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J Immunol published online 19 January 2015
http://www.jimmunol.org/content/early/2015/01/19/jimmunol.1401866
Unique Ligand-Binding Property of the Human IgM Fc Receptor

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The IgM Fc receptor (FcμR) is the newest FcR, and coligation of FcμR and Fas/CD95 on Jurkat cells with agonistic IgM anti-Fas mAb was shown to inhibit Fas-induced apoptosis. The ligand-binding activity of human FcμR was further examined. FcμR-mediated protection from apoptosis was partially blocked by addition of 10^4 molar excess of IgM or its soluble immune complexes, but it could be inhibited by addition of 10-fold excess of IgM anti-CD2 mAb. This suggests that FcμR binds more efficiently to the Fc portion of IgM reactive with plasma-membrane proteins than to the Fc portion of IgM in solution. The former interaction occurred in cis on the same cell surface, but not in trans between neighboring cells. This cis engagement of FcμR resulted in modulation of Ca^{2+} mobilization via CD2 on Jurkat cells or BCRs on blood B cells upon cross-linkage with the corresponding IgM mAbs. Several functional changes were observed with FcμR mutants: 1) significant increase in IgM ligand binding in the cytoplasmic tail-deletion mutant, 2) enhanced cap formation in FcμR upon IgM binding at 4°C with a point mutation of the transmembrane His to Phe, and 3) less protective activity of FcμR in IgM anti-Fas mAb-mediated apoptosis assays with a point mutation of the membrane-proximal Tyr to Phe. These findings show the importance of the cis engagement of FcμR and its critical role in receptor function. Hence, FcμR on B, T, and NK cells may modulate the function of surface proteins recognized by natural or immune IgM Abs on the shared membrane cell surface.

The Journal of Immunology, 2015, 194: 000–000.

Antibodies have dual binding activity: to Ag via their N-terminal variable regions, and to effector molecules such as FcRs via their C-terminal constant regions. FcRs are expressed by many different cell types, and their interaction with Abs can initiate a broad spectrum of effector functions essential in host defense. These functions include phagocytosis of Ab-coated microbes, lysosomal degradation of endocytosed immune complexes, Ab-dependent cell-mediated cytotoxicity, secretion of cytokines and chemokines, release of potent inflammatory mediators, regulation of Ab production by B cells, survival of plasma cells, and presentation of degradable as well as non-degradable Ags (1–7). These diverse functions depend on the Ab isotype and the cell type expressing the FcR. Structurally and functionally diverse FcRs, namely FcR for IgG (FcγRI/CD32, FcγRII/CD16, FcγRIII/CD16, FcγRIII/CD16, FcγRI/CD16, FcγRIV, FcRn), IgE (FcεRI, FceRII/CD23), IgA (FcαRII/CD89), and both polymeric IgA and IgM (Fcα/μR/CD351), have been characterized extensively at both the protein and genetic levels (1–5, 8–10).

It has long been a puzzle why an FcR for IgM (FcμR), the first Ig isotype to appear during phylogeny, ontogeny, and the immune response, has defined identification, despite extensive biochemical evidence of IgM Fc–binding proteins accumulated over decades (11–13). We previously successfully identified a cDNA encoding an authentic FcμR from cDNA libraries of human B-lineage cells using a functional cloning strategy (14). FCMR is a single-copy gene located on chromosome 1q32.2, adjacent to two other IgM-binding receptor genes, Fcα/μR and polymeric Ig receptor. The predicted FcμR is a transmembrane protein that consists of a single V-set Ig-like domain responsible for FcμR binding, an additional extracellular region with no known domain structure, a transmembrane segment containing a charged His residue, and a relatively long cytoplasmic tail (118 aa) containing three conserved Tyr and five conserved Ser residues. FcμR binds pentameric IgM with a surprisingly high avidity of ~10 nM as determined by Scatchard plot analysis, with the assumption of a 1:1 stoichiometry of FcμR to IgM ligand. Upon ligation of FcμR with IgM ligands, both Tyr and Ser residues in the cytoplasmic tail are phosphorylated (14), and receptors are rapidly internalized into lysosomal compartments (15). Unlike other FcRs, the expression of FcμR is restricted to lymphocytes: B, T, and NK cells (14, 16). This suggests potentially distinct functions of FcμR as compared with other FcRs that are mainly expressed by myeloid cells.

Alternatively, the FcμR was initially designated as Fas apoptotic inhibitory molecule 3 (FAIM3), because coligation of Fas and FcμR/FAIM3 with an agonistic IgM anti-Fas mAb prevented Fas-mediated apoptosis (17). Unlike the effect of IgM anti-Fas mAb, however, ligation of Fas with an agonistic IgG mAb induced apoptosis irrespective of the expression of FcμR/FAIM3 (14, 16, 18). Notably, coligation of Fas and FcμR/FAIM3 with the corresponding mouse IgG mAbs plus a secondary reagent [e.g., F(ab')2 fragments of anti-mouse γ Ab] had no demonstrable effects on the IgG anti-Fas mAb-induced apoptosis (14). This suggests that the antia apoptotic activity of FcμR/FAIM3 depends on usage of the IgM anti-Fas mAb, and not on physical proximity of two receptors induced by artificial coligation. However, because the FcμR-mediated inhibition of the IgM anti-Fas mAb-
induced apoptosis is a reproducible and well-defined function, we employed this antiapoptotic assay in the present study as one of the readouts to explore the mode of action of FcµR. In this study, we have defined the unique ligand-binding properties of FcµR and the key residues involved in its receptor function.

