor, a multipotent cell capable of differentiating into lymphocytes and

DC cells. Pro-B cells are influenced by stromal cells through interactions via cell adhesion molecules and soluble products such as IL-7 (10). During the pro-B cell stage of development, the recombinase genes RAG1 and RAG2 are expressed resulting in immunoglobulin heavy chain (IgH) gene rearrangement producing either a functional or non-functional IgH gene locus (11). The $CD34^{+}CD45^{+}CD19^{+}$ late stage pro-B cells thereby differentiate into precursor B cells (pre-B cells) that express a pre-B cell receptor. Pre-B cells lose expression of CD34 and express CD21, C3d/ Epstein Barr virus receptor, in addition to μ heavy chains which associate with surrogate light chain to form the pre-BCR receptor, an essential component for this stage of maturation (12). Light-chain gene (IgL) locus rearrangement is required for the continued differentiation of pre-B cells into the immature B cell stage of development. Upon successful rearrangement of the IgL gene locus by resting pre-B cells, surrogate light chains are replaced by IgL to form the B cell antigen receptor (BCR) (13). Immature B lymphocytes leave the bone marrow compartment before expressing both IgM and IgD as naïve B cells which have yet to encounter antigen. Naïve B cells circulate in the peripheral blood from which they migrate to the lymph nodes, intestinal lymphoid tissues, and spleen. The process of migration to these lymphoid tissues is regulated by the differential expression of cellular adhesion molecules.

Upon encountering antigen within lymphoid tissues, the naïve B cells undergo clonal expansion and differentiation to generate memory B cells and plasma cells through thymus-dependent antigen responses. Antigen binding causes the internalization of the BCR and subsequent antigen presentation to T_H cells as a complex with the class II MHC. The T cell receptor recognizes the class II MHC-peptide complex and along with

costimulatory receptor-ligand interactions causes the T cell to secrete B cell activating cytokines. The activated B cell undergoes a series of replications wherein mutations are introduced in the variable region of the BCR genes, resulting in a higher affinity for the antigen, a process referred to as somatic hypermutation (14, 15). In addition to somatic hypermutation, activated B cells may undergo class switching, associating their modified V_H genes with the constant region of another isotype (16). B cells further differentiate into memory B cells and plasma cells. The memory B cell population is an inactive, long lived cell that can quickly proliferate upon subsequent exposure to antigen. Plasma cells produce a soluble form of the immunoglobulin which reacts with potential pathogens.

A series of checkpoints in B cell development and maturation must be controlled in order to mount an effective immune response while eliminating autoreactive responses to self (17). The maturation of B lymphocytes from hematopoietic stem cells is divided into distinct stages characterized by rearrangement of Ig V(D)J genes and the expression or loss of surface proteins (18). At each stage of Ig loci rearrangement, B cells are actively selected against in order to eliminate self-reactive B cells. In the bone marrow immediately or after peripheral migration, B cells that express mIgM that binds with self antigen are eliminated by clonal deletion (19). However, even self-reactive B cells may escape clonal deletion by editing of their light chain genes. In addition to functional, non-self reactive Ig gene rearrangements, the progression from pre-B cells to mature B lymphocyte is regulated by the signaling threshold potential of the Pre-B cell receptor and the B cell receptor (20). The BCR is composed of two identical light chains of either the κ or λ type and two identical heavy chains of constant region type μ, α, δ, γ, or ε (21). BCR diversity is determined by the differences in variable N-terminal regions of the

heavy and light chains, namely the complementarity determining region (CDR). The tertiary structure of BCR brings the hypervariable CDR regions together to form the antigen binding site. The BCR cytoplasmic domain lacks independent intracellular signaling capacity and must associate non-covalently with a heterodimer of Igα and Igβ, transmembrane molecules that contain immunoreceptor tyrosine-based activation motifs (ITAM) which mediate downstream signaling events (22). Upon BCR cross-linking, Igα and Igβ ITAM tyrosines are phosphorylated by the Src family kinases Lyn, Fyn and Blk (8). The ITAM tyrosine phosphorylation of Igα and Igβ enhances the recruitment and activation of the downstream mediator of signaling events Syk, a tyrosine kinase (Figure 1) (9). Activation of Syk directs downstream signaling events leading to the activation of the MAP kinase signaling pathway, calcium mobilization, and phosphorylation of downstream signaling targets that ultimately result in B cell proliferation, altered gene expression, and differentiation, (23, 24).

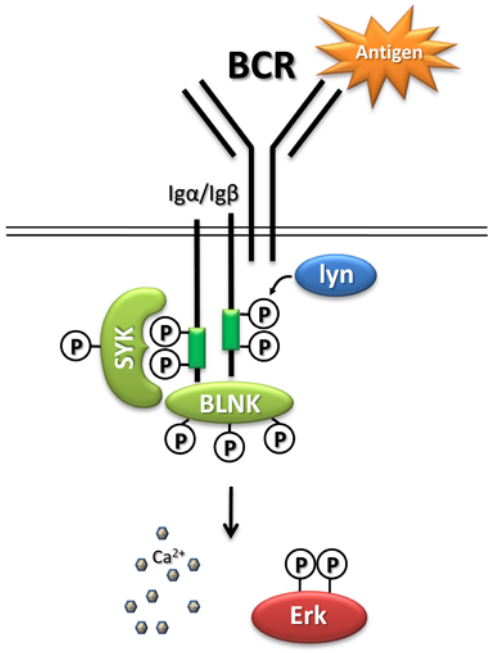

Figure 1. B cell receptor signaling cascade.

However, signaling through the B cell receptor can be significantly modified by enhancing and competing signals of coreceptors allowing for control over B cell signaling cascades. B cell coreceptor components include CD19, CD21, and CD81 that amplify activating signals through the BCR by creating an environment suitable for activation. CD19, with six cytoplasmic tyrosine residues, serves as a major substrate of protein tyrosine kinases such as Lyn (25). Upon cross-linking with the BCR, CD19 delivers Lyn to the BCR complex, amplifying activating signals. The B cell also has developed ways of limiting signaling through the B cell receptor. Resting B cells in the periphery express CD22, which constitutively associates with the BCR (26). CD22 contains six tyrosines in its cytoplasmic tail, four of which fall within immunoreceptor tyrosine-based inhibitory motifs (ITIM) and bind to the protein tyrosine phosphatase SHP-1, a well-known inhibitor of activation-promoting signaling cascades in hematopoietic cells. The balance between activating and inhibitory costimulation of BCR signaling events is delicate and when deregulated may shift B cells toward autoreactivity instead of tolerization. Disruption of CD22 leads to hyperactive B cell phenotype and an augmented thymus-independent immune response (27). Similarly, disruption of lyn leads to hyper-responsiveness to anti-IgM induced proliferation, auto-antibody production, and severe immune complex-mediated lupus-like nephritis (28).

The primary objectives of the research described in this dissertation were to evaluate the immunomodulatory role of FCRL5, a B cell specific member of the Fc receptor-like family of molecules and to define a novel family of conserved transmembrane proteins with fibronectin, immunoglobulin and leucine-rich repeat domains (FIGLER) with cytoplasmic signaling potential expressed in bone marrow and B

lineage cells. Although the FIGLER molecules do not have any apparent signaling motifs within their cytoplasmic domains, their structure appears to be consistent with their function as cellular adhesion molecules.

Fc Receptor-Like Molecule 5

Overview of Fc Receptor-Like Molecules

The immunoglobulin superfamily of genes includes both multi-gene and single-gene representatives, whose products represent an amazingly diverse array of functions from immune receptors to cartilage formation, reflecting the versatility of the shared common structure. Fc receptors (FcR) act as specific immunoglobulin superfamily cell-surface receptors for antigen-antibody complexes or aggregated immunoglobulins that bind a site in the Fc portion of the immunoglobulin molecule and may exhibit specificity for particular immunoglobulin classes. FcRs modulate cellular and humoral immunity in a variety of ways by coupling antibodies with effector cells. A family of FcR-like genes, Fc receptor-like molecules (*FCRL*) has joined the classical *FcR* genes on human chromosome 1 (29, 30).

The FCRL family were identified in our laboratory based on a 32 amino acid consensus sequence derived from Fc-binding portions of CD16, CD32, and CD64 as well as sequencing the translocation breakpoint (1;14)(q21;32) in a multiple myeloma cell line by the Dalla-Favera group (29, 30). Other groups have identified members of the FCRL family using a phylogenetic approach and by characterizing genes activated by anti-IgM cross-linking of a Burkitt's lymphoma cell line (31, 32). Eight human FCRL genes have been identified and are located at three distinct loci spanning a 4 Mb region of

chromosome 1q21-23. *FCRL1-5* are located in the centromeric most locus, *FCRL6* at a second locus, and *FCRLA* and *FCRLB* at a third distinct locus.

Each human FCRL family member has between 3 and 9 extracellular domains varying in sequence identity from 45% to 83% (30, 33, 34). The cytoplasmic domains of the FCRL family members with the exception of FCRL6 and FCRLA-B contain between 3 and 5 tyrosines indicating the presence of ITIM and ITAM motifs. The presence of multiple tyrosines-based activation and inhibitory motifs indicates that FCRL family members may have varied and diverse signaling potentials. ITAMs contain two repeats of the consensus sequence Y-X-X-L/I spaced by 6–8 amino acids (E/D)-X-XY-X-X-(L/I)-X6-8-Y-X-X-(L/I), while ITIMs feature a six amino acid consensus sequence (I/V/L/S)-X-Y-X-X-(L/V/I) (35, 36). Ligand binding to molecules containing ITAM motifs lead to phosphorylation of cytoplasmic tyrosines contained within the ITAM motif by src family kinases. The phosphorylation event leads to the recruitment of other signaling molecules that direct cellular activation events. Unlike ITAM motifs, ITIM motifs lead to cellular inhibition events upon tyrosine phosphorylation by providing a docking site for downstream phosphatases which contain SH-2 domains.

Overview of FCRL5

FCRL5 is the largest member of the family of Fc receptor-like molecules. The longest isoform of FCRL5 encodes a 977 amino acid type I transmembrane glycoprotein with nine extracellular Ig domains and 8 potential N-linked glycosylation sites (29, 30). The type I transmembrane isoform has a 23 amino acid transmembrane domain and a relatively short 104 amino acid cytoplasmic domain. Four FCRL family members,

including FCRL5 have extracellular Ig domains that possess a potential immunoglobulin binding site although experiments to confirm Fc binding are inconclusive. In its cytoplasmic domain, FCRL5 contains an ITAM-like sequence as well as two consensus ITIMs. The *FCRL5* gene consists of 17 exons. Through the use of differential mRNA splicing, FCLR5 displays several different splice variants including a 759 amino acid secreted isoform and a 592 amino acid glycosyl-phosphotidyl inositol (GPI)-linked isoform in addition to the transmembrane molecule that contains both transmembrane and cytoplasmic domains. A short 152 amino acid potentially secreted isoform of FCRL5 arises through the utilization of an early polyadenylation signal. Each isoform contains its own a unique 3' untranslated region.

FCRL5 expression occurs in B cells in a differentiation stage specific pattern. FCRL5 is expressed on naïve and memory B cells, and plasma cells in both tonsil and spleen (37). In peripheral blood, expression of FCRL5 on circulating $CD20^{+}$ B cells remains inconclusive which may be explained by variations in epitopes recognized by the different monoclonal antibodies against the receptor given the multiple splice variants (37, 38).

FCRL5 in B cell Malignancies

Chromosome 1q21 is a hotspot for abnormalities in B cell malignancies. FCRL5 has been detected on 8 B cell lymphoma cell lines, 6 of which were derived from non-Hodgekin's lymphoma (NHL) and one Burkitt's lymphoma cell line (30). In addition to cell lines, FCRL5 was detected on 27% to 39% of peripheral blood mononuclear cells in 5 patients afflicted with hairy cell leukemia (HCL) compared to the 4% to 8% detected

on PBMCs of normal donors (38). Recently, studies found elevated levels of soluble FCRL5 in the peripheral blood of patients with multiple myeloma (MM), chronic lymphocytic leukemia (CLL), and mantle cell lymphoma (MCL), corresponding to the percentage of plasma cells in the bone marrow aspirates in multiple myeloma and with white blood cell found in chronic lymphocytic leukemia (39). FCRL5 was also detected on the surface of MM, MCL, and CLL B cell malignancies. Surface and peripheral blood expression of FCRL5 was shown to correspond with tumor burden.

Recent studies have also shown that FCRL5 surface expression is also upregulated in B cells infected with Epstein-Barr virus (40). In this case, expression of FCRL5 is due to the formation of heterodimers by the viral protein EBNA2 heterodimerization with CBF1, which docks onto binding sites in the promoter regions of *FCRL5* and other target genes on chromosome 1q21. The EBNA2 protein thus replaces endogenous NOTCH as the transactivator unit for CBF1 heterodimers. Epstein-Barr virus has been implicated in the pathogenesis of several B-cell lymphoid proliferations.

Immunomodulatory Role of FCRL5

Here we present a potential immunomodulatory role for FCRL5 in the context of BCR complex signaling. By generating FcγRIIb/FCRL5 chimeric receptors, we were able to inhibit BCR-mediated calcium mobilization, intracellular tyrosine phosphorylation, and Erk kinase activation after coligation with the BCR. The inhibitory effect was mediated by dual ITIM recruitment of SHP-1, an SH-2 containing protein tyrosine phosphatase (41).

Fibronectin Immunoglobulin Leucine-Rich Repeat Molecules

Overview of FIGLER Molecules

In mouse B cell development, interleukin-7 (IL-7) is crucial to proliferation and development of B lymphocytes (42). In the absence of IL-7, murine B cells undergo G1 arrest suggesting IL-7 plays a major role in regulating the G1/S phase transition in murine B cell precursors (43-45). Whereas human T cell development appears to be regulated by the presence of absence of IL-7, human B cell development does not appear to be similarly affected. Recent studies showed that human hematopoietic progenitors develop into mature B cells after transplantation in immunodeficient mice (46). This finding suggests that the molecules essential for human B cell development are either present in the mouse or are supplied by the transplanted human cells.

Structure of Interleukin 7 Receptor

The interleukin 7 receptor (IL-7R) is a class I cytokine receptor, also known as a hematopoietin receptor for its ability to bind to cytokines classified as hematopoietins (47). The IL-7 receptor is a heterodimer composed of IL-7Rα, the specific receptor chain for IL-7 that can also recruit signaling molecules involved in anti-apoptotic signals, and γ_c. All class I cytokine receptors share several key structural similarities; the presence of four conserved cysteine residues, a tryptophan-serine-x-serine-tryptophan motif and fibronectin type III (FNIII) and Ig domains in the extracellular region of the receptor (48). All class I cytokine receptors also have signaling capability through intracellular tyrosine phosphorylation by Janus kinases (49). Binding of IL-7 to the alpha chain of the

IL-7 receptor induces dimerization of the IL-7Rα and γ_c chains of the IL-7 receptor complex (44). JAK1 and JAK3 phosphorylate the IL-7Rα and γ_c chains, respectively, recruiting signaling molecules such as STAT5, PI3 kinase, and Bcl2 to the complex (44, 45, 50, 51). STAT5 acts as a transcription factor in coordinating the activation of multiple downstream genes in both early B and T cell progenitors (50, 51).

Identification of Human FIGLER genes

We surmised that a cytokine/receptor pair similar to the IL-7/IL-7 receptor would be a good candidate for a lymphopoietic agent in the development of B lymphocytes from B cell progenitors. Using the common features of the cytokine receptors; Ig domains, FNIII domains, and signaling potential; we used a bioinformatic approach consisting of SMART, HMMER, and BLAST sequence analysis to identify nine human *fibronectin immunoglobulin leucine-rich repeat* (*FIGLER*) genes. Each encodes a type I transmembrane glycoprotein, with 6-12 leucine-rich repeats (LRRs), one C2 Ig domain, a single FNIII domain, a transmembrane region, and a tyrosine-containing cytoplasmic region with unknown signaling capacity (52).

