Molecular mechanism of IgM perception by IgM-specific receptor FcμR

Junyu Xiao (junyuxiao@pku.edu.cn)
Peking University  https://orcid.org/0000-0003-1822-1701

Yaxin Li
Peking University

Hao Shen
School of Life Sciences, Peking University
https://orcid.org/0000-0003-1083-6357

Ruixue Zhang
School of Life Sciences, Peking University

Yuxin Wang
Peking University

Chenggong Ji
Peking University

Chen Su
Peking University

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Molecular mechanism of IgM perception by IgM-specific receptor FcμR

Yaxin Li¹,²,⁵, Hao Shen¹,²,⁵, Ruixue Zhang³,⁵, Yuxin Wang¹,², Chenggong Ji¹,², Chen Su¹,², Junyu Xiao¹,²,⁴*

¹State Key Laboratory of Protein and Plant Gene Research, Peking University, Beijing, China.
²School of Life Sciences, Peking University, Beijing, China.
³Academy for Advanced Interdisciplinary Studies, Peking University, Beijing, China.
⁴Peking-Tsinghua Center for Life Sciences, Peking University, Beijing, China.
⁵These authors contributed equally to this work.

*Correspondence: Junyu Xiao (junyuxiao@pku.edu.cn).
Abstract

Immunoglobulin M (IgM) is the first antibody to emerge during embryonic development and humoral immune response\(^1\). IgM can exist in several distinct forms, including monomeric, membrane-bound IgM (mIgM) within the B-cell receptor (BCR) complex, pentameric and hexameric IgM in serum, and secretory IgM on mucosal surface. Fc\(\mu\)R/Toso/Faim3, the only IgM-specific receptor in mammals, recognizes different forms of IgM to regulate diverse immune responses\(^2\)-\(^4\). However, the underlying molecular mechanisms remain unknown. Here we delineate the structural bases of Fc\(\mu\)R-IgM interaction by crystallography and cryo-electron microscopy. We show that two Fc\(\mu\)R molecules interact with a C\(\mu\)4 dimer, suggesting that Fc\(\mu\)R can bind to mIgM with 2:1 stoichiometry. Further analyses reveal that both Fc\(\mu\)R-binding sites are accessible in the context of IgM-BCR. In contrast, the pentameric IgM can recruit four Fc\(\mu\)R to bind on the same side and therefore impose the formation of an Fc\(\mu\)R oligomer. One of these Fc\(\mu\)R molecules occupies the binding site of the polymeric immunoglobulin receptor/secretory component (pIgR/SC). Nevertheless, four Fc\(\mu\)R are recruited onto the other side of IgM in the presence of SC, which reconciles with, and further supports Fc\(\mu\)R's function in the retro-transport of secretory IgM. These results reveal intricate mechanisms of IgM perception by Fc\(\mu\)R.
Main

Among the five classes of antibodies in human, immunoglobulin M (IgM) is the first to emerge during embryonic development and humoral immune response. The predominant form of IgM in serum is a pentameric assembly (pIgM), with five IgM monomers joined together by the joining chain (J-chain). In the absence of J-chain, hexameric IgM can be formed. An Fc tailpiece plays a vital role in IgM polymer formation and J-chain incorporation. J-chain also facilitates the transport of pIgM to the mucosal surface by the polymeric immunoglobulin receptor (pIgR). On the other hand, the monomeric, membrane-bound IgM (mIgM) can serve as an integral component of the B-cell receptor (BCR) complex to function in antigen recognition and B-cell activation. A transmembrane segment is present in mIgM instead of the tailpiece for membrane localization.

Different immunoglobulin classes feature different Fc regions, which are perceived by specific Fc receptors to elicit distinct effector mechanisms. The aforementioned pIgR is a unique Fc receptor. It selectively recognizes the J-chain-containing pIgM, as well as the J-chain-containing dimeric/tetrameric/pentameric IgA, and then escort them to the mucosa. The ectodomain of pIgR, known as the secretory component (SC), remains bound to pIgM, resulting in the formation of the so-called secretory IgM (SIgM) complex. Two other IgM Fc receptors are present in human besides pIgR: FcαμR and FcμR. Both of them contain an immunoglobulin-like domain that resembles the D1 domain of pIgR, which is responsible for interacting with pIgM. Nevertheless, they function in distinct manners. FcαμR is involved in the internalization of IgM-coated microbes; and similar to pIgR, it binds both IgM and IgA. FcμR, on the other hand, binds IgM exclusively and is the only IgM-specific receptor identified to date.