Materials and Methods

Cells

The human leukemic T cell (Jurkat) and mouse thymoma (BW5174) lines, which stably expressed either only FCP as a control or both FCP and human FcµR, were prepared by retrovirus-mediated transduction as previously described (14) and were maintained in media containing puromycin at 1.5 and 0.75 μg/ml, respectively. BW5174 cells stably expressing human FcµR (without GFP) and wild-type (WT) Jurkat or BW5174 cells were maintained in media only as described (14). For Ca2+ mobilization, mononuclear cells isolated from healthy individuals by Ficoll-Hypaque density gradient centrifugation were enriched for B cells using a B cell isolation kit II (Miltenyi Biotec) with MACS. The frequency of CD19+ B cells in the resultant enriched fractions was >95% as determined by flow cytometry. This study involving human subjects was approved by the institutional review board of the University of Alabama at Birmingham.

Immunofluorescence analysis

Flow cytometric analysis of cell surface FcµR on transductants was performed by using biotin-labeled, receptor-specific mAb (HM14, γ1κ iso-type) and isotype-matched control mAb, along with PE-labeled streptavidin as described (14). For IgM binding, cells were incubated with biotin-labeled monoclonal antibodies (23) in PBS, washed, and then incubated with PE-labeled streptavidin. Stained cells were examined by an Accuri C6 flow cytometer (BD Biosciences), and flow cytometric data were analyzed with FlowJo software (Tree Star).

Apoposis assay

Apoposis assays were performed essentially the same as described before (14). In brief, FcµR+ or control Jurkat cells were cultured for 15 h with or without agonistic IgM anti-Fas mAb (CH11, 10 ng/ml; Millipore), along with various concentrations of inhibitors: 1) IgM mAbs specific for nitrophenyl (NP) hapten (B1-8) (19) or α3-1 dextran (clone 1-21) (20), 2) their soluble immune complexes with NP25-coupled BSA (molecular mass of ~70 kDa; Biosearch Technologies) or α3-1 dextran (molecular mass of ~1000 kDa), 3) FcµR-blocking (HM7) or nonblocking (HM3) mAbs (both γ2b2h), and 4) IgM mAb specific for the human CD2 (C373) or Jurkat TCR (C50S) (21). The latter two mAbs were provided by Dr. Arthur Weiss (Howard Hughes Medical Institute, University of California, San Francisco) and were purified from ascites by Sephacryl S300 gel filtration, and the remnants were purified by Ag-coupled affinity columns. For soluble immune complexes, IgM anti-NP and IgM anti-dextran mAbs and the corresponding Abs were premixed to reach a final concentration in the upper third or fourth of IgM mAbs with different molar ratios of Ab/Ag (1:10 to 1:10,000). Apoptotic cells were detected by staining with 7-aminoactinomycin D (7-AAD) and allophycocyanin-labeled annexin V (BD Biosciences) as described (14). For mixed cell cultures, FcµR+ or control Jurkat cells were cocultured with 10-fold excess number of FcµR+ cells and analyzed with FlowJo software (Tree Star).

Calcium mobilization

A mixture containing equal numbers of FcµR+ and WT Jurkat cells preloaded with 5 μM Indo-1/AM (Molecular Probes), a Ca2+-sensitive dye, was stimulated by various concentrations of IgM mAbs specific for CD2 or TCR or by 1 μM ionomycin as a positive control. The intracellular Ca2+ concentration ([Ca2+] i) in both cell populations were simultaneously measured by the ratio of emitted fluorescence of bound Ca2+ at 405 nm and free Ca2+ at 485 nm using an LSR II flow cytometer (BD Biosciences). In some experiments, an equal mixture of control GFP+ cells and WT Jurkat cells was also assessed similarly as controls. For blood B cells, MACS-enriched B cells (95%) were loaded with Fluo-4/AM (0.25 μM; Molecular Probes), tested, and then treated with a mixture of mouse IgM anti-human κ mAb (B29, 50 ng/ml) and mouse IgG2b anti-FcμR mAb (HM3 or HM3, 100 μg/ml). Changes in Fluo-4 fluorescence in the labeled cells following treatment were measured over real time with an Accuri C6 flow cytometer and analyzed by FlowJo software as described (22).