Identification of FIGLER Orthologs

BLASTN algorithmic search for homology was used in order to identify *FIGLER* orthologs in macaque, chimpanzee, orangutan, mouse, rat, dog, chicken, toad, and puffer fish. Additional Hidden Markov Model searching led to the identification of five potential new *FIGLER* orthologs in *Takifugu rubripes*. All FIGLER orthologs contained 6–12 LRR, a single C2 type Ig domain, a FN type III domain, a hydrophobic

transmembrane domain, and from one to seven cytoplasmic tyrosines. Comparative sequence analysis showed that the FIGLER orthologs shared between 38–99% overall amino acid identity with their human counterparts.

FC RECEPTOR-LIKE 5 INHIBITS B CELL ACTIVATION VIA SHP-1 TYROSINE PHOSPHATASE RECRUITMENT

by

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Abstract

The Fc receptor-like protein 5 (FCRL5) on B cells has both an immunoreceptor tyrosine-based activation motif (ITAM)-like sequence and two consensus immunoreceptor tyrosine-based inhibitory motifs (ITIM) in its cytoplasmic region. To evaluate its signaling potential, we expressed constructs for chimeric molecules composed of the cytoplasmic region of FCRL5 and the extracellular and transmembrane regions of the IgG Fc receptor FcγRIIB in a B cell line lacking an endogenous Fc receptor. Coligation of this fusion protein with the B cell receptor (BCR) inhibited BCR-mediated calcium mobilization, intracellular tyrosine phosphorylation, and Erk kinase activation. Our mutational analysis indicated that while tyrosines in both the inhibitory and activation motifs are phosphorylated after ligation, only those in ITIMs influence BCR-mediated signaling. This FCRL5 inhibitory effect was mediated through dual ITIM recruitment of the SH2 containing protein tyrosine phosphatase, SHP1, which in turn dephosphorylates the ITAM-based tyrosines in BCR Igα/Igβ heterodimers. A FCRL5 inhibitory effect on BCR signaling was likewise demonstrable for primary B cells. Although its ligand is presently unknown, we conclude that FCRL5 has the functional potential to serve as an inhibitory coreceptor on mature B cells in humans.

Introduction

B cell receptor (BCR) engagement initiates signaling cascades that lead to activation of the Ras-MAPK pathway, phosphatidylinositol-3-kinase, and phospholipase C γ (PLCγ) (1, 2). The BCR triggering ultimately induces gene expression patterns that can promote cell activation, apoptosis, or anergy, depending upon the balance of enhancing and inhibitory influences that vary according to the stage in B cell differentiation (3). Costimulatory or inhibitory coreceptors on B cells modulate BCR signaling to either enhance or attenuate downstream signaling cascades (4). Inhibitory coreceptors may dampen BCR signaling via an immunoreceptor tyrosine-based inhibition motif (ITIM) in their cytoplasmic region. When tyrosine phosphorylated, the ITIMs recruit protein tyrosine phosphatases and lipid phosphatases via SH2 domain binding to achieve downregulation or neutralization of BCR-induced activation (5). Conversely, the costimulatory receptors may have their own cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAM) or they may pair with a transmembrane protein containing one or more ITAMs, whose tyrosines are phosphorylated by src family kinases to allow the recruitment of signaling molecules that promote cellular activation (6). The balance between activating and inhibitory receptor influences can be complicated by their coexpression on individual B cells. Moreover, individual cell surface receptors may possess both activating and inhibitory motifs; their differential engagement, according to ligand specificity and affinity, may trigger inhibitory and/or activating signaling pathways in order to calibrate effector cell responses (7).

The recently recognized Fc receptor-like (FCRL) family includes five members that are preferentially expressed by B lineage cells, possess variable numbers of Ig

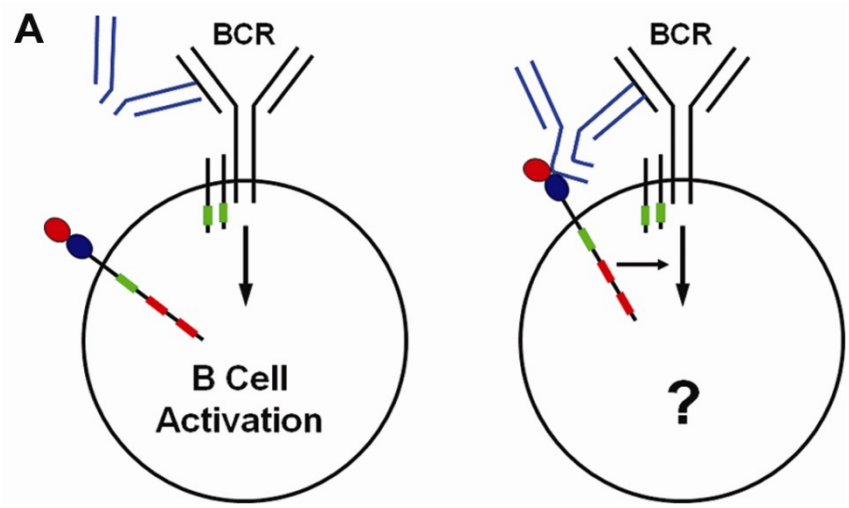
domains, and have either ITIMs, ITAMs, or both in their cytoplasmic tails (8-11). FCRL5 is the largest of the FCRL transmembrane proteins. In addition to its nine Ig-like extracellular domains and a transmembrane region, FCRL5 has a non-canonical ITAM-like consensus sequence and two canonical ITIMs in the cytoplasmic domain (8). Alternate isoforms have been identified, which include two secreted isoforms lacking transmembrane and cytoplasmic domains and a putative glycosyl-phosphatidyl inositol (GPI) linked form, both of which lack transmembrane and cytoplasmic domains (9, 12) . FCRL5 is found on most mature B cells with the highest levels being present on naïve and memory B cells and plasma cells (13). As for other FCRL family members, all of which have extracellular Ig domain sequences suggestive of immunoglobulin binding potential, an FCRL5 ligand has not yet been identified.

In this study, we assessed the signaling potential of FCRL5 by generating chimeric receptors encoding the intracellular domain of FCRL5 combined with the extracellular and transmembrane domains of FcγRIIb and evaluated their function in a B cell line. Our findings indicate that FCRL5 has the potential to inhibit BCR signaling through the recruitment of SHP-1 following tyrosine phosphorylation of its two ITIMs. FCRL5 on primary memory B cells was also shown to have inhibitory potential for BCR signaling when the two receptors were coligated. These findings have implications to understanding the pathogenesis of B cell malignancies, in which aberrant FCRL5 expression is often seen.

Results

FCRL5 Signaling Inhibits BCR-Mediated Protein Tyrosine Phosphorylation.

For these experiments, chimeric fusion proteins consisting of the FcγRIIb extracellular and transmembrane domains and the FCRL5 intracellular domain were expressed in the IgG-expressing A20-IIA1.6 mouse B cell line variant that lacks endogenous FcγRIIb. The inhibitory or activating potential of the FCRL intracellular domain was then examined by coligating the BCR with the chimeric FcγRIIb/FCRL5 receptor using intact anti-IgG antibodies. For comparison, BCR ligation was accomplished by treating the B cells with $F(ab')_2$ fragments of the anti-IgG antibodies. Using this model system, a panel of FcγRIIb/FCRL5 constructs with tyrosine to phenylalanine mutations of the ITAM-like and consensus ITIM sequences were expressed to determine their potential for modulating BCR-mediated signaling (Fig. 1). Transduced B cell populations expressing comparable levels of chimeric receptors were selected by fluorescence-activated cell sorting for use in these experiments (supplementary Fig. 1).



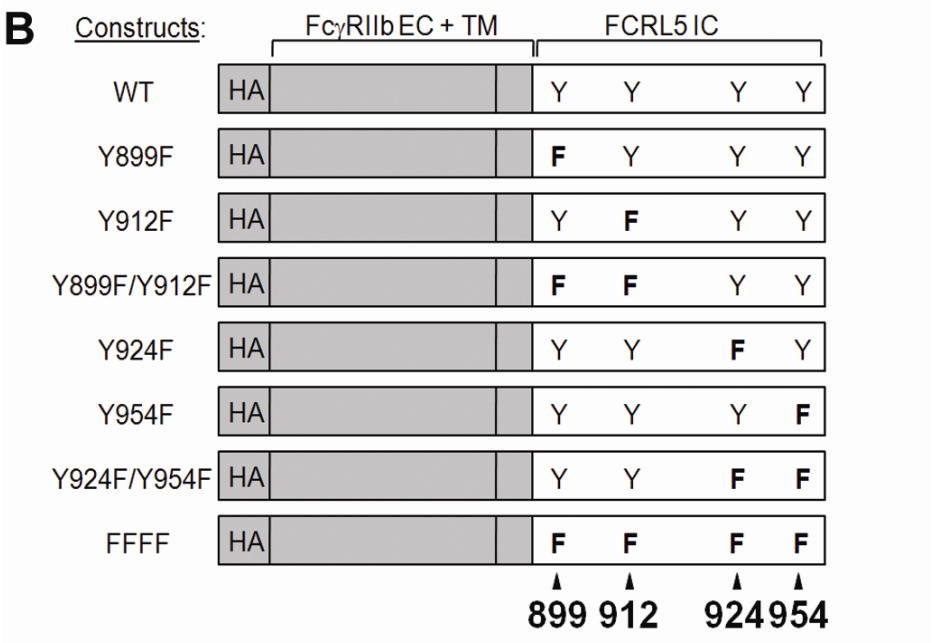


Figure 1. B cell stimulation models and constructs used to evaluate FCRL5 function. (A) Schematic illustration of exclusive BCR engagement using anti-IgG F(ab')$_2$ fragments results (left) and BCR coligation with FcγRIIb/FCRL5 chimeric receptor using intact anti-IgG antibodies (right). ITAM(-like) sequences are represented in green. ITIM sequences are represented in red. (B) FcγRIIb/FCRL5 chimeric constructs. FcγRIIb extracellular and transmembrane domains are shaded in gray. Tyrosines 899 and 912 correspond to a non-canonical ITAM. Tyrosines 924 and 954 correspond to two canonical ITIMs. Tyrosine to phenylalanine mutations are indicated by the letter F (bold).

BCR ligation alone, using the $F(ab')_2$ anti-Ig antibodies, induced rapid tyrosine phosphorylation of multiple intracellular proteins (Fig. 2), whereas ligation of the WT FCRL5 fusion protein with an anti-HA antibody failed to induce tyrosine phosphorylation of the fusion protein itself or of other intracellular proteins (data not shown). However, when the BCR was coligated with the WT chimeric receptor, whole cell tyrosine phosphorylation was greatly reduced. Notably, Erk 1/2 activation was reduced relative to that seen after BCR ligation alone. Following B cell stimulation with the intact anti-IgG antibodies, which bridge the BCR with FcγRIIb/FCRL5, the WT chimeric receptor was phosphorylated at increasing levels over a 30 minute period, whereas ligation of the BCR alone with anti-IgG $F(ab')_2$ did not induce phosphorylation of the WT chimeric molecule (Fig. 2, bottom panel). Longer chemiluminescent exposure of the Western blot indicated that the tyrosine phosphorylation of FCRL5 began before one minute after its ligation (data not shown). As anticipated, BCR coligation with the FFFF chimera, in which all of the cytoplasmic tyrosines were mutated, had no inhibitory effect on BCR-triggered protein tyrosine phosphorylation and Erk activation.

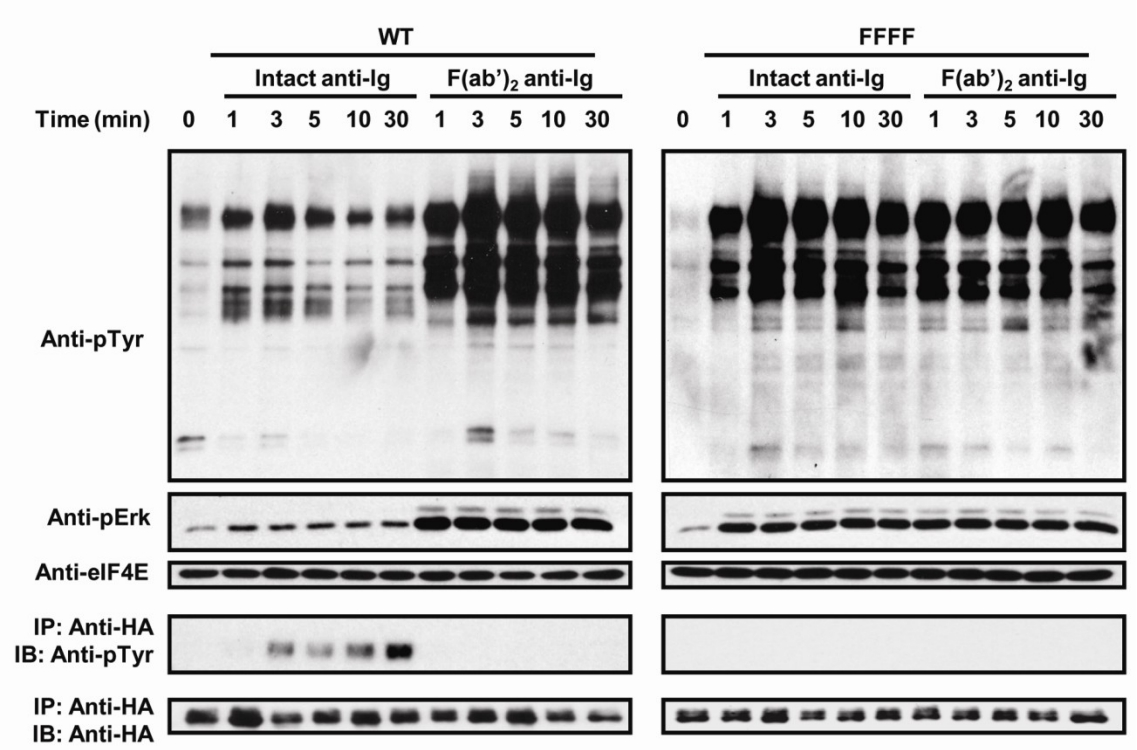


Figure 2. FcγRIIb/FCRL5 coligation inhibits BCR inhibited BCR-mediated protein tyrosine phosphorylation. WT or FFFF cells were treated with intact or $F(ab')_2$ fragments of anti-IgG antibodies and whole cell tyrosine phosphorylation was gauged over time by means of Western blot analysis using a phosphotyrosine antibody. Blots were reprobed with an anti-phospho Erk and anti-eIF4e antibodies to analyze Erk activation and verify equal protein loading. Immunoprecipitates of the chimeric receptor were analyzed by anti-phosphotyrosine and equal loading assured with an anti-HA control (bottom panel).

FCRL5 Inhibits BCR-Induced Ca^{2+} Mobilization.

We next examined the effect of FCRL5 co-engagement on calcium flux induced by BCR ligation on the A20-IIA1.6 B cells. Ligation of the BCR alone induced a characteristic wave of calcium mobilization, whereas BCR coligation with the WT FcγRIIb/FCRL5 chimeric receptor completely blocked BCR-induced calcium mobilization. When control B cells transduced with the "empty vector" were used, BCR

stimulation with either the F(ab')$_2$ anti-IgG or the intact anti-IgG antibodies induced the same calcium mobilization responses as observed for the non-manipulated cells (Fig. 3).

