

IgE^b immune complexes activate macrophages through Fc γ RIV binding

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Because functional analysis of Fc receptors (FcRs) relies heavily on mouse models, the identification of another Fc γ receptor is particularly noteworthy. We demonstrate that Fc γ RIV, identified here as the mouse ortholog of primate Fc γ RIII, required association of the FcR γ -chain for optimal expression and function on myeloid cells; its signaling potential was also enhanced by a cytoplasmic 'YEPP' motif that was able to recruit the adaptor molecule Crk-L and phosphatidylinositol-3-OH kinase. Unexpectedly, Fc γ RIV 'preferentially' bound immunoglobulin E antibodies of the 'b' allotype (IgE^b) as well as IgG2a and IgG2b antibodies. Ligation of Fc γ RIV by antigen-IgE^b immune complexes promoted macrophage-mediated phagocytosis, presentation of antigen to T cells, production of proinflammatory cytokines and the late phase of cutaneous allergic reactions. IgE^b antibody-mediated modification of macrophage responses may therefore influence mouse asthma models and strain-dependent differences in parasite susceptibility.

Immunoglobulin Fc receptors (FcRs) have a broad tissue distribution and serve a variety of immune defense functions¹. The classical FcRs modulate cellular and humoral immune responses by linking antigen-antibody complexes with effector cell function^{2,3}. The genes encoding these receptors, *Fcgr1* (encoding Fc γ RI, also called CD64), *Fcgr2b* (Fc γ RIIb, also called CD32), *Fcgr3* (Fc γ RIII, also called CD16) and *Fcer1a* (Fc ϵ RI), reside in the region of chromosome 1q21–23 in humans, whereas the syntenic region in mice is split between chromosomes 1 and 3 (refs. 4,5). High-affinity Fc γ RI (10^7 – 10^9 M⁻¹ Fc-binding affinity) binds monomeric immunoglobulin G (IgG) as well as antigen-antibody complexes, whereas Fc γ RIII and Fc γ RIIb have lower binding affinities (10^6 – 10^7 M⁻¹) and bind IgG mainly in antigen-antibody complexes². The relative affinities of FcRs for the different immunoglobulin isotypes are important variables for the 'fine-tuning' of humoral responses⁶, as are the activating versus inhibitory functions of the various FcRs. After binding their antibody ligands, Fc γ RI and Fc γ RIII deliver activating signals through an association with the FcR γ -chain (Fc γ), a transmembrane adaptor protein with an immunoreceptor tyrosine-based activation motif in its cytoplasmic domain². Fc γ RIIb instead uses its cytoplasmic immunoreceptor tyrosine-based inhibitory motif to initiate an inhibitory signaling cascade after ligation of the receptor by multivalent ligands, such as immune complexes. Coordination of the activating and inhibitory functions of FcRs is important for the successful initiation, amplification and termination of immune responses⁷. Functional imbalances due to genetic Fc γ R variations consequently predispose people to autoimmune disorders^{8,9}.

Yet another mouse FcR, Fc γ RIV, has been identified in a search of the mouse genome sequence databases for more family members^{5,10,11}. Fc γ RIV is encoded by the gene *Fcgr4* (also called *Fcgr3a*, *Fcrl3* and *CD16-2*)^{5,11,12}. Our phylogenetic analysis here confirmed that mouse Fc γ RIV is most closely related to human Fc γ RIII. Moreover, its cellular distribution pattern differed from that of mouse Fc γ RIII. We also found that Fc γ RIV cell surface expression and function was facilitated by association of the FcR γ -chain. In addition, Fc γ RIV has a cytoplasmic 'YEPP' motif that when tyrosine-phosphorylated associated with the proteins Crk-L (v-crk sarcoma virus CT10 oncogene homolog (avian)-like) and PI(3)K (phosphatidylinositol-3-OH kinase) to augment its signaling potential. In keeping with its sequence similarity to human Fc γ RIII, we confirm that mouse Fc γ RIV bound to antibodies of the IgG2a and IgG2b isotypes^{6,12}. Most notably, Fc γ RIV bound IgE antibodies of the 'b' allotype (IgE^b), and antigen-IgE^b antibody complexes initiated phagocytosis, antigen presentation and the production of proinflammatory mediators by macrophages.

RESULTS

Phylogenetic comparison of Fc γ RIV, Fc γ RIII and Fc γ RII

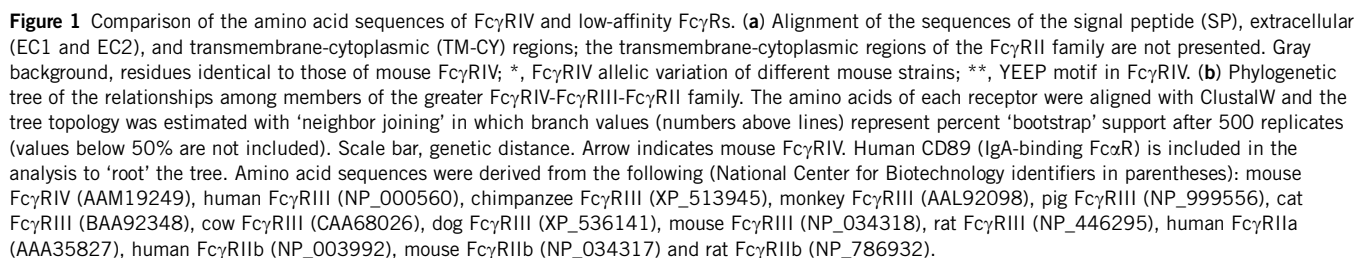
To compare the amino acid sequences of Fc γ RIV, Fc γ RIII and Fc γ RII in various mammals, we identified chimpanzee, monkey, pig, cat, cow and dog Fc γ RIII (Fig. 1a) through a search of the protein database of the National Center for Biotechnology Information using mouse Fc γ RIV and human Fc γ RIII as 'query' sequences. We identified rat Fc γ RIV by sequencing an IMAGE clone (6921627) and isolated