Previously known as Toso/Faim3, FcμR was first identified as an inhibitor of Fas-mediated apoptosis in T cells, before it was demonstrated to be the long-sought Fc receptor for IgM. FcμR knockout mice produce a myriad of phenotypes, suggesting that FcμR has critical functions in B-cell development and maturation, and immune tolerance (reviewed in [19,20]). FcμR is also involved in T cell activation and Th17 cell pathogenicity. Besides these lymphocyte-related functions, FcμR has also been implicated in the activation of myeloid cells and their responses to cancer, although this functional aspect is under debate. In addition, FcμR has a role in mucosal immunity, as it can convey the retro-transport of SIgM-antigen
complexes back from the mucosa to elicit immune responses \(^4\). Finally, Fc\(\mu\)R is highly expressed on chronic lymphocytic leukemia (CLL) B-cells \(^2,28-31\); and targeting Fc\(\mu\)R using either an IgM-toxin conjugate \(^32\) or the engineered chimeric antigen receptor T cells \(^33\) could provide new CLL treatment strategies.

Interestingly, Fc\(\mu\)R appears to be able to interact with all forms of IgM, including both pentameric and hexameric IgM, as well as mIgM within BCR \(^2,3,34,35\). It is involved in the internalization and transport of soluble IgM-antigen complexes to the lysosome, but not BCR, suggesting that it binds differently to polymeric and monomeric IgM \(^3,31,34\). Furthermore, as Fc\(\mu\)R can carry out the retro-transport of SC-containing SIgM \(^4\), it can bind pIgM together with pIgR/SC. Here we investigate the molecular mechanism of Fc\(\mu\)R-IgM interaction.

**Crystal structure of the Fc\(\mu\)R-D1:Fc\(\mu\)-C\(\mu\)4 complex**

The extracellular domain of Fc\(\mu\)R contains an immunoglobulin-like domain (Fc\(\mu\)R-D1) followed by an intrinsically disordered, highly O-linked glycosylated stalk region (Fig. 1a). We first present the crystal structure of Fc\(\mu\)R-D1 in complex with the Fc\(\mu\)-C\(\mu\)4 domain at 3.0 Å resolution (Fig. 1b, Extended Data Table 1). Surface plasmon resonance (SPR) experiment demonstrate that Fc\(\mu\)R-D1 binds Fc\(\mu\)-C\(\mu\)4 with high affinity, exhibiting a K\(_d\) value of 3.8 nM (Fig. 1c). The structure reveals a 2:1 assembly, in which two Fc\(\mu\)R-D1 bound to an Fc\(\mu\)-C\(\mu\)4 dimer from both sides. Fc\(\mu\)R-D1 adopts a typical immunoglobulin fold, and can be superimposed to pIgR/SC-D1 with an overall root-mean-square deviation of 1.3 Å. The interaction between Fc\(\mu\)R-D1 and Fc\(\mu\)-C\(\mu\)4 buries a total of 850 Å\(^2\) surface, and mainly focuses on Fc\(\mu\)-C\(\mu\)4 residues Asn465-Glu468 and Glu526 (Fig. 1d). For example, Arg45\(_{Fc\mu R}\) from the CDR-like loop 1 (CDR1) of Fc\(\mu\)R forms an ion pair with Glu468. In CDR2, Phe67\(_{Fc\mu R}\) packs against the aliphatic portion of Glu468, whereas Lys69\(_{Fc\mu R}\) coordinates Glu526. Thr110\(_{Fc\mu R}\) from CDR3 form hydrogen bonds with the main chain groups of Asn465 and Leu466, whereas Asp111\(_{Fc\mu R}\) interacts with Arg467. Thr57\(_{Fc\mu R}\) and Thr60\(_{Fc\mu R}\) from the C\(^\prime\) strand of Fc\(\mu\)R also mediate hydrogen bond contacts with Leu466-Arg467. A comparison of human Fc sequences shows that Asn465-Glu468 and Glu526 are uniquely present in Fc\(\mu\), rationalizing the specificity of Fc\(\mu\)R for Fc\(\mu\) (Extended Data Fig. 1a).

The binding mode between Fc\(\mu\)R-D1 and Fc\(\mu\)-C\(\mu\)4 is unique among the Fc-FcR complexes (Fig. 1e). The Fc receptors for IgG, including Fc\(\gamma\)RI/CD64, Fc\(\gamma\)RII/CD32, and
FcγRIII/CD16, form a 1:1 complex with Fcγ and bind to the lower hinge region on top of Cγ2. FcεRI, the IgE receptor, binds to Fcε in a similar fashion. In contrast, the IgA-specific receptor FcαRI/CD89 forms a 2:1 complex with Fcα and targets the Ca2-Cα3 junction. Here FcμR also binds Fcμ with a 2:1 stoichiometry; nonetheless, it interacts with the lower half of Cμ4 and approaches the Cμ4 dimer from a completely different angle. Notably, the C-termini of the two FcμR and two Fcμ molecules are located on the same side within the FcμR-D1:Fcμ-Cμ4 complex. Therefore, this structural arrangement would enable the interaction between FcμR and mIgM on the B-cell membrane. The long-awaited structure of IgM-BCR has been recently unveiled. Superposing the structure of the FcμR-D1:Fcμ-Cμ4 complex onto that of IgM-BCR reveal that the FcμR-binding site next to Igβ is completely exposed (Fig. 1f). The binding site next to Igα is largely accessible as well, although an Asn97-glycan on Igα may slightly interfere with the positioning of FcμR at this location (Fig. 1g).

