

Identity of the elusive IgM Fc receptor (Fc μ R) in humans

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Although Fc receptors (FcRs) for switched immunoglobulin (Ig) isotypes have been extensively characterized, FcR for IgM (Fc μ R) has defied identification. By retroviral expression and functional cloning, we have identified a complementary DNA (cDNA) encoding a bona fide Fc μ R in human B-lineage cDNA libraries. Fc μ R is defined as a transmembrane sialo-glycoprotein of \sim 60 kD, which contains an extracellular Ig-like domain homologous to two other IgM-binding receptors (polymeric Ig receptor and Fc α / μ R) but exhibits an exclusive Fc μ -binding specificity. The cytoplasmic tail of Fc μ R contains conserved Ser and Tyr residues, but none of the Tyr residues match the immunoreceptor tyrosine-based activation, inhibitory, or switch motifs. Unlike other FcRs, the major cell types expressing Fc μ R are adaptive immune cells, including B and T lymphocytes. After antigen-receptor ligation or phorbol myristate acetate stimulation, Fc μ R expression was up-regulated on B cells but was down-modulated on T cells, suggesting differential regulation of Fc μ R expression during B and T cell activation. Although this receptor was initially designated as Fas apoptotic inhibitory molecule 3, or TOSO, our results indicate that Fc μ R per se has no inhibitory activity in Fas-mediated apoptosis and that such inhibition is only achieved when anti-Fas antibody of an IgM but not IgG isotype is used for inducing apoptosis.

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Abbreviations used: 7-AAD, 7-aminoactinomycin D; APC, allophycocyanin; CLL, chronic lymphocytic leukemia; FAIM3, Fas apoptotic inhibitory molecule 3; FcR, Fc receptor; GPI, glycosylphosphatidylinositol; HRP, horseradish peroxidase; MFI, mean fluorescence intensity; MNC, mononuclear cell; pI, isoelectric point; pIgR, polymeric Ig receptor; PKC, protein kinase C; PLC, phospholipase C; SA, streptavidin.

IgM is the first Ig isotype to appear during phylogeny, ontogeny, and the immune response, and has been suggested as a first line of host defense to pathogens. Serum levels of IgM in mice raised under germ-free conditions are similar to those of mice maintained under conventional housing conditions (Haury et al., 1997). Thus, production of preimmune “natural” IgM antibody, primarily by CD5 $^{+}$ B-1 cells, is likely to be regulated by mechanisms unrelated to exogenous antigen specificity. In contrast, antigen-induced IgM production is mainly derived from conventional B-2 cells. The importance of both natural and induced IgM antibodies in immune responses has been established through recent studies of a mutant mouse strain in which B cells expressing surface IgM and IgD could switch and secrete IgG and IgA, but not IgM, antibodies (Boes et al., 1998a; Ehrenstein et al.,

1998). These mutant mice had impaired control of viral and bacterial infections because of inefficient induction of protective IgG antibody responses (Boes et al., 1998b; Ochsenbein et al., 1999; Baumgarth et al., 2000). Thus, these findings indicate that IgM antibody can profoundly influence immune responses and suggest that some of these effects are mediated by binding to effector molecules such as Fc receptor (FcR) and complement via its carboxyl-terminal regions.

Several FcRs, namely FcR for IgG (Fc γ RI/CD64, Fc γ RII/CD32, and Fc γ RIII/CD16), IgE (Fc ϵ RI), and IgA (Fc α R/CD89), have been characterized at both the protein and nucleic acid levels (Ravetch and Nimmerjahn, 2008).

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In contrast, FcR for IgM (Fc μ R) has defied genetic identification, although the existence of Fc μ R on B, T, NK, and phagocytic cells has been suggested for >30 yr with variable and conflicting results (Basten et al., 1972; Moretta et al., 1975; Lamon et al., 1976; Ferrarini et al., 1977; Moretta et al., 1977; Pichler and Knapp, 1977; Santana, 1977; Haegert, 1979; Reinherz et al., 1980; Uher et al., 1981; Sanders et al., 1987; Mathur et al., 1988a; Mathur et al., 1988b; Ohno et al., 1990; Nakamura et al., 1993; Pricop et al., 1993; Rabinowich et al., 1996). In addition to the aforementioned classical FcRs, several other receptors expressed on unique cell types also bind Ig molecules: (a) neonatal FcR for IgG (FcRn) on intestinal epithelium, placenta, and endothelium (Roopenian and Akilesh, 2007); (b) low affinity Fc ϵ R (Fc ϵ RII/CD23) on B cells and macrophages (Conrad, 1990); (c) polymeric Ig receptor (pIgR) on mucosal epithelium (Kaetzel, 2005); and (d) FcR for IgA and IgM (Fc α / μ R; Shibuya et al., 2000) on follicular dendritic cells (Kikuno et al., 2007). Although the latter two receptors bind polymeric IgA and IgM, their biochemical features and cellular distribution are distinct from those of the Fc μ R that we have previously characterized on B and T cells in humans (Sanders et al., 1987; Ohno et al., 1990; Nakamura et al., 1993). In this paper, we have identified a cDNA encoding a bona fide Fc μ R that is defined as transmembrane protein of ~60 kD expressed predominantly on B and T lymphocytes.

RESULTS

Molecular cloning of the Fc μ R

Our previous cellular and biochemical studies provided strong evidence for the existence of an Fc μ R that is expressed constitutively on chronic lymphocytic leukemia (CLL) B cells and inducibly on pre-B cell lines (Sanders et al., 1987; Ohno et al., 1990). To identify the gene encoding the putative Fc μ R, two different cDNA libraries from CLL B cells and a PMA-activated 697 pre-B cell line were constructed in a retroviral expression vector and then introduced into mouse T cell line BW5147. Transduced cells exhibiting IgM binding were enriched by FACS and subcloned. Many of the single cell-derived subclones from both cDNA libraries bound IgM (Fig. 1 A). RT-PCR analysis revealed that a DNA fragment of ~2 kb was specifically amplified only from IgM-binding subclones (Fig. 1 B), and their nucleotide sequence analyses defined an identical 1,173-bp open reading frame (CLL- and PMA-activated 697 pre-B cell-derived Fc μ R cDNA available from GenBank/EMBL/DDBJ under accession nos. GQ160900 and GQ160901, respectively; Fig. S1). Basic local alignment search technique database analysis revealed that the isolated Fc μ R cDNA was identical to that of the previously described human Fas apoptotic inhibitory molecule 3 (FAIM3; available from GenBank/EMBL/DDBJ under accession no. NM_005449), except for one nucleotide difference at a position reported as a synonymous single nucleotide polymorphism. FAIM3 was identified in a similar retroviral cDNA library-based functional assay as a potent inhibitor of Fas/CD95-induced apoptotic signaling and was

originally designated as TOSO, after a Japanese liquor drunk on New Year's day to celebrate long life and eternal youth (Hitoshi et al., 1998). Interestingly, however, the apoptosis in this functional assay was induced by ligation of Fas with a mouse mAb of IgM isotype (CH11). This immediately raised the possibility that the CH11 mAb bound to the Fas via its Fab μ portion and to the FAIM3/TOSO via its Fc μ portion, thereby bringing them in close physical proximity in a process reminiscent of that described in Fc γ RIIb-mediated inhibition of BCR signaling by intact IgG anti- μ antibodies (Tony and Schimpl, 1980; Ravetch and Nimmerjahn, 2008).

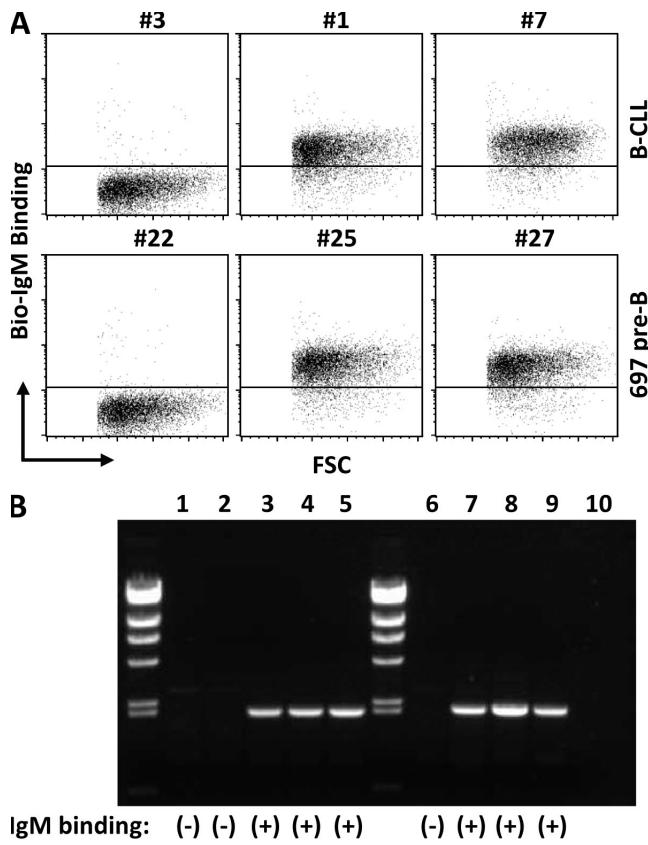


Figure 1. Isolation of IgM-binding subclones and identification of cDNA inserts. (A) Cells transduced by the retroviral expression construct containing CLL-derived (top) or PMA-activated 697 pre-B cell-derived (bottom) cDNA libraries were enriched for IgM binding by FACS and subcloned for limiting dilution. Three representative subclones from each library are shown for their IgM-binding activity or lack of binding, as determined by flow cytometry. (B) Agarose gel electrophoresis analysis of RT-PCR products. RNA isolated from nontransduced control BW5147 T cells (lane 1) and from IgM-binding (lanes 3–5 and 7–9) or IgM-nonbinding (lanes 2 and 6) subclones from CLL-derived (lanes 2–5) and PMA-activated 697 pre-B cell-derived (lanes 6–9) cDNA libraries were subjected to RT-PCR as described in Materials and methods. Amplified products were electrophoresed in 0.7% agarose and stained with ethidium bromide. Lane 10 is a PCR control without a first-strand cDNA template. *Hind*III-digested λ DNA was used as a size marker. The experiments were performed once for A and twice for B.