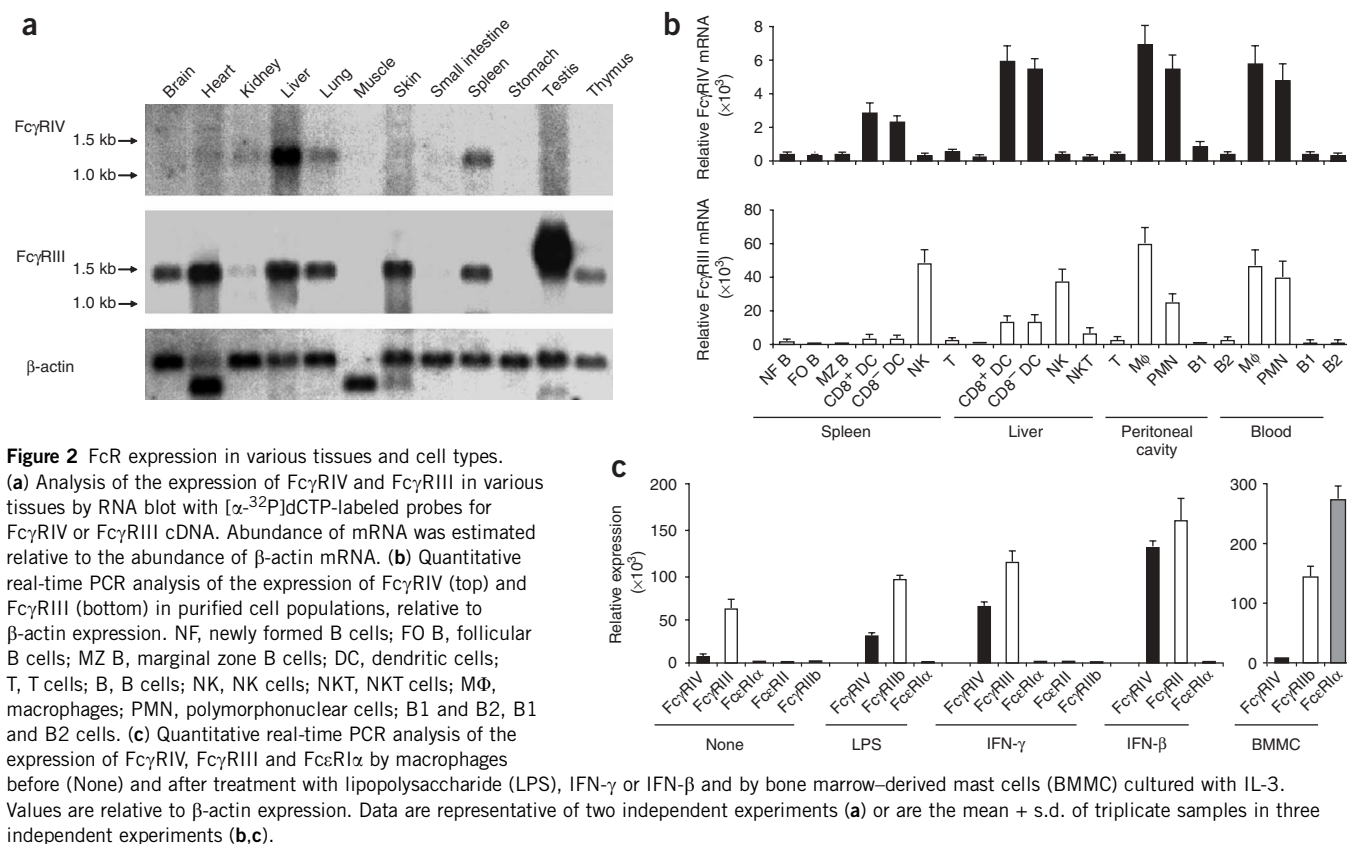
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we noted for rodent members of the FcγRIV family was the presence of a cytoplasmic YXXP ('YEEP') motif. This finding is potentially important because when the tyrosine residue in this motif is phosphorylated, it can serve as a binding site for the Src homology 2-containing molecules Crk, Nck and Abl¹³.

We easily detected *Fcgr4* transcripts of about 1.2 kilobases (kb) by RNA blot in samples from spleen and liver, but they were less prominent in lung, kidney and heart (**Fig. 2a**). *Fcgr3* seemed to be even more widely expressed, with transcripts of about 1.4 kb being evident in 8 of 12 tissues surveyed, including skin, heart and brain. In addition, *Fcgr3* transcripts of about 1.7 kb were prominent in the testis. We confirmed by sequence analysis that the *Fcgr4* transcript of about 1.2 kb and the *Fcgr3* transcript of about 1.4 kb were





full-length mRNA. In testis, the *Fcgr3* transcript of about 1.7 kb included two additional exons for the 5' untranslated region that were not present in *Fcgr3* transcripts from other tissues (data not shown). Quantitative PCR analysis indicated that both the *Fcgr4* and *Fcgr3* transcripts were relatively abundant in macrophages and polymorphonuclear leukocytes and were present in smaller amounts in dendritic cells. In contrast, *Fcgr3* was 'preferentially' expressed by natural killer (NK) cells. Neither FcR was expressed by the primary B cells and T cells we examined (Fig. 2b). Analysis of 18 cell lines representing B cells, T cells, NK cells and myeloid cells confirmed those patterns of *Fcgr4* and *Fcgr3* expression, except that *Fcgr3* expression was also present in NKT cell lines (Supplementary Table 1 online).

To test whether expression of FcγRIV, FcγRIII and FcεRI α is modulated by stimulation with lipopolysaccharide, interferon- γ (IFN- γ) or IFN- β , we isolated macrophages from the peritoneal cavity and derived mast cells from bone marrow cultures treated with interleukin 3 (IL-3). Transcription of both *Fcgr4* and *Fcgr3* was enhanced by stimulation of macrophages with lipopolysaccharide, IFN- γ and IFN- β (Fig. 2c, left), whereas *Fcer1a*, *Fcer2* and *Fcgr2b* transcripts were not present before or after macrophage stimulation. Conversely, the mast cells did not express *Fcgr4*, although they had abundant *Fcer1a* and *Fcgr3* mRNA (Fig. 2c, right). The mast cell expression of *Fcgr3* is in good agreement with the abrogation of IgG-dependent passive anaphylaxis and mast cell degranulation in FcγRIII-deficient mice¹⁴.

Facilitation of FcγRIV expression by FcR γ -chain

Because association with the FcR γ -chain is needed for cell surface expression of FcγRIII, we sought to determine whether FcγRIV also requires FcR γ for surface expression. For these experiments, we transduced mouse BW5147 thymoma and A20 IIA1.6 B cell lines

with retroviral constructs expressing either FcγRIV or FcγRIII with amino-terminal hemagglutinin tags, alone or together with FcR γ -chain constructs. We then assessed cell surface expression of FcγRIV or FcγRIII and their association with the FcR γ -chain by immunoprecipitation with FcγRIV-specific antibodies (Supplementary Fig. 1 online) followed by immunoblot analysis. Immunoprecipitation with anti-FcR γ followed by immunoblot of the immunoprecipitate with anti-hemagglutinin showed that FcR γ associated with FcγRIII of about 38 kilodaltons and FcγRIV of about 33 kilodaltons in cells transduced with FcγRIII-FcR γ and FcγRIV-FcR γ , respectively (Supplementary Fig. 2 online). Moreover, there was much more cell surface expression of FcγRIV by cells expressing FcγRIV and FcR γ -chain together (Supplementary Fig. 2). To determine the composition of FcγRIV on the surface of primary cells, we lysed biotinylated splenocytes before immunoprecipitation analysis with anti-FcγRIV. The FcR γ -chain of about 11 kilodaltons was associated with FcγRIV of about 33 kilodaltons in the immunoprecipitates of cell surface proteins (Supplementary Fig. 2). In conclusion, association with FcR γ is required for the optional cell surface expression and function of the ligand-binding FcγRIV.

FcγRIV recruits Crk-L and PI(3)K

To test the functional potential of the YEEP motif in the intracellular region of FcγRIV, we used cells transduced with FcγRIII-FcR γ or FcγRIV-FcR γ to determine whether the YEEP tyrosine residue becomes phosphorylated after receptor ligation. For receptor ligation we used monoclonal antibody 2.4G2 (to FcγR), which reacted with FcγRIV as well as with FcγRII and FcγRIII (Supplementary Fig. 3 online). After ligation with the antibody, wild-type FcγRIV was tyrosine-phosphorylated, whereas mutant FcγRIV (Y235W) and

