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Fc Receptor Homolog 3 Is a Novel Immunoregulatory Marker of Marginal Zone and B1 B Cells

Woong-Jai Won,*‡ Jeremy B. Foote,** Mary R. Odom,*‡ Jicun Pan,*§ John F. Kearney,*‡ and Randall S. Davis2*†‡§

Two members of the recently identified FcR homolog (FcRH) family in mice demonstrate preferential B cell expression. One of these, FcRH3, encodes a type I transmembrane protein with five extracellular Ig domains and a cytoplasmic tail with a consensus ITIM and a noncanonical ITAM. Analysis of full-length cDNAs from five different mouse strains defines two FcRH3 alleles. A panel of FcRH3-specific mAbs was generated to define its expression pattern and functional potential on B lineage cells. Although poorly detected on the majority of bone marrow or peripheral blood cells, FcRH3 was readily identified on splenic marginal zone (MZ) and MZ precursor B cells, but not on the bulk of newly formed B cells, follicular B cells, germinal center B cells, and plasma cells. In the peritoneal cavity, FcRH3 was found on B1 cells, and not on the majority of B2 cells. Consistent with its possession of an ITIM and ITAM-like sequence, FcRH3 was tyrosine phosphorylated following pervanadate treatment, and its coligation with the BCR inhibited calcium mobilization. These results suggest FcRH3 is a novel immunoregulatory marker of MZ and B1 B lineage cells. The Journal of Immunology, 2006, 177: 6815–6823.

B lymphocytes and their plasma cell (PC) progeny establish the humoral arm of adaptive immunity. Commitment to the B cell lineage and the early stages of B cell development take place in the bone marrow. Upon maturation, B cells emigrate to peripheral lymphoid tissues where they participate in host responses to antigenic challenge. There, B cells may undergo affinity maturation and maintain an Ab repertoire that equips the host for future pathogen encounters. Particulate blood-borne Ags first contact tissue-based immune system cells in a unique topographical blood-organ interface located in the marginal zone (MZ) of the spleen (1, 2). This region harbors intermediate-sized MZ B cells with a phenotype, Ig repertoire, and function distinct from the bulk of follicular (FO) B cells present in the spleen. Differing from their long-lived FO B cell neighbors, MZ B cells, which are also long-lived, possess a preactivated phenotype defined as IgMhiIgDloCD1d+CD11c+CD21hiCD23lo. There may also be positive selection for B cell clones into the MZ that express canonical Ig receptors that are valuable for responses against bacterial Ags, but may also have the potential to contribute to autoimmunity and malignancy (3–6). Once activated, these cells are capable of responding to Ag independently of T cell help and rapidly differentiate within 3 days into Ab-secreting cells (4, 7). Additionally, they may also participate in germinal center (GC) reactions to T-dependent Ags (8). The existence of somatically mutated memory B cells in the MZ has been documented in humans and rats (9–11), but not conclusively in mice (12–14); however, their function and the nature of their Ig repertoire suggest a role for MZ B cells in “natural immune memory” (15, 16).

Like MZ B cells, the CD5+ B1 B cells in the peritoneal cavity have similar function and may be enriched in clonal specificities for T-independent Ab responses (16–20). It is generally accepted that B1 cells are derived from the fetal liver, however, the relationship between B1a and B1b cell development remains unclear (21). Recent studies have demonstrated that B1a cells require an intact spleen for generation and maintenance (22). However, there is also evidence that B1a and B1b subsets may have distinct roles in innate and adaptive T-independent responses, respectively (23, 24). These biological features are suggestive of a common developmental pathway for MZ and B1 B cells; however, recent genetargeting studies of Notch-related family members have not completely supported this hypothesis, because mice conditionally deficient in Notch-2 and its downstream elements lack MZ B cells, but have normal numbers of B1 cells (reviewed in Ref. 25). Furthermore, mice deficient in the migration- and chemokine-related proteins Pyk and Lsc also lack MZ B cells, but possess a normal B1 B cell compartment (26, 27). It is clear that MZ and B1 B cells may share overlapping functional capabilities and contain clones with similar antigenic specificities, but these subsets occupy unique topographical niches (2, 28). This close relationship is further indicated by their partially shared phenotype including the recently defined marker CD9 (29) and now FcR homolog 3 (FcRH3).

Five FcR-like molecules provisionally termed FcRH demonstrate preferential B cell expression in humans and possess immunoregulatory potential by virtue of tyrosine-based inhibitory (ITIM) and/or activation-like (ITAM-like) motifs present in their cytoplasmic tails (30–33). Like their FcR relatives, the FcRH are members of the Ig superfamily. A close ancestral relationship with these “classical FcR” is supported by the
linked and similar genomic organization of their encoding genes on human chromosome 1, tyrosine-based immunoregulatory potential, and the variable usage of five types of Ig domains in all extended FcR family members (34). The expression of FcRH molecules primarily by B cells suggests that these receptors are positioned to operate at the interface of innate and adaptive humoral immunity.

The recent identification of three mouse FcRH orthologs with syntenic chromosomal positions and homologous, but diverse, protein products supports the conservation of the FcRH family in the mammalian immune system and will provide greater insight into the biological potential of these molecules (35). In mice, the FcR/ FcRh locus is split between chromosomes 1 and 3. The low-affinity FcR locus resides on chromosome 1, but FcRH1–3 are positioned near the FcγRI on chromosome 3. Like their human counterparts, mouse FcRH1 and FcRH3 are preferentially expressed by B cells and encode type I glycoproteins with uncharged transmembrane regions, and cytoplasmic tails with ITIM and ITAM-like consensus sequences. Although little expression is found in the bone marrow, FcRH1 is broadly transcribed in peripherally newly formed, MZ, and FO B cells in the spleen, and in B1 and B2 cells in the peritoneal cavity.