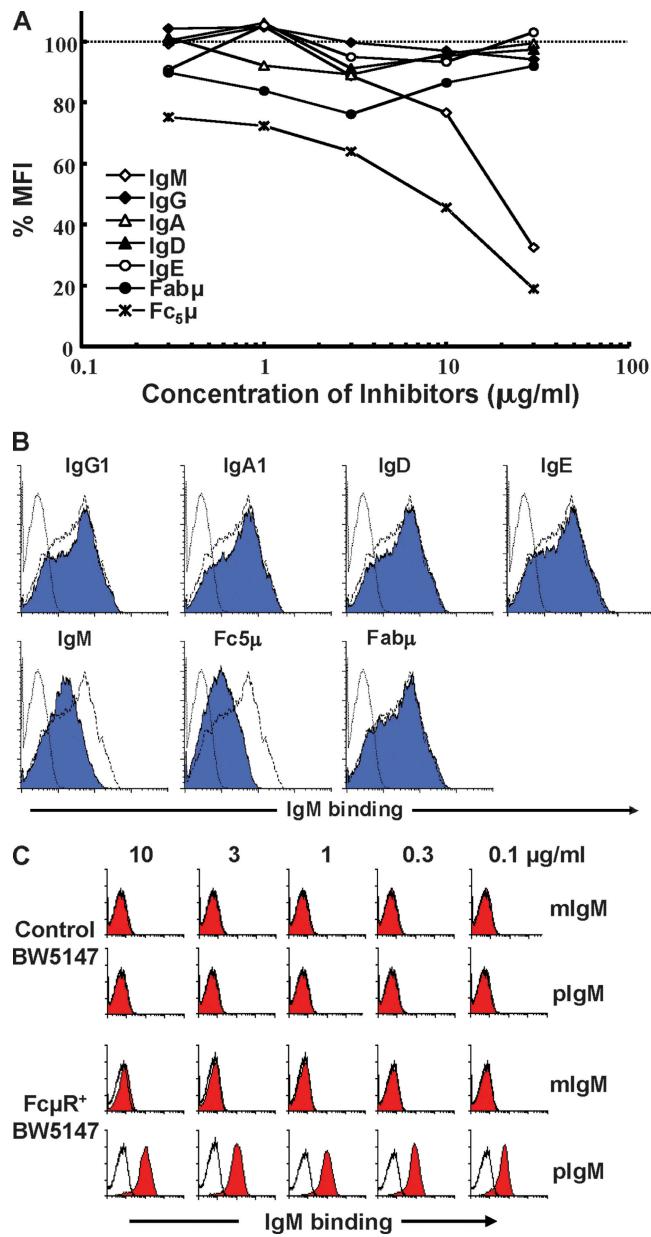


Figure 2. Evaluation of the Ig isotype specificity of the Fc μ R. (A) Fc μ R cDNA-transduced BW5147 T cells were preincubated with various concentrations of inhibitor paraproteins of human origin (IgM, IgG₁₋₄, IgA₁₋₂, IgD, IgE, Fab μ , and Fc₅μ) and incubated with 4 μ g/ml of biotin-labeled human IgM κ . Bound biotinylated IgM was detected by addition of PE-labeled SA. Stained cells were analyzed by flow cytometry. Results are expressed as the percent mean fluorescence intensity (MFI) estimated as follows: 100 \times [(X of IgM binding with inhibitors – X of background control)]/[X of IgM binding without inhibitors – X of background control]], where X indicates the MFI values. Because there were no significant differences among each subclass of IgG and IgA, the results from all four IgG subclasses and two IgA subclasses have been combined as IgG and IgA, and the mean values are presented for simplicity. (B) Representative binding inhibition profiles. Fc μ R⁺ BW5147 T cells were incubated first with an eightfold excess of the indicated inhibitor proteins and then with 4 μ g/ml of biotin-labeled human IgM κ . The dotted, dashed, and continuous lines indicate the immunofluorescence profiles for background con-

The FAIM3/TOSO gene encodes a bona fide Fc μ R

To reconcile the conflicting functions of FAIM3/TOSO and our functionally defined Fc μ R, an ~1.2-kb cDNA containing the protein coding region of FAIM3/TOSO/Fc μ R was PCR amplified from PMA-activated 697 pre-B cells, subcloned along with a GFP cDNA into the bicistronic retroviral vector and then transduced into BW5147 T cells. The resultant GFP⁺ transductants clearly exhibited IgM binding (Fig. S2), thereby confirming that FAIM3/TOSO is an IgM receptor. The Fc μ R cDNA is predicted to encode a 390-aa type I membrane protein (17-aa signal peptide, 236-aa extracellular region, 19-aa transmembrane segment, and 118-aa cytoplasmic tail). The N-terminal half of the extracellular region contains a single V-set Ig-like domain with homology to both the pIgR and Fc α / μ R (see next section), but the remaining extracellular region has no identifiable domain features (Fig. S3). There are no N-linked glycosylation motifs in the extracellular region, consistent with our previous biochemical characterization of the Fc μ R (Ohno et al., 1990). The mature core peptide is predicted to have an M_r of ~41 kD and an isoelectric point (pI) of ~9.9.

A quantitative inhibition immunofluorescence assay with various Ig isotypes and IgM fragments as inhibitors revealed that IgM and its Fc₅μ fragments consisting mostly of C μ 3/C μ 4 domains inhibited the binding of a biotin-labeled human IgM to Fc μ R⁺ BW5147 T cells in a dose-dependent manner, whereas the Fab μ fragments and other human Ig isotypes (IgG₁₋₄, IgA₁₋₂, IgD, and IgE) did not, thereby confirming the Fc μ specificity of the Fc μ R (Fig. 2 A). Fig. 2 B shows a representative inhibition profile for IgM binding with an eightfold excess of inhibitors. The inability of Fc μ R to bind polymeric IgA clearly indicates that Fc μ R is distinct from pIgR and Fc α / μ R, both of which are shown to bind IgM and polymeric IgA. Moreover, the lack of binding to aggregated IgG further confirms the unique IgM isotype specificity of this receptor. Interestingly, mouse IgM bound better to the human Fc μ R than human IgM, and essentially identical Fc μ R binding was observed with IgM κ and IgM λ ligands irrespective of the presence of Ca²⁺/Mg²⁺. The affinity of IgM/Fc μ R binding was estimated by Scatchard plot analysis using ¹²⁵I-labeled human IgM and Fc μ R⁺ BW5147 T cells. Assuming a 1:1 stoichiometry of pentameric IgM ligand to Fc μ R, this analysis revealed a strikingly high binding affinity of 10.8 \pm 9.2 nM (mean \pm SD from four experiments with two different human IgM myeloma proteins). Pretreatment of Fc μ R⁺ cells with neuraminidase slightly enhanced IgM binding, suggesting a role of sialic acid in this interaction, as reported previously by others

trois, IgM binding without inhibitors, and IgM binding with the test inhibitors, respectively. (C) Control and Fc μ R⁺ BW5147 cells were incubated with culture supernatants containing the indicated concentrations of monomeric (m) or pentameric (p) IgM anti-mouse RBC mAb before developing with biotin-labeled anti-mouse κ mAb and APC-SA. These experiments were performed at least twice.

(Pricop et al., 1993). Higher concentrations (>100-fold) were required for binding of IgM monomers to the Fc μ R $^+$ cells than IgM pentamers, indicating the importance of IgM ligand configuration (Fig. 2 C). Collectively, these results indicate that the previously identified FAIM3/TOSO is an authentic Fc μ R with exclusive and high affinity binding specificity for the Fc portion of IgM.

The Ig domain of Fc μ R is similar but distantly related to that of pIgR and Fc α /μR

FCMR is a single copy gene located on chromosome 1q32.2, adjacent to two other IgM-binding receptor genes, *PIGR* and *FCAMR*. The Ig-like domain of FAIM3/TOSO/Fc μ R is thought to be involved in the binding of agonistic IgM anti-Fas mAb (Hitoshi et al., 1998). A comparison of the protein sequence of the Ig-binding domains of Fc μ R, pIgR, and Fc α /μR to the pIgR structural data reported by Hamburger et al. (2004) provided some potential insight into ligand specificity (Fig. 3). In addition to a disulfide bond between Cys22 and Cys92 linking the two β sheets (B and F strands), a second disulfide bond between Cys38 and Cys46 linking the C and C' strands is also conserved in all three receptors.

Arg63 and Asp86 are also completely conserved, but Trp37 is found only in the pIgR and Fc α /μR. Several other residues (Gly6, Tyr24, Val29, Arg31, Lys35, Tyr55, and Leu101) are also conserved in pIgR and Fc α /μR but not in Fc μ R. A major difference between Fc μ R and the other two receptors is in the CDR1 region. The CDR1 of the pIgR from six different species consists of 9 aa (Pro25 to Thr33), and this is also the case in the Fc α /μR from two different species. In contrast, the corresponding region of the Fc μ R from seven different species consists of 5 aa and has a non-charged residue (Met, Leu, or Thr) at the position corresponding to Arg31, which has been shown to be solvent exposed and possibly to interact directly with polymeric IgA in the human pIgR (Hamburger et al., 2004). These results suggest a structural basis for the distinct mode of IgM interactions with Fc μ R versus pIgR and Fc α /μR.

Conserved Ser and Tyr residues in the cytoplasmic tail of Fc μ R

A charged His residue is adjacent to or within the putative 19-aa transmembrane segment of Fc μ R from all species examined except for the bovine (Fig. 4). The 118-aa cytoplasmic

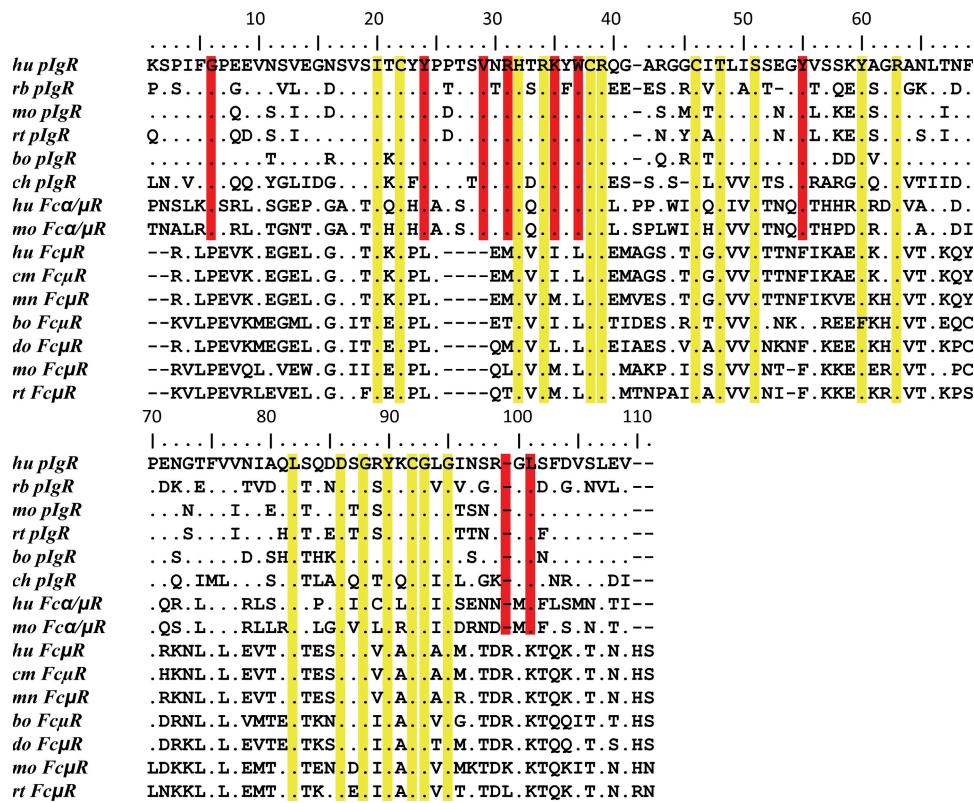


Figure 3. aa sequence alignment of IgM-binding receptors. The Ig-binding domains of pIgR, Fc α /μR, and Fc μ R from several species were aligned using the CLUSTAL W multiple alignment program (Thompson et al., 1994). aa identity is indicated by dots and gaps are indicated by dashes. Residues conserved in all three receptors and in pIgR and Fc α /μR are highlighted in yellow and red, respectively. The numbers indicate the aa position from the N terminus of the Ig-binding domain of human pIgR. These sequences are available from GenBank/EMBL/DBJ under the following accession nos.: pIgR of human (hu; P01833), rabbit (rb; P01832), mouse (mo; 070570), rat (rt; P15083), bovine (bo; P81265), and chicken (ch; AAP69798); Fc α /μR of human (AAL51154) and mouse (NP_659209); and Fc μ R of human (NP_005440), chimpanzee (cm; XP_001165341), monkey (mn; XP_001084243), bovine (XP_588921), dog (do; XP_547385), mouse (NP_081252), and rat (Q5M871).