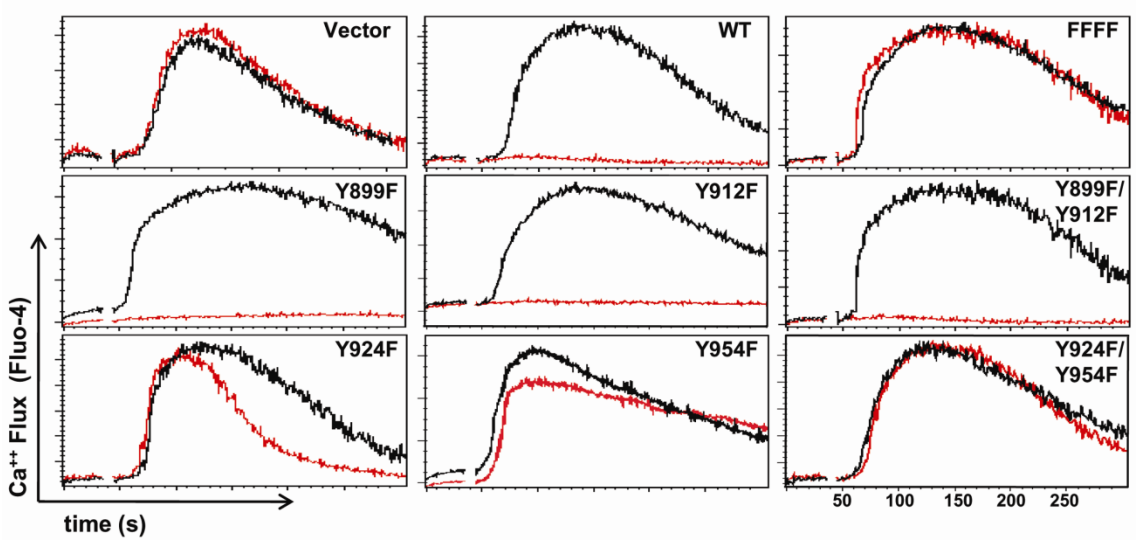


Figure 3. FcγRIIb/FCRL5 coligation inhibits BCR-triggered Ca^{2+} mobilization in B cells. Cells were preloaded with Fluo-4 NW for calcium flux evaluation and the FcγRIIb/FCRL5 chimeras were coligated (red) or not (black) with BCR. Note that mutation of the ITIM tyrosines 924 and 954 to phenylalanine restored BCR-induced calcium mobilization.

A panel of chimeric receptors with tyrosine to phenylalanine mutations was used to identify the cytoplasmic tyrosine residues responsible for the inhibitory effect of FCRL5. Coligation of the BCR complex with either the Y899F or Y912F chimeric receptors failed to block inhibition of calcium mobilization. Coligation of the BCR with the Y899F/Y912F double mutant chimera also did not inhibit calcium mobilization, suggesting that neither of the tyrosines in this ITAM-like motif affect the inhibitory activity observed for FCRL5 in this B cell model. In contrast, the Y924F tyrosine mutant affected the duration of the calcium flux, truncating the response with little effect on

maximal peak intensity in comparison to BCR-only induced calcium mobilization, whereas the Y954F mutant dampened the maximal peak intensity but not the duration of the calcium flux (Fig. 3). The Y924F/Y954F double mutant restored the normal pattern of BCR-induced calcium mobilization, as was observed for the "empty vector" control and the FFFF quadruple mutant. Notably, the tyrosine to phenylalanine mutations in both of these consensus ITIM motifs did not result in increased calcium flux relative to the BCR-only induced flux. Collectively, these results indicate that the two ITIMs contribute to the FCRL5 inhibition of BCR-mediated calcium mobilization. Moreover, the ITAM-like (Y899/Y912) consensus region has no obvious impact on the ability of FCRL5 to modulate the BCR-mediated calcium flux.

SHP-1 Binds a Tyrosine Phosphorylated Peptide Corresponding to the Membrane Distal ITIM of FCRL5.

Having demonstrated the FCRL5 inhibitory effect on BCR-induced intracellular tyrosine phosphorylation, Erk activation, and calcium mobilization, we sought to identify the inhibitory effector molecules. In these experiments, we used biotinylated synthetic phosphopeptides corresponding to the FCRL5 ITAM-like and two ITIM sequences as affinity reagents to probe A20-IIA1.6 cell lysates (Fig. 4). Following their tyrosine phosphorylation, ITIMs are known to serve as docking sites for molecules containing SH2 domains. Candidate inhibitory signaling molecules include the protein tyrosine phosphatases SHP-1 and SHP-2; and SHIP, an inositol polyphosphate phosphatase. When the tyrosines within ITAMs are phosphorylated by src family kinases, they may associate with Syk, a tyrosine kinase that resembles the classic src family kinases but

which lacks an SH3 domain, or a signaling intermediate containing both SH2 and SH3 domains such as PLCγ2. Of these downstream effector molecules, only SHP-1 was found to bind to the most membrane-distal ITIM peptide with phosphorylated tyrosine residue 954 (Fig. 4). None of the inhibitory candidates were found to bind the unphosphorylated synthetic peptides.

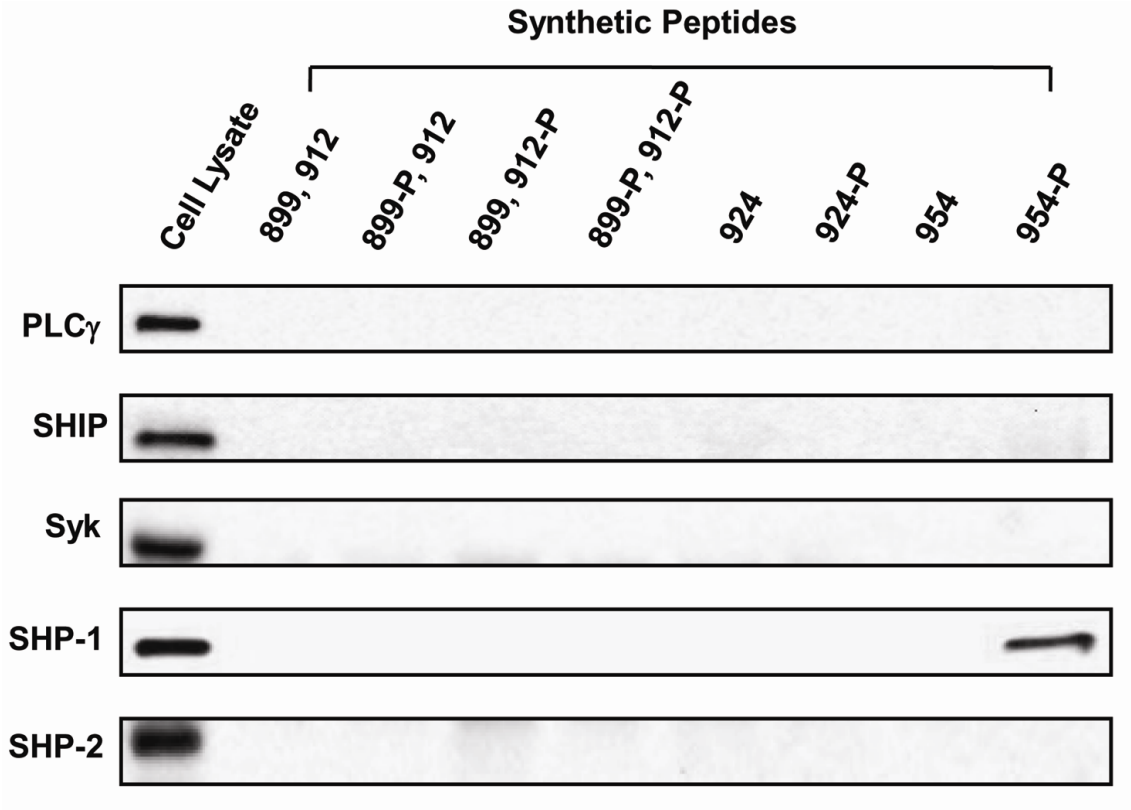


Figure 4. A phosphopeptide mimic of the membrane distal ITIM of FCRL5 binds SHP-1. The indicated phosphopeptides corresponding to each ITAM-like and ITIM region of FCRL were incubated with the B cell lysates and peptide precipitates were blotted with the indicated antibodies.

SHP-1 Binds to the FcγRIIb/FCRL5 Chimeric Receptor After BCR Coligation.

The phosphopeptide binding experiment and the kinetics of the calcium flux assay suggested the inhibitory effect of FCRL5 could be mediated by SHP-1. To determine whether SHP-1 can interact with the tyrosine-phosphorylated intracellular domain of the FcγRIIb/FCRL5 chimeric receptor after BCR coligation, we stimulated cells expressing each of the different mutants of FCRL5 with intact anti-IgG antibodies. After BCR coligation, we immunoprecipitated the FCRL5 chimeric receptors and probed Western blots of the immunoprecipitates with anti-phosphotyrosine antibodies and with antibodies to SHP-1, SHP-2, Syk, PLCγ2, and SHIP. Interestingly, all of the chimeric receptors were tyrosine phosphorylated after coligation with the BCR, with the sole exception of the FFFF mutant which lacks intracellular tyrosines (Fig. 5A). Moreover, the level of phosphorylation did not vary greatly between the mutant chimeric receptors. This analysis suggests that both the ITAM-like and ITIM regions of FCRL5 were phosphorylated during BCR coligation. When the immunoprecipitates were probed with antibodies against the candidate proteins, SHP-1 was found to be co-precipitated with the WT FcγRIIb/FCRL5, the Y899F and Y912F single mutants, and the Y899F/Y912F double mutant, but not with the Y924F, Y954F, Y924F/Y954F, or FFFF mutants. In contrast, the SHP-2 and SHIP phosphatases failed to associate with any of the FcγRIIb/FCRL5 chimeric receptors. Association of the activating kinases Syk and PLCγ2 also was not detected (data not shown). In summary, the ability of phosphorylated tyrosines 924 and 954 to promote SHP-1 binding suggests that FCRL5 associates with SHP-1 via an interaction with both ITIMs to achieve its inhibitory effect on intracellular tyrosine phosphorylation and calcium mobilization. While tyrosines 899

and 912 in the non-canonical ITAM are also phosphorylated after BCR coligation, they appear not to influence the FCRL5 modulating effect on BCR signaling.

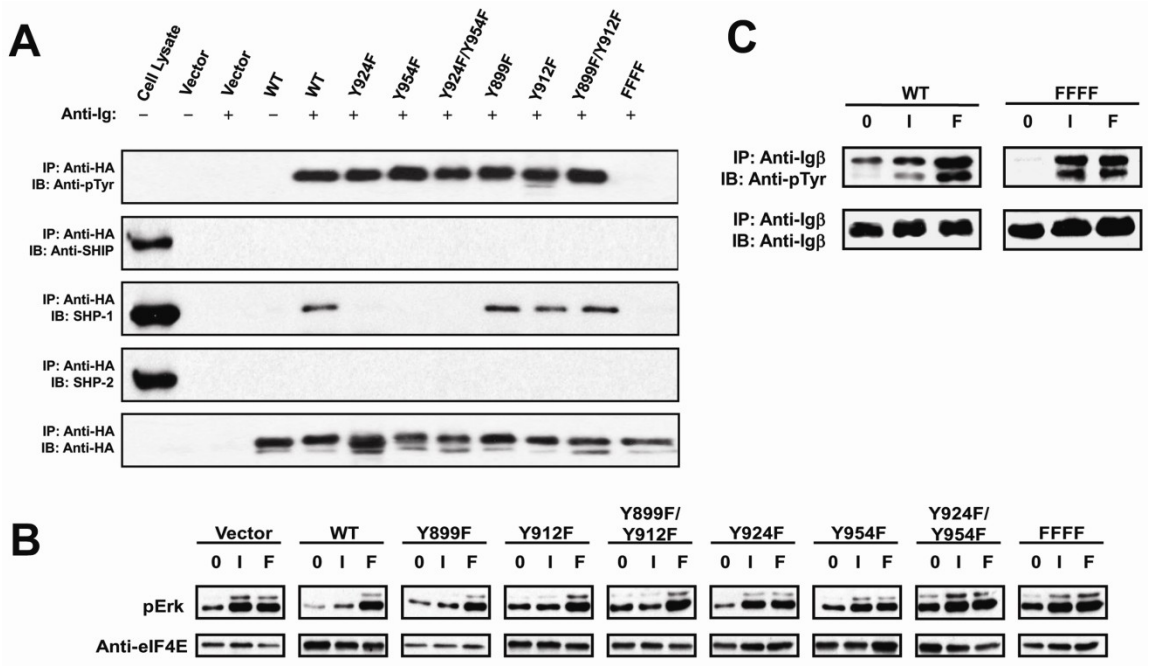


Figure 5. SHP-1 binding to the FcγRIIb/FCRL5 chimeric receptor after BCR coligation. (A) After stimulation of A20-IIA1.6 cells expressing the chimeric constructs, cell lysate immunoprecipitates by a HA tag antibody were analyzed for FcγRIIb/FCRL5 interaction with candidate signaling components by Western blotting. The blots were reprobed with anti-phosphotyrosine to assess phosphorylation of the individual receptors and also with anti-HA as gel loading control. (B) Evaluation of Erk activation. Cell lysates for the individual mutants were from cells that were untreated (0), stimulated with intact anti-IgG antibodies (I), or treated with $F(ab')_2$ fragments of anti-IgG (F). The membranes were probed with anti-phospho Erk antibodies and with anti-eIF4e to assess equivalent protein loading. (C) Attenuation of Igα/Igβ phosphorylation. Cell lysates from either WT or FFFF mutant FCRL5 were immunoprecipitated by anti-Igβ mAb and analyzed for tyrosine phosphorylation by Western blotting. The stripped blot was reprobed with anti-Igβ as a loading control.

Inhibition of Erk Activation Correlates with SHP-1 Association and Inhibition of BCR-Induced Calcium Flux.

The results shown in Figure 2 indicated that Erk phosphorylation is inhibited following BCR coligation with the WT FcγRIIb/FCRL5 chimeric receptor. We found that the Y924F, Y954F, and Y924F/Y954F mutants were capable of inhibiting Erk phosphorylation induced by BCR ligation, in keeping with their association with SHP-1 (Fig. 5B). In contrast, the non-SHP-1-associating Y899F, Y912F, Y899F/Y912F, and FFFF mutants were incapable of attenuating Erk activation upon coligation with the BCR. The differential ability of WT and the different FcγRIIb/FCRL5 mutants to alter Erk activation therefore parallels the effect on BCR-induced calcium mobilization and SHP-1 association. Since the tyrosines in the ITAM motifs of the Igα/Igβ components of the BCR complex are phosphorylated to trigger downstream signaling pathways that are activated by BCR ligation (22), they are logical targets for the FCRL5 associated SHP-1 tyrosine phosphatase. Accordingly, whereas BCR cross-linkage alone induced Igα and Igβ phosphorylation, WT FCRL5 coligation attenuated the Igα and Igβ tyrosine phosphorylation, and the FFFF mutant had no effect (Fig. 5C).

FCRL5 Coligation Attenuates BCR-mediated Signaling in Primary Memory B Cells.

Having demonstrated in a model B cell line that the FcγRIIb/FCRL5 receptor chimeras can inhibit tyrosine phosphorylation by recruiting SHP-1, we wished to see if the native FCRL5 receptor may function similarly in primary B cells. For these studies, memory B cells ($CD19^+$, IgD^-, $CD38^-$) were isolated by fluorescence-activated cell sorting of tonsil samples. After confirming that these cells express FCRL5

(Supplementary Fig. 2), we then subjected the isolated memory B cells to stimulatory conditions in which the BCR alone was cross-linked, the FCRL5 receptor alone was cross-linked, neither was cross-linked, or the BCR was coligated with the native FCRL5 receptor. The treated cells were then fixed and permeabilized before staining with a fluorochrome-labeled anti-phosphotyrosine antibody. BCR cross-linkage alone with anti-Ig $F(ab')_2$ fragments, or BCR cross-linkage coupled with treatment by a control irrelevant antibody led to a 10-fold shift in tyrosine phosphorylation (Fig. 6B). In contrast, cells stimulated with anti-FCRL5 $F(ab')_2$ fragments gave a signal comparable to that observed for the unstimulated population. However, tyrosine phosphorylation was significantly inhibited when FCRL5 was coligated with the BCR. When the memory B cells were pretreated with α-Bromo-4-hydroxyacetophenone 4-hydroxyphenacyl Br, a potent SHP-1 inhibitor, the inhibition of tyrosine phosphorylation by FCRL5/BCR colligation was abrogated. With the caveat that receptor coligation was achieved by non-physiological means, these results indicate that native FCRL5 receptors can engage SHP-1 to attenuate BCR triggered tyrosine phosphorylation following coligation of the two receptors.