Cryo-EM structure of the FcμR:Fcμ-J complex

Circulating IgM is present as pentameric and hexameric assemblies, and cell-based assay suggests that FcμR can bind to both forms with nanomolar affinity/avidity. Indeed, immobilized FcμR-D1 absorbs Fcμ-J, i.e., the pentameric IgM core containing an Fcμ pentamer and the J-chain, with extremely high affinity/avidity, with an apparent Kd of ~0.2 nM (Fig. 2a). The presence of antigen-binding fragments has no influence on this tight interaction, as FcμR-D1 binds to a recombinant full-length IgM (anti-CD20) with similar affinity. To gain further insights into the structural basis of FcμR-IgM interaction, we assembled the complex consisting of the entire ectodomain of FcμR and Fcμ-J, and performed cryo-electron microscopy (cryo-EM) analyses (Extended Data Fig. 2-3, Extended Data Table 2). Both 1:1 and 4:1 FcμR:Fcμ-J complexes were observed in the cryo-EM data, and their structures were separately determined at 3.4 and 3.7 Å resolutions, respectively.

In the 1:1 structure, a single FcμR-D1 occupies almost the same site on Fcμ-J as the D1 domain of pIgR/SC in SIgM. Similar to pIgR/SC-D1, this FcμR-D1 interacts with multiple regions in Fcμ-J. First, it binds to the Cμ4 domain of Fcμ1B in a similar manner as seen in the crystal structure. Here the ten Fcμ chains in the Fcμ pentamer are labeled as previously described, with the Fcμ chain attached to the C-terminal hairpin of J-chain as Fcμ1A. There is
no contact between FcμR-D1 and the Cμ3 domains or the Cμ3-Cμ4 junction, although the Cμ3 domains can be clearly visualized in the density map. This FcμR-D1 also interacts with the C-terminal region of the J-chain. For example, Met42_{FcμR}-His43_{FcμR} appear to pack on Tyr134_{J} (Extended Data Fig. 3j). Finally, Arg112_{FcμR} contacts the tailpiece of Fcμ5B (Extended Data Fig. 3k). Together, this composite interface involving residues from multiple chains ensures the highly selective binding of FcμR-D1 at this site (R1 site).

In the 4:1 FcμR:Fcμ-J structure, four FcμR molecules are arranged into a semi-circle, with one binding at the R1 site, whereas the other three binding sequentially to Fcμ2-4 (R2-R4 sites, Fig. 2c). Binding of FcμR to Fcμ5 is prevented by the β2-β3 loop of the J-chain. At the R2-R4 sites, FcμR mainly interacts with the Cμ4 domains of Fcμ2B, Fcμ3B, and Fcμ4B as in the crystal structure. The C-C' loop of Fcμ1B, Fcμ2B, and Fcμ3B from the adjoining Fcμ units also contributes slightly to the binding (Extended Data Fig. 3l). Importantly, the four FcμR molecules all bind on the same side of Fcμ-J. No particle class with FcμR molecules on both sides of Fcμ-J has been observed, despite the fact that FcμR is present in large excess during sample preparation (Extended Data Fig. 2-3). This is in sharp contrast to the crystal structure, in which two FcμR bind to both sides of the Cμ4 dimer. There is no significant contact among the four FcμR-D1 domains. Nevertheless, prominent densities are present both within the FcμR-D1 semi-circle and also below the FcμR-D1 layer (Fig. 2d). These densities could not be unambiguously modeled, but most likely belong to the stalk regions of FcμR, as the entire ectodomain of FcμR is used in the cryo-EM study. It is likely that the stalk region bridges intermolecular interactions between the four FcμR molecules and facilitate their binding on the same side of Fcμ-J.

**Cryo-EM structure of the FcμR:Fcμ-J-SC complex**

The fact that the R1 site overlaps with the binding site of pIgR/SC-D1 raises the interesting question of how FcμR enables the retro-transport of SC-containing SIgM. First, we confirm that FcμR can indeed bind to the core SIgM, which consists of Fcμ-J plus SC (Fig. 3a). Then we determined the cryo-EM structure of FcμR in complex with Fcμ-J-SC at 3.2 Å (Extended Data Fig. 4, Extended Data Table 2). We initially thought that FcμR may bind to the R2-R4 sites in this complex. To our surprise, four FcμR molecules now bind to the opposite side of Fcμ-J (R1'-R4' sites, Fig. 3b). The binding environments for FcμR at the R2'-R4' sites are highly similar to those of R2-R4 on the other side, involving Asn465-Glu468 in Fcμ4A, Fcμ3A,
and Fcµ2A; as well as the C-C' loop of Fcµ5A, Fcµ4A, and Fcµ3A from the adjacent Fcµ units. R1' site is slightly different. Besides mainly targeting Asn465-Glu468 in Fcµ5A, Arg112_Fcµ also contacts J-chain Ser65_J-Asp66_J, as well as Glu570 in the tailpiece of Fcµ1A (Extended Data Fig. 4g).