tail is composed of a basic aa-rich region, a Pro-rich region, two conserved Cys residues, and an acidic aa-rich region in all seven different Fc μ Rs. Of the Ser residues, five are completely conserved and an additional four are highly conserved among these Fc μ Rs, and some of them are potential sites for protein kinase C (PKC) phosphorylation (R/K₁₋₃-X₀₋₂-S/T-X₀₋₂-R/K₁₋₃ or R/K-X-S-Z-R/K, where Z represents a hydrophobic aa residue) or casein kinase 2 phosphorylation (S/T-X₂-D/E). Three Tyr residues are also completely conserved among these Fc μ Rs, but none of them (I/V-Y315-S/T-A-C, S-C-E/D-Y361-V-S, and S-D-D-Y385-I/V-N-V/I) match the immunoreceptor tyrosine-based activation motif (D/E-X₂-Y-X₂-L/I-X₆₋₈-Y-X₂-L/I), inhibitory motif (I/V-X-Y-X₂-L/V), or switch motif (T-X-Y-X₂-V/I). However, if phosphorylated, the most carboxyl tyrosine is a potential binding site (pY-X-N-X) for the Src homology 2 domains of growth factor receptor-bound protein 2 and growth factor receptor-bound protein 2-related adaptor protein, as observed in transmembrane adaptor proteins, including linker for activation of T cells and non-T cell activation linker (Horejsí et al., 2004). These findings indicate a quite distinct feature of the Fc μ R cytoplasmic tail compared with other FcRs, in which the Ig ligand binding chains are usually devoid of conserved Tyr residues except for Fc γ RIIA and Fc γ RIIB.

To determine whether these conserved Ser and Tyr residues are phosphorylated upon stimulation, Fc μ R⁺ BW5147 T cells were treated with a tyrosine phosphatase inhibitor, perva-nadate, or with preformed IgM immune complexes to cross-link Fc μ R. The Fc μ R was immunoprecipitated from the lysates of resting or activated cells and analyzed by immunoblotting with antibodies specific for phosphotyrosine or the

phosphoserine of PKC substrates. Phosphorylation of both serine and tyrosine residues was clearly demonstrated in perva-nadate-treated cells but not in untreated cells (Fig. 5 A). Interestingly, the serine-phosphorylated Fc μ R migrated at \sim 52 kD, whereas most of the tyrosine-phosphorylated Fc μ Rs migrated at \sim 60 kD and, to a lesser extent, at \sim 52 kD. When these membranes were reprobed with anti-Fc μ R mAb specific for its extracellular epitope, we found that in resting cells Fc μ R was present as a major band of \sim 60 kD, which is consistent with the M_r of the cell-surface Fc μ R (see Fig. 7), along with a minor band of \sim 45 kD, but in perva-nadate-treated cells the Fc μ R was resolved as a major band of \sim 52 kD together with multiple minor species of various sizes. When Fc μ R was cross-linked with preformed immune complexes consisting of IgM and F(ab')₂ fragments of anti- μ mAb, phosphorylation of both serine and tyrosine residues of the \sim 52 kD Fc μ R was also demonstrated as early as 3 min after ligation. The serine phosphorylation became more prominent at 30 min after ligation, whereas tyrosine phosphorylation was diminished by that time point (Fig. 5 B). In contrast to the effects seen with perva-nadate treatment, tyrosine-phosphorylated \sim 60-kD Fc μ R was not observed in the lysates of receptor-ligated cells. Reprobing of these membranes with anti-Fc μ R mAb revealed the presence of both \sim 60- and \sim 52-kD Fc μ R proteins as well as minor, but discrete, bands of \sim 45 and \sim 63 kD. Collectively, these findings suggest that the conserved serine and tyrosine residues seen in the cytoplasmic tail of Fc μ R are indeed potentially phosphorylated upon receptor ligation and that the phosphorylated Fc μ R protein migrates differently on SDS-PAGE compared with its unphosphorylated form.

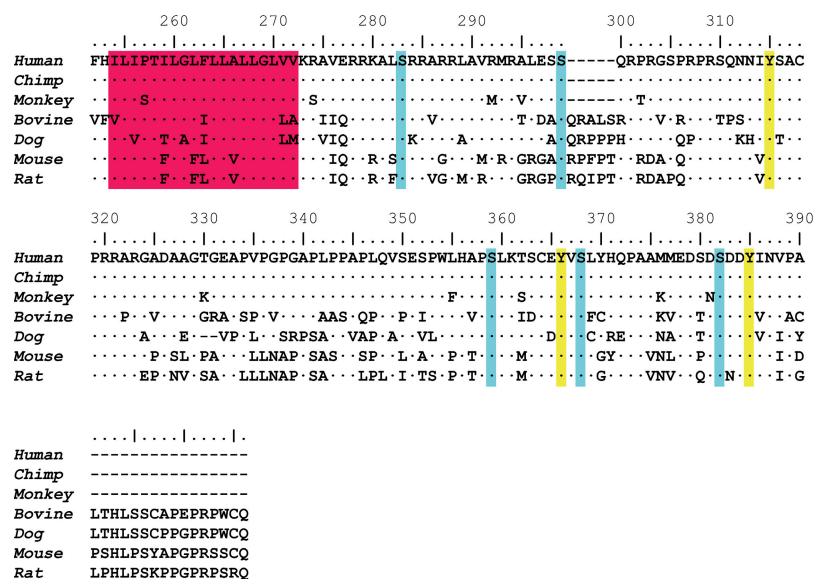


Figure 4. aa sequence alignment of the transmembrane and cytoplasmic regions of Fc μ Rs. aa sequences of the transmembrane segments and cytoplasmic tails of Fc μ R from seven different species are aligned. aa identity is indicated by dots, and a deletion is indicated by dashes. The predicted transmembrane region is highlighted in red. Conserved serine and tyrosine residues are also highlighted in blue and yellow, respectively. The numbers indicate the aa position from the first Met residue of human Fc μ R. The GenBank/EMBL/DDJB accession nos. for these Fc μ Rs are the same as those in Fig. 3. Chim, chimpanzee.

Fc μ R per se has no antiapoptotic activity

To determine whether Fc μ R inhibits Fas-mediated apoptosis as originally described for FAIM3/TOSO (Hitoshi et al., 1998), retroviral constructs containing both Fc μ R and GFP cDNAs or only the GFP cDNA were transduced into the apoptosis-prone Jurkat human T cell line. Cells expressing comparable levels of GFP were enriched from each transducent by FACS, and the Fc μ R/GFP transductant was found to express relatively high levels of cell-surface Fc μ R as determined by both receptor-specific mAbs (see next section) and IgM ligand binding (Fig. 6 A). The resultant Fc μ R⁺GFP⁺ or GFP⁺ Jurkat cells and nontransduced Jurkat cells as an additional control were then subjected to apoptosis assays using agonistic anti-Fas mAbs of the IgM or IgG₃ isotype. Cross-linkage of Fas with the IgM antibody induced robust early (annexin V⁺/7-aminoactinomycin D [7-AAD]⁻) and late (annexin V⁺/7-AAD⁺) apoptotic cells as well as dead cells (annexin V⁻/7-AAD⁺) in the nontransduced Jurkat cells and the GFP⁺ cells, but not in the Fc μ R⁺/GFP⁺ cells (Fig. 6 B). This result is consistent with the previously reported anti-apoptotic activity of FAIM3/TOSO (Hitoshi et al., 1998). It should be noted, however, that addition of control IgM of either human or mouse origin at a 100-fold molar excess into these cultures did not make the Fc μ R⁺/GFP⁺ cells susceptible to IgM anti-Fas mAb-induced apoptosis, suggesting that the simultaneous dual binding to Fas and Fc μ R (i.e., cis interaction) is dominant over the single binding to Fc μ R (i.e., trans interaction) in this apoptosis model (Fig. S4). Unlike the effect seen with IgM anti-Fas mAb, ligation of Fas recep-

tor with the IgG₃ antibody induced apoptosis in all three cell types, including the Fc μ R⁺GFP⁺ cells. Notably, ligation of Fc μ R and Fas with the corresponding mAbs either in the absence (i.e., separate ligation of each receptor) or presence of a common secondary reagent (i.e., coligation of both receptors) had no demonstrable effects on the IgG₃ anti-Fas mAb-induced apoptosis of Fc μ R⁺GFP⁺ cells. Essentially identical results using IgM versus IgG₃ anti-Fas mAb were also obtained with EBV-transformed B cell lines expressing both endogenous Fc μ R and Fas on their cell surface (unpublished data). Collectively, these findings indicate that Fc μ R has no intrinsic activity to inhibit Fas-mediated apoptosis, but they raise the interesting possibility that IgM anti-Fas autoantibody, if present in individuals with autoimmune disorders, could interrupt Fas-mediated signaling via Fc μ R in vivo.