FcµR mutants

FcµR cDNA constructs with point mutations (H253F, Y315F, Y366F, or Y385F) were generated by a QuickChange site-directed mutagenesis kit (Strategene) using nonmutated FcµR cDNA as a template and a set of primers: 1) 5'-GAAGGCAAGAGTTTCTACGTATCGCCAGCATT-3' and 5'-CTGGCGGGATACACGTAAGATGTT-3' for H253F; 2) 5'-CTCCCCAAAACACATCCTCCAGGGCTTCG-3' and 5'-GGGACGGCTGAGAATTGTGTGTGGAGGAG-3' for Y315F; 3) 5'-GACCACTGGTGAATTGTCACCTGCTCACAACAGCAG-3' and 5'-CTGGTGTTGAGACCTCACAATTCCAGCTGGTC-3' for Y366F; and 4) 5'-GAGACAGATGTCAGATGACCTCAGATCAGTCAGTCTAGTCTAG-3' and 5'-ACATGTTGAAGACATGCTAAGACTGCTAAGACTGCT-3' for Y385F (underlining indicates mutation sites), according to the manufacturer’s recommendation. For FcµR cytoplasmic tail deletion, an FcµR cDNA construct with a deletion of most of the cytoplasmic tail (A281–A390; ΔCy) but with an inclusion of the posttransmembrane basic aa-rich region (K273–K280) was generated by PCR using PrimerStar HS DNA polymerase (TaKaRa), FcµR cDNA as a template DNA, and a set of primers: 5'-GAATTCTGAAGACCTACGGACCATGACG-3' and 5'-CAGGAGACATTGTTGAAGACATGCTAAGACTGCTAAGACTGCT-3' for Y385F (underlining indicates mutation sites). The amplified PCR products of the expected size were gel purified and subcloned into the ZeroBlunt TOPO vector (Invitrogen). All plasmid DNA encoding FcµR mutants were digested with appropriate restriction enzymes, gel purified, and ligated into the pMX-PIE retroviral expression vector as described (14). After confirming the correct sequences with the expected mutation of the resultant cDNAs at both strands of DNA, they were transduced into 293TA packaging cell line before transducing Jurkat cells as described (14). GFP+ transductants were enriched by FACS before IgM binding, apoptosis, and Ca2+ mobilization analyses.

Epifluorescence microscopic analysis

Viable cells were incubated for 20 min on ice with Alexa Fluor 555–labeled, affinity-purified mouse monoclonal IgM (MD4 anti-hen egg lysozyme mAb) at 10 μg/ml in PBS with or without 0.1% NaN3, washed in PBS/1% FCS/0.1% NaN3, and cytospun onto glass slides prior to being air-dried and fixed in 95% ethanol/5% glacial acetic acid at −20°C. Some aliquots of cells were resuspended in warmed RPMI 1640/10% FCS and incubated for an additional 10 min at 37°C before cytospin centrifugation. The stained cells were examined under a Leica/Leitz DMRB microscope. Images were acquired with a Hamamatsu C4742 video camera and processed with Openlab software.

Results

Interaction of FcµR with soluble IgM immune complexes versus IgM bound to membrane components via the Fab region