Similar to the situation in the FcµR:Fcµ-J complex, only a maximum of four FcµR molecules are permitted to attach to Fcµ-J-SC. No FcµR is bound to Fcµ1A, even though the binding site appears exposed at first glance. Superposing an FcµR-D1:Fcµ-Cµ4 pair from the crystal structure to Fcµ1A suggests that binding of this 5th FcµR would lead to unfavorable packing with the R4' FcµR (Fig. 3c). Fcµ1A is tied to the C-terminal hairpin of the J-chain. It appears that this interaction leads to a slight tilting of Fcµ1 within the Fcµ-J plane. As a result, in order to bind to Fcµ1A, FcµR would have to approach the Fcµ-J platform with a slightly different angle when compared to the binding at the other sites, causing a clash with the R4' FcµR.

**FcµR mutants are defective in binding to IgM**

To assess the functional relevance of the molecular interactions described above, we first designed several FcµR-D1 mutants, and performed in vitro binding experiment. Indeed, the R45A mutant failed to pull down monomeric Fcµ (Fig. 4a). Similarly, R45A, F67A/K69A, N109A/R112A, R45A/F67A, R45A/R112A, and R45A/F67A/R112A all displayed greatly reduced or abolished interaction with Fcµ-J (Fig. 4b) and the Fcµ-J-SC complex (Fig. 4c). To further examine FcµR-IgM interaction in a cellular context, we fused wildtype (WT) FcµR or the R45A/F67A mutant to GFP and expressed them in HeLa cells. As shown in Fig. 4d, cells bearing WT FcµR readily absorbed Fcµ-J on surface, and also quickly internalized it at 37 °C, consistent with previous observations 13,31. In contrast, IgM binding and internalization were not observed in the R45A/F67A-expressing cells. We also generated HEK293T and Jurkat cell lines stably expressing WT FcµR or the R45A/F67A mutant. While their cell surface levels are similar, R45A/F67A-expressing cells captured significant less Fcµ-J, as evaluated by flow cytometry (Fig. 4e, Extended Data Fig. 5). Together, these data corroborate our structural analyses and demonstrate the critical roles of these FcµR residues in binding to IgM.

**Discussion**
Here we elucidate the structural bases of FcμR-IgM interaction, and our results shed light on how FcμR binds to mIgM, pIgM, and SIgM, respectively. We first show that FcμR can form a 2:1 complex with mIgM. Importantly, mIgM assembles with Igα/CD79A and Igβ/CD79B into the BCR complex, and plays a key role in B-cell activation. FcμR physically associates with IgM-BCR, and limits its cell surface delivery by retaining it in the Golgi apparatus. Indeed, a universal phenotype manifested by the Fcmr knockout mice is autoantibody production, demonstrating the critical function of FcμR in balancing BCR signaling. In light of the recently reported IgM-BCR structure, we show that the FcμR-binding sites are accessible in IgM-BCR (Fig. 1f). Future work is needed to determine how FcμR, particularly the stalk region and transmembrane segment of FcμR, mingles with Igα/Igβ on B-cell membrane to regulate BCR activity.

We further show that FcμR can form a 4:1 complex with both Fcμ-J and Fcμ-J-SC, albeit binding on opposite faces of the Fcμ-J platform. Among the eight different binding sites, the R1 site appears to have the highest affinity for two reasons: first, an FcμR molecule binds constantly at this site in the FcμR:Fcμ-J sample; and second, SPR analyses show that FcμR-D1 binds Fcμ-J and full-length IgM pentamer with extremely high affinity, whereas its affinity for the isolated Cμ4 dimer, or Fcμ-J-SC, is one order of magnitude lower. This higher affinity of FcμR for pIgM most likely reflects the stronger binding at the R1 site, since the binding environments at R2-R4 (and also R2'-R4') largely resemble that in the FcμR-D1:Fcμ-Cμ4 crystal structure. In contrast, intricate interactions between multiple Fcμ-J subunits and FcμR-D1 are present at the R1 site. The R1' site appears to be the second preferred site. When the R1 site is occupied by pIgR/SC, FcμR chooses to land on the other side of Fcμ-J instead of binding to the R2-R4 positions. Compared to the R2-R4/R2'-R4' sites, R1' site also features additional interactions mediated by Arg112FcμR to the J-chain and Fcμ1A tailpiece.