To determine if Fc μ R could also affect apoptosis mediated through the BCR, the same retroviral constructs as used for Jurkat cells were transduced into a mouse immature B cell line, WEHI231, and a human germinal center B cell line, Ramos, both of which are negative for Fc μ R expression and are known to undergo apoptosis after BCR cross-linking. However, unlike the Jurkat T cell line, no cell lines of either type that stably expressed both Fc μ R and GFP were obtained after multiple attempts in different laboratories (unpublished data), whereas control GFP⁺ cell lines were easily established. Even after enriching GFP^{hi} cells by FACS or by antibiotic selection, the established cell lines were found to express low levels of GFP and no cell-surface expression of Fc μ R. Flow cytometric analysis of these B cell lines shortly

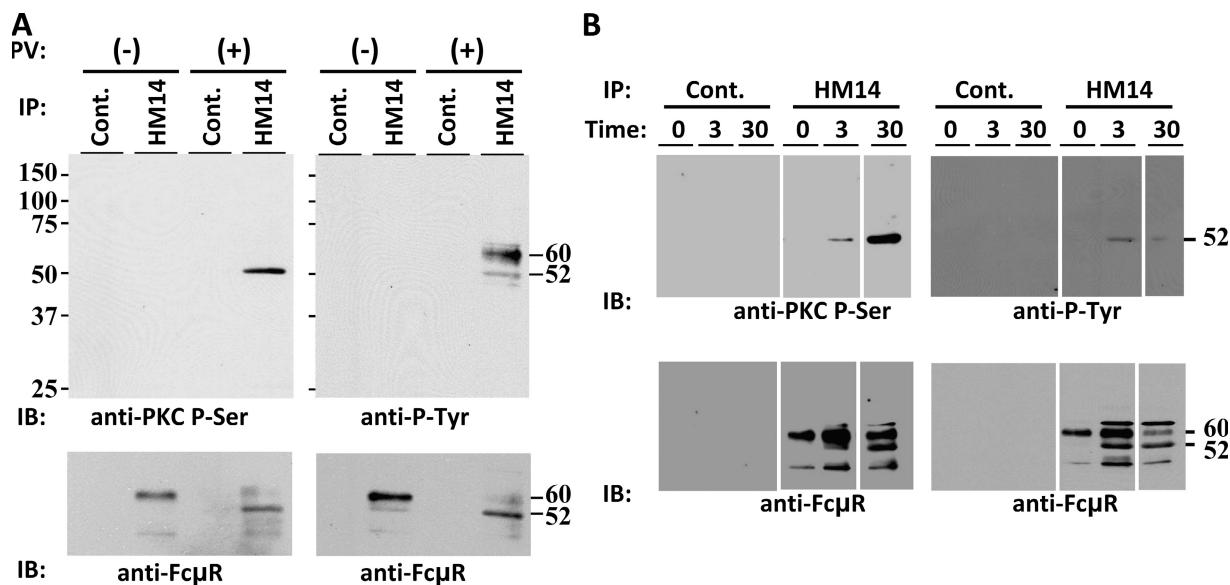


Figure 5. Tyrosine and serine phosphorylation of Fc μ R upon stimulation. (A and B) BW5147 T cells stably expressing human Fc μ R were incubated in the presence (+) or absence (−) of 100 μ M pervanadate for 15 min (A) or with the preformed IgM immune complexes for the indicated time periods (min) at 37°C (B) before cell lysis. Fc μ R was immunoprecipitated from cleared lysates with anti-Fc μ R (HM14) or control (Cont.) mAb-coupled beads, resolved on SDS-10% PAGE under reducing conditions, transferred onto membranes, and immunoblotted with rabbit antibody specific for phosphoserine of PKC substrates along with HRP-labeled goat anti-rabbit Ig antibody (anti-PKC P-Ser) or with HRP-labeled antiphosphotyrosine mAb (anti-P-Tyr) before visualization by ECL. After dissociating blotted antibodies, membranes were reprobed with biotin-labeled anti-Fc μ R mAbs along with HRP-labeled SA (anti-Fc μ R). These experiments were performed at least three times. M_r is shown in kilodaltons.

after transduction revealed that forced expression of Fc μ R resulted in down-modulation of their cell-surface IgM, presumably because of its ligation with the Fc μ R, thereby leading to loss of the Fc μ R⁺GFP⁺ cell population. Thus, these findings suggest that the ectopic expression of Fc μ R on WEHI231 and Ramos B cell lines triggers BCR-mediated apoptosis as a consequence of direct interaction between the Fc μ R and membrane-bound IgM molecules.

Fc μ R is an ~60-kD transmembrane protein

Two hybridoma mAbs specific for human Fc μ R, HM7 ($\gamma 2b\kappa$), and HM14 ($\gamma 1\kappa$) were established from mice immunized with Fc μ R⁺ BW5147 T cells and were used along with the IgM ligand for biochemical characterization of the receptor. The HM7 mAb appeared to recognize an epitope near the IgM ligand binding site, because HM7 antibody binding was significantly inhibited by preincubation of Fc μ R⁺ cells with IgM, whereas HM14 binding was not (unpublished data). Our earlier biochemical analysis revealed that Fc μ R on B-lineage cells could be attached to the plasma membrane via a glycosylphosphatidylinositol (GPI) linkage (Ohno et al., 1990; Nakamura et al., 1993), but the structure predicted by the cDNA is of a transmembrane protein. We thus reexamined this issue using a highly purified GPI-specific phospholipase C (GPI-PLC). After GPI-PLC treatment, the surface expression of the GPI-anchored Thy-1 on Fc μ R⁺ BW5147 T cells was reduced by ~65%, whereas surface Fc μ R levels were unaffected as determined by staining with both anti-Fc μ R mAbs and the IgM ligand (Fig. 7 A). As expected, levels of the control transmembrane glycoprotein

CD11a were also unaffected. We extended this analysis to the 697 pre-B cell line. Consistent with our previous IgM-binding results (Ohno et al., 1990), these cells do not constitutively express cell-surface Fc μ R, but its expression could be induced by PMA treatment (Fig. S5). After GPI-PLC treatment, the surface levels of Fc μ R and CD19 on PMA-activated 697 pre-B cells were unchanged, whereas the expression of GPI-anchored CD73 was reduced by ~50% (Fig. 7 B). Thus, these findings indicate that Fc μ R is an authentic transmembrane protein, consistent with the predicted structure encoded by the Fc μ R cDNA.

To determine the M_r of Fc μ R, we performed SDS-PAGE analysis of biotinylated cell-surface proteins that were precipitated from membrane lysates with anti-Fc μ R mAbs and IgM ligands. A major protein with an M_r of ~60 kD was precipitated from the Fc μ R-bearing but not control BW5147 T cells with both probes (Fig. 7 C). The same M_r estimate was obtained under both reducing and nonreducing conditions, indicating that there are no interchain disulfide linkages of Fc μ R with itself or other proteins. Removal of sialic acid residues with neuraminidase from the ~60-kD Fc μ R resulted in a decrease in M_r to ~50 kD. The cell-surface Fc μ R isolated from PMA-activated 697 pre-B cells and normal adult blood mononuclear cells (MNCs) had an identical M_r of ~60 kD, consistent with our previous size estimates (Sanders et al., 1987; Ohno et al., 1990; Nakamura et al., 1993). An additional minor band of ~40 kD was occasionally identified in the precipitates from membrane lysates of Fc μ R⁺ BW5147 T cells with anti-Fc μ R mAbs irrespective of detergents used (NP-40, digitonin, or CHAPS). The molecular identity of this 40-kD

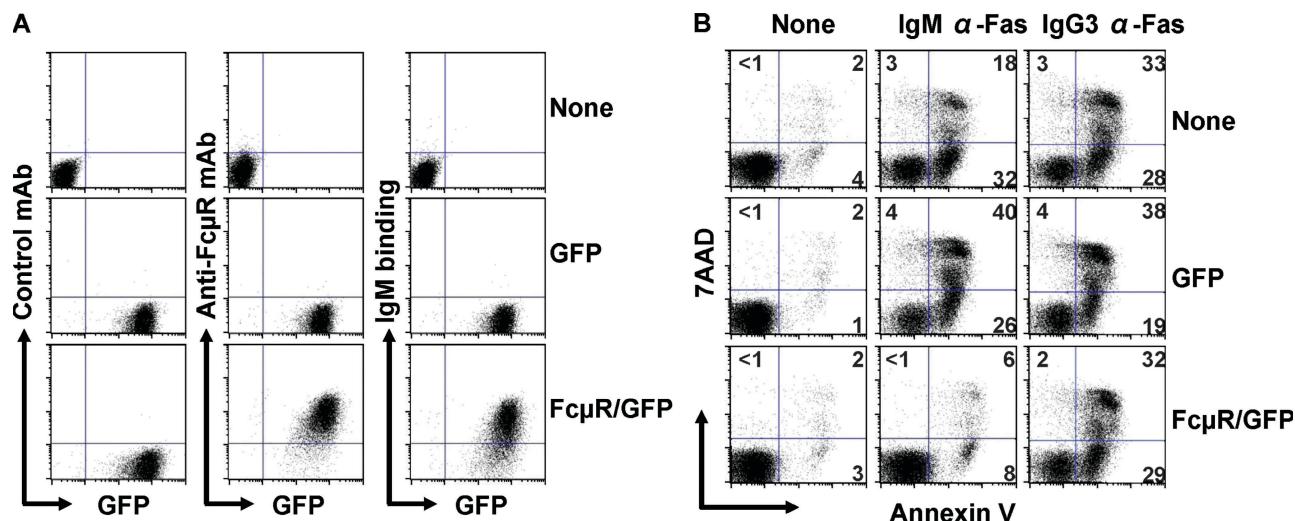


Figure 6. Role of Fc μ R in Fas-mediated apoptosis of Jurkat T cells. (A) Jurkat cells transduced without (none) or with the bicistronic retroviral construct containing GFP cDNA only (GFP) or both Fc μ R and GFP cDNAs (Fc μ R/GFP) were incubated with biotin-labeled isotype-matched control mAb (left), HM14 anti-Fc μ R mAb (middle), or human IgM (right), and then with APC-SA before analysis by FACSCalibur. Note the comparable levels of GFP in both GFP and Fc μ R/GFP transductants, and the expression of Fc μ R on the Fc μ R/GFP transductant as determined by anti-Fc μ R reactivity and IgM ligand binding. (B) These three cell lines were incubated at 37°C for 24 h with agonistic anti-human Fas mAbs of mouse IgM κ (CH11 clone; 10 ng/ml) or IgG 3κ isotype (2R2 clone; 0.3 μ g/ml). Cells were stained with 7-AAD and APC-labeled annexin V before identification of early (annexin V⁺/7-AAD⁻) and late (annexin V⁺/7-AAD⁺) apoptotic and dead (annexin V⁻/7-AAD⁺) cells by FACSCalibur. Note the resistance of Fc μ R/GFP transductant to IgM but not IgG 3κ anti-Fas mAb-induced apoptosis. Numbers indicate percentages of cells. These experiments were performed more than three times.

protein is presently unknown. Although the predicted pI of Fc μ R is \sim 9.9, the \sim 60-kD Fc μ R was resolved into a spot with a pI of \sim 5 by two-dimensional gel electrophoresis analysis (unpublished data), consistent with our previous finding that Fc μ R is sialylated (Ohno et al., 1990).

Fc μ R is predominantly expressed by both B and T lymphocytes

To determine the cellular distribution of Fc μ R, we first conducted RT-PCR analysis of various tissues and a panel of representative cell lines. Fc μ R transcripts were restricted to hematopoietic and lymphoid tissues, including the blood, bone marrow, tonsils, spleen, and appendix. Fc μ R transcripts were detected in both CD4 $^+$ and CD8 $^+$ T cells from blood as well as in all subsets of tonsillar B cells, although the transcript levels appeared highest in the follicular and memory B cells (Fig. S6, top). Among the cell lines, 697 pre-B cells expressed Fc μ R transcripts, although they did not constitutively express cell-surface Fc μ R protein (Fig. S5). Another pro-/pre-B cell line (REH) and some B cell lines (Ramos and the EVB-transformed line BDB-14.4) also contained Fc μ R mRNA (Fig. S6, bottom).