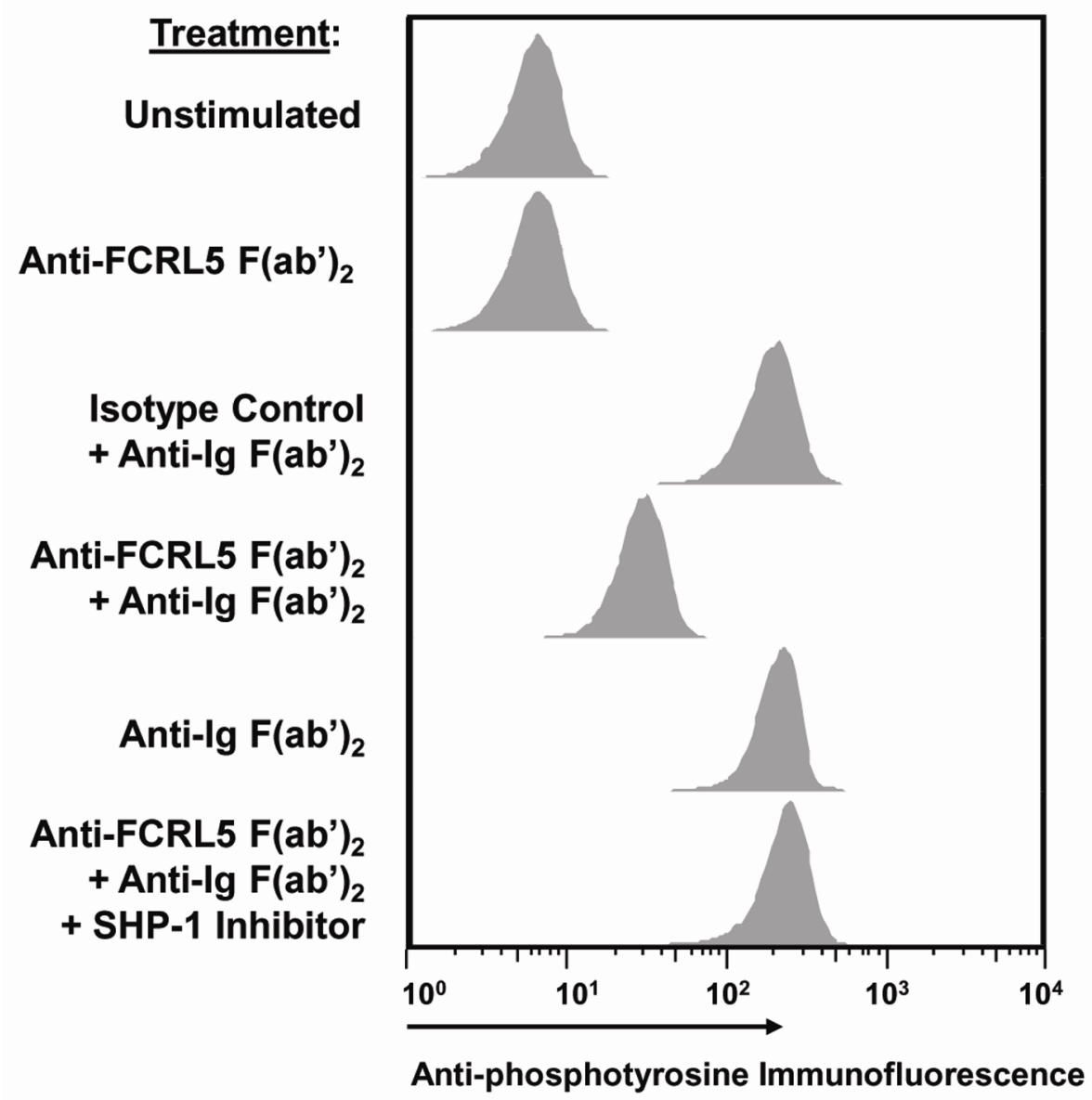


Figure 6. FCRL5 attenuates tyrosine phosphorylation in tonsillar memory B cells after coligation with the BCR. Tonsillar memory B cells were treated as indicated for 10 min or preincubated with a SHP-1 inhibitor before treatment. Fixed cells were permeabilized, and the level of intracellular tyrosine phosphorylation was measured by flow cytometric analysis by using a fluorescein-conjugated phosphotyrosine antibody.

Discussion

This analysis of FCRL5 signaling capability establishes its inhibitory potential for modulating the BCR-mediated activation of B cells. A dominant role is shown for the two FCLR5 ITIMs, whereas no functional activity is revealed for the ITAM-like motif. By expressing wild type and mutated versions of FcγRIIb/FCRL5 chimeric receptors in B cells lacking endogenous FcγRIIb, we could show that the tyrosine residues in the two ITIMs are essential for the inhibitory function of FCRL5. Following its coligation with the BCR, WT FcγRIIb/FCRL5 fusion protein inhibited calcium mobilization, whole cell tyrosine phosphorylation, and Erk activation. Conversion of tyrosines in the non-canonical ITAM-like motif to phenylalanine had no demonstrable effect on B cell activation, and mutation of the ITIM tyrosines eliminated the inhibitory effect of the FcγRIIb/FCRL5 protein. The inhibitory function of FCRL5 involved SHP-1 tyrosine phosphatase recruitment and a reduction in tyrosine phosphorylation of Igα/Igβ ITAMs following FCRL5 and BCR coligation. These results accord with previous studies of SHP-1 function (18) to implicate the BCR Igα/β signaling units as important substrates for the FCRL5/SHP-1 mediated inhibition of BCR signaling in this model system.

Our mutational analysis of the intracellular tyrosines in the FCRL5 signaling domain indicates that both ITIM tyrosines, residues 924 and 954, contribute to the attenuation of BCR signaling. While a synthetic phosphopeptide mimic of the most membrane-distal ITIM could be shown to bind SHP-1, an immunoprecipitation analysis of B cell lysates suggested that both ITIMs are needed for optimal SHP-1 binding, in that mutation of the tyrosine in either of the ITIMs eliminated detectable SHP-1 association. In comparison to the calcium mobilization pattern observed following BCR ligation

alone, we observed a truncated response in B cell transductants wherein tyrosine 924 was mutated and a diminished calcium flux response when tyrosine 954 in the second ITIM was mutated. The cellular tyrosine phosphorylation response was also attenuated in primary B cells following colligation of the BCR with FCRL5, and this inhibitory effect was abrogated by pre-treatment with a SHP-1 inhibitor.

While these findings unambiguously indicate the FCRL5 potential for inhibiting B cell activation by BCR-mediated signaling, our studies notably employed artificial means to coligate the two receptors. Under physiological conditions, IgG antibodies could possibly bridge antigen-bound BCR with FCRL5, given that the two membrane distal Ig domains of FCRL5 share sequence similarity with classical Fcγ receptors (8) and preliminary evidence has been reported for FCRL5 binding of heat-aggregated IgG (9). However, the absence of confirmatory evidence for an Fc receptor function suggests either a relatively low IgG binding affinity or that FCRL5 has other natural ligands. An unambiguous definition of a FCRL5 ligand(s) thus remains an important goal.

Enhanced FCRL5 expression has been observed in B lineage malignancies and in B cells infected with Epstein-Barr virus (EBV) (9, 12, 23, 24). Interestingly, FCRL5 was identified initially as an Ig superfamily gene located near the breakpoint of a chromosome 1q21 translocation event in a myeloma cell line (9). Moreover, upregulated FCRL5 expression is frequently associated with a 1q21 translocation abnormality in both B cell non-Hodgkins lymphomas (B-NHL) and multiple myelomas (MM) (25-27). Patients with MM, chronic lymphocytic leukemia (CLL), and mantle cell lymphoma (MCL) have been shown to have elevated levels of the soluble FCRL5 isoform. Their serum levels correspond with the tumor burden (12), and the tumor cells express

transmembrane FCRL5 as well. Cell surface FCRL5 expression is also upregulated in EBV-infected B cells (23). This is due to the formation of CBF1/RBPJκ heterodimers , which dock onto binding sites in the promoter regions of *FCRL5* and other target genes. EBV-infected cells, the EBNA2 protein replaces endogenous NOTCH as the transactivator unit for CBF1 heterodimers. While these clues suggest that FCRL5 over-expression contributes to the pathogenesis of B cell malignancies, our results pose the question of how heightened expression of an inhibitory receptor can foster lymphomagenesis. It would be desirable to have a mouse model to address this issue, but mice only have two *FCRL1-5* gene family homologs, F*crl1* and F*crl5*, and neither shares high sequence homology with its human counterpart. Notably, mouse Fcrl5 has only one consensus ITIM (28). Mouse models thus are unlikely to be very helpful in discerning the roles of the FCRL5 isoforms in lymphomagenesis.

FCRL5 is expressed at highest levels on mature B cells, memory B cells, and plasma cells, none of which are in a proliferative mode, and it is downregulated on the proliferative B cells in germinal centers. The inhibitory FCRL5 on the resting B cells could participate in the delicate balance that checks cell cycle progression while allowing the basal level of constituitive BCR signaling needed for B cell survival (3). However, transcripts are expressed for multiple FCRL5 isoforms, including transmembrane, secreted, and GPI-anchored versions, by both normal B cells and B cell lines (9, 24) as another complicating feature in deducing the biological roles of FCRL5. The function of the soluble FCRL5 isoform, which is elevated in patients with MM, CLL, and MCL (12), is presently unknown. Theoretically, the soluble isoform could compete with transmembrane FCRL5 for a natural ligand thereby abrogating its inhibitory function to

allow unimpeded BCR mediated proliferation and cell survival. The soluble isoform of FCRL5 potentially could also modulate a ligand-bearing immunocompetent cell needed for tumor detection and clearance. In a similar scenario, soluble FCRL5 could play a role in EBV infection by serving either to block the ligand for the transmembrane FCRL5 isoform or as a modulating factor for cytotoxic T cells that eliminate EBV infected B cells. The speculative nature of these considerations further emphasizes the need to determine the natural FCRL5 ligand(s) in order to understand its function in normal B cell physiology, malignancies and EBV infection.

In conclusion, our studies show that FCRL5, like FCRL4 (16, 29), has potent inhibitory potential for BCR-mediated signaling. However, whereas FCRL4 expression is confined to a tissue-based subpopulation of memory B cells (27), FCRL5 is expressed throughout B cell differentiation and therefore could have a broader influence on B cell responses to both endogenous and exogenous antigens.

Methods

Cells and Antibodies.

A20-IIA1.6 B cells and BW5147 T cells were maintained in RPMI medium 1640 supplemented with 10% FCS, 25 mM HEPES, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5.0% CO_2. BOSC23 cells were grown in DMEM supplemented with 10% FCS, 2 mM L-glutamine, and 100 U/ml penicillin and 100 μg/ml streptomycin. Anti-hemagglutinin (HA) antibody 12CA5 was obtained from Roche Diagnostics (Mannheim, Germany), anti-PLCγ2, anti-SHP-1, anti-SHP-2, anti-SHIP, anti-Syk, anti-pErk (Tyr 204) and anti-eIF4E from Santa

Cruz Biotechnology (Santa Cruz, CA), horseradish peroxidase-coupled anti-phosphotyrosine antibodies 4G10 from Upstate Biotechnologies (Lake Placid, NY), whole Ig and $F(ab')_2$ fragments against Ig from Zymed (Carlsbad, CA), anti-phosphotyrosine PY20 R-PE-conjugated for phosphospecific flow cytometry and anti-IgD-PE and anti-CD38-FITC from BD Biosciences (Franklin Lakes, NJ), and anti-Igβ was produced in our lab.

Production of monoclonal anti-FCRL5 antibodies.

Hybridoma clones producing monoclonal anti-FCRL5 antibodies were generated by hyperimmunizing BALB/c mice with BW5147 cells expressing full-length FCRL5 and extracellular FCRL5-Fc fusion protein (10 μg/injection) before fusion of regional lymph node cells with the Ag8.653 plasmacytoma cell line (14). Hybridoma supernatants were screened by ELISA for FCRL5 antibody (5C3) activity, the specificity of which was determined by cell surface immunofluorescence reactivity with BW5147 cell lines expressing FCRL1-5. Hybridomas producing anti-FCRL5 antibody were subcloned by limiting dilution and the antibody isotype was determined by an indirect capture ELISA (Zymed, San Francisco, CA). $F(ab')_2$ fragments were prepared using the ImmunoPure $F(ab')_2$ Preparation Kit (Pierce, Rockford, IL).

Generation of Chimeric FcγRIIb/FCRL5 Constructs.

The wild type and mutant chimera proteins were generated by fusing the extracellular and transmembrane domains of FcγRIIb to the intracellular domain of FCRL5 as previously described (15, 16). Site-directed mutagenesis was performed

according to standard protocols. Wild type and mutated cDNAs encoding the intracellular domain of FCRL5 were subcloned into pBluescript and verified by DNA sequencing. Clones were fused with cDNAs encoding the extracellular and transmembrane domains of HA-tagged murine FcγRIIb and cloned into pMX-PIE, a retroviral expression vector which expresses the gene of interest upstream of an internal ribosomal entry site and the enhanced green fluorescent protein (EGFP) (17).

Transfection of BOSC23 Cells and Generation of A20-IIA1.6 Cells Expressing Chimeric Receptors.

BOSC23 cells were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. The virus containing supernatant was passed through a 0.2 μm filter, mixed with polybrene to a final concentration of 5 μg/ml and added to 2 x 10^6 A20-IIA1.6 cells as described previously (16). Transduced A20-IIA1.6 cells were selected in medium containing puromycin (1.5 μg/ml) for 4 days followed by fluorescence-activated cell sorting (FACS; Becton Dickinson) to enrich for EGFP/chimeric receptor expressing cells.

Cellular Activation, Western Blotting, Affinity Precipitation, and Immunoprecipitation.

To examine the effects of the chimeric receptor on BCR-induced signaling, 5 x 10^6 cell aliquots were washed twice with PBS and incubated for 2 hours in medium lacking FCS and supplemented with 20 mM Hepes (pH 7.2) before stimulation with intact anti-IgG antibodies (25 μg/ml) or anti-IgG $F(ab')_2$ fragments (16.6 μg/ml). Western blotting and immunoprecipitations were performed following standard protocols. Briefly, samples were lysed with M-PER cellular lysis buffer (Pierce) supplemented with a

Protease Inhibitor Cocktail (Roche Applied Sciences), and phosphatase inhibitors Na_3VO_4 (0.2 mM), Na_2MoO_4 (1 mM), and β-glycero-phosphate (5 mM). Proteins in cell lysates were quantified by using the Bicinchoninic Acid Solution (BCA) reagent (Pierce). Whole cell lysates were treated with 20 µl of 50% slurry of anti-HA sepharose beads (Roche Applied Sciences) and incubated for 1 hour at 4°C. After brief centrifugation, the supernatants were removed and the beads were washed 5 times with 1 ml M-PER buffer and boiled before being subjected to SDS/PAGE followed by transfer to nitrocellulose membranes (MSI, Westboro, MA) which were probed with the indicated antibodies, and the proteins were visualized using the ECL reagent (Amersham Pharmacia Biosciences, Piscataway, NJ).

Calcium Mobilization Assay.

Cells (5×10^6) were washed twice in Hanks' balanced salt solution (HBSS) (with Ca^{2+} and Mg^{2+}), then resuspended in 500 µl Fluo-4 NW assay buffer (Invitrogen) and incubated at 37°C for 30 minutes followed by 30 minutes at room temperature. Two hundred fifty-microliter aliquots of the cells were analyzed by flow cytometry after addition of 25 µg/ml intact anti-IgG or 16.6 µg/ml $F(ab')_2$ fragments of anti-IgG. Data analysis was carried out using the Flowjo software package (Treestar, Ashland, OR).

Phosphopeptide Immunoprecipitation Assay.