Our results strongly suggest that four FcμR molecules tend to cluster on one side of the pentameric IgM platform. Once an FcμR binds to the preferred R1 or R1' site, it may help recruit the other three molecules to the same side of Fcμ-J via the stalk region, as suggested by the unmodeled densities in the 4:1 FcμR:Fcμ-J complex (Fig. 2d). Formation of receptor clusters is a common means to initiate signaling events. We envision that the formation of an FcμR tetramer, implemented by associating with IgM, would bring the transmembrane segments and cytosolic regions of FcμR together to trigger downstream effector functions. Interestingly, binding of a 5th
FcμR is not allowed on either side of the IgM pentamer: on the R1-R4 side, it is hindered by the β2-β3 loop of J-chain; whereas on the R1'-R4' side, the modest tilting of Fcμ1 caused by its alliance with J-chain deters the binding. Therefore, in addition to preventing IgM hexamer formation, J-chain appears to break another layer of symmetry, allowing only four FcμR molecules to bind to an IgM pentamer. In this regard, it remains to be determined whether six FcμR molecules can bind simultaneously to an IgM hexamer, and whether this would lead to a different signaling outcome.

Compared to human FcμR, the mouse ortholog binds IgM more weakly. Differences at two key sites may contribute to this different binding behavior. First, mouse FcμR lacks Asn66, which may impact the positioning of Phe67, a key residue for all the binding sites described above. Second, Arg112 is replaced by a Lys in mouse, which may affect the binding at the R1 (and R1') site.

In summary, we have delineated the complex mechanism of IgM perception by its specific receptor FcμR. These results are important steps forward for further understanding the elusive effector functions of IgM.
Methods

Cell culture

Sf21 and High Five cells were maintained using a non-humidified shaker at 27°C in the SIM-SF and SIM-HF media (Sino Biological), respectively. HEK293F cells were cultured in a humidified shaker with 5% CO₂ and 55% humidity at 37°C in SMM 293T-I (Sino Biological). HeLa and HEK293T cells were cultured in DMEM (Gibco) supplemented with 1% penicillin-streptomycin (Gibco) and 10% fetal bovine serum (FBS, Gibco) in a humidified incubator at 37 °C with 5% CO₂. Human Jurkat T cells were cultured in RPMI-1640 medium supplemented with 2 mM GlutaMAX (Gibco), 15% FBS, 10 mM HEPES (Gibco), 1 mM Sodium Pyruvate (Gibco), 1% MEM Non-Essential Amino Acids Solution (100×, Gibco), and 1% penicillin-streptavidin at 37 °C and 5% CO₂.

Protein expression and purification

For expression in insect cells, DNA fragments encoding the FcμR-D1 domain (residues 18-124) or FcμR-ECD (residues 18-251) were cloned into the pFastBac vector with a N-terminal melittin signal peptide followed by a His₁₀ tag. Bacmids were generated using the Bac-to-Bac system (Invitrogen). Recombinant baculoviruses were generated and amplified using the Sf21 insect cells. For protein production, Hive Five cells were infected at a density of 1.5-2.0 million cells per mL. After 48 h, the conditioned media were collected by centrifugation and dialyzed to exchange into the binding buffer (25 mM Tris-HCl, pH 8.0, 150 mM NaCl). The recombinant proteins were then isolated using Ni-NTA affinity purification and eluted with the binding buffer supplemented with 500 mM imidazole. The FcμR-D1 domain and FcμR-ECD were further purified using a Superdex 75 column (GE Healthcare) and Superdex 200 increase column (GE Healthcare), respectively. The final buffer used for the gel filtration is the same to the binding buffer. FcμR-D1 mutants were expressed and purified similarly to WT FcμR-D1.

The Fcμ-J complex was expressed and purified as previously described. For expression of the Fcμ-Cμ4 domain in HEK293 cells, the DNA fragment encoding Fcμ residues 446-558 was cloned into a modified pcDNA vector with a N-terminal IL-2 signal peptide and a C-terminal His₈ tag. The plasmid was transiently transfected into HEK293F cells using polyethylenimine (PEI, Polysciences). Four days following transfection, the Fcμ-Cμ4 domain was purified from the conditioned media similarly to FcμR-D1. For the pull-down assay, constructs of Fcμ with a
N-terminal twin-strep tag were transiently transfected into HEK293F cells and cultured for four days. The protein was retrieved from the conditioned medium using the Streptactin resin (Smart Lifesciences), and then further purified using a Superdex 200 Increase column in the buffer containing 25 mM Tris-HCl, pH 7.4, and 150 mM NaCl.