In our previous studies apoptosis-prone human Jurkat cells stably expressing FcµR were shown to be protected from Fas/CD95-mediated apoptosis when ligated with an agonistic IgM anti-Fas mb (CH11), but not when ligated with an agonistic IgG anti-Fas mAb or Fas ligand (14, 18). Notably, coligation of FcµR and Fas with the corresponding IgM Abs plus a common secondary reagent could not prevent IgG anti-Fas mAb-induced apoptosis (14). To determine whether this apoptosis protection mediated by FcµR is affected by IgM ligands, IgM mAbs specific for NP (clone B1-8) or α3-1 dextran (clone 1-21) and their immune complexes were initially used as inhibitors in the apoptosis assays. As expected, addition of IgM anti-Fas mAb at 10 ng/ml induced robust apoptosis of control GFP+ cells, but not GFP+/FcµR+ cells as determined by visualizing early (annexin V+/−-AAD+) and late (Annexin V+/−-AAD+) apoptotic cells (Fig. 1A, upper second column). The addition of both types of IgM mAbs at 10 ng/ml (upper third column; not shown for anti-dextran mAb) or their soluble immune complexes (upper fourth and lower first columns) did not affect the FcµR-mediated protection, suggesting the inability of such IgM molecules to block the interaction of the Fc portion of IgM Fas mb with FcµR. The failure to inhibit protection was observed with wide ranges of Ab/Ag molar ratios (Fig. 1B, Supplemental Fig. 1). However, addition of both types of IgM at 10 ng/ml (Fig. 1B, Supplemental Fig. 1) and their immune complexes with (Fig. 1A, lower second column, 1B, Supplemental
FIGURE 1. Role of FcμR in IgM anti-Fas mAb–induced apoptosis. (A) Representative flow cytometric profiles. Jurkat cells stably expressing GFP only (GFP) as a control or both GFP and human FcμR (GFP/FcμR) were cultured with (+) or without (−) agonistic IgM anti-human Fas mAb (clone CH11, 10 ng/ml) in the presence or absence of the inhibitors with the indicated concentrations (ng/ml) in parentheses: IgM anti-NP mAb (B1-8) or its soluble complexes with NP25-BSA, IgM anti-dextran mAb (1-21, not shown) or its soluble complexes with dextran, IgM anti-CD2 mAb (C373), and IgG2b blocking (bl; HM7) or nonblocking (nb; HM3) anti-FcμR mAb. The Ab/Ag molar ratios were 1:10 \(^2\) and 1:10 \(^3\) in the upper fourth and fifth columns and 1:1 and 1:10 \(^2\) in the lower first and second columns. After culture, cells were stained with allophycocyanin–annexin V and 7-AAD before identification of early apoptotic (annexin V+/7-AAD\(^{-}\)), late apoptotic (annexin V+/7-AAD\(^{+}\)), and dead (annexin V\(^{-}\)/7-AAD\(^{-}\)) cells by flow cytometric analysis. Numbers in each quadrant indicate percentages of cells. (B) Effects of IgM and its immune complexes on FcμR-mediated antiapoptotic activity. GFP+ (open columns) or GFP+/FcμR+ (filled columns) cells were cultured with IgM anti-Fas mAb (10 ng/ml) in the presence of the indicated concentrations (ng/ml) of IgM anti-NP mAb alone or with NP25-BSA as indicated for Ab/Ag molar ratios. Apoptotic (early and late) cells were similarly assessed and percentage relative apoptosis was determined as follows: 100 \(\times\) [(% anti-Fas–induced apoptotic cells with or without inhibitors in GFP\(^{+}\) or FcμR\(^{+}\)/GFP\(^{+}\) cell line) − (% spontaneous apoptotic cells in the corresponding cell line)] \(\div\) [(% anti-Fas mAb–induced apoptotic cells without inhibitors in GFP\(^{+}\) cell line) − (% spontaneous apoptotic cells in GFP\(^{+}\) cell line)]. Results are shown as means ± 1 SD from three to five independent experiments. The dotted line indicates percentage apoptosis of FcμR\(^{+}\)/GFP\(^{+}\) cells in the presence of IgM anti-Fas mAb without inhibitors. (C) Efficient inhibition of IgM anti-CD2 mAb in FcμR-mediated antiapoptotic activity. GFP\(^{+}\) (open columns) or GFP\(^{+}\)/FcμR\(^{+}\) (filled columns) cells were similarly cultured with the indicated doses (ng/ml) of IgM anti-CD2 mAb along with IgM anti-Fas mAb (10 ng/ml) before assessment of apoptotic cells. Results are shown as means ± 1 SD from three independent experiments. The dotted line indicates percentage apoptosis of FcμR\(^{+}\)/GFP\(^{+}\) cells in the presence of IgM anti-Fas mAb without IgM anti-CD2 mAb. Results using a Student \(t\) test comparison of anti-Fas mAb–induced apoptosis in the absence and presence of inhibitors are indicated as \(*p<0.05, **p<0.01, and ***p<0.001.\)
Fig. 1) partially but significantly blocked the FcμR-mediated protection. All three IgM preparations (CH11, B1-8, and 1-21) were found to bind equally to FcμR cells (not shown), ruling out the possibility that the CH11 IgM has a unique posttranslational modification to bond more efficiently the FcμR compared with others. In contrast, addition of FcμR-blocking IgG2b mAb (HM7), but not FcμR nonblocking IgG2b mAb (HM3), at 10^4 ng/ml specifically inhibited the interaction of the Fc portion of IgM Fas mAb with FcμR, thus permitting the FcμR+ cells to become apoptotic (Fig. 1A, lower fourth and fifth columns). These findings indicate that soluble IgM immune complexes are not good competitors in the interaction of FcμR with the Fc portion of IgM anti-Fas mAb, although FcμR is shown by Scatchard plot analysis to bind pentameric IgM with a surprisingly high avidity of ~10 nM (14). This also suggests the possibility that FcμR may bind more efficiently to the Fc portion of IgM, which is attached to plasma membranes via its Fab region, than to the Fc portion of free IgM or its immune complexes in solution.

To test this hypothesis, we employed additional IgM mAbs reactive with CD2 or TCR on the surface of Jurkat cells as potential competitors for the interaction of IgM Fas mAb with FcμR. Addition of IgM anti-CD2 mAb (C373) at 10^4 ng/ml efficiently inhibited FcμR-mediated protection, thereby permitting the FcμR+ cells to undergo apoptosis (see Fig. 1A, lower third column, 1C). The anti-CD2 mAb alone did not induce apoptosis of either of cell types (not shown). Similar results were also obtained with an IgM anti-TCR mAb (C305, not shown). Thus, these findings suggest that FcμR binds more efficiently to IgM mAbs, which attach to cell surface components via their Fab regions, than to IgM immune complexes in solution.