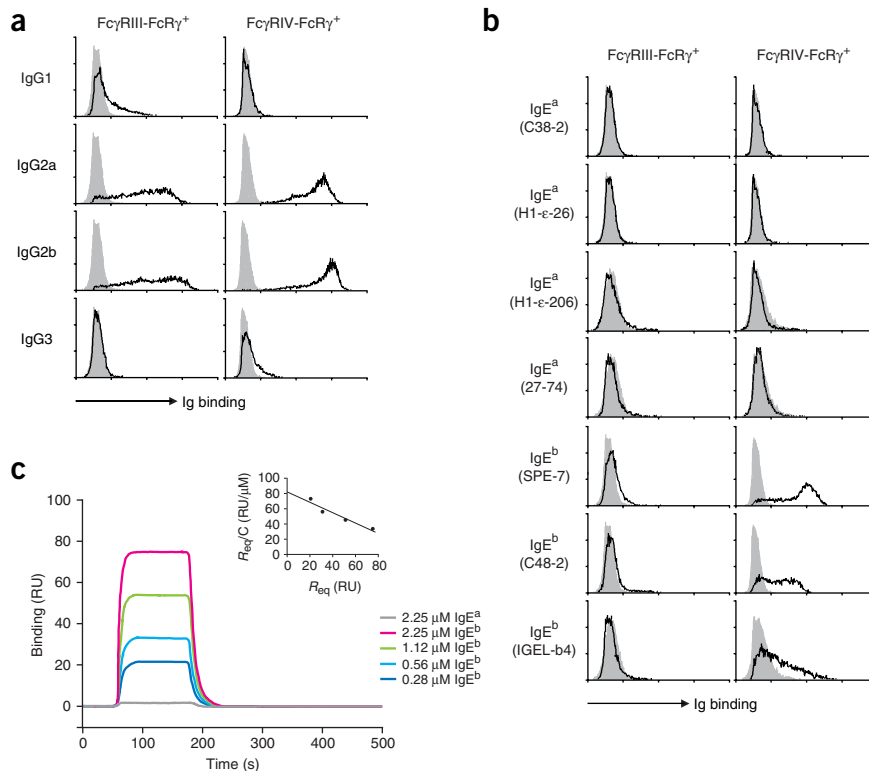


Figure 3 Comparative analysis of the mouse immunoglobulin isotype-binding specificities of FcγRIII and FcγRIV. **(a)** Flow cytometry of the binding of FcγRIV or FcγRIII to IgG1, IgG2a, IgG2b and IgG3. FcγRIV or FcγRIII was expressed on BW5147 cells by transduction together with FcRγ; after confirmation of cell surface expression of FcγRIV and FcγRIII by flow cytometry with anti-hemagglutinin, transduced cells were incubated with mouse IgG1, IgG2a, IgG2b or IgG3, followed by staining with phycoerythrin-conjugated antibodies to the IgG molecules (black lines). Results are presented as 'bi-exponential' histograms. Transduced cells stained with only secondary antibodies are presented as controls (shaded histograms). **(b)** Binding of FcγRIII or FcγRIV to mouse IgE^α and IgE^β, assessed by flow cytometry of cells transduced with FcγRIII-FcRγ or FcγRIV-FcRγ and incubated with mouse IgE^α or IgE^β (black lines). Shaded histograms, background control staining. **(c)** Surface plasmon resonance analysis of the binding of IgE to FcγRIV. IgE^α or IgE^β was injected at a flow rate of 50 μl/min over the FcγRIV-coated surface (100 resonance units (RU)) until an equilibrium state was reached. Inset, Scatchard plot of the binding of IgE^β to FcγRIV (solid line is a linear fit). R_{eq}, resonance units at equilibrium; C, concentration of IgE^β. Data are representative of four **(a)** or three **(b)** experiments or were reproduced in three independent experiments **(c)**.

wild-type FcγRIII were not (**Supplementary Fig. 4** online). To identify intracellular proteins that may associate with FcγRIV, we incubated lysates of A20 IIA1.6 B cells and P815 mastocytoma cells with phosphorylated peptides corresponding to the YEPP motif of FcγRIV. Both Crk-L and PI(3)K were bound by the peptide containing tyrosine-phosphorylated YEPP, whereas neither was bound by this peptide in its unphosphorylated form (**Supplementary Fig. 4**). These results suggest a potentially important function for the tyrosine-phosphorylated YEPP motif in FcγRIV.

Immunoglobulin-binding capacity of FcγRIV and FcγRIII

To compare the Fc-binding profiles of FcγRIV-FcRγ and FcγRIII-FcRγ, we incubated cells bearing these receptor complexes with mouse or human immunoglobulins of various isotypes for analysis by flow cytometry. We found that FcγRIV bound mouse IgG2a and IgG2b (**Fig. 3a**) and also bound human IgG1, IgG3 and IgG4, although not as well (**Supplementary Fig. 5** online). FcγRIII bound mouse IgG1, IgG2a and IgG2b (**Fig. 3a**) and also bound human IgG1 and IgG3 (**Supplementary Fig. 5**). Unexpectedly, FcγRIV bound mouse IgE^β but not IgE^α, whereas neither IgE allotype bound to FcγRIII (**Fig. 3b**). Control thymoma cells of mouse origin bound human and mouse IgM and human IgA (**Supplementary Fig. 5** and data not shown), in keeping with published evidence of thymocyte expression of mRNA encoding the FcR for IgM and IgA ('Fcα/μR')¹⁵.

We used extracellular regions of recombinant FcγRIV and FcγRIII in surface plasmon resonance analysis to verify binding of the various mouse and human immunoglobulin isotypes and to estimate binding affinities. When we assessed histidine-tagged FcγRIV or FcγRIII attached by means of a histidine-specific antibody on the sensor chip for binding of immunoglobulin in solution, we found that FcγRIV bound to mouse IgG2a and IgG2b and human IgG1 and IgG3 with intermediate affinity, whereas FcγRIII bound to mouse

IgG1, IgG2a and IgG2b with low affinity (**Supplementary Table 2** online). The binding affinities of FcγRIII and FcγRIV for mouse IgG2b that we estimated were slightly higher than those reported before¹², probably because different preparations of recombinant proteins and antibodies were used. A more notable difference was the finding that IgG2a and IgG2b bound to FcγRIV with about 30-fold higher affinity than they bound to FcγRIII. Notably, FcγRIV bound IgE^β with relatively low affinity ($6.8 \times 10^5 \text{ M}^{-1}$) but did not bind IgE^α (**Fig. 3c** and **Supplementary Table 2**). The FcγRIV immunoglobulin-binding profile, therefore, is substantially different from the mouse FcγRIII immunoglobulin-binding profile.

Antigen-IgE^β complexes activate macrophages through FcγRIV

We explored the functional consequences of the binding of IgE^β antibodies by FcγRIV in view of knowledge that association with the FcR γ-chain endows FcγRIII with endocytic and phagocytic potential¹⁶. We isolated peritoneal macrophages (CD11b⁺ Gr-1⁺CD45R⁻) by flow cytometry from C57BL/6, BALB/c, *Fcgr3*^{-/-} and *Fcer1g*^{-/-} mice (**Supplementary Fig. 6** online) and incubated the macrophages with sheep red blood cells (SRBCs) as immune complexes with IgG2b, IgE^α or IgE^β antibodies. Macrophages from C57BL/6 and BALB/C mice that were treated in this way were able to phagocytize both IgE^β immune complexes and IgG2b immune complexes, but neither bound nor phagocytized IgE^α immune complexes (**Fig. 4a** and data not shown). Pretreatment of macrophages with FcεRIα and FcεRII Fab antibody fragments had no effect on the phagocytosis of IgE^β or IgG2b immune complexes (data not shown). Notably, phagocytosis of IgG2b immune complexes was slightly lower in *Fcgr3*^{-/-} macrophages than in wild-type macrophages, whereas phagocytosis of IgE^β immune complexes was unaltered in the absence of FcγRIII. Pretreatment with monoclonal antibody 2.4G2 or with anti-FcγRIV inhibited the phagocytosis of IgE^β immune complexes by