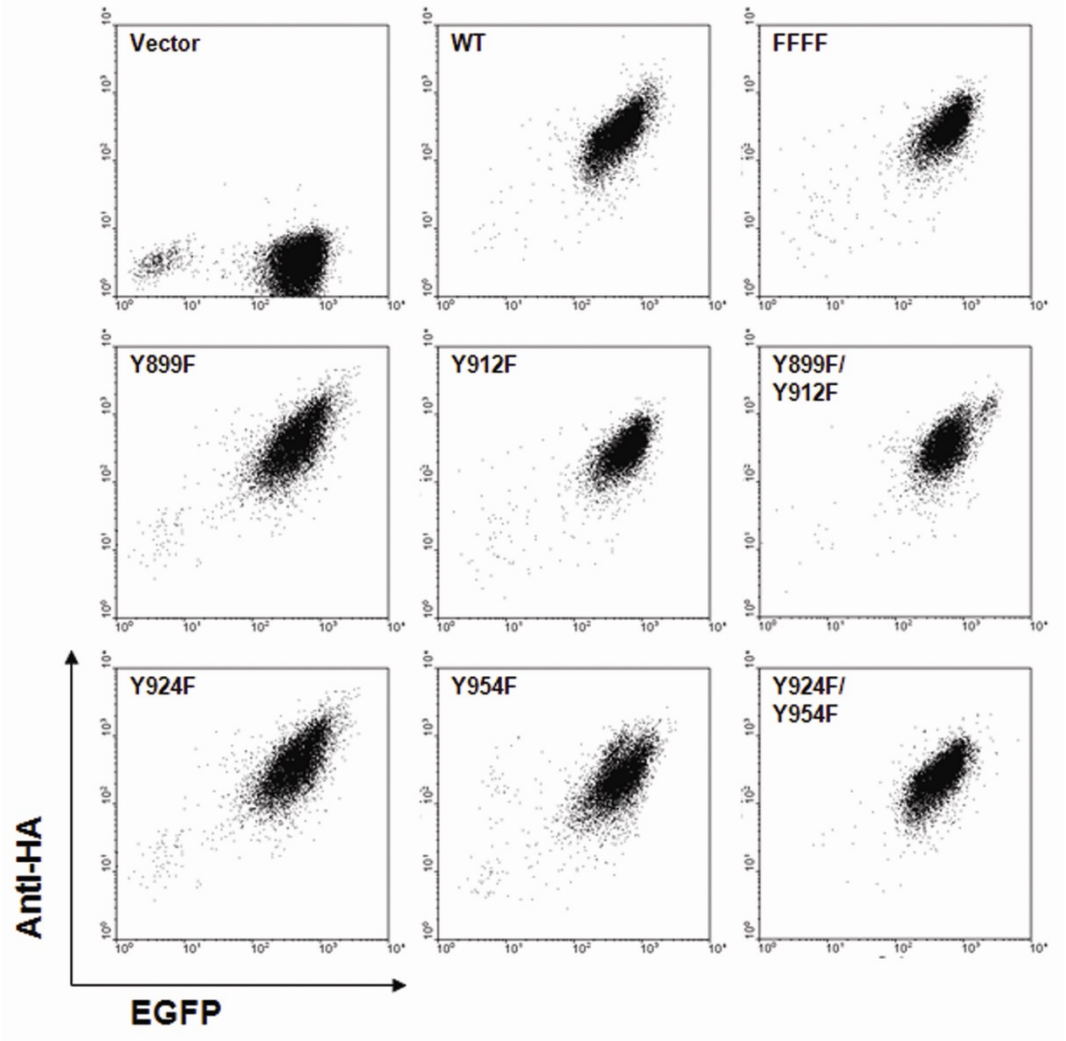
A20-IIA1.6 cells (5×10^6) were lysed with M-PER lysis buffer. Biotinylated phosphopeptides or control peptides (Alpha Diagnostic International, San Antonio, TX) were added to these lysates at a final concentration of 5 µg and incubated at 4°C for 30

minutes. Peptide-protein complexes were recovered using streptavidin-conjugated beads. (Amersham Pharmacia Biosciences). The precipitates were then washed 5 times with M-PER lysis buffer and resuspended in SDS-PAGE sample buffer. After boiling, the precipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The blots were probed with the indicated antibodies and the proteins were visualized using the ECL reagent.

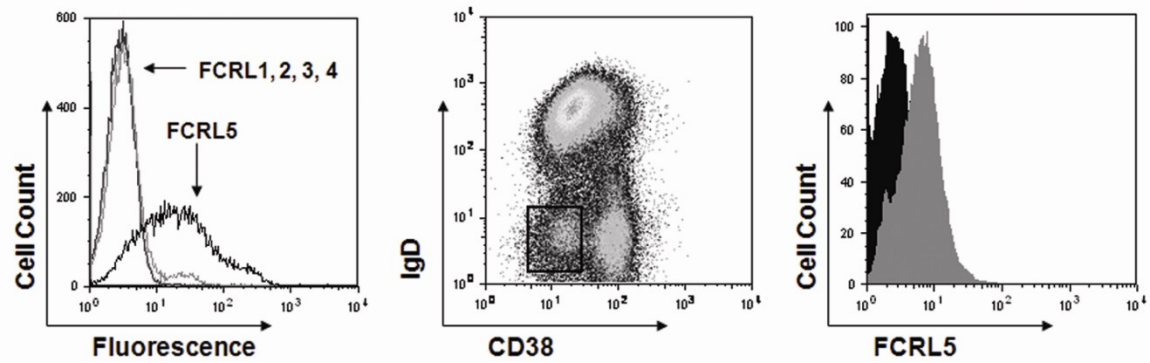
Phosphospecific Flow Cytometric Analysis of Primary Tonsillar Cells.

Phosphospecific flow cytometry was performed using a modified, previously reported protocol (18). Tonsillar memory B cells ($CD19^+$, $CD38^-$, IgD^-) purified by fluorescence-activated cell sorting (19, 20) were incubated at 37°C in a CO_2 incubator for 2 hours before stimulation. The cells (1×10^6 aliquots) were then treated for 15 minutes on ice with anti-FCRL5 (5C3) $F(ab')_2$ fragments before stimulation with rabbit anti-Ig $F(ab')_2$ fragments (Zymed) for 10 minutes at 37°C in a CO_2 incubator. Cells were also treated with 200 μM of α-Bromo-4-hydroxyacetophenone 4-Hydroxyphenacyl Br (Calbiochem, San Diego, CA), a cell-permeable, protein tyrosine phosphatase inhibitor (21). Finally, the treated cells were fixed with 50 μl of BD Phosflow Fix Buffer I (BD Biosciences) at 37°C for 10 minutes, permeabilized with BD Phosflow Perm Buffer II (BD Biosciences) for 30 minutes at 4°C, and then stained with R-PE-conjugated anti-phosphotyrosine PY20 before FACS analysis.

Supplementary Figures



Supplementary Figure 1. Comparative analysis of FcγRIIb/FCRL chimeric protein expression in transduced A20-IIA1.6 cells. Cells transduced with either "empty vector" or the indicated constructs were stained with a HA tag antibody and examined by flow immunocytometry for intracellular enhanced green fluorescent protein (EGFP) and cell surface expression of the different chimeric receptors.



Supplementary Figure 2. Analysis of anti-FCRL5 monoclonal antibody specificity. Monoclonal FCRL5 antibody specificity was determined by immunofluorescence analysis of BW5147 cells expressing the indicated FCRL family members (Left). Memory B cells ($CD19^{+}$, IgD^{-}, $CD38^{-}$) from tonsil samples (Center; the cells in the indicate gate) were analyzed for FCRL5 surface expression (Right).

Endnotes

Author contributions

C.L.H., G.R.A.E., and M.D.C. designed research; C.L.H. and R.J.B. performed research; R.S.D. contributed new reagents/analytic tools; C.L.H., G.R.A.E., and M.D.C. analyzed data; and C.L.H., R.S.D., and M.D.C. wrote the paper.

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A CONSERVED GENE FAMILY ENCODES TRANSMEMBRANE PROTEINS WITH FIBRONECTIN, IMMUNOGLOBULIN AND LEUCINE-RICH REPEAT DOMAINS (FIGLER)

by

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Format adapted for dissertation

Abstract

Background

In mouse the cytokine interleukin-7 (IL-7) is required for generation of B lymphocytes, but human IL-7 does not appear to have this function. A bioinformatics approach was therefore used to identify IL-7 receptor related genes in the hope of identifying the elusive human cytokine.

Results

Our database search identified a family of nine gene candidates, which we have provisionally named fibronectin immunoglobulin leucine-rich repeat (FIGLER). The FIGLER 1–9 genes are predicted to encode type I transmembrane glycoproteins with 6–12 leucine-rich repeats (LRR), a C2 type Ig domain, a fibronectin type III domain, a hydrophobic transmembrane domain, and a cytoplasmic domain containing one to four tyrosine residues. Members of this multichromosomal gene family possess 20–47% overall amino acid identity and are differentially expressed in cell lines and primary hematopoietic lineage cells. Genes for FIGLER homologs were identified in macaque, orangutan, chimpanzee, mouse, rat, dog, chicken, toad, and puffer fish databases. The non-human FIGLER homologs share 38–99% overall amino acid identity with their human counterpart.

Conclusions

The extracellular domain structure and absence of recognizable cytoplasmic signaling motifs in members of the highly conserved FIGLER gene family suggest a trophic or cell adhesion function for these molecules.

Background

Interleukin-7 (IL-7) is a non-redundant cytokine required for the generation of B and T lineage cells in mice [1-5]. Although IL-7 is essential for T cell development in humans, human B cell development is unaffected by the absence of IL-7 or its receptors [6-8]. Despite extensive research, the predicted IL-7 equivalent for human B lymphopoiesis has so far eluded identification. An important clue, provided by recent studies showing that human hematopoietic progenitors develop into mature B cells after transplantation in immunodeficient mice, suggests that the molecules essential for human B cell development are either present in the mouse or can be provided by the transplanted human cells [9,10]. In seeking a human B lymphopoietic cytokine/receptor pair, we reasoned that novel or orphan human receptors with structural features resembling those of the IL-7 receptor would be good candidates. A common feature of many cytokine receptors is the presence of Ig domains, fibronectin (FN) type III domains, and potential signaling capability [11]. Ig domains define members of the Ig superfamily, which is the largest family of mammalian cell surface molecules, comprising approximately half of the leukocyte cell surface glycoproteins [12]. FNIII domains are often found in molecules with adhesive function and can act as a spacer to ensure the correct positioning of functional sites [13].

Using bioinformatic searches for transmembrane proteins with Ig domains, FNIII domains, and signaling potential, nine human genes were identified that fulfilled the search criteria. These encode type I transmembrane glycoproteins, with 6–12 leucine-rich repeats (LRRs), one C2 Ig domain, one FNIII domain, a transmembrane domain, and a tyrosine containing cytoplasmic domain. The genes have been provisionally named *fibronectin immunoglobulin leucine-rich repeat* (*FIGLER*) *1–9*. In contrast to the known

cytokine receptors, the predicted FIGLER molecules have a unique domain structure, marked by the N-terminal LRRs and an unusual genomic organization. Two previously described molecules that combine LRR, Ig and FNIII domains with unknown signaling capacities and function are included in this family, namely the photoreceptor-associated LRR superfamily member (PAL) and the neuronal leucine-rich repeat protein 3 (NLRR3) [14-22]. Here, we describe the features and expression patterns of the human FIGLER family members and identify multiple non-human orthologs.

Results

Identification of human FIGLER genes

Over 3 000 nucleotide and amino acid sequences of hypothetical proteins, as defined by the NCBI database, were analyzed by SMART and BLAST to determine domain structure and sequence similarity to known molecules. The initial screening of the human NCBI Genome Database led to the identification of a hypothetical gene that was predicted to encode a protein with IL-7 receptor-like structure in that it possessed both Ig and FNIII domains. The predicted amino acid sequence was then used to search NCBI's BLAST protein database, leading to the identification of eight other related molecules in humans (Figure 1 and Table 1). Based on analysis using the SMART database, each of these proteins is predicted to contain 6–12 LRR, one C2 Ig domain, one FNIII region, one hydrophobic transmembrane region and one to four cytoplasmic tyrosines. These molecules were provisionally named fibronectin immunoglobulin leucine-rich repeat (FIGLER) 1–9. Although the *FIGLER* genes are dispersed in the genome, the predicted amino acid sequences of the nine FIGLER molecules share 20–

47% overall amino acid identity. Tyrosines are present in each of the FIGLER cytoplasmic regions, although they are not located within currently recognizable signaling motifs. Further analysis of the predicted amino acid sequences indicated that *FIGLER 5* and *FIGLER 9* correspond to the previously described *NLRR3* and *Pal* genes [16,21,22].

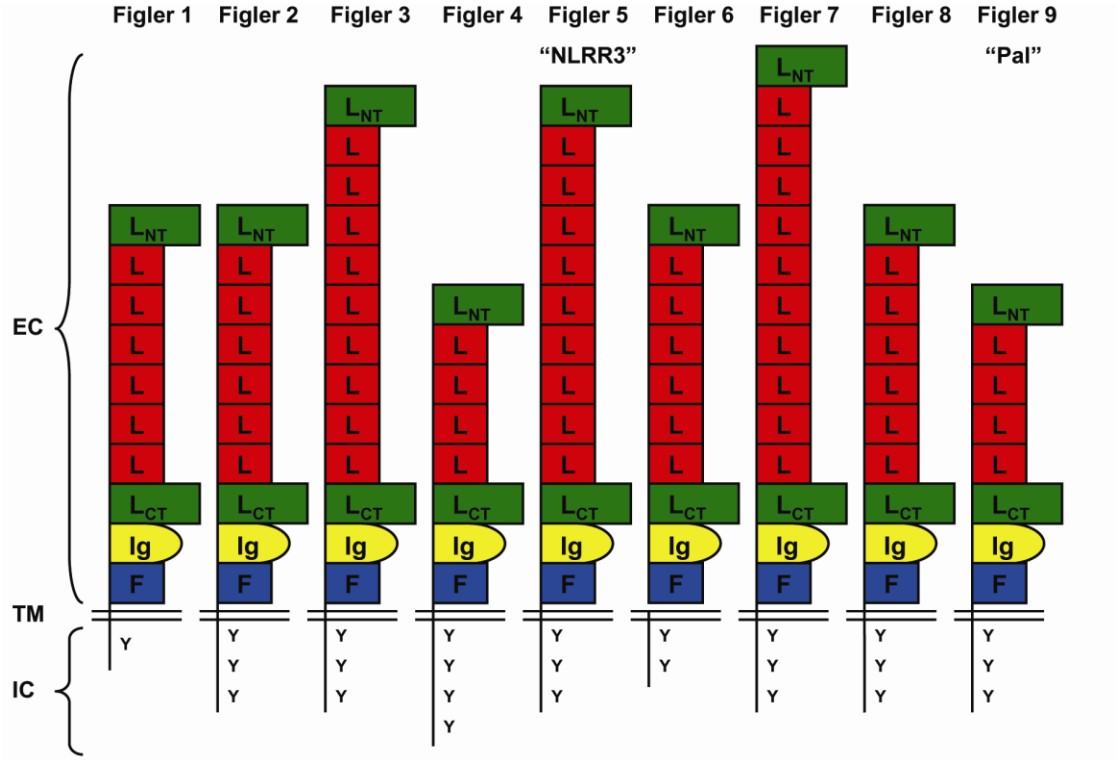


Figure 1. Human FIGLER molecules. Schematic representation of human FIGLER molecules 1–9 detailing extracellular motifs and intracellular tyrosines. L_{NT}, N-terminal leucine-rich repeat; L, leucine-rich repeat; L_{CT}, C-terminal leucine-rich repeat; Ig, C2 type Ig domain; FN, FN type III domain.

Table 1
Percentage amino acid identity. Pairwise analysis of each FIGLER domain was performed using the Megalign CLUSTALW method algorithm, with FIGLER 1 serving as the index of comparison. Percent amino acid identities are indicated and aligned in relation to the FIGLER 1 domains. The identity percentage scoring employed here did not penalize for shortened cytoplasmic tails or the presence of < 8 LRRs.