Having shown that FcRH3 transcripts are highly enriched in splenic MZ B cells and peritoneal cavity derived B1 cells (35), we developed a panel of specific monoclonal anti-FcRH3 Abs (mAb) and found that its expression is confined primarily to a precursor population of MZ and MZ B cells, and B1 B cells. FcRH3 immunoregulatory potential is suggested by its capacity for tyrosine phosphorylation and its ability to inhibit calcium release from BCR-activated B cells.

Materials and Methods

Mice

129SveEv were purchased from Taconic Farms. C57BL/6j, BALB/cJ, CBA/CaJ (sir control), CBA/CAH (sir mutant), and NZB/BINJ mice were purchased from The Jackson Laboratory. All mice were maintained in our animal facilities at the University of Alabama at Birmingham (UAB; Birmingham, AL). All studies and procedures were approved by the UAB Institutional Animal Care and Use Committee. Vp181-X-C57BL/6 IgVHp1 transgenic mice were bred at UAB and have been previously described (3). Unless otherwise stated, 8- to 12-wk-old female mice were used for staining and RNA isolation.

Immunizations

To examine the expression of FcRH3 on PCs and peanut lectin agglutinin-positive (PNA +) GC B cells, C57BL/6j (or BALB/cJ) mice were immunized by intraperitoneal injection of 80 μg of 10% sheep RBC (SRBC; Colorado Serum) i.v. as previously described (36). Fisher rats (The Jackson Laboratory) were immunized at 3- to 4-day intervals over a 3-week period and popliteal nodes were fused with the mouse plasmacytoma Ag8.653 and plated in 96-well plates (37). At 10–14 days after fusion, hybridoma supernatants were collected for screening by staining of BALB/c splenic B cells and hemagglutinin (HA)-tagged FcRH3 transductants. FcRH3 allotypic Abs were generated by immunizing BALB/c mice with FcRH3-BL/6 transductants using a scheme similar to that described above.

Cells

Cells were grown in RPMI 1640 medium supplemented with 10% FCS, 2 mM l-glutamine, 100 U/ml penicillin/streptomycin, and 50 μM 2-ME (Invitrogen Life Technologies). BOSC23 cells were maintained in DMEM supplemented with 10% FCS, 2 mM l-glutamine, and 100 U/ml penicillin/streptomycin.

Retroviral transductants

HA-tagged cDNA constructs for vector only, FcRH1 (for HA-tagged control), BALB/c-derived FcRH3 (FcRH3-BALB/c), and C57BL/6-derived FcRH3 (FcRH3-BL/6) were generated using standard PCR cloning techniques as described previously (33). Amplified products representative of the extracellular, transmembrane, and cytoplasmic regions including the endogenous splice codons were cloned into the pDisplay (Invitrogen Life Technologies) vector in-frame with the Ig e-chain leader and HA tag sequences. DNA was sequenced and subcloned into the pMX-PLE retroviral expression vector which expresses the gene of interest upstream of an internal ribosomal entry site element and the enhanced GFP (33). After retroviral packaging in BOSC23 cells, the virus-containing supernatant was used to transduce the BW5147 mouse T or WEHI231 B cell lines, which were then puronycine selected and analyzed by FACS (FACScan; BD Biosciences) to detect the HA-tagged receptor-expressing cells.

Immunoprecipitation (IP), Western blot analysis, and tyrosine phosphorylation

Vector-only transductants or those expressing FcRH1, FcRH3-BL/6 or FcRH3-BALB/c, were lysed in 1% Nonidet P-40 lysis buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, and protease inhibitors (Sigma-Aldrich), PMSF (40 μg/ml), leupeptin (5 μg/ml), aprotinin (5 μg/ml), and pepstatin (1 μg/ml), and incubated with the anti-HA-Ab 12CA5 (Roche Diagnostics) or the indicated anti-FcRH3 mAbs before IP with Protein G beads (Pierce) (33). Equal amounts of whole cell lysates were added after Bradford assay quantitation with the BCA reagent (Pierce). Eluted materials were resolved by 7.5% SDS-PAGE under reducing conditions, immunoblotted (Western blot) for the HA tag and HRP-labeled goat anti-mouse Ig polyclonal Abs (DakoCytomation), and visualized by ECL (Amersham Biosciences) after exposure to film. For tyrosine phosphorylation studies, cells were treated with 0.1 mM Na3VO4 (Sigma-Aldrich) for the indicated time points, lysed, immunoprecipitated, and electrophoresized for resolution of tyrosine phosphorylation with the anti-phosphotyrosine Ab 4G10 (Upstate Biotechnology).