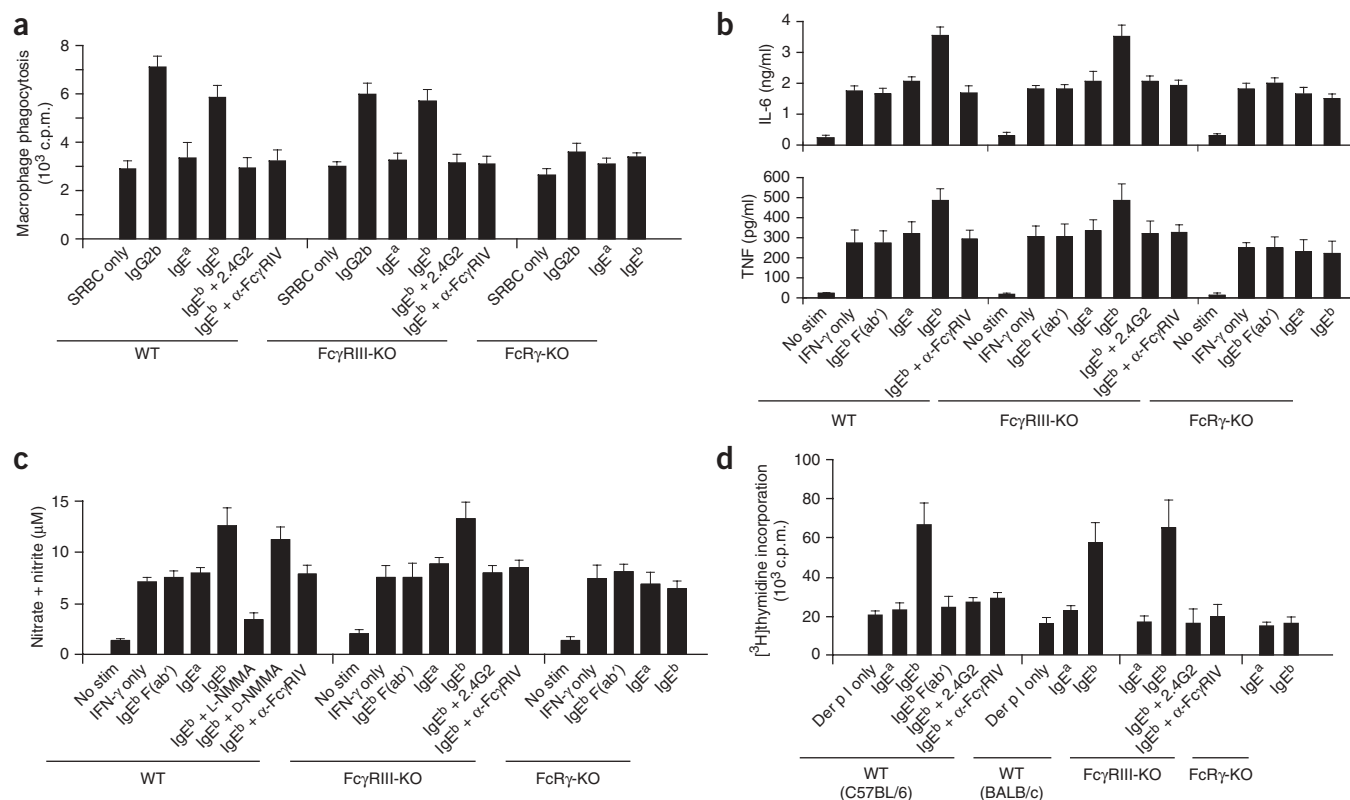


Figure 4 FcγRIV-IgE^b induces phagocytosis, the production of proinflammatory mediators and the presentation of exogenous antigen. **(a)** Incorporation of IgE^b immune complexes into C57BL/6 (WT), *Fcgr3*^{-/-} (FcγRIII-KO) or *Fcer1g*^{-/-} (FcγRγ-KO) peritoneal macrophages stimulated with IFN-γ before incubation with ⁵¹Cr-labeled SRBC-immunoglobulin immune complexes in culture medium containing FcεRIα and FcεRII Fab antibody fragments, assessed as incorporation of SRBCs into macrophages, measured with a γ-counter. + 2.4G2, addition of antibody; + α-FcγRIV, addition of FcγRIV Fab antibody fragments. **(b)** ELISA of cytokine secretion by C57BL/6, *Fcgr3*^{-/-} and *Fcer1g*^{-/-} macrophages in response to stimulation with IgE^b immune complexes. Peritoneal macrophages were left unstimulated (No stim) or were stimulated with IFN-γ, then were incubated with SRBC-immunoglobulin immune complexes in culture medium containing FcεRIα and FcεRII Fab antibody fragments. TNF, tumor necrosis factor. **(c)** Nitrate and nitrite production by C57BL/6, *Fcgr3*^{-/-} or *Fcer1g*^{-/-} macrophages in response to stimulation with IgE^b. Peritoneal macrophages were stimulated and incubated as described in **b** and nitrate plus nitrite was measured in culture supernatants. **(d)** Mediation of IgE^b-facilitated antigen presentation by FcγRIV. C57BL/6 (WT (C57BL/6)), BALB/c (WT (BALB/c)), *Fcgr3*^{-/-} or *Fcer1g*^{-/-} mice sensitized with Der p extract were challenged intranasally 5 d later with *D. pteronyssinus*; splenic macrophages and T cells were cultured for 48 h with Der p I, Der p I-IgE^a or Der p I-IgE^b in medium containing FcεRIα and FcεRII Fab antibody fragments; [³H]thymidine uptake during the final 16 h was measured. Data are the mean + s.d. of triplicate samples from three (**a,c,d**) or two (**b**) independent experiments.

wild-type and *Fcgr3*^{-/-} macrophages. There was also no phagocytosis of the immune complexes by *Fcer1g*^{-/-} macrophages. These findings collectively demonstrate that the FcγRIV-FcγR receptor complex on macrophages can induce the phagocytosis of IgE^b immune complexes.

FcγRI and FcγRIII, which share FcR γ-chain association with FcγRIV, are known to be critical in inflammation triggered by IgG immune complexes². To evaluate the ability of FcγRIV to mediate inflammatory responses through IgE binding, we incubated wild-type and *Fcgr3*^{-/-} macrophages with immune complexes of SRBCs with IgE antibodies of either allotype. The IgE^b immune complexes induced macrophage production of tumor necrosis factor, IL-6 and nitric oxide, but the IgE^a immune complexes did not (Fig. 4b,c). This IgE^b immune complex-triggered production of proinflammatory mediators was abrogated in *Fcer1g*^{-/-} mice and was inhibited by pretreatment of wild-type and *Fcgr3*^{-/-} macrophages with anti-FcγRIV. These findings demonstrate that ligation of the FcγRIV-FcγR receptor complex by IgE^b immune complexes elicits a proinflammatory response by macrophages.

To determine whether FcγRIV ligation can promote the presentation of antigens coated by IgE^b antibodies, we incubated T cells from allergen-challenged mice with macrophages bearing antigen-IgE^b-FcγRIV and then analyzed proliferative responses. We sensitized and

challenged wild-type (C57BL/6 and BALB/c), *Fcgr3*^{-/-} (C57BL/6) and *Fcer1g*^{-/-} mice (C57BL/6) with a *Dermatophagoides pteronyssinus* allergen (called 'Der p' here) to induce the production of antigen-specific IgE antibodies. After antigen challenge, serum IgE titers were similar in all the mice. We isolated splenic macrophages and T cells for analysis and found that the Der p-IgE immune complexes induced a T cell proliferative response in cultures containing T cells and macrophages from both wild-type and *Fcgr3*^{-/-} mice, whereas stimulation with the IgE^a immune complex or the IgE^b Fab immune complex did not elicit this T cell response (Fig. 4d). Furthermore, the IgE^b immune complexes did not induce a T cell response for cells from *Fcer1g*^{-/-} mice. Pretreatment of cells from C57BL/6 and *Fcgr3*^{-/-} mice with 2.4G2 or anti-FcγRIV also prevented the T cell response. These findings indicate that the FcγRIV-FcγR receptor complex on macrophages can participate in antigen presentation to T cells when engaged by antigen-antibody immune complexes of the IgE^b allotype.