	Amino acid identity (%)							
FIGLER	2	3	4	5	6	7	8	9
LRR	53.1	33.6	28.1	32.1	62.2	35.0	56.6	27.9
Ig C2	55.4	36.9	31.8	38.5	64.6	41.5	52.3	32.9
FNIII	48.8	13.4	12.2	17.1	47.6	15.9	54.4	13.0
IC	39.2	6.8	13.2	13.5	29.7	17.6	36.5	11.4
EC	36.3	25.8	19.0	25.6	53.7	29.0	48.4	24.5
Overall	33.0	22.5	20.8	24.4	47.3	25.3	43.6	22.6

Cellular expression of human FIGLER

As this study was directed initially toward identification of genes that might influence human B lymphopoiesis, RT-PCR analysis of *FIGLER* gene expression was performed on fetal bone marrow B lymphocyte subpopulations, bone marrow stromal cell lines, fetal thymus, and the non-B cell fraction in bone marrow. PCR products were cloned and sequenced to confirm their identity (data not shown). *FIGLER 9* (*Pal*) served as a negative control gene in these experiments, as it was previously shown to be retina-specific (Figure 2A) [16]. The analysis indicated that *FIGLER 1*, *2*, *3*, and *5* mRNA transcripts were expressed in primary B lineage cells. *FIGLER 1* expression began at the immature $sIgM^+$ B cell stage. *FIGLER 2* expression was detected at low levels in pro-B cells and in the $CD34^+CD19^-$ cells, a heterogeneous population that includes hematopoietic stem cells. *FIGLER 3* expression was initiated at the pro-B cell stage, and *FIGLER 5* expression began at the pre-B cell stage. *FIGLER 1* and *5* were also expressed

by non-B lineage BM cells. Only *FIGLER 2* was found to be expressed in human stromal cell lines. *FIGLER 4*, *6*, *7* and *8* could not be detected in any of the bone marrow-derived cells examined, despite multiple attempts and the use of three or more different gene-specific primer pairs per gene. To examine the *FIGLER* gene expression pattern further, we analyzed representative hemopoietic cell lines, B, T, myeloid and erythroid (Figure 2B). The up-regulation of *FIGLER 1* expression observed as a function of normal B cell development was also observed in representative B lineage cell lines, although the OB5 pre-B cell line had relatively high levels compared to the normal pre-B cells. *FIGLER 1* and *2* were expressed weakly in the thymus and in the Jurkat T cell line, whereas they exhibited reciprocal expression patterns in myeloid cell lines. The K562 erythroid cell line did not express any of the *FIGLER* genes. Surprisingly, given their robust expression in primary cells of bone marrow origin, *FIGLER 3* and *5* were not detected in any of the cell lines tested, nor were *FIGLER 6*, *7*, and *8* (data not shown). As the NCBI EST profile database did not contain bone marrow or spleen expression data for the *FIGLER* molecules, this constitutes the first evidence for expression of these molecules in the bone marrow and B lineage cells in particular.

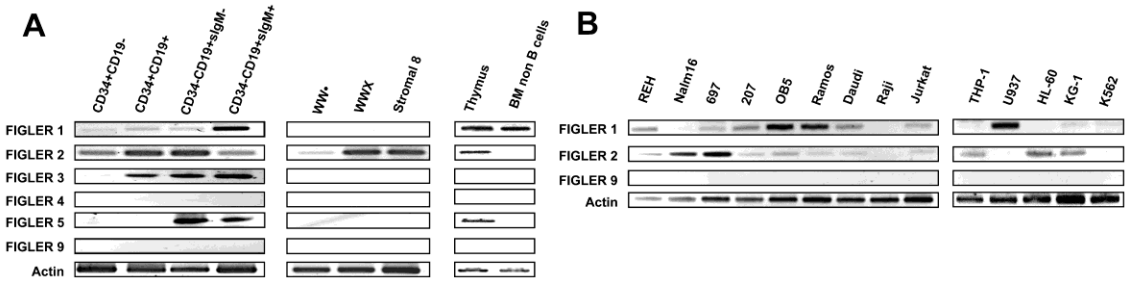


Figure 2. Analysis of FIGLER expression in hematopoietic cells and cell lines. (A) RT-PCR analysis of human FIGLER 1-5 mRNA transcripts. Panel 1, primary bone marrow B lineage cells: $CD34^{+}CD19^{-}$, hemopoietic stem cells and other early hemopoietic cells; $CD34^{+}CD19^{+}$, pro-B cells; $CD34^{-}CD19^{+}IgM^{-}$, pre-B cells; and $CD34^{-}CD19^{+}IgM^{+}$ B cells. Panel 2, bone marrow-derived stromal cell lines. Panel 3, primary thymus and bone marrow non-B lineage cells. (B) RT-PCR analysis of FIGLER 1, 2 and 9 mRNA transcripts in human pro-B (REH, Nalm16), pre-B (697, 207, OBS), B (Ramos, Daudi, Raji), T (Jurkat), myeloid (THP-1, U937, HL-60, KG-1), and erythoid (K562) cell lines. FIGLER 9 (Pal), a retina specific protein, served as a negative control and β-actin served as a loading control for all these analyses. PCR product identity was confirmed by sequencing as described in the Methods section.

Identification of non-human FIGLER homologs

Putative *FIGLER* orthologs were found in macaque, chimpanzee, orangutan, mouse, rat, dog, chicken, toad, and puffer fish NCBI databases using the BLASTN algorithmic search for homology to the identified human *FIGLER* gene sequences (Tables 2–5). Hidden Markov model searching of GenBank led to the identification of five potential new *FIGLER* orthologs in *Takifugu rubripes*, although little information is available about these molecules except amino acid sequence (data not shown).

Table 2

Percentage amino acid identity comparison of primate with human FIGLER.

Amino acid identity with human FIGLER (%)					
Species	Macaque	*Pongo* (orangutan)		*Pan* (chimpanzee)	
FIGLER	2	5	1	8	9
LRR	100	99.1	100	62.7	97.7
(Number)	(8)	(11)	(8)	(7)	(5)
Ig C2	98.8	100	100	68.2	100
FNIII	97.6	97.6	98.8	33.7	100
IC	98.7	100	98.6	39.9	95.7
(Number of Tyr)	(2)	(2)	(1)	(5)	(2)
EC	99.4	99.7	99.8	49.7	98.7
Entire molecule	99.4	98.6	99.7	47.2	98.4

Table 3

Percentage amino acid identity comparison of rodent with human FIGLER.

Amino acid identity with human FIGLER (%)										
Species	Mouse							Rat		
FIGLER	1	2	4	5	6	7	9	2	5	8
LRR	100	97.5	93.5	80.8	95.5	93.4	81.5	98.7	92.6	66.7
(Number)	(8)	(8)	(6)	(10)	(18)	(12)	(6)	(8)	(10)	(8)
Ig C2	96.9	96.3	88.2	84.1	95.4	92.6	87.1	96.9	88.2	61.5
FNIII	95.2	98.8	95.1	67.1	98.8	82.9	83.5	93.1	92.8	62.5
IC	85.1	84.4	58.3	76.0	94.9	84.0	67.1	82.2	64.4	41.4
(Number of Tyr)	(1)	(3)	(3)	(3)	(1)	(3)	(2)	(3)	(3)	(4)
EC	97.2	97.4	89.6	59.8	96.9	91.4	75.1	95.1	90.0	57.6
Entire molecule	95.8	94.9	56.3	54.0	96.5	87.7	71.6	92.7	87.2	56.5

Table 4
Percentage amino acid identity comparison of Tetra and Xenopus with human FIGLER.

Amino acid identity with human FIGLER (%)						
Species		*Tetra* (puffer fish)			*Xenopus* (African clawed toad)	
FIGLER	2	3	8	9	2	3
LRR	78.7	74.8	67.8	54.3	68.3	64.7
(Number)	(8)	(11)	(8)	(6)	(8)	(11)
Ig C2	75.8	69.6	63.6	56.5	70.3	69.6
FNIII	74.4	87.8	68.4	70.1	45.0	46.3
IC	42.7	75.3	55.0	41.9	34.8	38.5
(Number of Tyr)	(5)	(5)	(2)	(3)	(4)	(7)
EC	77.4	83.1	62.0	49.7	39.0	60.1
Entire molecule	67.1	75.1	63.1	49.6	38.9	59.2

Table 5
Percentage amino acid identity comparison of dog, cow, and chicken with human FIGLER.

Amino acid identity with human FIGLER (%)							
Species		Dog		Cow		Chicken	
FIGLER	2	5	8	1	4	8	9
LRR	99.1	97.9	60.1	99.2	64.4	97.8	68.9
(Number)	(6)	(5)	(4)	(8)	(2)	(4)	(4)
Ig C2	98.4	90.0	59.7	100	84.3	95.5	77.1
FNIII	98.5	94.0	34.4	97.6	64.1	95.0	69.2
IC	88.5	83.3	54.2	78.2	60.0	55.6	32.2
(Number of Tyr)	(2)	(3)	(6)	(1)	(3)	(1)	(1)
EC	98.9	95.2	65.5	97.2	52.5	96.0	54.4
Entire molecule	96.1	94.1	62.4	95.1	44.8	94.5	67.9

As was the case for their human counterparts, these genes were found to be located on different chromosomes. All FIGLER orthologs contained 6–12 LRR, a single C2 type Ig domain, a FN type III domain, a hydrophobic transmembrane domain, and from one to seven cytoplasmic tyrosines. The predicted amino acid sequences of the FIGLER homologs shared 38–99% overall identity with their human counterpart. Phylogenetic tree analysis was performed to cluster the non-human FIGLER molecules to their nearest human FIGLER homolog (Figure 3). The alternative names, chromosomal locations, predicted amino acid length, and accession numbers of the human *FIGLER* and non-human *FIGLER* molecules are listed in Tables 6 and 7.

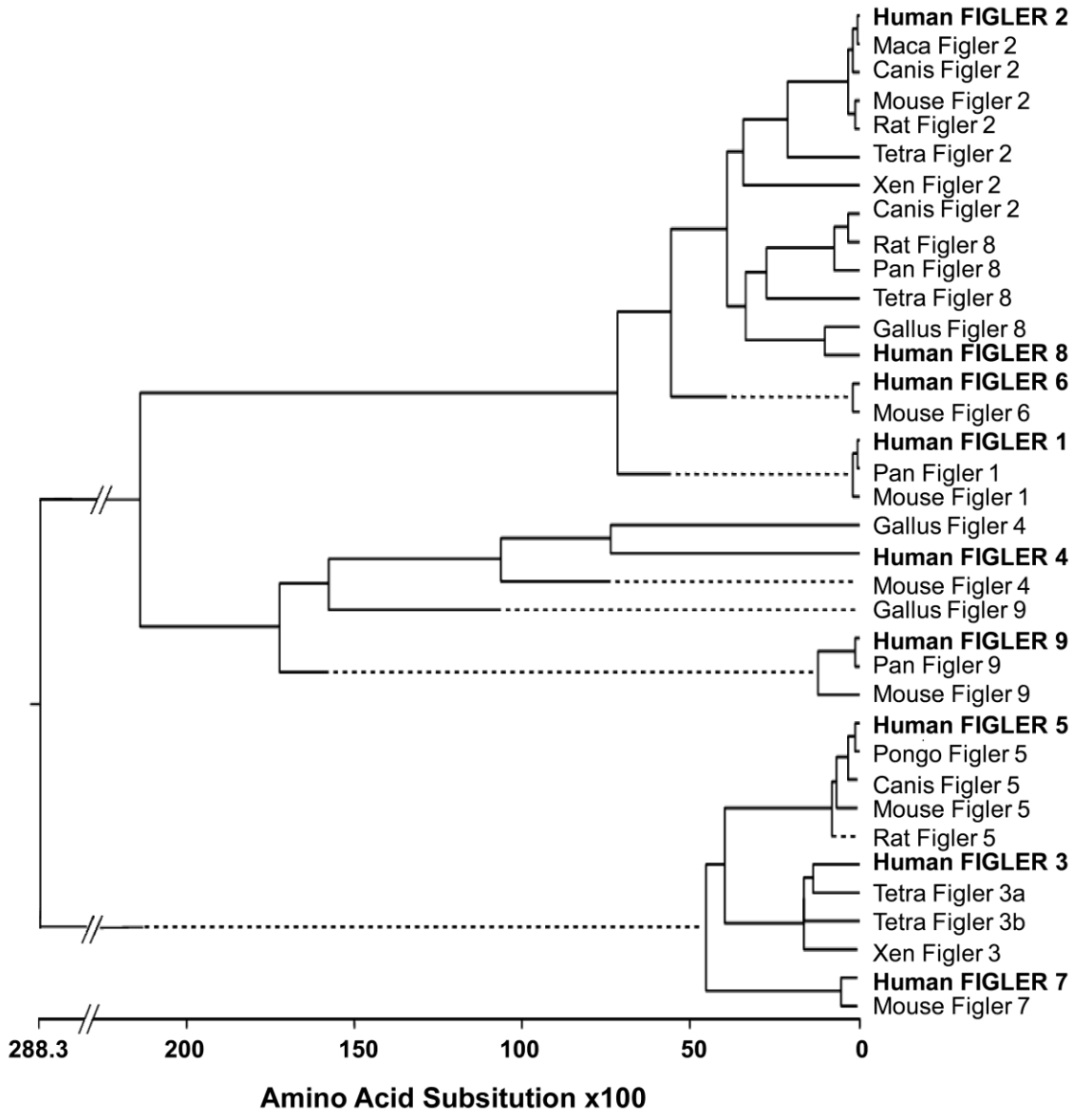


Figure 3. Phylogenetic analysis of the *FIGLER* family of molecules. Phylogenetic analysis of the molecule coding amino acid sequence region of the FIGLER family members. The CLUSTALW algorithm was used for multiple sequence alignment of divergent sequences after the variable length LRR regions were masked out.

Table 6
Human and non-human FIGLER alternative names, chromosomal locations, predicted protein lengths and NCBI GenBank accession numbers.