Flow cytometry

All Abs were acquired from BD Biosciences unless specified. Spleen and peritoneal cavity cells were isolated and stained with FITC-conjugated anti-mouse CD23, B220, PNA (Jackson ImmunoResearch Laboratories), goat anti-mouse IgG1 (Southern Biotechnology Associates (SBA)), or goat anti-mouse IgA (SBA); PE conjugated anti-mouse CD5, CD1d, IgD, HSA, IgM, syndecan-1, goat anti-mouse Ig (SBA), or goat anti-rat Ig (SBA); Alexa 647-conjugated CD21; PE-Cy7-conjugated B220 or IgM; and biotinylated goat anti-mouse IgM (SBA). Biotinylated or Alexa 647-conjugated CD9 (M3Z clone) (29) and FcRH3 (M2, 9D10, or 3B7 clones) mAbs were generated in our laboratory. Biotinylated mAbs were incubated with streptavidin (SA)-allophycocyanin or SA-Pacific Blue (Molecular Probes) as a secondary reagent. Anti-mouse CD21 (7G6 clone) was conjugated with Alexa 647 in our laboratory and PE was conjugated commercially. Propidium iodide (Sigma-Aldrich) was used to exclude dead cells. For cytoplasmic staining, cells were incubated with 1% paraformaldehyde in PBS overnight and permeabilized with 0.2% Tween 20 in PBS for 40 min (38). Cells were analyzed using FACS Calibur (BD Biosciences) or LSR II (BD Biosciences) flow cytometers and plotted with WinMDI (The Scripps Research Institute) or FlowJo (Tree Star) software.

Cell sorting and in vitro mitogenic stimulation

To examine the induction of FcRH3 protein on the cell surface of primary mouse B cell subsets, total spleen B cells obtained after RBC lysis were AutoMACS enriched by staining with magnetic beads conjugated with anti-CD43 to deplete most myelid, dendritic cells, and T cells in mouse spleen. These cells were stained with FITC-conjugated anti-CD23 and PE-conjugated anti-CD21, and sorted using a MoFlo cell sorter to isolate spleen MZ and FO B cells (Cytomation). The sterile-sorted MZ and FO B cells were cultured for 1–3 days with LPS (Sigma-Aldrich) or anti-CD40 (BD Biosciences). FcRH3 expression was determined by flow cytometry using FcRH3-specific mAbs.

DNA sequencing and allelic characterization

Primers used in end-to-end amplification to generate the full-length FcRH3 cDNAs were as follows: forward 5’-GGTGGAGCTACATCCATTGGGAG

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CAAG-3′ and reverse 5′-CAGGCCCAATAGAAGATCAGG-3′. Each amplification reaction underwent initial denaturation at 94°C for 30 s followed by 30 cycles of denaturation at 94°C for 5 s, annealing and extension at 68°C for 4 min, and final extension at 72°C for 6 min. PCR products were ligated into the pCR2.1 TOPO T/A vector (Invitrogen Life Technologies). Inserts were sequenced on both strands by the dideoxy chain termination method using Sequencher 4.6 and an automated sequencer (LiCor). Nucleotide and amino acid sequence alignment was analyzed with the DNASTAR software package and homology searches were performed using the basic local alignment search tool (39).

Calcium flux experiments

Cells (5 × 10^6) were washed twice in HBSS with 1 μM Ca^{2+} and Mg^{2+} and resuspended in 1 mL of HBSS before addition of Fluo-4 AM and SNAP-1 (Molecular Probes). After incubation for 40 min at 37°C, cells were washed in HBSS twice and resuspended for analysis by a FACSCalibur flow cytometer (BD Biosciences). Calcium mobilization was measured before and after receptor ligation using the Fluo-4 fluorescent calcium indicator dye (33). Biotinylated Abs included goat anti-mouse Ig (SBA), anti-FcRh3 (M22 clone), anti-FcγRIII (2.4G2 clone; BD Biosciences), and rat IgG2b,κ (BD Biosciences) followed by egg avidin (Jackson Immunoresearch Laboratories) and used at a final concentration of 10 μg/mL. To assay Ca^{2+} flux in primary mouse B cells, single-cell-suspended whole spleen cells were labeled with Indo-1/AM in HBSS with 1 μM Ca^{2+} and Mg^{2+} and, then, stained with FITC-CD23 and PE-CD21. Calcium mobilization was then monitored after cross-linking the BCR and FcRh3 on a FACStarPlus cell sorter (BD Biosciences). Ca^{2+} flux was plotted by gating CD23^{low}CD21^{high} cells as MZ B cells.

Immunofluorescence analysis of tissue sections

Tissue sections were processed and viewed as previously described (7). Frozen sections from the spleens of V_{H}81X-C57BL/6 IgV_{H} transgenic mice (3) were stained with Moma-1 (rat, IgG2a, κ; a gift from Dr. G. Kraal, Amsterdam, The Netherlands) developed with goat anti-rat IgG-AMCA (Jackson Immunoresearch Laboratories), blocked with normal rat serum (Pel-Freeze), washed, and then stained with a mixture of goat anti-mouse IgM-PE (SBA) and anti-FcRh3-FITC (M22 clone). Rabbit anti-mouse FITC (Molecular Probes) was used to amplify the M22-FITC signal.

Results

Identification of two FcRh3 alleles in mice

Due to the high frequency of polymorphisms in FcR and other Ig-like family members (40, 41), we determined whether polymorphic alleles exist for FcRh3 among different mouse strains. RT-PCR amplification and sequencing of full-length FcRh3 cDNAs was performed on RNA isolated from the spleens of C57BL/6, BALB/c, 129, NZB, and CBA mouse strains. This analysis indicated that FcRh3-BALB/c, 129, NZB, and CBA strains share a common molecular mass of 50 to 70 kDa whereas the FcRh3-BL/6 strain expresses a band at 68 kDa (Fig. 2). All three strains express a glycosylated band at 100 kDa, whereas 3B7 has specificity for the BL/6-derived FcRh3 allele.