**Surface plasmon resonance (SPR)**

SPR experiments were performed using the Biacore T200 (GE Healthcare). For this purpose, 800-1100 resonance units (RU) of FcμR-D1 domain were firstly immobilized on a CM5 Chip (Cytiva). Serial dilutions of purified Cμ4, Fcμ-J, anti-CD20 IgM, or Fcμ-J-SC were then injected, ranging in concentration from 160 nM to 0.125 nM (2-fold dilutions). The SPR results were then analyzed with Biacore Evaluation Software and fitted using a 1:1 binding model.

**Crystallization and structure determination**

To obtain the FcμR-D1:Fcμ-Cμ4 complex for crystallization, purified Fcμ-Cμ4 was incubated with excess FcμR-D1 overnight on ice. The complex was then purified on a Superdex 75 column and eluted with the final buffer. The purified FcμR-D1:Fcμ-Cμ4 complex was concentrated to 6-7 mg/mL for crystallization. Diffraction-quality crystals were grown at 18°C by the sitting-drop vapor diffusion method, using 1:1 ratio of protein:reservoir solution containing 0.1 M Ammonium citrate tribasic (pH 7.0) and 12% (w/v) PEG 3,350. For data collection, crystals were transferred into the solution containing 0.1 M Ammonium citrate tribasic (pH 7.0), 14% (w/v) PEG 3,350, and 24% ethylene glycol before being flash-frozen under liquid nitrogen. The diffraction data were collected at the National Facility for Protein Science Shanghai (beamline BL19U). Data were processed using HKL2000 (HKL Research). The crystal structure was determined by molecular replacement using the program Phaser. The Fcμ-Cμ4 domain in the published structure of Fcμ-J-SC (PDB ID: 6KXS) and the model of FcμR-D1 domain generated by Swiss-Model were used as search models. The structural model was then manually adjusted in Coot and refined using Phenix. The final structure was validated with the wwPDB server.

**Cryo-EM sample preparation, data collection and processing, and model building**
To obtain the FcμR:Fcμ-J complex for cryo-EM analyses, purified Fcμ-J and FcμR-ECD were mixed in an 1:10 molar ratio and incubated on ice for 2 h. After that, the complex was further purified using the Superose 6 increase column and eluted with the final buffer. The FcμR:Fcμ-J-SC complex was obtained similarly.

For cryo-EM analysis, the FcμR:Fcμ-J or FcμR:Fcμ-J-SC complex was concentrated to 0.6 mg/mL and cross-linked with 0.05% glutaraldehyde (Sigma) for 10 mins at 20°C. Cryo-grids were prepared using the Vitrobot Mark IV (FEI). Briefly, 4 μL protein sample was applied onto the holy-carbon gold grids (Quantifoil, R1.2/1.3) or graphene oxide coated holy-carbon gold grids (EMR, R1.2/1.3), blotted with filter papers (Whatman No.1) at 4 °C and 100% humidity, and then plunged into the liquid ethane. The grids were first screened using a 200 kV Talos Arctica microscope equipped with a Ceta camera (FEI). Data collection was carried out using a Titan Krios electron microscope (FEI) operated at 300 kV. Movies were recorded on a K2 summit direct electron detector (Gatan) for FcμR:Fcμ-J complex and K3 summit direct electron detector (Gatan) for FcμR:Fcμ-J-SC complex.

Movie frames were motion-corrected and dose-weighted using the MotionCor2 program. CTF correction were performed using Gctf. The rest of the image processing was performed in RELION or cryoSPARC. High-quality micrographs were selected manually, and particles were autopicked by template picking. Particles were initially subjected to several rounds of 2D to exclude inaccurate particles and then further suffered 3D classifications for choosing the correct conformation. The favorite classes were selected for 3D refinement for generating the final 3D reconstruction. The local resolution map was analyzed using ResMap and displayed using UCSF ChimeraX.

The cryo-EM structure of Fcμ-J or Fcμ-J (PDB ID:6KXS), and the FcμR-D1 crystal structure were docked into the cryo-EM density map using UCSF Chimera. The structural models were then adjusted using Coot, and refined using the real-space refinement in Phenix.

**Pull-down assay**

A twin-strep tag is present on the N-terminal of Fcμ. To examine the interaction between the Fcμ monomer or Fcμ-J and FcμR-D1, 40 μg of monomeric Fcμ or Fcμ-J was mixed with purified WT FcμR-D1 or mutants, and incubated on ice for 1 h. The mixtures were then incubated with StrepTactin resins (Smart Lifesciences) in the binding buffer supplemented with
0.1% Triton X-100 at 4 °C for another hour. After incubating at 4 °C for another hour with mild rotation, the StrepTactin beads were spun down and washed three times using the binding buffer. Proteins retained on the beads were then eluted using the binding buffer supplemented with 10 mM desthiobiotin (IBA Lifesciences).