IgE-FcγRIV in cutaneous allergic reactions

Mice passively sensitized with monoclonal IgE antibodies undergo biphasic cutaneous inflammation after epicutaneous antigen challenge¹⁷. This inflammatory reaction features an immediate-phase

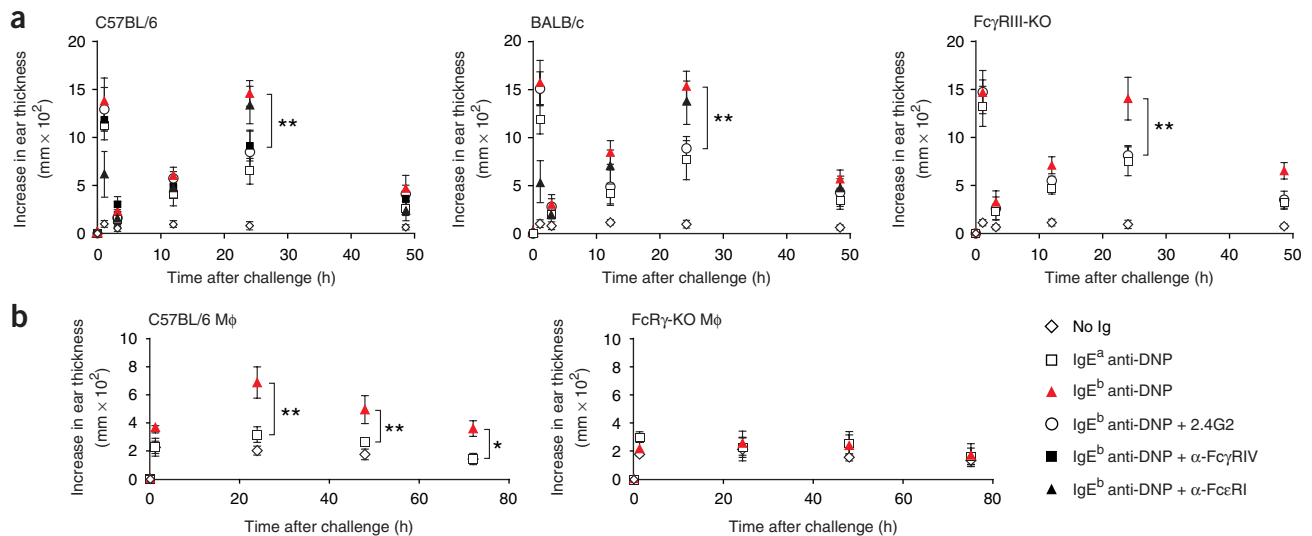


Figure 5 Induction of late-phase cutaneous reactions by macrophage activation mediated by IgE^b-FcγRIV. **(a)** IgE-mediated cutaneous inflammation via FcγRIV in C57BL/6, BALB/c and *FcγRIII-KO* mice passively sensitized intradermally with saline (No Ig), IgE^a anti-DNP or IgE^b anti-DNP; cutaneous reactions were elicited by the application of DNFB and ear thickness was then measured. + 2.4G2, + α-FcγRIV or + α-FcεRI (key), treatment with 2.4G2, anti-FcγRIV or anti-FcεRI before sensitization. **(b)** Cutaneous inflammation by IgE-ligated macrophages via FcγRIV. Macrophages purified from DNFB-sensitized C57BL/6 and *FcεR1g^{-/-}* mice were treated with saline, IgE^a anti-DNP or IgE^b anti-DNP and were injected intradermally into ear pinnae of C57BL/6 or *FcεR1g^{-/-}* mice; mice were then challenged by the application of DNFB and ear thickness was then measured. *, $P < 0.05$, and **, $P < 0.01$, IgE^b versus IgE^a. Data represent the mean ± s.e.m. for five mice at each time point from two independent experiments.

peak at 1 h and a late-phase peak at 24 h. To determine whether ligation of FcγRIV with antigen-IgE immune complexes influences either phase of this biphasic response, we primed mice with IgE^a antibody to dinitrophenyl (IgE^a anti-DNP) and IgE^b anti-DNP and then induced a cutaneous reaction by application of 2,4-dinitro-1-fluorobenzene (DNFB) to the ear. Immediate- and late-phase cutaneous reactions were elicited to the same extent in wild-type and *FcγR3^{-/-}* mice sensitized with the IgE^b antibody (Fig. 5a). Although the intensity of the immediate reaction was essentially the same in mice sensitized with IgE^a or IgE^b, the intensity of the late-phase reaction was lower in wild-type and *FcγR3^{-/-}* mice sensitized with IgE^a than in mice sensitized with IgE^b. Moreover, pretreatment with 2.4G2 or anti-FcγRIV inhibited the late phase of the cutaneous reaction in wild-type and *FcγR3^{-/-}* mice sensitized with IgE^b. Conversely, pretreatment with anti-FcεRI substantially reduced the immediate phase of the cutaneous allergic reaction, whereas it had no observable effect on the late phase. These findings suggested that FcγRIV is an important contributor to allergic inflammation in mice that produce IgE^b antibodies to the inciting antigen.

To verify the function suggested for macrophage FcγRIV in IgE^b-triggered cutaneous inflammation, we adoptively transferred primed macrophages into unsensitized mice. We prepared macrophages bearing FcγRIV ligated to IgE^b anti-DNP and injected them intradermally into the ears of wild-type and *FcεR1g^{-/-}* mice before subsequent challenge with DNFB. We used IgE^a-treated macrophages as a control for this assay. We transferred the macrophages from wild-type and *FcεR1g^{-/-}* mice into mice with the corresponding genetic background in these experiments. Although IgE^a-treated macrophages and IgE^b-treated *FcεR1g^{-/-}* macrophages did not confer cutaneous inflammatory responsiveness in recipient mice, IgE^b-treated macrophages from wild-type mice supported the induction of cutaneous inflammation, with the reaction peak at 24 h (Fig. 5b and data not shown). Parallel experiments consisting of treatment with IgE anti-trinitrophenyl (anti-TNP) and challenge with picryl chloride produced the same outcome (data not shown). The composite results of these

experiments suggest a prominent function for macrophage FcγRIV in the IgE^b-facilitated late phase of cutaneous allergic reactions.

DISCUSSION

The evidence presented here has indicated that the newly identified FcγRIV on myeloid-lineage cells is structurally, functionally and physically distinct from FcRs described before. Our data have also demonstrated a previously unknown functional difference between allelically encoded IgH constant-region structures, in this case IgE^a and IgE^b. Our phylogenetic analysis of FcγRs indicated that FcγRIV in mice, rats and hamsters is more closely related to the FcγRIII homologs in other mammals than to the rodent FcγRIII receptors linked to FcγRIV before; the rodent FcγRIII receptors being more similar to FcγRII. This analysis favors the idea that rodent FcγRIV receptors and non-rodent FcγRIII receptors share a common ancestry. Although the extracellular regions of mouse FcγRIII and FcγRIIb may derive from a common ancestral gene, their signal peptide, transmembrane and cytoplasmic regions apparently reflect intergenic recombination^{4,11,18}. Although both receptors have cell-activating functions, mouse FcγRIII differs from human FcγRIIa in having a negatively charged transmembrane residue that promotes the association of FcR γ-chains for the transduction of activation signals, whereas the human FcγRIIa has its own cytoplasmic immunoreceptor tyrosine-based activation signaling motif. Notably, rodent FcγRIV differs from other known FcRs in having a cytoplasmic YEEP motif, which is a known potential binding site for Src homology 2-containing adaptor proteins¹³. Accordingly, we found that crosslinking of FcγRIV induced tyrosine phosphorylation of this motif and association with Crk-L and PI(3)K. FcγRIV, therefore, may directly recruit Src homology 2-containing adaptor molecules to facilitate its signal transduction function through its FcRγ partner.