A panel of specific mAbs recognize distinct FcRh3 allotypes

HA-tagged FcRh BW5147 transductants demonstrating moderate levels of surface expression after staining by flow cytometric analysis with anti-HA Abs were used as immunogens and to define specificities of FcRh3 Abs. Two FcR3-specific mAbs, M22 (rat γ2b), and 9D10 (rat γ2c), were found to react by cell staining and IP with both BALB/c and C57BL/6 strain-derived forms of FcRh3, but not FcRh3 transductants (Fig. 2). These studies also revealed that the allelic variations observed in the FcRh3-BALB/c and BL/6 cDNAs translated into differences in the M_{r} of the HA-tagged proteins, ~95 and ~100 kDa, respectively (Fig. 2B). The increased molecular mass of FcRh3-BL/6 is likely the result of additional glycosylation conferred by the extra consensus N-linked glycosylation site, as is also suggested by the broad character of the FcRh3 band.

A third mouse Ab, 3B7 (γ1c), generated in BALB/c mice immunized with the FcRh3-BL/6 transductant, demonstrated reactivity by flow cytometry and IP with FcRh3-BL/6 transductants, but not with the FcRh3 or FcRh3-BALB/c transductants, indicating this mAb recognizes an allelic epitope distinct from that present on FcRh3-BALB/c (Fig. 2B). None of the three mAbs reacted by Western blotting. All three mAbs were found to be specific for unique FcRh3 epitopes by binding inhibition studies (data not shown). These data indicate that M22 and 9D10 recognize FcRh3 epitopes common to the two known mouse alleles, whereas 3B7 has specificity for the BL/6-derived FcRh3 allele.

![FIGURE 1. Allelic differences in FcRh3 among different mouse strains.](http://www.jimmunol.org/)

**FIGURE 1.** Allelic differences in FcRh3 among different mouse strains. (A) Allelic differences in FcRh3 among different mouse strains. (B) Calcium mobilization in FcRh3-BL/6 transductants demonstrating moderate levels of surface expression after staining by flow cytometric analysis with anti-HA Abs were used as immunogens and to define specificities of FcRh3 Abs. Two FcR3-specific mAbs, M22 (rat γ2b) and 9D10 (rat γ2c), were found to react by cell staining and IP with both BALB/c and C57BL/6 strain-derived forms of FcRh3, but not FcRh3 transductants (Fig. 2). These studies also revealed that the allelic variations observed in the FcRh3-BALB/c and BL/6 cDNAs translated into differences in the M_{r} of the HA-tagged proteins, ~95 and ~100 kDa, respectively (Fig. 2B). The increased molecular mass of FcRh3-BL/6 is likely the result of additional glycosylation conferred by the extra consensus N-linked glycosylation site, as is also suggested by the broad character of the FcRh3 band. A third mouse Ab, 3B7 (γ1c), generated in BALB/c mice immunized with the FcRh3-BL/6 transductant, demonstrated reactivity by flow cytometry and IP with FcRh3-BL/6 transductants, but not with the FcRh3 or FcRh3-BALB/c transductants, indicating this mAb recognizes an allelic epitope distinct from that present on FcRh3-BALB/c (Fig. 2B). None of the three mAbs reacted by Western blotting. All three mAbs were found to be specific for unique FcRh3 epitopes by binding inhibition studies (data not shown). These data indicate that M22 and 9D10 recognize FcRh3 epitopes common to the two known mouse alleles, whereas 3B7 has specificity for the BL/6-derived FcRh3 allele.
FcRH3 is a discrete marker of MZ and B1 B cells

To further define the FcRH3 expression pattern, the MZ2 and 9D10 mAbs were used in a flow cytometry assay on a panel of cell lines. Of the 38 cell lines tested, FcRH3 was only detected on WEHI231, a B cell line, NFS203, a MZ lymphoma-derived cell line, and the CH31 B1 B cell line (Table I). In multicolor immunofluorescence analyses, FcRH3 expression was determined on primary cells from bone marrow (BM), peripheral blood, spleen, lymph node, Peyer’s patches, and the peritoneal cavity (PEC) of BALB/c and C57BL/6 mice. FcRH3 was not detected on non-B lineage cells including myeloid cells, T cells, NK cells, and macrophages. In the BM, where FcRH3 was identified on <5% of the cells, it was not appreciably detected among the pro-B, pre-B, transitional, immature, or mature B cell populations.

In accord with the detection of FcRH3 transcripts in MZ B cells (35), and to a lesser extent in newly formed B cells in the spleen, examination of splenic B cell subsets from BALB/c mice revealed a high level of FcRH3 surface expression on CD21<sup>high</sup>CD23<sup>low</sup> MZ B cells, but not on CD21<sup>mid</sup>CD23<sup>high</sup> FO B cells (Fig. 3A). FcRH3 was also readily identified on B220<sup>+</sup>CD5<sup>+</sup> B1 B cells in the spleen (data not shown), but was not identified in abundance on other splenic B cells, including newly formed immature or transitional B cells (data not shown). In mice immunized with SRBC, FcRH3 was not significantly expressed on PNA<sup>+</sup> GC B cells, IgM<sup>high</sup> PCs, or on other class-switched PC (data not shown). These findings demonstrate that FcRH3 is a novel marker of MZ B cells and, like CD9, can distinguish these cells from their FO counterparts (29).