To examine the interaction between Fcμ-J-SC and FcμR-D1, the Flag tag on the N-terminal of SC was used to perform the pull-down experiment with the Flag agrose (Sigma-Aldrich). The experimental process was similar to the strep pull-down assay described above, except that the binding proteins were eluted using 200 μg/mL Flag peptide. The results were analyzed by immunoblotting using antibodies for the strep tag (HuaxingBio, HX1816) and His tag (TransGen Biotech, HT501).

**Confocal fluorescence microscopy**

HeLa cells that grown on coverslips were transfected with indicated constructs using PEI and cultured for 24 h. Twin-strep tagged Fcμ-J complex was firstly incubated with P-phycoerythrin (PE)-labeled streptavidin (Invitrogen) on ice for 30 min and was then added to cells culture to a final concentration of 15 μg/mL. After incubation on ice or at 37°C for an additional 30 min, cells were washed twice using ice-cold or 37°C PBS and fixed with 4% paraformaldehyde. The final coverslips were washed twice and mounted on slide with fluorescence mounting medium (Dako). Images were acquired using a Nikon Live SR CSU W1 confocal microscope equipped with the 100X/1.4 objective. Images were processed using Fiji (ImageJ) with brightness and contrast adjustment for the whole image.

**Preparation of FcμR stable cell lines and flow cytometry**

The GFP only, FcμR-GFP and the R45A/F67A-GFP mutant were cloned into the pQXCIP vector. These constructs were transfected into the HEK293T cell line together with the helper plasmid pCL10A1 using X-tremeGENE 9 (Roche). 2 d later, the culture supernatants containing viruses were collected and filtered, and were used to infect the Jurkat cells. Fresh media with 4 μg/mL puromycin were added to the remaining HEK293T culture to screen for the stable HEK293T cell lines overexpressing FcμR and the mutant. For the Jurkat T cell line, polybrene (Sigma-Aldrich) was added to a final concentration of 10 μg/mL before infecting cells at a ratio of 1 × 10⁶ cells/mL of the supernatant. Transfection was performed by centrifugation at
500 g for 90 min. The Jurkat cells expressing comparable levels of GFP were maintained in media containing puromycin at 1 µg/mL and enriched three times from each transductant using a BD FACS Aria III cell sorter (BD Biosciences).

Flow cytometric analyses of cell surface levels of FcµR were performed using the PE-labeled anti-FcµR mAb (HM14, BD Biosciences). To examine IgM binding, cells were incubated with twin-strep tagged Fcµ-J complex at a concentration of 15 µg/mL for 30 min on ice, washed, and then incubated with PE-labeled streptavidin. Stained cells were measured by the CytoFLEX S system (Beckman Coulter), and the flow cytometric data were analyzed with FlowJo software (Tree Star) and GraphPad Prism (v8.0.2).
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47


Acknowledgments

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Authors Contributions

J.X. conceived and supervised the project. Y.L. and H.S. carried out the structural studies. Y.L. and R.Z. performed the biochemical and cellular experiments, with the help of Y.W., C.J., and C.S. J.X. wrote the manuscript, with inputs from all authors.

Competing interests

The authors declare no competing interests.

Data Availability

Cryo-EM density maps of FcμR:Fcμ-J (1:1 and 4:1) and FcμR:Fcμ-J-SC have been deposited in the Electron Microscopy Data Bank with accession codes EMD-34085, EMD-34086, and EMD-34074. The corresponding structural coordinates have been deposited in the Protein Data Bank (PDB) with accession codes 7YTC, 7YTD, and 7YSG. Crystal structure of FcμR-D1:Fcμ-Cμ4 has been deposited in PDB with accession code 7YTE.
Figure 1. Crystal structure of the FcμR-D1:Fcμ-Cμ4 complex.

a. Schematic representations of FcμR and Fcμ.

b. Overall structure of the 2:1 FcμR-D1:Fcμ-Cμ4 complex.

c. SPR analyses of FcμR-D1:Fcμ-Cμ4 interaction.

d. The FcμR-D1:Fcμ-Cμ4 interface.

e. Comparison of Fcγ-FcγRI (PDB ID: 4W4O), Fcε-FcεRI (PDB ID: 1F6A), Fcα-FcαRI (PDB ID: 1OW0), and Fcμ-FcμRI (a composite model).

f. A model of two FcμR molecules bound to an IgM-BCR complex (PDB ID: 7XQ8).

g. The Asn97-glycan on Igα may slightly interfere with the binding of FcμR in its vicinity.
**Figure 2. Cryo-EM structure of the FcμR:Fcμ-J complex.**

a. SPR analyses of the interactions between FcμR-D1 and Fcμ-J or anti-CD20 IgM.

b. Overall structure of the 1:1 FcμR:Fcμ-J complex. The S1gM core complex is shown on the right for comparison, with the D1 domain of SC highlighted in yellow.

c. Structure of the 4:1 FcμR:Fcμ-J complex. The β2-β3 loop of the J-chain prevents the binding of FcμR to Fcμ5.