Cis engagement of FcμR with IgM anti-Fas mAb on the same cell surface

To determine whether the engagement of FcμR with IgM attached to plasma membrane occurs in trans or cis (see Fig. 2A), we performed mixed cell cultures of FcμR+ (or control) Jurkat cells plus a 10-fold excess of mouse BW5147 cells stably expressing human FcμR. When the Fc portion of IgM anti-Fas mAb binds FcμR in trans between the excess of cocultured FcμR+ BW5147 cells, then FcμR+ Jurkat cells will become apoptotic. Alternatively, when the Fc portion of IgM anti-Fas mAb must bind FcμR in cis on the same cell surface to perform this protective function, FcμR+ Jurkat cells will still be protected against IgM anti-Fas mAb-mediated apoptosis even in the presence of a 10-fold excess number of FcμR+ BW5147 cells. As shown in Fig. 2B, addition of excessive FcμR+ BW5147 cells did not affect the FcμR-mediated protection from apoptosis (second versus fourth column), suggesting predominance of the protective mechanism involving a cis interaction of the Fc portion of IgM anti-Fas mAb with FcμR on the same cell surface. Addition of excessive FcμR+ BW5147 cells did not spontaneously induce apoptosis of either of FcμR+ or control Jurkat cells in the absence of anti-Fas mAb (third column). Collectively, these findings show that although FcμR binds soluble IgM pentamers at a high avidity (14), FcμR binds more efficiently to the Fc portion of IgM Ab when it reacts with a membrane component on the same cell surface. This finding implies that FcμR can modulate the functional activity of lymphocyte cell surface receptors or molecules recognized by either natural or immune IgM Abs.

Calcium mobilization by coligation of FcμR with CD2 or BCR by IgM mAbs

To explore this possibility, we compared Ca2+ mobilization in cells with a single ligation of CD2 or TCR versus the coligation of both FcμR and CD2 or TCR. An equal mixture of WT and FcμR+ Jurkat cells was preloaded with the Ca2+ dye Indo-1/AM and simultaneously stimulated by various concentrations of IgM mAbs specific for CD2 or TCR. Although it has been well documented that induction of CD2-mediated Ca2+ mobilization in T cells requires ligation of CD2 with two different IgG mAbs (23–25), the IgM anti-CD2 mAb (C373) alone clearly induced Ca2+ mobilization (Fig. 3A). Notably, the rise in [Ca2+]i occurred signifi-
cantly faster in FcR\(^{+}\) cells than in WT cells when ligated with the anti-CD2 mAb at three different concentrations (3, 10, and 30 \(\mu\)g/ml, data at 3 and 30 \(\mu\)g/ml are not shown). A similar difference, but to a much less extent, was also observed with the cross-linkage of TCR with IgM mAb (C305) (not shown). In contrast, the ionomycin-induced [Ca\(^{2+}\)]\(_i\) rise occurred at the same time in both cell types. These findings suggest that the coligation of FcR\(\mu\) with CD2 or TCR induces a more rapid increase in [Ca\(^{2+}\)]\(_i\), than does the ligation of CD2 or TCR alone.

Because we found that freshly prepared blood T cells, unlike Jurkat cells, were not activated by the C373 anti-CD2 mAb (not shown), we switched to using a mitogenic IgM anti-\(\kappa\) mAb (END-SC1) and examined Ca\(^{2+}\) mobilization induced by BCR cross-linkage in the presence of FcR\(\mu\)-blocking (HM7) or nonblocking (HM3) mAbs. As shown in Fig. 2B, IgM anti-\(\kappa\) mAb–induced Ca\(^{2+}\) mobilization was indistinguishable in the absence or presence of FcR\(\mu\) nonblocking mAb (HM3). In contrast, the presence of FcR\(\mu\)-blocking mAb (HM7) diminished the IgM anti-\(\kappa\) mAb–induced Ca\(^{2+}\) mobilization, suggesting that FcR\(\mu\) provides a stimulatory signal upon BCR cross-linkage with IgM mAbs.

**Key residues in the FcR\(\mu\) transmembrane and cytoplasmic tail responsible for receptor functions**

In FcRs and in other paired Ig-like receptors, it is generally observed that when the ligand-binding \(\alpha\)-chain has a short cytoplasmic tail with no signal-transmitting potential, then it contains a charged residue in the transmembrane segment that facilitates noncovalent association with another transmembrane protein—containing ITAMs. This association allows for the transmission of activating signals to cells as seen in Fc\(\gamma\)RI, Fc\(\gamma\)RII, Fc\(\varepsilon\)RI, and Fc\(\varepsilon\)RII. Alternatively, when the ligand-binding \(\alpha\)-chain has a conventional hydrophobic transmembrane segment, then it usually has a long ITAM-containing cytoplasmic tail (26). This recruits the phosphatase SHIP upon phosphorylation to attenuate the signals as in Fc\(\gamma\)RII. However, FcR\(\mu\) is unusual in having both features: a charged His residue (H253) in the transmembrane region and a long cytoplasmic tail containing three conserved Tyr (Y315, Y366, Y385) and five conserved Ser residues, features that have been conserved in multiple species (see Fig. 4A) (14). These characteristics suggest a dual signaling ability of FcR\(\mu\): one from a potential adaptor protein noncovalently associating with the FcR\(\mu\) via the H253 residue, and the other from its own Tyr and/or Ser residues in the cytoplasmic tail.