Analysis of peritoneal B1 cells revealed high FcRH3 expression on B220<sup>+</sup>CD5<sup>+</sup> B1a cells and B220<sup>+</sup>CD5<sup>-</sup> B1b cells, but little expression on B220<sup>high</sup>CD5<sup>-</sup> B2 type B cells (Fig. 3B). Similar results were obtained when Mac-1 staining was used in the analysis (data not shown). FcRH3 was not appreciably detected on lymph node or Peyer’s patch-derived B cells.

Immunohistology of spleen sections from unimmunized wild-type (WT) mice showed that FcRH3<sup>+</sup> cells are concentrated in the splenic MZ, where most are IgM<sup>+</sup>, with much less frequent FcRH3<sup>+</sup> cells identified in the red pulp and follicles (Fig. 3C).

**Table I. FcRH3 surface expression on selected cell lines**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Stage and Lineage</th>
<th>FcRH3</th>
</tr>
</thead>
<tbody>
<tr>
<td>BaF3</td>
<td>Pro-B, IL-3 dependent</td>
<td>–</td>
</tr>
<tr>
<td>D19</td>
<td>Pro-B, IL-7 dependent</td>
<td>–</td>
</tr>
<tr>
<td>Scid7</td>
<td>Pro-B, IL-7 dependent</td>
<td>–</td>
</tr>
<tr>
<td>40E1</td>
<td>Pre-B</td>
<td>–</td>
</tr>
<tr>
<td>38C13</td>
<td>Pre-B, IL-7 dependent</td>
<td>–</td>
</tr>
<tr>
<td>I8.81</td>
<td>Pre-B</td>
<td>–</td>
</tr>
<tr>
<td>38B9</td>
<td>Pre-B</td>
<td>–</td>
</tr>
<tr>
<td>70Z/3</td>
<td>Pre-B</td>
<td>–</td>
</tr>
<tr>
<td>I1H6A</td>
<td>Pre-B</td>
<td>–</td>
</tr>
<tr>
<td>BC77</td>
<td>Pre-B-IM B</td>
<td>–</td>
</tr>
<tr>
<td>WEHI231</td>
<td>Immature B</td>
<td>+</td>
</tr>
<tr>
<td>L1-2</td>
<td>Mature B</td>
<td>–</td>
</tr>
<tr>
<td>K46</td>
<td>Mature B</td>
<td>–</td>
</tr>
<tr>
<td>X16C8.5</td>
<td>Mature B</td>
<td>–</td>
</tr>
<tr>
<td>IlA1.6</td>
<td>Mature B</td>
<td>–</td>
</tr>
<tr>
<td>CH12</td>
<td>Mature B1</td>
<td>–</td>
</tr>
<tr>
<td>CH31</td>
<td>Mature B1</td>
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<tr>
<td>NFS203</td>
<td>Mature B (MZ B lymphoma)</td>
<td>+</td>
</tr>
<tr>
<td>A20</td>
<td>Differentiated B</td>
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</tr>
<tr>
<td>Ag8.563</td>
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</tr>
<tr>
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<tr>
<td>S1A wild</td>
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<tr>
<td>BW5147.3</td>
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</tr>
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<td>Yac-1</td>
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<tr>
<td>WEHI3</td>
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<tr>
<td>HTX-1</td>
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</tr>
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<td>J774A.1</td>
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</tr>
<tr>
<td>EOC13</td>
<td>Macrophage-like</td>
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<td>P815</td>
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</tr>
<tr>
<td>LTK</td>
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<tr>
<td>T220</td>
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<tr>
<td>NIH3T3</td>
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</tr>
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<tr>
<td>BMST2</td>
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<td>–</td>
</tr>
<tr>
<td>OP42</td>
<td>Stromal</td>
<td>–</td>
</tr>
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<sup>a</sup> – Absence of FcRH3 surface staining; +, presence of surface FcRH3.

**FIGURE 3.** FcRH3 is preferentially expressed by splenic MZ and PEC B1 B cells. A, Eight-week-old old female BALB/cJ splenocytes were stained with anti-CD21-PE, CD23-FITC, FcRH3-biotin (MZ2 clone), or CD9-biotin (MZ3 clone), followed by SA-allophycocyanin, and analyzed with a FACS-Calibur instrument. B, Peritoneal cavity cells prepared from 8-wk-old female BALB/cJ mice were stained with anti-CD5-PE and B220-FITC, and FcRH3 and CD9 mAbs as above. C, Immunohistology of the spleen from an unimmunized VH81X-IgH C57BL/6 mouse stained with IgM (red), MOMA-1 (blue), and FcRH3 (MZ2 clone) (green). Note IgM<sup>+</sup>FcRH3<sup>+</sup> cells in the MZ stain yellow.
These findings correlate well with the FACS analysis of isolated FcRH3+ cells and indicate that FcRH3 is selectively expressed on MZ B cells in the spleen.

Collectively these results indicate that FcRH3 is a marker of splenic MZ and B1 B cells and in the peritoneal cavity marks both B1a and B1b cell subpopulations.