An association with FcR polymorphisms has been noted for certain allergic, infectious and autoimmune diseases in humans and mice^{8,9,19},

but whether immunoglobulin polymorphisms can influence antibody binding by FcRs to favor disease pathogenesis has remained unclear^{20,21}. We found unexpectedly that FcγRIV 'preferentially' bound antibodies of the IgE^b allotype. IgE^a and IgE^b antibodies have five amino acid differences in their constant (C) regions²². Two of these differences are located in the C_ε3 domain (S248P and N260H) and three are in the C_ε4 domain (G346E, Q355H and R419H). Modeling of the mouse IgE C_ε3-C_ε4 domains of 'a' and 'b' allotypes with FcγRIV, with the human FcγRIII and IgG1 complex as a structural template (Protein Data Bank accession number 1t83; ref. 23), suggests that residue 260 is located near the FcγRIV-IgE interface, where it could participate in the binding of FcγRIV and IgE^b. The polymorphic FcγRIV residue at position 57 (isoleucine for C57BL/6 and BALB/c mice and threonine for CBA, NBZ, NOD and CZECHII mice) is located in the middle of extracellular region 1, well away from the predicted FcγRIV-IgE interface. Sialylation of the immunoglobulin Fc regions may modify their binding affinities with FcRs²⁴, but the finding of similar sialylation ratios for IgE^a and IgE^b antibodies (about 70% for each) suggests that the difference in the binding of IgE^a and IgE^b to FcγRIV is not determined by sialylation status.

FcεRII (also called CD23) binds IgE with a relatively low affinity (about 1×10^6 M⁻¹)²⁵, similar to the affinity of FcγRIV for IgE^b (6.8×10^5 M⁻¹). Nevertheless, FcεRII has a demonstrable regulatory effect on allergic inflammation in mice^{26,27}. Our data indicate that allergic responses may involve the binding of IgE^b to the FcγRIV-FcγR complex, given that we found that engagement of the macrophage FcγRIV-FcγR complex by the IgE^b immune complex triggered the production of proinflammatory mediators and antigen presentation to T cells. Although antigen-IgE immune complexes interact with FcεRI to initiate antigen presentation by human macrophages²⁸, we did not detect expression of FcεRIα or *Fcer2* by mouse macrophages before or after IFN-γ stimulation. These findings suggest that the mouse IgE^b-FcγRIV interaction and the human IgE-FcεRIα interaction may have similar functions in antigen presentation by macrophages.

When sensitized allergic people are challenged with an allergen, they undergo an inflammatory response called 'immediate hypersensitivity'^{29,30}. Passive sensitization of mice with monoclonal IgE antibodies leads to a biphasic inflammatory response after epicutaneous antigen challenge¹⁷. When we examined the functions of macrophages and their FcγRIV in this biphasic cutaneous response, we found that the FcγRIV-bound IgE^b antibodies promoted the late phase of the reaction. The demonstration that IgE polymorphism can influence allergic responses suggests that responses mediated by IgE-FcγRIV merit consideration in evaluating mouse models of allergic inflammation.

IgE^b-mediated inflammatory responses by macrophages may also contribute to the strain-dependent differences noted for parasitic susceptibility, given that a strong IgE response is induced by helminth parasites, including schistosomiasis³¹, and that IgE antibodies are critical in immunity to helminth infection³². An essential function for the FcR γ-chain in defense against intestinal nematodes has been established³³, and the nitric oxide and tumor necrosis factor products of activated macrophages participate in the killing of schistosomes^{34–36}. After penetrating the skin, the schistosomal cercariae transform into schistosomula, which enter blood vessels en route to the lungs, heart and liver. Because FcγRIV-bearing macrophages may contribute to schistosomal resistance in all those tissues, it is notable that mouse strains that are relatively resistant to schistosomal infection (C57BL/6 and SJL/J) produce antibodies of the IgE^b allotype, whereas relatively susceptible strains of mice (BALB/c, C3H/HeN and CBA/J) produce

antibodies of the IgE^a allotype^{37–40}. The physiological variation in inflammatory responses promulgated by IgE antibodies of the 'b' allotype versus the 'a' allotype suggests the need for reevaluation of mouse models of parasitic as well as allergic diseases.

METHODS

Cells. The BW5147 T cell and A20 IIA1.6 B cell lines were maintained in RPMI 1640 medium supplemented with 10% (vol/vol) FCS, 2 mM L-glutamine, 25 μM 2-mercaptoethanol and 100 U/ml of penicillin-streptomycin. BOSC23 cells were grown in DMEM supplemented with 10% (vol/vol) FCS, 2 mM L-glutamine and 100 U/ml of penicillin-streptomycin. Mouse RAW8.1 (pro-B cell), 70Z/3 (pre-B cell), WEHI-231 (immature B cell), A20 (B cell), X16C8.5 (B cell), EL4 (T cell), NKT, 2C12 (NKT cell), NIH3T3 (fibroblast) and WEHI-3 (myeloid cell) cell lines have been described⁴¹. The LNK5E6 NK cell line⁴² was cultured in RPMI medium with 10% (vol/vol) FCS, 500 U/ml of recombinant human IL-2, amphotericin B and gentamycin. The JAWSII immature dendritic cell line, MPRO neutrophil cell line, IC-21 cell line and PU5-1.8, P388D and RAW264.7 macrophage cell lines were from American Tissue Culture Collection and were cultured according to recommendations of the American Tissue Culture Collection. Mast cells were obtained over 6 weeks from bone marrow cultures (C57BL/6) containing IL-3 (100 U/ml).

Mice. C57BL/6 mice, BALB/c mice, *Fcgr3*^{-/-} mice (C57BL/6 background)¹⁴ and *Fcer1g*^{-/-} mice (C57BL/6 background)⁴³ were from the Jackson laboratory. All mice were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Antibodies. The hemagglutinin antibodies 12CA5 and 3F10 were from Roche Diagnostics; anti-FcγR (06-727) was from Upstate Biotechnology; and the streptavidin-horseradish peroxidase conjugate was from Amersham Pharmacia Biotech. Mouse IgM (TEPC 183), IgA (TEPC 15), IgG1 (MOPC 21), IgG2a (UPC 10), IgG2b (MOPC 141), IgG3 (I 3784) and IgE^b (anti-DNP; SPE-7) and human IgM (I 8135), IgA (I 2636), IgG1 (I 5154), IgG2 (I 5404), IgG3 (I 5654) and IgG4 (I 4639) were from Sigma. Mouse antibodies H1-e-206 and H1-e-26 (IgE^a anti-DNP) have been described⁴⁴. Two other IgE^a antibodies, 27-74 (anti-dansyl) and C38-2 (anti-TNP), and the IgE^b antibody C48-2 (anti-TNP) were from BD Pharmingen. Anti-FcγR (2.4G2) was from BD Pharmingen. Anti-FcεRIα (MAR-1) was from eBiosciences.