To determine which part of the FcR\(\mu\) molecule is responsible for these potential functions, we made FcR\(\mu\) cDNA constructs with point mutations (H253F, Y315F, Y366F, or Y385F) or a deletion of most of the cytoplasmic tail (A281–A390; \(\Delta\)Cy) and expressed them in Jurkat T cells. After enriching GFP\(^{+}\) cells by FACS, the surface expression of FcR\(\mu\) as determined by reactivity with receptor-specific mAbs was comparable among the nonmutant and mutant FcR\(\mu\) transductants (Fig. 4B), suggesting that any introduced mutation does not affect the surface expression of FcR\(\mu\) on Jurkat cells. Remarkably, however, the IgM ligand-binding activity was significantly increased by the \(\Delta\)Cy mutant (Fig. 4C, 4D), suggesting the influence of the FcR\(\mu\) cytoplasmic tail on its ectodomain-mediated ligand binding.

Next, the fate of IgM ligands after binding to FcR\(\mu\) on these transductants was examined by epifluorescence microscopy. IgM binding at 4˚C in the presence of Na\(\mathrm{N}\)O\(_3\), an inhibitor of energy-dependent contraction of actin filament, was observed in a weak patchy staining pattern at the peripheral rim of transductants regardless of their mutations. However, when incubated at 4˚C without Na\(\mathrm{N}\)O\(_3\), distinct IgM binding, marked by a more intense and localized or capping pattern, was observed with the H253F mutant (Fig. 4Eb) as compared with all others except for the \(\Delta\)Cy mutant, which exhibited a more broadly localized capping pattern (Fig. 4Ea for nonmutated). This suggests an important role of the His\(^{253}\) residue in the anchoring of FcR\(\mu\) in the transmembrane. After washing and incubating at 37˚C for 10 min without Na\(\mathrm{N}\)O\(_3\), the IgM/FcR\(\mu\) complexes were internalized and localized in intracellular locations, presumably endosomal or lysosomal compartments, in the FcR\(\mu\) nonmutated (Fig. 4Ec), H253F (Fig. 4Ed), and Y315F transductants (not shown), but not in other mutants (Y366F, Y385F, and \(\Delta\)Cy, not shown). These results are consistent with previous findings reported by others that two C-terminal–conserved Tyr residues were involved in receptor-mediated endocytosis (15).

The FcR\(\mu\)-mediated protection from apoptosis was significantly diminished in the FcR\(\mu\) Y315F and \(\Delta\)Cy mutants, as the frequencies of apoptotic cells in these mutants were indistinguishable from those in the control transductant (Fig. 4F). Other FcR\(\mu\) mutants (H253F, Y366F, and Y385F) still had significant protective activity compared with controls (GFP\(^{+}\)), but the extent of their protective activity was significantly lower than that of the nonmutated FcR\(\mu\). These findings suggest that the membrane-proximal Tyr residue (Y315) is largely responsible for, but other Tyr residues also contribute minimally to, the FcR\(\mu\)-mediated protection. Notably, the rapid rise in IgM anti-CD2 mAb–induced [Ca\(^{2+}\)]\(_i\), observed in the FcR\(\mu\) nonmutant was abolished by all of the FcR\(\mu\) mutants, suggesting that both the cytoplasmic tail including all three Tyr residues and the transmembrane His residue are involved in signaling mediated by coligation of CD2 and FcR\(\mu\).

**Discussion**

The present study is focused on the IgM ligand-binding property of FcR\(\mu\), and several remarkable features of the FcR\(\mu\) have been identified. First, although FcR\(\mu\) binds soluble pentameric IgM at a relatively high avidity of \(\sim 10\) nM (14), FcR\(\mu\) actually interacts more efficiently with the Fc portion of IgM Abs when bound to plasma membranes via their Ag-binding Fab regions, and this interaction occurs in cis on the same cell surface. This conclusion is based on the functional assays rather than actual ligand-binding assessments. Second, the Ca\(^{2+}\) mobilization induced by cross-linkage of CD2 or BCRs with IgM mAbs differs between the ligation of CD2 or BCR alone and the coligation of FcR\(\mu\) and CD2 or BCR. Third, several remarkable functional changes are observed.