**FcRH3 is expressed on an early MZ precursor**

Aiolos-deficient mice (42) and Notch-2 conditional knockout animals (43) lack a population of precursor MZ B cells within the T2 compartment (44). Additionally, the characterization of a proposed MZ precursor population (45), prompted an evaluation of FcRH3 expression on these cells for possible insight into its developmental expression pattern. By multicolor flow cytometric analysis, four subsets of B cells can be defined as T1 (IgM^high^IgD^low^CD21^low^CD23^low^CD1d^low^), follicular-FO (IgM^low^IgD^high^CD21^high^CD23^low^CD1d^low^), pre-MZ (IgM^high^IgD^high^CD21^high^CD23^high^CD1d^high^), and MZ B cells (IgM^high^ IgD^low^CD21^high^CD23^high^CD1d^high^) (Fig. 4A). Counterstaining with the FcRH3 (9D10) mAb revealed intermediate levels of expression on pre-MZ and MZ B cells, whereas very low levels were observed for the majority of cells in the T1 and FO^+^T2/3 subsets (Fig. 4B). These data indicate that pre-MZ B cells express FcRH3.

FcRH3 expression was also examined in CD19^−/−^ mice, because they lack appreciable numbers of MZ B cells (4, 24, 46, 47), and in Btk-nonfunctional xid mice, which have a MZ B cell repertoire deficit (4). In contrast to WT littermates, CD19^−/−^ mice (BL/6 background) lacked CD21^high^CD23^low^ MZ B cells, but had normal numbers of CD21^high^CD23^−^ splenocytes enriched with the MZ precursor phenotype (45) (Fig. 5A). Despite the paucity of MZ B cells in CD19^−/−^ mice, FcRH3 was clearly identified on the CD21^high^CD23^−^ population, whereas a minor population of FO B cells was found to express FcRH3 at greater frequency than in WT C57BL/6 mice. Interestingly, the presence of FcRH3^+^ FO B cells appeared to be strain specific, because few were identified in BALB/c mice. Given its distinct expression on the MZ B cell precursor population, this data indicates that FcRH3 may serve as a marker of MZ B cell commitment.

Btk-nonfunctional xid CBA/CaJ and CBA/CaHN mice generate near normal numbers of MZ B cells (4), but respond poorly to T-independent Ags (48). FcRH3 expression was found to be dramatically downregulated on the CD21^high^CD23^low^ and CD21^high^CD23^−^ cells

**FIGURE 4.** FcRH3 is expressed by precursor MZ B cells. A, Ten-week-old female C57BL/6 splenocytes were stained with anti-B220-PE Cy7, CD23-FITC, goat anti-mouse IgM-biotin plus SA-Pacific blue, CD21-Alexa 647, or PE to define four populations: T1, FO^+^T2/3, Pre MZ, and MZ B cells. B, Relative expression levels of distinguishing markers on the four subsets counterstained with anti-IgD-PE, CD1d-PE, HSA-PE, or FcRH3 (9D10 clone)-Alexa 647. These results are representative of three experiments from four mice analyzed with a LSR2 (BD Biosciences) instrument. Similar results were obtained with the BALB/c strain.

**FIGURE 5.** FcRH3 defines precursor and MZ B cell subsets in the spleen and B1 cells in different mouse strains. A, Spleen cells from BALB/c, C57BL/6, CD19^−/−^, CBA/CaJ, and CBA/CaHN mice were stained using anti-CD21-PE, anti-CD23-FITC, and anti-FcRH3 (9D10 clone)-Alexa 647. Contour plots display live lymphocytes and the three gates distinguish MZ, precursor MZ (MZP), and FO B cell populations that are displayed by histograms for each respective strain. *, Gating of the CD21^high^CD23^−^ population includes Carsetti T2 (44) and MZP cells. B, PEC-derived cells isolated by lavage were stained with anti-CD5-PE, anti-B220-FITC, and anti-FcRH3 (9D10 clone)-Alexa 647 (+, ND, not detectable). The numbers shown in each histogram indicate the average number of FcRH3^+^ cells from at least three mice.
FcRH3 expression is dependent on normal BCR signaling via btk.

**FctRH3 expression on PEC B cell subsets from normal and mutant mice**

Although CD9 differentially segregates PEC B1 cells in WT BALB/c and C57BL/6 mice in a strain-dependent fashion (29), CD5 B220 B1a and CD5 B220 B1b cells did not demonstrate significant differences in FcRH3 staining in these strains and a minority of CD5 B220 B1b cells were FcRH3 reactive (−25%) (Fig. 5B). In CBA/CaHN (xid) mice that have a striking decrease in B2 cells in BALB/c, C57BL/6J, and CBA/CaJ mice (data not shown). In CD19-deficient mice, which lack CD5 B220 B1a and CD5 B220 B1b cells did not demonstrate significant differences in FcRH3 staining in these strains and a minority of CD5 B220 B1b cells were FcRH3 reactive (−25%) (Fig. 5B). In CBA/CaHN (xid) mice that have a striking decrease in the number of B1b cells and no apparent B1a cells (49), we were unable to analyze significant numbers of B1 cells to make a definitive conclusion about FcRH3 expression. However, similar to splenic B cells in xid mice (Fig. 5A), a great reduction in FcRH3 surface staining was seen on B2 cells compared with B2a/CaJ control mice (Fig. 5B). This indicates that btk is also required for FcRH3 expression on B2 cells. Interestingly, FcRH3 B2 cells are intermediate in size compared with larger B1 and smaller FcRH3− B2 cells in BALB/cJ, C57BL/6J, and CBA/CaJ mice (data not shown). CD19-deficient mice, which lack CD5 B1a cells and have severely reduced numbers of CD5 B1b cells (24, 50, 51), FcRH3 was difficult to identify (data not shown). Thus, FcRH3 appears to be a marker of peritoneal B1 cells, but varies among strains with respect to its expression on a subset of B2 cells.