Identification of rat and hamster FcγRIV receptors. Golden Syrian hamster cDNA (BioChain), modified by ligation of adaptors (Marathon cDNA amplification kit; Clontech), was used in PCR for 5' and 3' rapid amplification of cDNA ends. Gene-specific primers were as follows: forward, 5'-CAGTGT GACCCTCAGGTGCCAGGGCACC-3', and reverse, 5'-GGGGTCCCCCTCC TGGAACACAGGCCG-3'. Products of the rapid amplification of cDNA ends were ligated into the pCR2.1 TOPO T/A vector (Invitrogen) and were sequenced with an automated sequencer (LiCor). Rat FcγRIV was identified by sequencing of the IMAGE clone 6921627 (Invitrogen).

RNA blot. A Mouse Poly(A)⁺ RNA Northern Blot (Origene) and a FirstChoice Northern Blot Mouse Blot (Ambion) were hybridized with the following gene-specific probes derived from their respective cDNA, amplified by PCR and labeled with [α -³²P]dATP or [α -³²P]dCTP: a 549-base pair fragment (nucleotides 569–1117) corresponding to the transmembrane-cytoplasmic 3' untranslated region of FcγRIV, and a 198-base pair fragment (nucleotides 48–245) corresponding to the signal peptide-extracellular 1 region of FcγRIII. Probes were labeled with Strip-EZ DNA (Ambion) or Rediprime II and ProbeQuant G-50 microcolumns (Amersham Pharmacia Biotech). Membranes were hybridized as described⁴¹.

Immunofluorescence and cell sorting. Mouse spleen, liver and blood cells were stained with indocarbocyanine-conjugated anti-CD21 (ref. 41); fluorescein isothiocyanate-conjugated anti-CD19 (1D3), anti-CD11c (HL3) or anti-CD11b (Mac-1; M 1/70); phycoerythrin-conjugated anti-CD23 (FcεRII; B3B4), anti-CD8 (53-6.7), anti-CD49b (DX5) or anti-Gr-1 (RB6-8C5); or allophycocyanin-conjugated anti-CD4 (GK 1.5; all from BD Pharmingen). Primary cells were differentially sorted on a MoFlo (Cytomation) as follows: newly formed B cells (CD19⁺CD21⁺CD23⁻), follicular B cells (CD19⁺CD21⁺CD23⁺), marginal zone

B cells (CD19⁺CD21^{hi}CD23^{lo}), NK cells (DX5⁺CD11c⁻), dendritic cells (DX5⁻CD11c⁺), macrophages (CD11b⁺Gr-1⁻) and neutrophils (CD11b⁺Gr-1⁺). Peritoneal cells obtained by saline lavage of C57BL/6 mice were stained with allophycocyanin-conjugated anti-CD19, phycoerythrin-conjugated anti-CD5, fluorescein isothiocyanate-conjugated anti-CD11b and biotin-conjugated anti-Gr-1, followed by staining with streptavidin-peridinin chlorophyll protein (all from BD PharMingen) and sorting by flow cytometry to a purity of over 98%.

Quantitative mRNA analysis. Total cellular RNA extracted from mouse cells with the RNeasy kit (QIAGEN) was primed with oligo(dT) primers and was reverse-transcribed with SuperScript III (Invitrogen) into single-stranded cDNA. Gene-specific primers for real-time PCR were as follows: *Fcgr4* forward, 5'-ATCTTCAGCATCCTTTCGTATA-3', and reverse, 5'-TTGATGCCACGGTG GAAAC-3'; *Fcgr3* forward, 5'-TCCAAGCCTGTCACCATCAC-3', and reverse, 5'-GGGAGAAAGCAGTGTGGTACC-3'; and *Actb* (encoding β -actin) forward, 5'-GCTCTGGCTCCTAGCACCAT-3', and reverse, 5'-GCCACCGATCCACAC AGAGT-3'. Assay-on-Demand Gene Expression Products (Applied Biosystems) were used for other genes. Real-time PCR was set up according to the manufacturer's instructions with the 2 $\times$ SYBR Green or TaqMan PCR Master Mix and was done on a 7900HT Sequence Detection System (Applied Biosystems). Values are normalized to expression of *Actb*. Real-time PCR analysis used material from three or more independent 'sorts'.

Generation and transfection of Fc γ RIV and Fc γ RIII expression vectors. The cDNA containing the full-length open reading frame encoding mouse Fc γ RIV, Fc γ RIII, Fc γ or CD8 was amplified by PCR from the respective IMAGE clones (Invitrogen) or with FirstChoice RACE-Ready Mouse Spleen cDNA (Ambion). PCR products were ligated into the pCR2.1 or pCRII TOPO T/A vector (Invitrogen) and were sequenced. A second round of PCR was done for subcloning of Fc γ RIV or Fc γ RIII cDNA, lacking the sequence encoding the signal peptide, into the pDisplay vector (Invitrogen) in-frame with a hemagglutinin sequence tag. The cDNA encoding each chimeric protein was excised from pDisplay and was subcloned into the pMX retrovirus vector⁴⁵, which is modified with a puromycin-resistance gene and sequence encoding enhanced green fluorescent protein downstream of an internal ribosomal entry site element. Fc γ cDNA was amplified and was cloned into the pcDNA4-myc-His vector (Invitrogen) for the addition of a c-Myc-histidine tag and was subcloned into the pMX vector modified with sequence encoding mouse CD8 downstream of an internal ribosomal entry site element. Transfection of BOSC23 cells was done as described⁴⁵. Supernatant containing retrovirus was added to 1 $\times$ 10⁶ BW5147 or A20 IIA1.6 cells. Transduced cells were incubated for 4 d in medium containing puromycin (1.5 μ g/ml) and were sorted with a MoFlo.

Production of recombinant Fc γ RIV. The cDNA encoding amino acids 21–81 or amino acids 130–190 of the Fc γ RIV extracellular region was amplified by PCR, was purified by agarose gel electrophoresis, was digested with *Bam*HI and *Hind*III and was ligated into the pET-24b expression vector in-frame with a six-histidine tag and the basic region of human immunodeficiency virus 1 transactivator protein (amino acids 47–57). After plasmids were sequenced, Rosetta (DE3) *Escherichia coli* cells (Novagen) were transformed with the plasmids and proteins were induced by the addition of 1 mM isopropyl- β -D-thiogalactopyranoside and were purified on an affinity column (nickel-nitrilotriacetic acid resin; QIAGEN).

Production of polyclonal antibodies. A New Zealand White rabbit was 'hyperimmunized' with the recombinant Fc γ RIV extracellular proteins (six times, 50 μ g each) over a 2-month period. Antiserum was collected 7 d after the final immunization and was affinity-purified with recombinant Fc γ RIV coupled to *N*-hydroxysuccinimide-activated sepharose (Amersham Pharmacia Biotech). Antibodies were digested with papain or pepsin to create Fab or F(ab')₂ fragments, respectively.

Evaluation of Fc γ RIV expression and immunoglobulin binding. BW5147 and A20 IIA1.6 cells were retrovirally transduced with Fc γ RIV or Fc γ RIII either alone or together with Fc γ constructs. Cell surface expression of Fc γ RIV and Fc γ RIII was assessed by flow cytometry with anti-hemagglutinin and binding of immunoglobulins was assessed after incubation with 50–100 μ g/ml of mouse IgM, IgA, IgG1, IgG2a, IgG2b, IgG3 or IgE or human IgM, IgA, IgG1, IgG2,

IgG3 or IgG4. Cells were counterstained with phycoerythrin-conjugated polyclonal goat anti-mouse immunoglobulin, goat anti-human immunoglobulin (Southern Biotechnology Associates) or monoclonal anti-mouse IgE (eBiosciences).