**FIGURE 3.** Ca\(^{2+}\) mobilization by IgM mAbs against CD2 or Ig \(\kappa\). (A) An equal mixture of WT (GFP\(^{+}\), blue lines) and FcR\(\mu\)–GFP\(^{+}\) (red lines) Jurkat cells preloaded with Ca\(^{2+}\) dye Indo-1/AM were stimulated with IgM anti-CD2 (C373) mAb at 10 \(\mu\)g/ml or by 1 \(\mu\)M ionomycin at the time point indicated by arrows. The [Ca\(^{2+}\)]\(_i\) levels were assessed by the 405/485-nm fluorescence ratio in each viable cell population using an LSR II flow cytometer. This is a representative result from four independent experiments. (B) Fluo-4–loaded blood B cells were treated with IgM anti-human \(\kappa\) mAb (END-SC1, 10 \(\mu\)g/ml) in the absence (red line) or presence of IgG2b, FcR\(\mu\)–blocking HM7 (blue line), or nonblocking HM3 (green line) mAb (100 \(\mu\)g/ml) at the time point indicated by an arrow. The [Ca\(^{2+}\)]\(_i\) levels were assessed by fluorescence intensity during a 5-min period. Representative result from three independent experiments.
with FcμR mutants: 1) increased IgM ligand binding by the FcμR ΔCy mutant, 2) enhanced cap formation of the FcμR H253F mutant even at 4°C upon IgM ligand binding, and 3) less protective activity of the FcμR Y315F mutant in IgM anti-Fas mAb–mediated apoptosis.

The preferential interaction of FcμR with IgM attached to plasma membrane via its Fab region was initially notified in our previous reconcilement of antiapoptotic activity of Toso, the plasma membrane via its Fab region was initially notified in our previous reconcilement of antiapoptotic activity of Toso, the plasma membrane via its Fab region was initially notified in our previous reconcilement of antiapoptotic activity of Toso, the plasma membrane via its Fab region was initially notified in our previous reconcilement of antiapoptotic activity of Toso, the plasma membrane via its Fab region was initially notified in our previous reconcilement of antiapoptotic activity of Toso, the plasma membrane via its Fab region was initially notified in our previous reconcilement of antiapoptotic activity of Toso, the plasma membrane via its Fab region was initially notified in our previous reconcilement of antiapoptotic activity of Toso, the plasma membrane via its Fab region was initially 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In the present study, we have defined more quantitatively the ligand-binding property of FcμR. The interaction of FcμR with the Fc portion of IgM anti-Fas mAb in Jurkat cells was not inhibited by addition of 10^2-fold excess of IgM mAbs or their soluble immune complexes, but it was blocked by addition of 10^3-fold excess of IgM mAbs or immune complexes, 10^3-fold excess of FcμR-blocking mAb (HM7, γ2bc isotype), or 10-fold excess of IgM anti-CD2 or anti-TCR mAb binding to the Jurkat cells. The interaction of FcμR with the Fc of IgM anti-Fas mAb occurred in cis on the same cell surface and not in trans between neighboring cells, as evidenced by the fact that adding 10-fold excess of FcμR+ BW5147 cells (of mouse origin) did not affect FcμR-mediated protection from IgM anti-Fas mAb–induced apoptosis. The preferential cis engagement of FcμR is thus distinct from the trans engagement of FcγRIIB, an inhibitory FcγR, in death receptor–mediated apoptosis. The interaction of agonistic IgG mAbs against death receptors, including Fas/CD95, with FcγRIIB is essential for the death receptor–mediated apoptosis and occurs in trans, but not in cis (27–29). The cis engagement of FcμR is significant in that FcμR has the po-