Next, we examined cell-surface Fc μ R expression by immunofluorescence analysis using receptor-specific mAbs and IgM ligands. In normal adult blood samples, Fc μ R was clearly expressed on CD19 $^+$ B cells and on the CD4 $^+$ and CD8 $^+$ T cells, although there was no discrete demarcation between Fc μ R $^+$ and Fc μ R $^-$ T cells (Fig. 8 A). The intensity

of staining with the HM14 mAb was higher than with the HM7 mAb (unpublished data), an observation consistent with the finding that the HM7 epitope is sensitive to IgM ligand binding; given its high affinity, the Fc μ R is likely to be occupied by IgM in vivo. Clearly, mAb reactivity was a more sensitive assay for the detection of Fc μ R than ligand binding using biotin-labeled human IgM, although the sensitivity of the ligand-binding assay could be increased by using mouse IgM κ , biotin-labeled rat anti-mouse κ mAb and streptavidin (SA)-PE. In addition to B and T cells, CD56 $^+$ /CD3 $^-$ NK cells also expressed Fc μ R at relatively low density. Other blood cell types, CD14 $^+$ monocytes, CD13 $^+$ granulocytes, erythrocytes, and platelets, did not express Fc μ R at detectable levels (Fig. S7).

Notably, overnight culture of blood MNCs in IgM-free media enhanced Fc μ R expression especially by T cells (Fig. 8 B), consistent with our previous IgM-binding data (Nakamura et al., 1993). Curiously, this enhancement was more evident for the cell preparations from the tonsils and spleen than from blood. Freshly isolated tonsillar MNCs, including B and T cells, had no reactivity with either anti-Fc μ R mAbs or IgM ligands, but after overnight culture, there was clear-cut expression of Fc μ R on the surface of the CD19 $^+$ B cells and the CD4 $^+$ and CD8 $^+$ T cells (Fig. 8 C). Most follicular (IgD $^+$ /CD38 $^-$) and memory (IgD $^-$ /CD38 $^-$) B cells expressed Fc μ R, whereas only a small subpopulation of the germinal center (IgD $^-$ /CD38 $^+$) and pregerminal center (IgD $^+$ /CD38 $^+$) B cells expressed Fc μ R, consistent with our RT-PCR data. Many

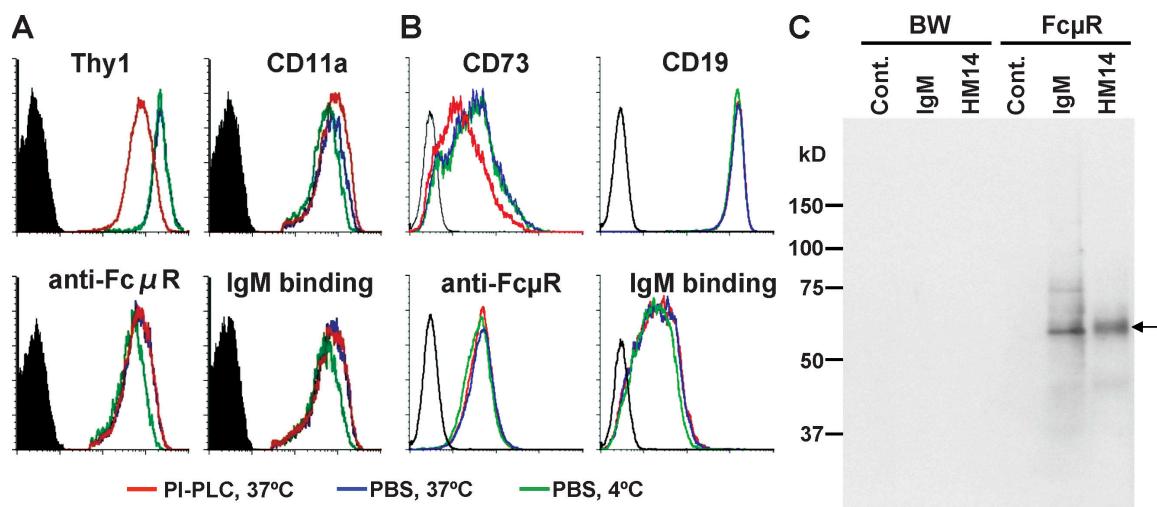


Figure 7. Biochemical characterization of Fc μ R molecules. (A and B) GPI-PLC treatments. BW5147 T cells stably expressing human Fc μ R (A) and PMA-activated 697 pre-B cells (B) were incubated with PBS (blue) or 10 U/ml GPI-PLC (red) for 30 min at 30°C, and then examined for the expression of Fc μ R by anti-Fc μ R mAb or IgM binding along with the expression of Thy-1 and CD11a (A) or of CD73 (ecto-5'-nucleotidase) and CD19 (B). A control sample was kept on ice during this treatment without GPI-PLC (green). Note the significant reduction in MFI of Thy-1 and CD73 but not of CD11a, CD19, Fc μ R, and IgM-binding profiles after GPI-PLC treatment. (C) SDS-PAGE analysis of cell-surface proteins. Plasma membrane proteins on control (BW) and Fc μ R-bearing BW5147 T cells (Fc μ R) were labeled with biotin, quenched, and incubated with mouse γ 1 κ control (Cont.) or anti-Fc μ R (HM14) mAbs or mouse IgM κ ligand before washing and solubilization in 1% NP-40 lysis buffer containing protease inhibitors. The mAb-bound cell-surface proteins were captured by addition of beads coupled with rat anti-mouse κ mAb (187.1 clone) and resolved on SDS-10% PAGE under nonreducing (not depicted) and reducing conditions, followed by transfer onto membranes, blotting with HRP-SA, and visualization by ECL. The same results were obtained with the HM7 anti-Fc μ R mAbs. The experiments were performed 3 times for A and B and >10 times for C. The arrow indicates Fc μ R. The experiments were performed 3 times for A and B and >10 times for C.

CD4⁺ T cells and the majority of CD8⁺ T cells clearly expressed cell-surface Fc μ R after culture. After overnight culture, the proportion of Fc μ R⁺ B, CD4⁺ T, CD8⁺ T, and NK cells in the spleen was similar to that in blood samples (unpublished data). In adult bone marrow, a small subpopulation (~21%) of the CD19⁺/surface IgM⁻ pro-/pre-B cells expressed low levels of Fc μ R on their cell surface, whereas ~42% of the CD19⁺/surface IgM⁺ B cells expressed slightly higher levels of Fc μ R, indicating that Fc μ R expression begins at the pro-/pre-B cell stage in B-lineage differentiation (Fig. 8 D). No Fc μ R expression was observed on myeloid cells even after overnight culture in IgM-free media. In contrast to the Fc μ R phenotype of humans, our initial immunofluorescence analysis of mouse splenocytes with a receptor-specific mAb revealed that Fc μ R was expressed by B220⁺ B cells but not by CD3⁺ T cells or Mac-1⁺ macrophages (unpublished data).

To further examine the effects of cellular activation on surface Fc μ R expression, blood MNCs were activated with various stimuli. Treatments of blood B cells with anti- μ mAb or PMA for 24 h resulted in an ~2.2-fold increase in the cell-surface Fc μ R level in comparison to that on B cells cultured in media only (unpublished data). In contrast, treatment of blood T cells with anti-CD3 mAb or PMA for 24–72 h reduced the cell-surface Fc μ R level by ~90%, suggesting that signaling through antigen receptors on B and T cells has distinct modulating effects on Fc μ R expression. Consistent with previous observations (Ferrarini et al., 1977; Pichler and Knapp, 1977; Sanders et al., 1987), there was enhanced Fc μ R expression by CLL B cells from three randomly selected patient blood samples (Fig. S8). Collectively, these findings indicate that, in striking contrast to other FcRs, Fc μ R is predominantly expressed by cells of the adaptive immune system. Moreover, the cell-surface levels of Fc μ R