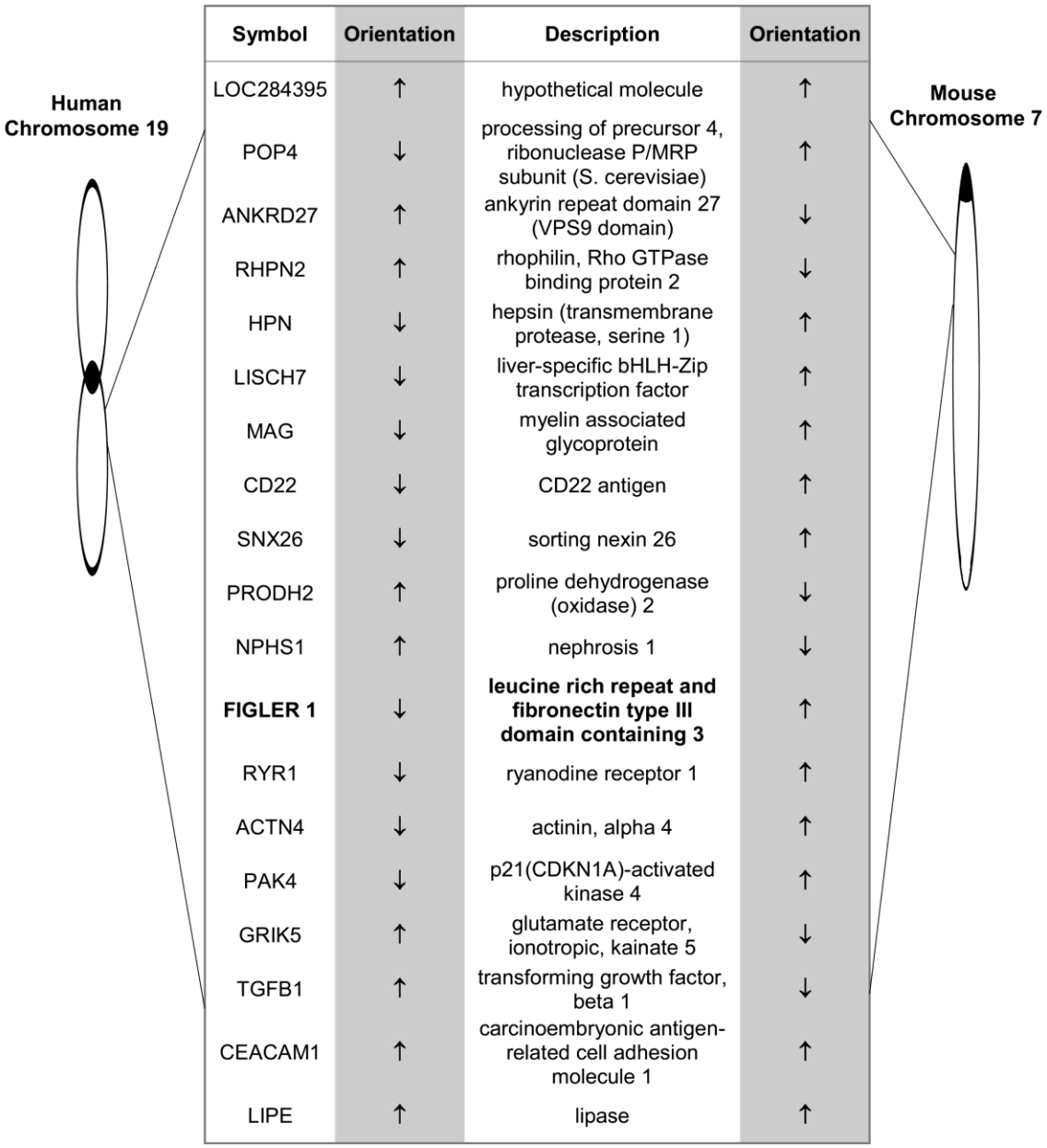
Species	Gene name (FIGLER)	Alternative name	Chromosome location	Predicted protein length (amino acids)	Protein accession number
Homo Sapiens	1	LRFN3	19q13.12	628	NP_078785
	2	LRFN2	6p21.2	789	NP_065788
	3	LRRN1	3p26.2	716	NP_065924
	4	FLJ44691	4q25	496	NP_940908
	5	LRRN3	7q31.1	708	NP_060804
	6	LRFN4	11q13.2	635	NP_076941
	7	LRRN5	1q32.1	713	NP_963924
	8	LRFN5	14q21.1	719	NP_689660
	9	PAL/ LRRC21	10q23	623	NP_056428
Macaca fascicularis	2	LRFN2	–	789	BAB39323
Mus musculus	1	LRFN3	7	626	NP_780687
	2	mKIAA 1246	17	824	BAC65758
	4	–	3	679	XP_143529
	5	LRRN3/ LRFN5	12	707	NP_034863
	6	LRFN4	19	636	NP_700437
	7	LRRN2	1	730	AAQ74241
	9	–	14	618	NP_666357
Rattus norvegicus	2	–	9q11	804	XP_236914
	5	NLRR3	6q21	707	NP_110483
	8	–	1q21	766	XP_344875

Table 7
Non-human FIGLER alternative names, chromosomal locations, predicted protein lengths and NCBI GenBank accession numbers.

Species	Gene name (FIGLER)	Alternative name	Chromosome location	Predicted protein length (amino acids)	Protein accession number
Gallus Gallus	4	–	4	1009	XP_420649
	8	–	5	559	XP_421485
	9	–	6	884	XP_426489
Tetraodon nigroviridis	2	–	10	794	CAF99016
	3	–	11	715	CAG06728
	8	–	16	574	CAG08917
	9	–	17	660	CAF98662
Pongo pygmaeus	5	LRRN3/ NLRR-3	–	708	CAH93434
Pan troglodytes	1	–	19	628	XP_524229
	8	–	19	795	XP_512991
	9	–	10	775	XP_521533
Canis familiaris	2	–	12	779	XP_538906
	5	–	14	708	XP_539523
	8	–	1	730	XP_541626
Bos taurus	1	LRFN3	18	628	NM_001076959
Xenopus laevis	2	LOC496079	–	722	AAH87496
	3	XNLRR-1	–	718	BAA28530

The chromosomal regions of the human and mouse *FIGLER* genes were compared to further examine their relatedness. The positions of neighboring upstream and downstream genes were found to be highly conserved, but the regions containing the mouse *FIGLER* genes were inverted relative to the orientation of human *FIGLER* chromosomal regions. This is illustrated for *FIGLER 1* and mouse *FIGLER 1* (Figure 4). By contrast, the non-mouse *FIGLER* genes have the same chromosomal orientation as their human *FIGLER* counterparts (data not shown).

As the known *NLRR3* (*FIGLER 5*) and *Pal* (*FIGLER 9*) genes have been shown to have an unusual three exon structure, we analyzed the predicted exon structure of the entire *FIGLER* gene family (Figure 5). Most of the human *FIGLER* and non-human *FIGLER* genes have the same atypical 3 exon structure, in which exon 1 contains the 5' untranslated region; exon 2 encodes the signal peptide, LRR, Ig domain, and part of the FNIII domain; and exon 3 encodes the remaining FNIII region, transmembrane region, cytoplasmic tail, and 3' untranslated region. Human *FIGLER 3*, *5*, and 7, chimpanzee *FIGLER 5,* mouse *FIGLER 5* and *7*, rat *FIGLER 8,* and dog *FIGLER 5* are encoded by a single exon. Interestingly, chimpanzee and chicken *FIGLER 9* and chicken FIGLER 4 have a more standard exon organization, in which exon 1 contains the 5'untranslated region, exon 2 encodes the LRRs, exon 3 contains the Ig domain, and exon 4 encodes the FNIII region, transmembrane domain, cytoplasmic tail and 3'UTR.



Symbol	Orientation	Description	Orientation
LOC284395	↑	hypothetical molecule	↑
POP4	↓	processing of precursor 4, ribonuclease P/MRP subunit (S. cerevisiae)	↑
ANKRD27	↑	ankyrin repeat domain 27 (VPS9 domain)	↓
RHPN2	↑	rhophilin, Rho GTPase binding protein 2	↓
HPN	↓	hepsin (transmembrane protease, serine 1)	↑
LISCH7	↓	liver-specific bHLH-Zip transcription factor	↑
MAG	↓	myelin associated glycoprotein	↑
CD22	↓	CD22 antigen	↑
SNX26	↓	sorting nexin 26	↑
PRODH2	↑	proline dehydrogenase (oxidase) 2	↓
NPHS1	↑	nephrosis 1	↓
FIGLER 1	↓	**leucine rich repeat and fibronectin type III domain containing 3**	↑
RYR1	↓	ryanodine receptor 1	↑
ACTN4	↓	actinin, alpha 4	↑
PAK4	↓	p21(CDKN1A)-activated kinase 4	↑
GRIK5	↑	glutamate receptor, ionotropic, kainate 5	↓
TGFB1	↑	transforming growth factor, beta 1	↓
CEACAM1	↑	carcinoembryonic antigen-related cell adhesion molecule 1	↑
LIPE	↑	lipase	↑

Figure 4. Human/mouse homology map for *FIGLER 1*. The chromosomal locations and transcriptional orientation of the human and mouse FIGLER 1 and flanking genes were determined using the NCBI GenBank Mapview Database. Black ovals denote the centromere.

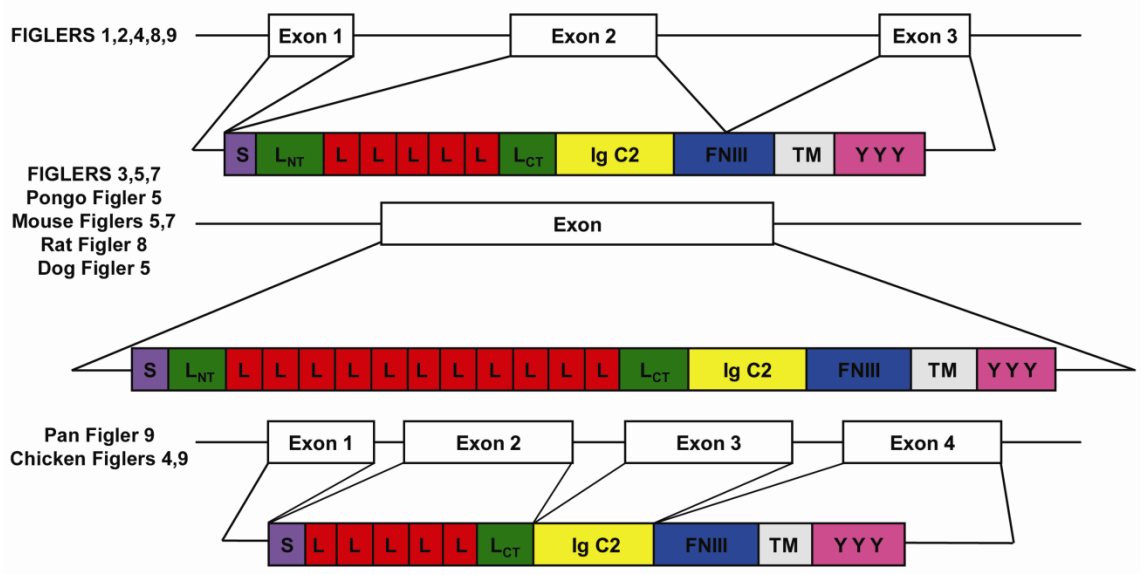


Figure 5. Predicted genomic structure and protein product of human and non-human FIGLER molecules. S, Signal Peptide; L_{NT}, N-terminal leucine-rich repeat; L, leucine-rich repeat domain; L_{CT}, C-terminal leucine-rich repeat, Ig, C2 type Ig domain; FNIII, FN type III domain; TM, hydrophobic transmembrane domain; Y, tyrosine in predicted cytoplasmic domain. Non-human FIGLER genes not listed in this schematic have the same three exon structure as human FIGLER 1, 2, 4, 8, and 9.

Discussion

The nine human orphan receptors identified here were named FIGLER to emphasize their unique combination of FN, Ig, and LRR domains. The tyrosines present in their cytoplasmic regions may indicate signaling potential, although they were not found to be embedded in known consensus signaling motifs. The *FIGLER* genes encode molecules that share 20–47% amino acid identity and similar structure despite their widespread distribution in the genome. Although our initial goal was to identify a novel IL-7-like receptor, the FIGLER molecules differ from known cytokine receptors in that they have LRR domains on the N-terminal end of the protein. The LRRs are

characterized by specific leucine and asparagine residue spacing and are found in proteins with diverse functions and cellular distribution [14,15,17,21,22]. LRRs also are found in other molecules that are expressed by B cells, such as Toll-like receptors 9 and 10 [23]. As we sought to identify molecules that could potentially influence human B lymphopoiesis, *FIGLER* expression was analyzed in hematopoietic and lymphoid tissues. *FIGLER 1*, *3*, and *5* mRNA expression was found to be up-regulated as a function of human B cell development, whereas *FIGLER 2* was expressed throughout development and in non-B lineage cells.

The identification of *FIGLER* relatives in macaque, cow, chimpanzee, orangutan, mouse, rat, dog, chicken, toad and puffer fish that share 38–99% amino acid identity to their human counterparts indicates that this is a conserved gene family. All of the members encode type I transmembrane proteins, and each gene is located on a different chromosome, except for the chimpanzee *FIGLER 1* and *8*, which are both located on chromosome 19. Based on human *FIGLER* and mouse *FIGLER* gene chromosomal analysis, it is likely that the human and non-human *FIGLER* genes are homologs that were derived from common ancestral genes. Intriguingly, the mouse FIGLER genes are located on inverted chromosomal segments relative to their human syntenic regions, although *FIGLER* genes in other species have the same orientation as their human counterparts.

As the known *NLRR3* (*FIGLER 5*) and Pal (*FIGLER 9*) genes have been shown to have an unusual predicted exon structure, we determined the exon structure of the entire *FIGLER* gene family [16,21,22]. Most of the human *FIGLER* and non-human *FIGLER* genes have the same atypical 3 exon structure, in which exon 1 contains the 5’

untranslated region; exon 2 encodes the signal peptide, LRR, Ig domain, and part of the FNIII domain; and exon 3 encodes the remaining FNIII region, transmembrane region, cytoplasmic tail, and 3' untranslated region. Ig domains are almost universally encoded by a single exon, which in part is thought to have accounted for their ready dispersal in the genome and widespread utilization in cell surface glycoproteins with multiple functions. Human *FIGLER 3*, *5*, and *7,* and chimpanzee *FIGLER 5,* mouse *FIGLER 5,* mouse *FIGLER 7*, rat *FIGLER* 8, and dog *FIGLER 5* are all intronless genes, suggesting their integration into the genome via reverse transcription. Nevertheless we have demonstrated cellular expression for all but *FIGLER 4* and *6-8*, and all of these genes are predicted to encode an intact open reading frame. Finally, the chimpanzee and chicken *FIGLER 9* genes and chicken *FIGLER 4* have a more typical organization, and the Ig domain is encoded by a separate exon.

Conclusions

The functions of the *FIGLER* gene products are presently unknown. FIGLER 5 and 9 are identical to the previously identified Pal and NLRR3. Pal is a type 1 transmembrane protein that is preferentially expressed in the retina, where it is up-regulated during photoreceptor outer segment development. As for the other FIGLER family members, Pal lacks recognizable signaling motifs in its cytoplasmic tail and is predicted to act as a trophic factor receptor or adhesion molecule [16]. NLRRs were identified through screening of a mouse brain cDNA library, and homologs have been found in *Xenopus*, rat, and human brain tissue [18-20,24,25]. All of the NLRR members are expressed predominately in the central nervous system [24]. Of the three known mouse NLRRs,

only NLRR3 is up-regulated with brain injury, thereby suggesting its involvement in injury recognition or repair [26]. While several of the FIGLER molecules described here are expressed in B lineage cells and the expression of FIGLER 1, 3, and 5 is developmentally regulated within this lineage, the possibility that these molecules are involved in the interaction between B lineage cells with the supportive bone marrow microenvironment remains speculative.

Methods

Database and search strategies

The nucleotide sequence, amino acid sequence and domain structure of the human IL-7 receptor α chain (NM_002185) was used in the basic local alignment search tool (BLAST) and simple modular architecture research tool (SMART) database searches for novel IL-7 receptor α chain relatives [27-29]. The National Center for Biotechnology Information (NCBI), European Molecular Biology Laboratory (EMBL), Ensembl, and DNA database in Japan were all queried, including the expressed sequence tags (EST) and high throughput genomic sequences (HTGS) databases. The predicted human *FIGLER* sequences were used to identify homologs in other species using BLASTN algorithmic and hidden Markov model (HMM) searches of the NCBI and GenBank databases. HMM hits were filtered through Pfam according to their domain composition. The NCBI genome database was used to align the predicted mRNA sequences to the genomic sequences and determine intron-exon boundaries. The human BLAST-like alignment tool (BLAT) database (University of California at Santa Cruz) and Megalign CLUSTALW method algorithm (Windows version 3.12e; DNASTAR, Madison, WI,

USA) were used to design primers and determine amino acid identity among the newly identified FIGLER molecules. The molecular coding amino acid sequence region phylogenetic tree analysis of the FIGLER molecules as listed in the GenBank database was conducted using the CLUSTALW method algorithm of Megalign [30]. The variable length LRR regions were masked out during phylogenetic analysis in order to compensate for sequence length.

Cell lines

Human stromal cell lines included the WW•, WWX, and stromal 8 lines, which were derived from bone marrow samples in our laboratory. B lineage cell lines were REH and Nalm 16 (pro B cell); 697, 207, and OB5 (pre B cell); and Ramos, Daudi, and Raji (B cell). Myeloid cell lines included THP-1 (monocytic), HL-60 (promyelocytic), U937, and KG-1 (myelocytic). The Jurkat T cell line and K562 erythroid cell lines were also used in this analysis.