d. Densities in the 4:1 FcμR:Fcμ-J complex (grey) that could belong to the FcμR stalk region.
Figure 3. Cryo-EM structure of the FcμR:Fcμ-J-SC complex.

a. SPR analyses of the interactions between FcμR-D1 and Fcμ-J-SC.


c. FcμR bound to Fcμ1A would lead to unfavorable packing with the R4' FcμR.
Figure 4. FcμR mutants display reduced binding to IgM.

a. FcμR-D1 R45A does not bind to the monomeric Fcμ.

b. FcμR-D1 mutants display greatly reduced or abolished interaction with Fcμ-J.

c. FcμR-D1 mutants also do not bind Fcμ-J-SC.

d. HeLa cells expressing FcμR-R45A/F67A do not bind and internalize twin-strep tagged Fcμ-J, as examined by confocal fluorescence microscopy. Fcμ-J binding is examined using PE-labeled streptavidin.

e. Flow cytometry analyses suggest that HEK293T and Jurkat cells stably expressing FcμR-R45A/F67A exhibit reduced binding to Fcμ-J. Cell surface FcμR levels are examined using the HM14 anti-FcμR antibody. The frequencies of positive cells are plotted as means ± 1 SEM from three independent experiments. Statistical analysis was performed by two-tailed unpaired Student t test. **p <0.01, *** p < 0.001, **** p < 0.0001, when compared with WT cells.
Extended Data Fig. 1. Sequence alignment of antibody Fc sequences and FcμR.

a. Sequence alignment of human antibody Fc sequences. Fcμ residues that are recognized by FcμR are highlighted.

b. Sequence comparison between human FcμR and the mouse protein.
Extended Data Fig. 2. Purification of the FcμR:Fcμ-J and FcμR:Fcμ-J-SC complexes for cryo-EM.


b. SDS-PAGE analyses of the FcμR:Fcμ-J complex.


d. SDS-PAGE analyses of the FcμR:Fcμ-J-SC complex.
Extended Data Fig. 3. Workflow for the cryo-EM 3D reconstructions of the FcμR:Fcμ-J complex.

a. A representative raw cryo-EM image.
b. Representative 2D classes.
c. Flow chart for image processing.
d. Gold standard Fourier shell correlation (FSC) curves with estimated resolutions of the 1:1 FcμR:Fcμ-J complex.
e. FSC curves of the 4:1 FcμR:Fcμ-J complex.
f. Euler angle distribution of the classified particles for the 1:1 FcμR:Fcμ-J complex.
g. Euler angle distribution of the classified particles for the 4:1 FcμR:Fcμ-J complex.
h. Resolution estimations of the final map of the 1:1 FcμR:Fcμ-J complex.
i. Resolution estimations of the final map of the 4:1 FcμR:Fcμ-J complex.
j. FcμR-D1 interacts with the C-terminal region of the J-chain at the R1 site.
k. FcμR-D1 interacts with the tailpiece of Fcμ5B at the R1 site.
l. At the R2-R4 sites, besides mainly binding to the Cμ4 domains of Fcμ2B, Fcμ3B, and Fcμ4B, FcμR also slightly interacts with the C-C' loop of Fcμ1B, Fcμ2B, and Fcμ3B from the adjoining Fcμ units. Shown here is a copy of the FcμR-D1:Fcμ-Cμ4 pair from the crystal structure (green) superposed to Fcμ2B, illustrating the close contact between the C-C' loop of Fcμ1B and the R2 FcμR.
Extended Data Fig. 4. Workflow for the cryo-EM 3D reconstructions of the FcμR:Fcμ-J-SC complex.

a. A representative raw cryo-EM image.
b. Representative 2D classes.
c. Flow chart for image processing.
d. Gold standard Fourier shell correlation (FSC) curves with estimated resolutions.
e. Euler angle distribution of the classified particles.
f. Resolution estimations of the final map.
g. Arg112_FcμR contact J-chain Ser65-J-Asp66, as well as Glu570 in the tailpiece of Fcμ1A.
Extended Data Fig. 5. Gating strategy for the detection of FcμR expression and IgM binding by flow cytometry.

1. Gating strategy for analyzing the cell surface FcμR levels in 293T cells.
2. Gating strategy for analyzing the cell surface FcμR levels in Jurkat cells.
3. Gating strategy for analyzing IgM binding in 293T cells.
4. Gating strategy for analyzing IgM binding in Jurkat cells.
Extended Data Table 1. Crystal Data collection and refinement statistics.

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Each dataset was collected from a single crystal. Values in parentheses are for highest-resolution shell.
Extended Data Table 2. Cryo-EM data collection, refinement and validation statistics.

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**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- Cu4d1valreportfull.pdf
- IgMFCMRvalreportfullP1.pdf
- IgM4FCMRvalreport.pdf
- slgMFCMRvalreport.pdf