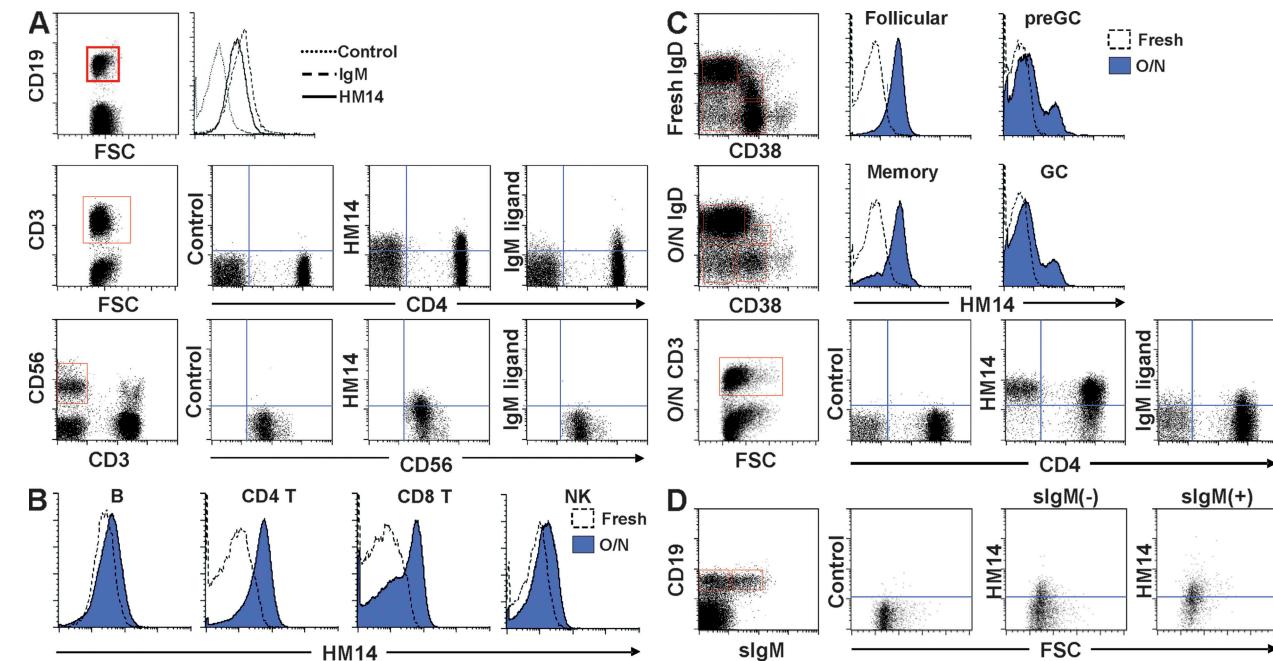


Figure 8. Immunofluorescence analysis of cell-surface Fc μ R expression in various tissues. (A–D) MNCs from blood (A and B), tonsils (C), and bone marrow (D) were first incubated with aggregated human IgG to block Fc γ Rs and then with biotin-labeled HM14 anti-Fc μ R mAb along with the appropriate fluorochrome-labeled mAbs specific for CD19, IgM, IgD, CD38, CD3, CD4, CD8, or CD56. For IgM ligand binding, mouse IgM κ and biotin-labeled rat anti-mouse κ mAbs were sequentially added to MNCs without preincubation with aggregated IgG. The bound biotin-labeled reagents were detected by addition of SA-PE. Essentially the same results were obtained with the HM7 anti-Fc μ R mAb (not depicted). Because the results of the Fc μ R expression by CD3⁺/CD8⁺ and CD3⁺/CD4⁺ T cells were essentially the same, the CD8 data were omitted for simplicity. The cell populations indicated by the red boxes were gated and examined for their reactivity with the HM14 anti-Fc μ R mAb and IgM ligand. Biotin-labeled irrelevant mAbs of the $\gamma 1\kappa$ (for HM14) or $\gamma 2\kappa$ (for HM7) isotype were used as controls. The analysis was performed with freshly prepared cell preparations (A and D; labeled Fresh) or with cells cultured overnight in IgM-free media (O/N; B and C). Because the immunofluorescence profiles of freshly prepared tonsillar B cells with anti-Fc μ R mAbs and isotype-matched control mAbs as well as these of overnight-cultured B cells with the isotype-matched control mAbs were all essentially the same, only the results of freshly prepared and overnight-cultured B cells with anti-Fc μ R mAbs are shown in C (top) for simplicity. CD19⁺ B cells in tonsils (C) were analyzed for Fc μ R expression as follicular/naive (IgD⁺/CD38⁻), pregerminal center (preGC; IgD⁺/CD38⁺), germinal center (GC; IgD⁻/CD38⁺), and memory (IgD⁻/CD38⁻) cells. The frequency (%) of Fc μ R⁺ cells in each cell type among 10 different blood samples was 62 \pm 18 for CD19⁺ B cells, 62 \pm 13 for CD4⁺ T cells, 43 \pm 23 for CD8⁺ T cells, and 19 \pm 11 for CD56⁺ NK cells (means \pm SD). The frequencies (%) of Fc μ R⁺ cells over the background staining with isotype-matched control mAbs in three tonsillar samples were 31 \pm 7 for follicular/naive, 15 \pm 3 for preGC, 10 \pm 3 for GC, and 30 \pm 12 for memory B cells, and 34 \pm 6 for CD4⁺ T and 51 \pm 7 for CD8⁺ T cells (means \pm SD). The experiments were performed >10 times for A and B, 3 times for C, and 2 times for D.

are sensitive to IgM ligand concentration, tissue milieu, and cellular activation status.

DISCUSSION

We have identified for the first time a bona fide $\text{Fc}\mu\text{R}$ cDNA in humans. By using receptor-specific mAbs and IgM ligands, $\text{Fc}\mu\text{R}$ is defined as an ~ 60 -kD transmembrane sialoglycoprotein. Its predicted structure consists of a single V-set Ig-like domain with homology to the Ig-binding domains of pIgR and $\text{Fc}\alpha/\mu\text{R}$, an additional extracellular region with no known domain features, a transmembrane segment containing a charged His residue, and a relatively long cytoplasmic tail carrying conserved Tyr and Ser residues. Pentameric IgM and its $\text{Fc}_5\mu$ fragments bound cell-surface $\text{Fc}\mu\text{R}$ on transductants, but the $\text{Fab}\mu$ fragments and other Ig isotypes did not, thereby confirming the $\text{Fc}\mu$ specificity of this receptor. The affinity of the $\text{Fc}\mu\text{R}$ for its IgM ligand is strikingly high, ~ 10 nM. Despite the initial designation of $\text{Fc}\mu\text{R}$ as an anti-apoptotic protein FAIM3/TOSO, $\text{Fc}\mu\text{R}$ per se had no inhibitory activity in Fas-mediated apoptosis, and such inhibition was only achieved when agonistic anti-Fas antibody of an IgM but not IgG isotype was used for inducing apoptosis. The cell types expressing $\text{Fc}\mu\text{R}$ were predominantly B and T cells and not phagocytes; hence, the cellular distribution of $\text{Fc}\mu\text{R}$ is quite distinct from that of other FcRs. The surface $\text{Fc}\mu\text{R}$ levels on those lymphocytes were susceptible to IgM ligand concentration, tissue milieu, and cellular activation.

The *FCMR* gene is found to be in an appropriate location on chromosome 1q32.2 adjacent to two other IgM-binding receptor genes (*PIGR* and *FCAMR*). Although the ligand-binding domains of these three receptors are similar to each other, $\text{Fc}\mu\text{R}$ seems to be the most distantly related among the group based on the following findings. (a) Many residues are well conserved in pIgR and $\text{Fc}\alpha/\mu\text{R}$ but not in $\text{Fc}\mu\text{R}$. (b) The length of the CDR1 region, which is predicted to contact the Ig ligands, is shorter in $\text{Fc}\mu\text{R}$ (5 aa) than in pIgR and $\text{Fc}\alpha/\mu\text{R}$ (9 aa). (c) Within the CDR1 region, there are two charged residues: Arg31 conserved in both pIgR and $\text{Fc}\alpha/\mu\text{R}$, and His32 conserved in all three receptors. Although Arg31 is predicted to be solvent exposed and to interact directly with polymeric IgA (Hamburger et al., 2004), $\text{Fc}\mu\text{Rs}$ from seven different species have a noncharged residue at the corresponding position. (d) $\text{Fc}\mu\text{R}$ recognizes only the IgM isotype, whereas both pIgR and $\text{Fc}\alpha/\mu\text{R}$ in humans bind polymeric IgA and IgM (Kaetzel, 2005; Kikuno et al., 2007). These findings thus suggest that the interaction of $\text{Fc}\mu\text{R}$ with its IgM ligand is distinct from that of pIgR and $\text{Fc}\alpha/\mu\text{R}$ with IgM and polymeric IgA. In this regard, the finding that $\text{Fc}_5\mu$ fragments mostly consisting of the $\text{C}\mu 3/\text{C}\mu 4$ domains inhibit IgM binding to $\text{Fc}\mu\text{R}$ suggests that $\text{Fc}\mu\text{R}$ recognizes a molecular configuration on IgM that is conferred by the $\text{C}\mu 3/\text{C}\mu 4$ domains. In contrast, pIgR recognizes the C-terminal domain $\text{C}\mu 4$ (Kaetzel, 2005).

The finding that the $\text{Fc}\mu\text{R}$ cDNAs identified in two cDNA libraries from PMA-activated 697 pre-B cells and CLL B cells encode a transmembrane but not a GPI-linked

protein was unexpected, because in our previous biochemical analysis, the $\text{Fc}\mu\text{R}$ expressed on such pre-B cells was sensitive to GPI-PLC, whereas the $\text{Fc}\mu\text{R}$ on blood T cells was resistant (Ohno et al., 1990; Nakamura et al., 1993). An intensive search for an alternatively spliced transcript encoding a GPI-linked form of $\text{Fc}\mu\text{R}$ was unsuccessful but led to identification of an $\text{Fc}\mu\text{R}$ splice variant lacking the transmembrane exon that may encode a soluble form of $\text{Fc}\mu\text{R}$ with an M_r of ~ 34 kD (unpublished data). Reexamination of the susceptibility of $\text{Fc}\mu\text{R}$ to GPI-PLC treatment yielded an unequivocal result: the expression of $\text{Fc}\mu\text{R}$ on both PMA-activated 697 pre-B cells and T cell transductants was unchanged after GPI-PLC treatment, whereas the surface expression of GPI-anchored CD73 or Thy-1/CD90 was reduced by 50–65%. Thus, the discrepancy is likely caused by the fact that the GPI-PLC available for our studies in 1990 contained residual contaminating protease activity.

Unlike other FcRs, the $\text{Fc}\mu\text{R}$ has a relatively long cytoplasmic tail containing three conserved tyrosine and five to nine conserved serine residues. We found that some of these tyrosine and serine residues are targets for phosphorylation after $\text{Fc}\mu\text{R}$ ligation with IgM immune complexes or pervanadate treatment. Intriguingly, the phosphorylated $\text{Fc}\mu\text{R}$ was found to migrate on SDS-PAGE faster than the unphosphorylated form. One possible explanation for this is that such phosphorylation may cause a global structural change of $\text{Fc}\mu\text{R}$ leading to increased mobility on SDS-PAGE. Although phosphorylated proteins usually migrate slower than their unphosphorylated forms, CD45 on a myeloid cell line in fact exhibits enhanced mobility on SDS-PAGE after PMA-induced phosphorylation (Buzzi et al., 1992). Another explanation may be proteolytic cleavage in the cytoplasmic tail of $\text{Fc}\mu\text{R}$ after receptor ligation, as has been observed in the $\text{Fc}\gamma\text{RIIA}$ on platelets (Gardiner et al., 2008). $\text{Fc}\gamma\text{RIIA}$ ligation on platelets leads to activation of both the metalloprotease that targets the collagen receptor GPVI to shed its ectodomain and the intracellular calpain that cleaves the cytoplasmic tail of $\text{Fc}\gamma\text{RIIA}$ to remove the immunoreceptor tyrosine-based activation motif-containing stub, suggesting a novel mechanism for platelet dysfunction by $\text{Fc}\gamma\text{RIIA}$ after immunological insult including IgG autoantibodies to platelets. The precise mechanism for the enhanced migration of phosphorylated $\text{Fc}\mu\text{R}$ awaits further investigation.

Nucleotide sequence analysis indicated that $\text{Fc}\mu\text{R}$ and FAIM3/TOSO are identical, but we have clearly shown that the antiapoptotic activity of $\text{Fc}\mu\text{R}$ in Fas-bearing Jurkat cells is only observed when agonistic anti-Fas mAb of an IgM but not IgG₃ isotype is used and that the $\text{Fc}\mu\text{R}$ expression itself does not prevent Fas-mediated apoptosis. Addition of a 100-fold molar excess of control IgM into these cultures did not convert the $\text{Fc}\mu\text{R}^+\text{GFP}^+$ cells from resistance to sensitivity to IgM anti-Fas mAb-induced apoptosis. Even when $\text{Fc}\mu\text{R}$ and Fas on $\text{Fc}\mu\text{R}^+$ cells were brought into close physical proximity by ligation with a common secondary antibody, $\text{Fc}\mu\text{R}$ did not inhibit Fas-mediated apoptosis. Notably, Hitoshi et al. (1998) found that a FAIM3/TOSO deletion

mutant lacking most of its cytoplasmic tail could still inhibit apoptosis mediated by IgM anti-Fas mAb, implying that FAIM3/TOSO might act indirectly through noncovalent association with another cell-surface protein. This might be relevant to our finding that an additional membrane protein of ~40 kD often coprecipitated with the ~60-kD Fc μ R from membrane lysates of Fc μ R $^+$ cells and that there is a charged His residue, which could be involved in electrostatic association with other proteins, adjacent to the transmembrane segment of Fc μ R. We therefore propose that the original designation of this gene as *FAIM3/TOSO* should be reconsidered and that renaming it *FCMR* would be more appropriate in keeping with its true physiological role.