Human bone marrow and thymus primary cell isolation

Human adult bone marrow was obtained from resected ribs of healthy renal transplant donors in accordance with policies established by the University of Alabama at Birmingham Institutional Review Board. The resected ribs were processed within 24 h of obtainment, and lymphoid cells were isolated by Ficoll-Hypaque gradient centrifugation (Mediatech, Herndon, VA, USA). B lineage cells were isolated from the bone marrow samples using a MACS B cell Isolation Kit (Miltenyl Biotec, Auburn, CA, USA). The isolated primary B lineage cells were resuspended in FACS buffer (FACS buffer: PBS

plus 2% FBS) and incubated for 20 min at 4 °C with CD34-APC, CD19-PE, and IgM-FITC labeled antibodies (BD Biosciences, Palo Alto, CA, USA). The cells were then sorted based on their differential CD34, CD19, and IgM expression into four B lineage populations: $CD34^{+}19^{-}$, $CD34^{+}19^{+}$, $CD34^{-}19^{+}IgM^{-}$, and $CD34^{-}19^{+}IgM^{+}$, using a MoFlo instrument (Cytomation, Fort Collins, CO, USA). Human fetal bone marrow and thymus samples used for RT-PCR analysis were obtained in accordance with policies established by the University of Alabama at Birmingham Institutional Review Board. Lymphocytes from 12- to 29-week fetal bone marrow and thymus were obtained by sedimentation at 100*g* over a lymphocyte separation medium for 30 min at room temperature. The cells recovered at the interface were washed in PBS containing 5% FCS and 2 to 4×10^{7} cells. B lineage cells were isolated from the bone marrow and thymus samples using a MACS B cell Isolation Kit.

Reverse transcription (RT)-PCR

Total RNA was isolated from isolated populations of primary fetal bone marrow B lineage cells, primary fetal thymic B lineage cells, hematopoietic cell lines, and stromal cell lines using TRIzol (Invitrogen, Carlsbad, CA, USA). cDNA was generated from the isolated mRNA using Superscript First Strand Synthesis System for RT-PCR, per the manufacturer's suggestions (Invitrogen). The following gene specific primers were used to amplify each gene via PCR using Platinum Taq DNA Polymerase (Invitrogen): FIGLER 1, forward 5'-CTGCTAGGCAACTCAAGC-3' and reverse 5'-GATAGGCCGCTGATCCG-3'; FIGLER 2, forward 5'- GCTACTTCTGGCATGTGC-3' and reverse 5'-ACCACTGTCCTGAGATGT-3'; FIGLER 3, forward 5'-

CAGTACAGCCCTTGCTG-3' and reverse 5'-CCACATGTAATAGCTTG-3'; FIGLER 4, forward 5'-CTCGTGGTGACCAGTACT-3' and reverse 5'-AGCTTCTGTCACGTCTGC-3'; FIGLER 5, forward 5'-CAGCAATGCTCTCAGTGC-3' and reverse 5'-TCGAGCACTTTGCGCAG-3'; FIGLER 9, forward 5'-TCTCAATGCAGCTGCAGC-3' and reverse 5'-GCTGGCACATCTCAGTTC-3'. Each amplification reaction underwent an initial denaturation of 94 °C for 2.5 min, followed by 35 cycles of annealing at 55 °C for 1 min, extension at 72 °C for 1 min, denaturation at 94 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR products were separated by agarose gel electrophoresis, and their identity was verified via sequencing after cloning into a TOPO TA pCR2.1 Vector (Invitrogen). DNA sequencing was accomplished for both strands by the dideoxy chain termination method using Thermo Sequenase (Amersham Biosciences Corp, Piscataway, NJ, USA), per the manufacturer's suggestions, and an automated sequencer (LiCor, Lincoln, NE, USA).

Endnotes

Authors' contributions

DLM conducted the primary database searches, analysis, RT-PCR experiments, and manuscript preparation. CLH contributed to database searches, analysis, experimental design, interpretation, and manuscript preparation. PDB contributed to the experimental design, analysis, interpretation, and manuscript preparation. MDC contributed to the experimental design, interpretation, and manuscript preparation. All authors have read and approved the final manuscript. *DLM and CLH contributed equally to this work.

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CONCLUSIONS

B cell development and maturation is a highly controlled process closely regulated by signaling events through the B cell antigen receptor. Signals transmitted through the BCR function as regulatory mechanisms for cell survival, apoptosis, proliferation, cellular progression, and antibody repertoire selection. Control of B cell development and BCR signaling is critical in allowing the B lymphocyte to mount an effective response against invading pathogens. Our work presented in this dissertation has furthered the understanding of the role of FCRL5 as a potential regulator of B cell receptor signaling and laid the foundation for further exploration of the evolutionarily conserved FIGLER molecules and their role in the life cycle of B cells.

Fc Receptor-Like Molecule 5

Prior to the experiments presented in this dissertation, little was known about the signaling potential of FCRL5 or any FCRL family member containing both ITAM and ITIM signaling motifs. By creating FcγRIIb/FCRL5 chimeric receptors and expressing them in a cell line lacking endogenous FcγRIIb, we were able to identify the tyrosine residues that contribute to the inhibitory signaling effect of FCRL5 upon coligation with the B cell receptor. When coligated with the BCR, the WT FcγRIIb/FCRL5 acted as an inhibitor of calcium mobilization, whole cell tyrosine phosphorylation, Erk activation, and Igα/Igβ heterodimer phosphorylation. WT FcγRIIb/FCRL5 was shown to associate

with SHP-1 as demonstrated by immunoprecipitation of the chimeric receptor following stimulation.

Mutational analysis of cytoplasmic tyrosines in the FCRL5 signaling motifs indicates that the two ITIM regions containing tyrosine residues 924 and 954 are functionally important in the attenuation of BCR signaling. A phosphopeptide corresponding to the most membrane-distal ITIM was shown to precipitate SHP-1. However, immunoprecipitation experiments suggest that both ITIMs are necessary for SHP-1 association given that mutation of either membrane tyrosine resulted in no detectable level of SHP-1 association. When left unmutated, both of the ITIM regions working in tandem associate with SHP-1. In comparison to BCR-only induced calcium mobilization, calcium flux using the individual ITIM mutants resulted in truncation of the response duration when tyrosine 924 was mutated and a decrease of maximal peak intensity when tyrosine 954 was mutated. The ITAM-like signaling motif tyrosines, although phosphorylated in our system following coligation with the BCR, did not appear to contribute to the overall signaling response of FCRL5.

Using tonsillar memory B cells, we demonstrated that the native FCRL5 receptor may play a similar inhibitory role in primary cells. By examining the effects of BCR coligation with FCRL5 by phosphospecific flow cytometry in tonsillar memory B cells, we showed that the overall level of whole cell tyrosine phosphorylation was greatly attenuated when FCRL5 was ligated with the BCR. Furthermore, this attenuating effect of whole cell tyrosine phosphorylation was shown to be mediated by SHP-1 as demonstrated by preincubation with α-Bromo-4-hydroxyacetophenone 4-Hydroxyphenacyl Br, a powerful inhibitor of SHP-1, prior to stimulation with anti-

FCRL5 and anti-Ig $F(ab')_2$ fragments. Addition of the SHP-1 inhibitor resulted in the restoration of whole cell tyrosine phosphorylation to levels comparable to BCR-only induced response.

Recently, data has shown FCRL5 expression is upregulated on B cell lineage malignancies and Epstein-Barr virus infected cells (30, 38-40). In the case of multiple myeloma, chronic lymphocytic leukemia, and mantle cell lymphoma, elevated levels of the soluble isoform of FCRL5 have been detected in blood samples corresponding to tumor burden (39). In addition to the soluble isoform, cell surface expression of FCRL5 has also been shown on malignant B cells. In cells infected with Epstein-Barr virus, FCRL5 surface expression has been shown to be upregulated by an interaction of EBNA2 with CBF1 leading to translation of 1q21, a region associated with chromosome abnormalities in B cell malignancies such as B cell non-Hodgkin's lymphoma (B-NHL) and multiple myeloma (40).

However, a fundamental question must be addressed. Why would a B cell malignancy maintain surface expression of an inhibitory receptor? FCRL5 is not the first case of an inhibitory molecule expressed on B cell malignancies. Previous reports indicate that FcγRIIb is expressed on Burkitt's B cell lymphoma cell lines and this inhibitory receptor has also been shown to mediate antibody-dependent cellular cytotoxicity. Analysis of surface expression in tonsil and spleen indicate that FCRL5 is expressed by most naïve, memory, and plasma cell B cells and down regulated in germinal center populations (37). In B cell malignancies, FCRL5 expression may be associated with retention of cell surface markers at the onset of lymphomagenesis.

FCRL5 was identified by one group of investigators as a result of a translocation event of chromosome 1q21 (30). Deregulation of FCRL5 expression is commonly associated with the 1q21 translocation abnormality in B-NHL and MM. FCRL5 expression on B cell malignancies may be an inevitable result of gene expression deregulation of the region spanning the chromosomal translocation event. With no readily apparent B cell signaling capacity, the soluble isoform of FCRL5 may serve as an effector molecule for other lymphocytic cells involved in tumor cell detection and clearance. The unknown role of the soluble FCRL5 isoform emphasizes the need to identify the natural ligand(s) of FCRL5.

In EBV infection, EBNA2 has been shown to induce FCRL5 surface expression through the host DNA binding protein CBF1. Again, we must question why EBNA2 would cause a downstream interaction leading to the induction of FCRL5 on potentially tumorogenic B cell. It is important to note that the recent study of EBNA2 induced FCRL5 expression does not necessarily indicate lymphomagenesis is the result of either FCRL5 expression or EBNA2 activity. FCRL5 could be employed in the viral life cycle of EBV, without correlation with lymphomagenesis. We have shown that both whole cell tyrosine phosphorylation as well as Erk activation is significantly decreased upon BCR coligation with our FcγRIIb/FCRL5 chimeric receptor. In EBV infection, inhibition of the BCR signaling cascade is associated with maintaining viral latency (53). In lymphoblastoid cell lines (LCL), the constitutively phosphorylated EBV protein LMP2A has been shown to block BCR signaling and Erk activation by associating with Lyn and Syk, diverting them from participating in BCR signaling cascades and targeting them for later degradation. However, short term expression of LMP2A induces the viral lytic life

cycle in B cells and appears to be correlated with the ability of LMP2A to exclude the BCR complex from entering lipid rafts (54). Although such dominant-negative signaling effects have yet to be demonstrated, a similar role may be played by FCRL5 in EBV infection by constituitive association with SHP-1 near or away from the BCR. Although further research is needed to fully understand the complexity of FCRL5 induction and signaling in lymphoma and EBV infection, FCRL5 may provide an attractive target for immunotherapy given its potent inhibitory potential and expression on leukemias and lymphomas.

FIGLER Molecules

The FIGLERs represent an interesting family of conserved fibronectin containing, leucine-rich repeat, transmembrane molecules. Using bioinformatic searches for proteins similar to the structure of interleukin-7, we identified nine human transmembrane proteins with Ig domains, FNIII domains, and signaling potential. In addition to the human *FIGLER* genes, we identified homologs in macaque, orangutan, chimpanzee, mouse, rat, dog, chicken, toad, and puffer fish databases. RT-PCR analysis of *FIGLER* expression in hematopoietic cells and cell lines provided the first evidence for expression of the FIGLER molecules in bone marrow and B lineage cells.

In mice, IL-7 plays a critical role in the generation of B and T lymphocytes. In humans, however, B cell development is unimpeded in the absence of IL-7 or its receptor. Our intent was to identify a molecule that might serve as the human equivalent of the IL-7 receptor in the murine B cell developmental system. Unlike classic cytokine receptors, the FIGLER molecules have leucine-rich repeats in the N-terminal domain of

the receptor. The IL-7 receptor normally associates with Janus kinases and is phosphorylated upon cytokine-receptor binding. Phosphorylation of the IL-7 receptor leads to the association and activation of STAT5, a transcription factor that regulates many aspects of cell survival, growth, and differentiation. Although the FIGLER molecules have intracellular tyrosines, they lack a currently recognizable signaling motif. Although continued work is needed in determining their role in the life cycle of B cells, we suspect they may serve as cell adhesion molecules given their extracellular domain structure and lack of recognizable signaling motifs.

The structural organization of the *FIGLER* genes differs significantly from that of the *IL-7α receptor*. The human *FIGLERs* have an unusual 3 exon, with exon 1 encoding the 5' untranslated region, exon 2 encoding the signal peptide, LRR, Ig domain, and part of the FNIII domain, and exon 3 encoding the remainder of the molecule and 3' untranslated region (52). The *IL-7α receptor* gene consists of 8 exons with exon 1 encoding the 5' untranslated region, signal peptide, and amino terminus of the mature peptide, exons 2-6 encoding the remainder of the extracellular domain, transmembrane domain, and a portion of the cytoplasmic domain, exons 7 and 8 encode the remainder of the cytoplasmic domain and the 3' untranslated region (55).

Our transcriptional analysis also indicated that human FIGLER molecules 1, 2, 3 and 5 are expressed in primary B lineage cells, whereas FIGLERs 1 and 5 were also expressed in non-B lineage bone marrow cells and FIGLER 2 was expressed in human stromal cell lines. The RT-PCR analysis of human FIGLER expression pattern represents the first evidence that the FIGLER molecules are expressed in bone marrow and B lineage cells.

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APPENDIX

INSTITUTIONAL REVIEW BOARD PROTECTION OF HUMAN SUBJECTS APPROVAL LETTER

OMB No. 0990-0263
Approved for use through 11/30/2008

Protection of Human Subjects
Assurance Identification/IRB Certification/Declaration of Exemption
(Common Rule)

Policy: Research activities involving human subjects may not be conducted or supported by the Departments and Agencies adopting the Common Rule (56FR28003, June 18, 1991) unless the activities are exempt from or approved in accordance with the Common Rule. See section 101(b) of the Common Rule for exemptions. Institutions submitting applications or proposals for support must submit certification of appropriate Institutional Review Board (IRB) review and approval to the Department or Agency in accordance with the Common Rule. Institutions must have an assurance of compliance that applies to the research to be conducted and should submit certification of IRB review and approval with each application or proposal unless otherwise advised by the Department or Agency.

1. Request Type	2. Type of Mechanism	3. Name of Federal Department or Agency and, if known, Application or Proposal Identification No.
[ ] ORIGINAL [X] CONTINUATION [ ] EXEMPTION	[ ] GRANT [ ] CONTRACT [ ] FELLOWSHIP [ ] COOPERATIVE AGREEMENT [ ] OTHER:_____	

4. Title of Application or Activity	5. Name of Principal Investigator, Program Director, Fellow, or Other
Comparison of B Cell Differentiation in Mice and Humans (AI39816)	COOPER, MAX D

6. Assurance Status of this Project *(Respond to one of the following)*

[X] This Assurance, on file with Department of Health and Human Services, covers this activity:
Assurance Identification No. FWA00005960, the expiration date 09/19/2010 IRB Registration No. IRB00000726

[ ] This Assurance, on file with *(agency/dept)*_____, covers this activity.
Assurance No._____, the expiration date_____ IRB Registration/Identification No._____*(if applicable)*

[ ] No assurance has been filed for this institution. This institution declares that it will provide an Assurance and Certification of IRB review and approval upon request.

[ ] Exemption Status: Human subjects are involved, but this activity qualifies for exemption under Section 101(b), paragraph_____.

7. Certification of IRB Review (Respond to one of the following IF you have an Assurance on file)

[X] This activity has been reviewed and approved by the IRB in accordance with the Common Rule and any other governing regulations.
by: [ ] Full IRB Review on (date of IRB meeting) _____ or [X] Expedited Review on (date) 11-07-07
[ ] If less than one year approval, provide expiration date _____

[ ] This activity contains multiple projects, some of which have not been reviewed. The IRB has granted approval on condition that all projects covered by the Common Rule will be reviewed and approved before they are initiated and that appropriate further certification will be submitted.

8. Comments	Title X001020003
Protocol subject to Annual continuing review. HIPAA Waiver Approved?: No	Comparison of B Cell Differentiation in Mice and Humans (AI39816)

IRB Approval Issued: 11-09-07

9. The official signing below certifies that the information provided above is correct and that, as required, future reviews will be performed until study closure and certification will be provided.	10. Name and Address of Institution
11. Phone No. *(with area code)* (205) 934-3789 12. Fax No. *(with area code)* (205) 934-1301 13. Email: smoore@uab.edu	University of Alabama at Birmingham 701 20th Street South Birmingham, AL 35294

14. Name of Official	15. Title
Marilyn Doss, M.A.	Vice Chair, IRB

16. Signature	17. Date
Marilyn Doss	11-07-07

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