**FcRH3 expression on activated B cells**

Sorted MZ and FO B cells were cultured in the presence of LPS or anti-CD40 and FcRH3 surface expression was determined by flow cytometry at baseline, and after 1 or 3 days. FcRH3 was modestly up-regulated on MZ B cells following 24 h of LPS stimulation, but after 3 days, when the majority of MZ B cells are differentiating into plasmablasts (52), its expression declined (Fig. 6). In contrast, little FcRH3 expression was detected on resting FO B cells, but after 1 day of culture it was up-regulated on a minor population of cells and the level remained constant for 3 days. In similar assays, CD9 was previously found to be induced on MZ B cells following LPS stimulation, but compared with FcRH3 modulation, CD9 expression is maintained on activated MZ B cells as they differentiate and become PCs (29). CD9 was also identified on a subset of FO B cells upon LPS stimulation. Compared with the modulation of FcRH3 expression by LPS, anti-CD40 had little effect on FcRH3 expression in sorted MZ B cells, however, a small proportion of FcRH3− cells appeared among the bulk of FO B cells with prolonged culture. This contrasts with the lack of FcRH3 expression on SRBC-induced PNA+ GC B cells (data not shown) and Id-positive sydencan1+ IgM+ PCs derived from anti-phosphorylcholine Ig transgenic mice (M167) immunized with *Streptococcus pneumoniae* (R36a) (53) (data not shown). These data indicate that FcRH3 is down-modulated as MZ B cells differentiate into PCs, but is up-regulated on a small population of FO B cells after LPS or anti-CD40 stimulation in vitro.

FcRH3 is tyrosine phosphorylated and can inhibit BCR-mediated calcium flux in activated B cells

One of the characteristic features of FcRH family members is the presence of ITAM-like and/or consensus ITIM sequences in their cytoplasmic regions implying immunoregulatory potential (31). To determine whether FcRH3 is tyrosine phosphorylated, vector-only control or HA-tagged FcRH3-BL/6 BW5147 transductants were treated with the potent phosphatase inhibitor, pervanadate, immunoprecipitated with anti-HA, and immunoblotted with either anti-phosphotyrosine or positive control 12CA5 anti-HA mAb (33) (Fig. 7A). The results demonstrate that in its equilibrium state in these cells, FcRH3 is not tyrosine phosphorylated, but after phosphatase inhibition the balance is shifted and phosphorylation can be detected at 1 min, and is significantly amplified after 10 min of treatment. FcRH3 was also found to be tyrosine phosphorylated in the WEHI231 B cell line (data not shown). By virtue of its tyrosine phosphorylation, these results suggest that FcRH3 has immunoregulatory potential.

To determine whether FcRH3 ligation can influence B cell activation, WEHI231 B cells transduced with FcRH3-BL/6 were ligated with biotinylated anti-FcRH3 mAbs (MZ2, 9D10, or 3B7) and then cross-linked with egg avidin. Ligation of FcRH3 alone did not significantly affect calcium mobilization (data not shown). To determine whether FcRH3 could modulate BCR-mediated calcium flux, the transductants were incubated with biotinylated anti-FcRH3 mAbs together with biotinylated goat anti-mouse Ig polyclonal Abs and then cross-linked with egg avidin. Although BCR ligation alone resulted in a robust calcium flux that was not diminished by BCR ligation in the presence of an isotype-matched control mAb, coligation of FcRH3 with the BCR resulted in a marked dampening of BCR-induced intracellular calcium mobilization, similar to that seen for another inhibitory receptor on these cells, FcγRIIB (Fig. 7B). We then assayed whether FcRH3 had similar effects on calcium mobilization in primary splenic MZ B cells. Consistent with the results observed in the WEHI231 cell line, cross-linking the BCR and FcRH3 diminished calcium mobilization in MZ B cells (Fig. 7C). This experiment demonstrates a potential inhibitory influence of the FcRH3 receptor on B cells.

**Discussion**

The analysis of full-length cDNAs from five different mouse strains defined two FcRH3 alleles. Of particular interest is the C57BL/6-encoded alloform that possesses 10 nonsynonymous SNPs in its extracellular region. One of these polymorphisms, T376N, confers an additional N-linked glycosylation consensus sequence not present in the four other strains analyzed. The additional glycosylation implied by this substitution is evident in the migration of this BL/6 alloform at a higher molecular mass relative to its BALB/c counterpart. The significance of these extracellular differences is underscored by the unique reactivity of the 3B7 mouse anti-mouse mAb that binds to an epitope on the C57BL/6 alloform that is absent from FcRH3-BALB/c. It is unclear at this point whether the amino acid and glycosylation differences have any functional consequences, although it is noteworthy that the cytoplasmic domains encoded by the two alleles do not differ in amino acid composition. Thus, allelic differences may alter ligand binding but are unlikely to affect downstream signaling events.
FcRH3 is not a classic Ig receptor. The presence of two alleles in heat-aggregated mouse Ig, but none was detected regardless of deficien the gene. Activating and inhibitory sig
tivities in FcRH3 expression are observed in CD9 deficient animals, where there are few if any MZ CD21^high^CD23^low^ B cells (4). Fourth, in 11b mice FcRH3 is significantly down-regulated despite ample numbers of CD21^high^CD23^+^ and CD21^high^CD23^low^ cells. This suggests that FcRH3 expression is dependent on an intact BCR-signaling pathway through btk for its normal expression on splenic B cells.