Another reason to rename this gene is that several groups (Pallasch et al., 2008; Proto-Siqueira et al., 2008) have recently reported that *FAIM3/TOSO* is overexpressed in CLL, a heterogeneous leukemia thought to originate from antigen-stimulated B cells that escape normal cell-death mechanisms. The interpretation of this finding by both groups is that the resistance of CLL B cells to death mechanisms may result from the enhanced expression of “antiapoptotic” FAIM3/TOSO molecules. However, enhanced Fc μ R expression by CLL B cells had been consistently observed by many investigators using either rosetting or immunofluorescence methods (Ferrarini et al., 1977; Pichler and Knapp, 1977; Sanders et al., 1987). Although the mechanism for enhanced Fc μ R expression on CLL cells is unclear, it may result from chronic antigenic stimulation as supported by (a) reduced levels of membrane IgM, IgD, and CD79/Ig α /Ig β on CLL cells and (b) polyreactivity of CLL-derived IgM molecules (Chiorazzi et al., 2005). In this regard, our finding that treatment of normal blood B cells with anti- μ antibody down-modulates membrane IgM and up-regulates Fc μ R cell-surface expression is consistent with the hypothesis that CLL cells are being activated by certain common antigens; thereby, antigen-driven proliferation may provide an alternative mode of survival of the leukemic cells.

The finding that the major cell types expressing Fc μ R are the adaptive immune cells, both B and T lymphocytes, is remarkable, because FcRs for the switched Ig isotypes (Fc γ Rs, Fc ε RI, and Fc α R) are expressed by various hematopoietic cells, including phagocytes, and are thought to be central mediators coupling the innate and adaptive immune responses (Nimmerjahn and Ravetch, 2008). Intriguingly, Fc μ R is the only FcR constitutively expressed on T cells, which are generally negative for the expression of other FcRs. The expression of Fc μ R by both CD4 $^+$ and CD8 $^+$ T cells is consistent with an early report that T cells forming rosettes with IgM-coated erythrocytes included both cell types (Reinherz et al., 1980). For B cells, Fc μ R is the only IgM-binding receptor expressed. Although the initial report indicated that Fc α / μ R is expressed on B cells (Shibuya et al., 2000), our subsequent analyses revealed that the major cell type expressing Fc α / μ R was a follicular dendritic cell in both humans (Kikuno et al., 2007) and mice (unpublished data). A small subpopulation of blood CD56 $^+$ /CD3 $^-$ NK cells was also found to express Fc μ R,

consistent with the results previously reported by others (Pricop et al., 1993; Rabinowich et al., 1996). The physiological relevance of such restricted cellular expression of Fc μ R may be related to unique features of the IgM ligand, such as its early appearance during immune responses, the pentameric configuration of its secreted form, and its potency in complement activation.

Many investigators had previously noticed the instability of IgM binding by B, T, and NK cells (Moretta et al., 1977; Nakamura et al., 1993; Pricop et al., 1993). We also found that the cell-surface Fc μ R levels were sensitive to extracellular IgM concentration, tissue milieu, and cellular activation status. This vulnerability could explain why Fc μ R was limited to an operationally defined entity for such a long time. As is the case with many other receptors, the detection of Fc μ R with IgM ligands was much less efficient than with anti-Fc μ R mAbs. Short-term culture in IgM-free media enhanced the cell-surface expression of Fc μ R on T cells and, to a lesser extent, on B and NK cells. Remarkably, this phenomenon was much more pronounced with cells from tonsils and spleen; cell-surface Fc μ R was not detectable on freshly isolated B and T cells from these organs but easily demonstrated after overnight culture in IgM-free media. Many other cell-surface antigens were detectable in those freshly isolated preparations, ruling out an artifact of tissue manipulation. To our knowledge, the IgM concentration in the interstitial spaces of such intact tissues has never been determined. If this *in vivo* down-modulation of Fc μ R is solely dependent on the extracellular concentration of IgM and not on the tissue microenvironment (e.g., proteases) or cellular activation status, then the interstitial IgM concentration in secondary lymphoid tissues is perhaps higher than in blood. In this regard, it is noteworthy that IgM-producing plasma cells are in the immediate vicinity of B and T cells within these lymphoid tissues.

Although both B and T cells express Fc μ R, there is a striking difference in their response after antigen receptor ligation. Fc μ R expression on B cells was up-regulated after treatment with anti- μ mAb, whereas its expression on T cells was down-modulated after treatment with anti-CD3 mAb. The response to PMA was also different in the B and T cells, suggesting that the difference might be attributed to the downstream events involving PKC. The role of PKC in internalization of cell-surface receptors including TCR has been clearly demonstrated (Cantrell et al., 1985; Minami et al., 1987; Bonefeld et al., 2003). PKC is a conserved family of 11 serine/threonine protein kinases, and most cell types express multiple isozymes of PKC (Spitaler and Cantrell, 2004). PKC α , β , δ , ε , η , θ , and ζ are known to be present in lymphocytes. Interestingly, the disruption of the gene encoding a single PKC isozyme expressed in both B and T cells (e.g., PKC β $^{-/-}$, PKC ζ $^{-/-}$, and PKC δ $^{-/-}$) often caused a selective immunological abnormality in only one of the cell types, suggesting compensatory or complementary functions of other PKC isozymes in the other cell type (Spitaler and Cantrell, 2004). Based on the consensus sequence motifs, there are several potential Ser residues available for phosphorylation by PKC in

the human Fc μ R, particularly R-R-K-A-L-S283-R-R or A-P-S359-L-K. Thus, it seems possible that Fc μ R expressed on B and T cells may have distinct influences on their respective antigen receptor-mediated signaling.

With regard to the function of Fc μ R on B cells, it has been shown that passive administration of IgM antibody, in contrast to IgG, enhances the subsequent antibody response to relevant antigenic challenge (Hjelm et al., 2006). Recent studies with mice unable to produce the soluble form of IgM have clearly demonstrated the importance of secreted IgM in development of protective IgG antibody responses to viral and bacterial infections, presumably through both complement and Fc μ R systems (Boes et al., 1998a; Boes et al., 1998b; Ehrenstein et al., 1998; Ochsenbein et al., 1999; Baumgarth et al., 2000). The complement cleavage products C3dg and C3d attach covalently to antigen and cross-link both CD21 (CR2) and BCR on the membrane of B cells, thereby facilitating the B cell response to low concentrations of antigen despite the typically low affinity of BCR during the primary immune response (Fearon and Carter, 1995; Fearon and Carroll, 2000). The enhancing or adjuvant activity of C3d has been demonstrated with various antigens, although recently the inhibitory activity of C3d has also been reported for certain antigens (Bergmann-Leitner et al., 2006). Given that IgM antibody is a first line of host defense, it is reasonable to propose that Fc μ R may contribute to enhancement of B cell responses by interacting with BCR and CD21/CD19/CD81 via IgM–antigen–C3d complexes. Another potential role for Fc μ R on B cells is antigen presentation. The functional significance of Fc μ R on T cells has been the subject of considerable speculation (Moretta et al., 1977; Mathur et al., 1988b; Nakamura et al., 1993). It seems possible that Fc μ R on T cells may interact with the IgM BCR or IgM/antigen complexes on B cells to facilitate T and B cell interactions, thereby enhancing B cell activation. Fc μ R may also trigger cytotoxic T cells in IgM antibody–dependent cell-mediated cytotoxicity. The true physiological roles of Fc μ R, however, will become apparent with further studies, including analysis of the immunological phenotypes in Fc μ R-deficient mice. Although the molecular nature of the Fc μ R has long been elusive, its final unveiling in this study reveals a receptor full of intriguing aspects and opens new avenues of investigation.

MATERIALS AND METHODS

Construction of retroviral cDNA libraries. The cDNA libraries were constructed by a cDNA synthesis kit (Agilent Technologies) using poly(A)⁺ RNA isolated by an Oligotex mRNA purification kit (QIAGEN) from (a) blood MNCS from a patient with CLL and (b) the 697 pre–B cell line preactivated with 10 nM PMA for 8 h, as previously described (Kubagawa et al., 1997). The EcoRI/XbaI-digested and size-fractionated cDNAs were ligated into the pMXsΔN/S retrovirus vector, in which the 1,328-bp NotI/SalI fragment containing an IRES and a GFP cDNA was removed from the original pMXsIG vector (Kitamura et al., 2003). The ligated cDNA constructs were used to transform XL2-Blue MRF' ultracompetent cells (Agilent Technologies). The titer of the cDNA library was \sim 10⁵ and \sim 10⁶ CFU/ μ g mRNA for CLL and 697 pre–B cells, respectively, and the mean size of the insert DNA in these libraries was \sim 1.6 kb.

Transfection, transduction, and screening. The cDNA libraries were transfected into the ecotropic retroviral packaging cell line BOSC23 with FuGENE 6 (Roche). 2 d later, the culture supernatants containing viruses were collected and filtered, and polybrene (Sigma-Aldrich) was added to a final concentration of 10 μ g/ml before infecting the mouse thymoma line BW5147 at a ratio of \sim 3 \times 10⁵ cells/ml of supernatants. After 2 d, infected BW5147 T cells were incubated with biotin-labeled human IgM κ and then with antibiotin microbeads (Miltenyi Biotec) or PE-labeled SA (Southern Biotech) before sorting IgM-binding cells by MACS or FACS, respectively. Enrichment of IgM-binding cells was repeated three times for MACS and once for FACS within the interval of \sim 3 d, and the final FACS-sorted cells were cloned by limiting dilution.

Identification of cDNA inserts and sequencing. Total RNA isolated from single-cell derived, IgM-binding and -nonbinding subclones was converted to first-strand cDNA with a primer (5'-CCCTTTCTGGAGAC-TAAAT-3') corresponding to the 3' vector sequence flanking the cloning site and SuperScript II RT (Invitrogen). The resultant first-strand cDNAs were used as template DNAs in PCR amplification with PrimeSTAR HS DNA polymerase (Takara Bio Inc.) and a set of primers corresponding to the 5' and 3' flanking vector sequences of the cloning site, as previously described (Arase et al., 2001). Amplified PCR products were subcloned into the ZeroBlunt TOPO vector (Invitrogen) before sequencing analysis was performed at our institutional sequencing core facility using a DNA analyzer (model 3730xl) and DNA Sequencing Analysis Software (version 5.2; both from Applied Biosystems).

Preparation of Fc μ R stable transductants. Total RNAs isolated from PMA-activated 697 pre–B cells, CLL B cells, and tonsils were similarly converted to first-strand cDNA with an oligo(dT)₁₈ primer, and the resultant first-strand cDNAs were used as template DNAs for amplification of Fc μ R cDNA with a set of primers (forward, 5'-AGATCTAGAAGGGACAATG-GACT-3', and reverse, 5'-GAATTCTCAGGCAGGAACATTGATGT-3'; underlined portions indicate BglII and EcoRI sites). Amplified products of the expected size of \sim 1.2 kb were subcloned into the BamHI and EcoRI sites of the pMXsPIE retroviral vector that contains a GFP cDNA and *Streptomyces alboniger* puromycin-N-acetyltransferase cDNA (a gift of A. Mui; DNAX, San Francisco, CA; Ehrhardt et al., 1999). After confirming sequence identity, the ligated Fc μ R cDNA construct and the empty vector were similarly transduced in BW5147 T cells, and the GFP⁺ cells in both transductants were enriched by FACS and in the presence of 1 μ g/ml puromycin. For Fc μ R⁺ Jurkat cells, both the Fc μ R/GFP and GFP-only constructs were transfected into the 293T-A amphotropic packaging cell line before transducing the human Jurkat T cell line. GFP⁺ cells were enriched three times by FACS before establishing the stable cell lines. In some experiments, both Fc μ R/GFP and GFP constructs were similarly transfected into an appropriate packaging cell line and transduced into WEHI231 mouse B cells and Ramos human B cells.