FIGURE 4. Effects of FcμR mutations on receptor function. (A) Predicted protein structure of FcμR. The human FcμR cDNA encodes type I transmembrane protein that consists of a single V-set Ig-like domain (oval shape), an additional extracellular region with no known structure, a transmembrane segment (between two thick lines) containing a charged His residue (●), and a relatively long cytoplasmic tail containing three conserved Tyr residues (○). Point mutations are indicated and the extent of the deletion of the cytoplasmic tail is shown by the black bracket. Hatch marks indicate exon boundaries in the FCMR gene. (B-E) Cell surface levels and IgM ligand binding. Jurkat cells stably expressing only GFP (Cont.) or both GFP and FcμR were stained with biotin-HM14 anti-FcμR mAb or PBS (shaded) for ligand binding (B) and for IgM ligand-binding activity (C). The mean fluorescence intensity (MFI) of FcμR levels was determined as MFI of anti-FcμR mAb minus MFI of control mAb (B) and the MFI of IgM binding as MFI of IgM minus MFI of PBS (C). Results are shown as the MFI ± 1 SD from three to five independent experiments. *p < 0.05 when compared with nonmutated (NM) FcμR. (D) Representative flow cytometric profiles. FcμR NM (blue) and ΔCy (red) cells were stained with biotin-HM14 anti-FcμR (open) or control (shaded) mAb for cell surface expression of FcμR (left) and with biotin-IgM myeloma (open) or PBS (shaded) for ligand binding (right). Because profiles with isotype-matched mAb or PBS were the same between FcμR NM and ΔCy cells, only one shaded profile is shown. (E) Representative epifluorescence microscopic images. The FcμR NM (a and c) and H253F (b and d) cells were incubated with Alexa Fluor 555–IgM (without NaN3) on ice (a and b), washed, and then incubated for additional 10 min at 37°C (c and d) before cytacentrifugation. Fluorescence images were combined with phase-contrast cell images. Scale bars, 10 μm. (F) FcμR-mediated protection from apoptosis. FcμR NM or mutant and control Jurkat cells were incubated with CH11 anti-Fas mAb as described in the Fig. 1 legend, and the frequencies of early and late apoptotic cells are plotted as means ± 1 SD from three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 when compared with FcμR/GFP+ control cells.
potential to modulate the functional activity of lymphocyte surface receptors on the same cell when they are recognized by natural or immune IgM Abs. The physiological relevance of this cist engagement may be related to unique features of the IgM ligand and its receptor. In idiotype network theory, BCRRs (or their secreted products) are proposed to be directed toward the variable regions of other BCRRs or their Ig molecules. Two thirds of the newly generated B cells in bone marrow are now known to react with self-antigens such as dsDNA, ssDNA, insulin, and LPS (30). According to our estimates, approximately a fourth of the IgM-containing culture supernatants from 96 different EBV-transformed neonatal B cell lines react with lymphocyte surface components (H. Kubagawa, K. Honjo, and Y. Kubagawa, unpublished observations). IgM anti-lymphocyte Abs are often present in individuals with autoimmune diseases or chronic viral infections, such as HIV-1, and recognize many different surface Ags (e.g., CD45, CD175/Tn, CD3ε, CD4, chemokine receptors, sphingosine-1-phosphate receptor 1), and some of those Abs regulate T cell–mediated inflammatory responses in vitro (31–41). Unlike FCs for switched Ig isotypes, the expression of FCμR is restricted to lymphocytes, namely, B, T, and NK cells (14). Therefore, it is highly probable that FCμR on B, T, and NK cells preferentially binds the Fc portion of IgM Ab reactive with their cell surface Ags or receptors, thereby modulating the function of their target Ags or receptors. Supporting this idea, the CD2-mediated Ca2+ mobilization after cross-linkage with IgM mAb was clearly faster in FCμR+ than control Jurkat cells. The Ca2+ mobilization induced by cross-linkage of BCR on blood B cells with IgM anti-Ig mAb was significantly different in the presence of an FCμR blocker (HM7) versus an FCμR nonblocker (HM3). The HM7 blocker diminished BCR-mediated Ca2+ mobilization, whereas the HM3 nonblocker did not. Unlike the coligation of FCμR and Fas receptor, FCμR provides a stimulatory signal upon coligation with CD2 or BCR.

Mutational analyses of FCμR revealed several remarkable alterations in its function. Whereas nonmutated and mutant FCμR transductants expressed comparable levels of cell surface FCμR as judged by receptor-specific mAbs, the IgM ligand-binding activity was significantly increased in the ΔCy mutant. This suggests either the formation of oligomeric FCμR due to its presumably mobile nature within the plasma membrane or the inside-out regulation of ligand-binding activity of FCμR by its cytoplasmic tail. Given the precedent of inside-out signaling regulation in the integrin family of cell surface adhesion molecules (42) and the FcRα integrin family of cell surface adhesion molecules (42) and the tail. Given the precedent of inside–out signaling regulation in the

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15. Van, B., A. David, and A. Westner, 2011. Toso, the FcμRα receptor, is highly expressed on chronic lymphocytic leukemia B cells, internalizes upon IgM binding, shuttles to the lysosome, and is downregulated in response to TLR activation. J. Immunol. 187: 4040–4050.

Acknowledgments
We thank Dr. Arthur Weiss for the gift of IgM mAbs specific for CD2 (C373) or Jurkat TCR (C305) and for valuable suggestions, Dr. Peter Burrows for critical reading of the manuscript, Enid Keyser for FACS sorting, and Marion Spell for Ca2+ mobilization.

Disclosures
The authors have no financial conflicts of interest.

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SUPPLEMENTAL FIGURE 1. Effects of IgM anti-dextran mAb and its immune complexes on FcμR-mediated anti-apoptotic activity. Control GFP+ (white columns) or FcμR+/GFP+ (black columns) Jurkat cells were cultured with the indicated concentrations (ng/ml) of IgM anti-dextran mAb (clone 1-21) along with or without α1-3 dextran Ag as indicated Ab/Ag molar ratios in the presence of agonistic IgM anti-Fas mAb (clone CH11; 10 ng/ml). Apoptotic (early and late) cells were assessed and % relative apoptosis was determined as described in the Fig. 1 legend. Results are shown as the mean ± 1 SD from 3 – 7 independent experiments. The increase in apoptosis in even control cells at 10^5 ng/ml may be due to some toxic effects in the IgM anti-dextran mAb preparation or α1-3 dextran. * P < 0.05, ** P < 0.01, and *** P < 0.001, respectively.