These findings indicate that FcRH3 is a novel marker of MZ and B1 B lineage cells that possesses immunoregulatory potential. Until the characterization of CD9 as a surface molecule that distinguishes MZ from FO B cells, the identification and separation of these subsets was complicated by the battery of markers needed to differentiate them. Despite its ubiquitous expression on other hemopoietic and nonhemopoietic cells, CD9 has substantially facilitated delineation of these B cell subsets. Now FcRH3 simplifies this discrimination even further because of its B cell-restricted expression. Although FcRH3 and CD9 expression patterns are similar, at this time the function of both molecules on MZ B cells remains unclear. Recent analysis of CD9-deficient mice indicates normal numbers of MZ and B1 B cells, suggesting a nonessential role for CD9 in generation or maintenance of these cell types (our unpublished observations and Ref. 36). Furthermore, no differences in FcRH3 expression are observed in CD9^−/−^ mice (our unpublished observations).

The functional role of FcRH3 may become clear when mice deficient in this gene are generated. Activating and inhibitory signaling properties have been demonstrated for two human FcRH family members, FcRH1 (32) and FcRH4 (33), that bear ITAM-like or ITIM consensus sequences, respectively. Furthermore,
SPAP2, a FcRH3 counterpart in humans which possesses a consens
sensus ITAM and ITIM, has been shown to recruit the protein
kinases Syk and Zap70 and protein phosphatases SHP-1 and
SHP-2 following pervanadate stimulation, respectively (57). Like
SPAP2, mouse FcRH3 possesses ITIM and an ITAM-like se
quences implying that it could have dual functional potential.
Here, we show that FcRH3 can be tyrosine phosphorylated and
inhibit calcium mobilization in activated B cells. Although it
is currently unclear whether both of these tyrosine-based sequences are func
tional, the biological potential of these motifs is intriguing.
The observation that signaling elements differ between B1 cells
present in the spleen vs peritoneal cavity suggests that FcRH3
function may differ by cell type, location, and the nature of Ag
receptor engagement realized by FcRH3-expressing cells (58, 59).
Although no effect on calcium flux was observed when FcRH3 was
ligated alone, concomitant ligation with the BCR in the WEHI231
B cell line and primary MZ B cells resulted in decreased calcium
flux similar to that of BCR-FcγRIIB ligation. Its potential for ty
rosine phosphorylation and inhibitory function on calcium mobiliza
tion implies that the FcRH3 ITIM could be constitutively ac
tive in MZ B cells. It is thus possible that the expression of FcRH3
on these preactivated cells could repress cellular activation until
the appropriate stimulus overcomes the cell’s inhibitory threshold.
Although other scenarios could also be considered, the distinct
signaling features of this receptor should prove very interesting in
future studies.

It is intriguing that CD9 and FcRH3 share the distinction of
marking MZ and B1 B cells. Recent studies have indicated that
MZ B cell development is linked to functional BCR and non-BCR-
signaling pathways and that recruitment into this developmental
pathway may begin at the immature B cell stage (45, 60, 61). In
this study, FcRH3 expression was observed on <5% of transitional B
cells using the AA4.1 marker for analysis. Similarly, in freshly
isolated FO B cells from BALB/c mice, neither FcRH3 transcripts
(35) nor surface expression are apparent. However, in C57BL/6
and CBA/CaJ mice, we consistently observe a small subset (∼12–
14%) of FcRH3+ cells present among the CD21lowCD23high popu
lation that is almost twice as abundant in CD19−/− mice, but did
not differ in forward scatter from the rest of the FO B cell popu
lation. Moreover, FcRH3+ cells numbered ∼25% of the B2 cells
from the peritoneal cavity, but were expanded 2-fold in CBA/CaJ
mice. These B2 cells do not stain positively for Mac-1 (data not
shown), but have increased forward scatter relative to the rest of
the B2 population. This result suggests that FcRH3+ B2 cells could
be a more activated subpopulation or might possibly repre
sent a recirculating subset of FcRH3+ splenic FO B cells. Addi
tionally, FcRH3, as well as CD9 (29), expression becomes evident
on a subset of sorted FO B cells after culture in the presence of
LPS. In the context of MZ B cell development, it appears that
FcRH3 expression increases as differentiating cells attain a more
activated status. What distinguishes the FcRH3+ FO B cell popu
lation from other FO B cells remains unclear, perhaps these cells
represent a subset destined for MZ development. The differentia
tion of MZ B cells from the FO compartment has been recently
suggested in a model from the Allman group (45). Perhaps FcRH3
and CD9 identify a similar subset of FO B cells with MZ features
or otherwise mark cells that are committed for participation in
T-independent responses. However, in contrast to CD9, which per
sists on PCs, FcRH3 disappears from the cell surface as MZ B cells
differentiate into plasmablasts. Thus, FcRH3 appears to be a com
mitment marker for MZ B cell development and could be an
indicator of positive selection for this cell population. Future studies
of FcRH3 expression and function maybe helpful for better un
derstanding what drives MZ B cell and B1 B cell development.

Note added in proof. FcRH3 has now been designated FCRL5 in
recently established nomenclature (62).

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