Ig ligands and binding assay. Human IgM myeloma proteins were purified from serum samples by euglobulin fractionation and Sephadryl S-300 gel filtration column chromatography (GE Healthcare; Ohno et al., 1990). The Fc μ and Fab μ fragments were prepared from a human IgM κ by hot trypsin digestion (Plaut and Tomasi, 1970; Ohno et al., 1990; Nakamura et al., 1993). Other human myeloma IgS of each isotype (γ 1, γ 2, γ 3, γ 4, α 1, α 2, δ , and ϵ) and mouse myeloma IgM were purchased from EMD and Sigma-Aldrich. The purity of IgM, its fragments, and other myeloma IgS was confirmed by SDS-PAGE under both reducing and nonreducing conditions. Protein concentration was determined by absorbance at 280 nm with an extinction coefficient of 1.4 as 1 mg/ml. For the binding inhibition assay, Fc μ R⁺ BW5147 T cells were incubated with various concentrations of Ig preparations along with a constant amount of biotin-labeled IgM κ , washed, and incubated with PE-labeled SA to determine the bound IgM.

Production of hybridoma mAbs. BALB/c mice were hyperimmunized subcutaneously with BW5147 T cells expressing human Fc μ R, and regional lymph node cells were fused with the Ag8.653 plasmacytoma line, as previously

described (Kikuno et al., 2007). Hybridoma clones producing IgG mAbs reactive with $\text{Fc}\mu\text{R}^+$ BW5147 T cells, but not with $\text{Fc}\alpha/\mu\text{R}^+$ BW5147 T cells, control BW5147 T cells, and pIgR $^+$ FT-29 cells were selected and subcloned by limiting dilution. Two human $\text{Fc}\mu\text{R}$ -specific mAbs, HM7 ($\gamma 2\kappa$) and HM14 ($\gamma 1\kappa$), were selected in this study. Their $\text{F}(\text{ab}')_2$ fragments were prepared by digestion with lysyl endopeptidase (Yamaguchi et al., 1995) and pepsin (Maruyama et al., 1985) for HM7 and HM14, respectively.

Flow cytometric analysis of cells. Blood MNCs were isolated by Ficoll-Hypaque density gradient centrifugation. Granulocytes were isolated from erythrocyte pellets by differential sedimentation in 1.5% dextran in PBS. MNCs were also prepared from long bone, tonsil, and spleen tissues obtained from our institutional tissue procurement service. Approval for use of these human materials in this investigation was obtained from the University of Alabama at Birmingham Institutional Review Board. Cells were first incubated with aggregated human IgG to block $\text{Fc}\gamma\text{Rs}$ and then stained with biotin-labeled anti- $\text{Fc}\mu\text{R}$ mAbs along with fluorochrome-labeled mAbs specific for CD3, CD4, CD8, CD19, CD14, CD56, CD10, or CD13. PE-labeled SA was used as a developing reagent for biotinylated mAbs. Controls included isotype-matched irrelevant mAbs labeled with the corresponding fluorochromes or biotin. In some experiments, biotin-labeled $\text{F}(\text{ab}')_2$ fragments of anti- $\text{Fc}\mu\text{R}$ mAbs were used. Stained cells were analyzed with a FACSCalibur instrument (BD). For GPI-PLC treatment, 10^6 cells were incubated for 45 min at 30°C in 10 mM Hepes/HBSS (without Ca^{2+} and Mg^{2+}) containing 10 U/ml GPI-PLC (Sigma-Aldrich). After treatment, cells were washed and examined for $\text{Fc}\mu\text{R}$ and other cell-surface antigens by FACSCalibur. For neuraminidase treatment, 5×10^6 cells/ml in HBSS were incubated with 50 U/ml neuraminidase (New England Biolabs, Inc.) at 37°C for 45 min before washing and immunofluorescence analysis.

Cell-surface biotinylation and immunoprecipitation analysis. Plasma membrane proteins on 10^7 viable cells were labeled with 1 ml sulfo-NHS-LC-biotin (0.1 mg/ml; Thermo Fisher Scientific) in 0.15 M NaCl/0.1 M Hepes (pH 8) for 30 min at 25°C. After washing, biotinylated cells were incubated with 10 μl anti- $\text{Fc}\mu\text{R}$ or isotype-matched control mAbs or mouse IgM κ ligand (50 $\mu\text{g}/\text{ml}$) for 20 min on ice, washed, and lysed in 200 μl of 1% NP-40 lysis buffer containing protease inhibitors (Sanders et al., 1987; Ohno et al., 1990; Nakamura et al., 1993). Cleared lysates were either transferred to 96-well plates precoated with 20 $\mu\text{g}/\text{ml}$ of rat anti-mouse κ mAb (clone 187.1; Yelton et al., 1981) or incubated with rat anti-mouse κ mAb-coupled beads, and the bound materials were dissociated and separated by SDS-PAGE under reducing and nonreducing conditions, followed by transfer to membranes, blotting with horseradish peroxidase (HRP)-SA, and visualization by ECL (GE Healthcare), as previously described (Kikuno et al., 2007). In some experiments, the anti- $\text{Fc}\mu\text{R}$ mAb-bound materials were resuspended in 7 M urea/2 M thiourea/4% CHAPS/40 mM dithiothreitol/0.5% ampholite (pH 3–10)/40 mM Tris-HCl (pH 8.8) and subjected to two-dimensional gel electrophoresis analysis, as previously described (Ohno et al., 1990).

Immunoblot analysis. To determine the phosphorylation status of Tyr and Ser residues in $\text{Fc}\mu\text{R}$, 3×10^7 cells serum starved for 1.5 h in RPMI 1640/20 mM Hepes media were treated with 100 μM pervanadate for 15 min at 37°C, lysed in 1 ml of 1% NP-40 lysis buffer with protease/phosphatase inhibitors, and immunoprecipitated with Sepharose 4B beads coupled to HM14 anti- $\text{Fc}\mu\text{R}$ mAb or AM3 anti- $\text{Fc}\alpha/\mu\text{R}$ mAb as an isotype-matched control. The bound materials were dissociated with 0.1 M glycine-HCl buffer (pH 2.8) in 0.5% NP-40, immediately neutralized with 1 M Tris, and resolved on SDS-10% PAGE before transfer onto membranes. After soaking with 5% nonfat milk, membranes were immunoblotted with HRP-labeled antiphosphotyrosine mAb (4G10; Millipore) or rabbit antibody specific for phosphoserine of PKC substrates (Cell Signaling Technology) along with HRP-labeled goat anti-rabbit Ig antibody (SouthernBiotech) as a developing reagent. For receptor ligation, serum-starved cells were incubated with 50 μl of the preformed IgM immune complexes, an equal mixture of human IgM κ myeloma protein (100 $\mu\text{g}/\text{ml}$) and $\text{F}(\text{ab}')_2$ fragments of anti-human μ mAb with specificity for the $\text{C}\mu 1$ domain (50 $\mu\text{g}/\text{ml}$), at 37°C for 0, 3, and

30 min before solubilizing in 200 μl of 1% NP-40 lysis buffer. The cleared lysates were subjected to immunoprecipitation with HM14 or AM3 mAb-coupled beads, and the bound materials were similarly analyzed by immunoblotting. Immunoblotted membranes were visualized by ECL. After dissociating the blotting antibodies, the membranes were reblotted with biotin-labeled anti- $\text{Fc}\mu\text{R}$ mAbs (HM14 and HM7) to confirm the phosphorylation of Tyr and Ser residues of $\text{Fc}\mu\text{R}$.

Apoptosis assay. 4×10^5 cells/ml were cultured for 24 h in RPMI 1640 containing 10% FCS, penicillin/streptomycin, and 5×10^{-5} M 2-ME in the presence or absence of either of the agonistic anti-Fas mAbs, CH11 (10 ng/ml; mouse $\mu\kappa$ isotype; Millipore) or 2R2 (300 ng/ml; mouse $\gamma 3\kappa$; Invitrogen), washed twice with PBS, and incubated with 7-AAD and allophycocyanin (APC)-labeled annexin V for detecting apoptotic cells according to the manufacturer's recommendation (BD). In some experiments, 100-fold molar excess of human or mouse IgM myeloma protein as a ligand was added in these cultures. In other experiments, cells were preincubated with the 2R2 anti-Fas mAb (300 ng/ml) and either $\text{F}(\text{ab}')_2$ fragments or the intact form of the HM14 anti- $\text{Fc}\mu\text{R}$ mAb (50 $\mu\text{g}/\text{ml}$) for 20 min at 4°C, washed, and cultured in the presence or absence of $\text{F}(\text{ab}')_2$ fragments of goat anti-mouse κ antibodies (50 $\mu\text{g}/\text{ml}$) overnight at 37°C.

Scatchard plot analysis. 2×10^6 $\text{Fc}\mu\text{R}^+$ BW5147 T cells were incubated in triplicate with serial dilutions of ^{125}I -labeled IgM with a specific activity of $\sim 1.6 \times 10^{17}$ cpm/mol in 30 μl PBS containing 3% FCS and 0.2% sodium azide for 1.5 h at room temperature before washing and aspirating unbound IgM by centrifugation. Some tubes contained a 200-fold molar excess of cold IgM to determine the amounts of nonspecific binding of ^{125}I -labeled IgM to cells. The numbers of IgM molecules specifically bound per cell were plotted on the x axis against the ratio of bound to free IgM on the y axis, and the apparent dissociation constant was obtained by dividing the number of receptors per cell by the bound/free ratio at the y-axis intercept, as previously described (Lowenthal et al., 2001).

Online supplemental material. Fig. S1 shows the nucleotide sequence of the human $\text{Fc}\mu\text{R}$ cDNA. Fig. S2 shows the definition of FAIM3/TOSO as an $\text{Fc}\mu\text{R}$. Fig. S3 shows the predicted protein structure of human $\text{Fc}\mu\text{R}$. Fig. S4 shows the effects of $\text{Fc}\mu\text{R}$ ligation on anti-Fas antibody-mediated apoptosis in Jurkat T cells. Fig. S5 shows the expression of cell-surface $\text{Fc}\mu\text{R}$ on 697 pre-B cell line before and after PMA stimulation. Fig. S6 shows FCMR gene expression analyzed by RT-PCR. Fig. S7 shows the lack of $\text{Fc}\mu\text{R}$ expression by monocytes, granulocytes, erythrocytes, and platelets. Fig. S8 shows enhanced $\text{Fc}\mu\text{R}$ expression on CLL cells. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20091107/DC1>.

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