Cell surface biotinylation, immunoprecipitation, affinity precipitation and immunoblot. Transduced cells were lysed with 1% Nonidet P-40 lysis buffer before immunoprecipitation with anti-hemagglutinin or normal rabbit IgG and were separated by SDS-PAGE in reducing conditions. Proteins transferred to polyvinylidene difluoride membranes were blotted with horseradish peroxidase-labeled anti-hemagglutinin or anti-Fc γ R. Horseradish peroxidase-labeled goat anti-rat immunoglobulin (Amersham Pharmacia Biotech) or goat anti-rabbit immunoglobulin (Southern Biotechnology Associates) was used to develop membranes for visualization by enhanced chemiluminescence (Amersham Pharmacia Biotech). Spleen cells (5 $\times$ 10⁷) were biotinylated for 1 h at 4 °C and were lysed with 1% Nonidet P-40 lysis buffer before immunoprecipitation with adsorbed polyclonal anti-mouse Fc γ RIV or normal rabbit IgG. Immunoprecipitates were separated by SDS-PAGE in reducing conditions, were transferred to polyvinylidene difluoride membranes and were blotted with horseradish peroxidase-labeled streptavidin, anti-Fc γ RIV or anti-Fc γ before incubation with horseradish peroxidase-labeled goat anti-rabbit immunoglobulin for development and visualization by enhanced chemiluminescence. For affinity precipitation, cell lysates from A20 IIA1.6 and P815 (mastocytoma) cells were incubated with the biotinylated peptide corresponding to the YEEP motif of mouse Fc γ RIV (residues 223–247) or the version of this peptide phosphorylated at Tyr235. Peptide precipitates were separated by SDS-PAGE and were analyzed by immunoblot with anti-Crk-L or anti-PI(3)K and chemiluminescence.

Surface plasmon resonance analysis. A BIAcore 3000 (BIAcore) instrument was used to study the interaction between mouse Fc γ RIII and Fc γ RIV with mouse or human immunoglobulins as described⁴⁶. A monoclonal antibody to a five-histidine tag (Penta-His; QIAGEN) was attached to a CM5 sensor chip (6,000 resonance units) with a standard amine-coupling kit (BIAcore). Mammal-derived recombinant histidine-tagged Fc γ RIV or Fc γ RIII (R&D Systems) was attached to the chip at the manufacturer's recommended concentration (100 resonance units). Binding of immunoglobulin to the chip was measured in duplicate assays at a flow rate of 50 μ l/min in buffer consisting of 10 mM Hepes, pH 7.4, 150 mM NaCl, 3.4 mM EDTA and 0.005% (wt/vol) surfactant P20. IgE^a anti-TNP (C38-2; BALB/c) and IgE^b anti-TNP (C48-2; C57BL/6), prepared by fusion with the mouse F0 myeloma cell line, were used for IgE-binding analysis.

Macrophage stimulation, FcR-mediated phagocytosis and cytokine secretion analysis. Peritoneal cells stained with fluorescein isothiocyanate-conjugated anti-CD11b and biotin-conjugated anti-Gr-1 followed by staining with streptavidin-phycoerythrin-Texas red (all from BD PharMingen) were also stained with allophycocyanin-conjugated anti-B220 (anti-CD45R; RA3-6B2; BD PharMingen) for identification of B-1 cells. CD11b⁺Gr-1⁻B220⁻ macrophages, representing about 5% of peritoneal cavity cells (Supplementary Fig. 6), were isolated from C57BL/6, BALB/c, *Fcgr3*^{-/-} or *Fcer1g*^{-/-} mice by sorting with a MoFlo. Sorted cells (over 98% pure) were cultured for 48 h in DMEM containing IgG-free FCS and IFN- γ (100 U/ml). For quantitative real-time PCR, peritoneal macrophages from C57BL/6 mice were cultured for 48 h with lipopolysaccharide (1 μ g/ml), IFN- γ (100 U/ml) or IFN- β (100 U/ml).

Phagocytosis was assayed by a standard method⁴⁷. A 50% (vol/vol) Alsever's mixture of SRBCs (Colorado Serum Company) was washed and treated with 2,4,6-trinitrobenzenesulfonic acid (Sigma) to create TNP-conjugated SRBCs. TNP-conjugated SRBCs, Na₂⁵¹CrO₄ and IgE anti-TNP or IgG anti-TNP were mixed and incubated together for 1 h at 37 °C for opsonization and labeling of SRBCs. Labeled SRBC immune complexes were added to culture medium containing 10 μ g/ml of Fc ϵ RI α and Fc ϵ RII Fab antibody fragments and were incubated for 20–30 min at 37 °C in a 96-well plate with IFN- γ -stimulated peritoneal macrophages (5 $\times$ 10⁴). Macrophages were washed with buffer consisting of 0.15 M NH₄Cl, 1 mM KHCO₃ and 0.1 mM EDTA, pH 7.2, and were lysed with 0.5% (wt/vol) SDS. Lysates were collected and were assayed on a γ -counter (Perkin Elmer). For analysis of the production of tumor necrosis

factor, IL-6 and nitric oxide, macrophages were stimulated with IFN- γ and were incubated for 12 h with immune complexes of SRBCs and IgE^a, IgE^b or IgE^b in culture medium containing Fc ϵ RI α , Fc ϵ RII and Fc γ RIIb Fab antibody fragments. Supernatants were used to measure the concentrations. For analysis of the production of nitric oxide, 10 μ M L-NMMA (N^G-monomethyl-L-arginine monoacetate salt; Sigma) or D-NMMA (N^G-monomethyl-D-arginine monoacetate salt; Sigma) was added to culture medium in control experiments. Tumor necrosis factor and IL-6 in culture supernatants were measured with enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (Pierce). The concentration of nitrate plus nitrite was measured with the Nitrate/Nitrite Colorimetric Assay kit (Cayman Chemical) according to the manufacturer's instructions.

Antigen presentation. Allergen sensitization was accomplished as described⁴⁸. Intraperitoneal injection of 80 allergy units of *D. pteronyssinus* (Greer Laboratories) in alum suspension (1:4 (vol/vol) Imject; Pierce) was done at weekly intervals (six times). At 5 d after the last injection, mice (C57BL/6, BALB/c, *Fcgr3*^{-/-} and *Fcer1g*^{-/-}) were challenged intranasally with 50 allergy units of *D. pteronyssinus*. This was followed within 10 min by intravenous injection of saline, 2.4G2 or Fc γ RIV Fab antibody fragments (40 μ g/100 μ l). The allergen challenge and these treatments were repeated on a daily basis for 5 d. Spleen cells of treated mice were then stained with anti-CD11b, anti-Gr-1, anti-CD8, anti-B220 (CD45R), anti-DX5 and anti-Ter119 before macrophages (CD11b⁺Gr-1⁻B220⁻) and T cells (all negative) were sorted by flow cytometry. Serum IgE was measured by capture ELISA with an OptEIA ELISA Set (BD PharMingen) or mouse IgE ELISA quantification kit (Bethyl Laboratories). Der p I (Indoor Biotechnologies) pretreated with 2,4,6-trinitrobenzenesulfonic acid was allowed to react with IgE^a or IgE^b anti-TNP to make Der p I-IgE immune complexes. Mitomycin C-treated macrophages were incubated with saline, 2.4G2 or Fc γ RIV Fab antibody fragments (20 μ g/ml). Macrophages and T cells of the same mouse strain were mixed and were incubated for 48 h with Der p I, Der p I-IgE^a, or Der p I-IgE^b in culture medium containing Fc ϵ RI α and Fc ϵ RII Fab antibody fragments (20 μ g/ml). [³H]thymidine incorporation during the final 16 h of culture was measured.

IgE-mediated cutaneous inflammation. This cutaneous reaction was induced essentially according to an established method¹⁷. C57BL/6, BALB/c or *Fcgr3*^{-/-} mice were passively sensitized by intravenous injection of 15 μ g IgE^a anti-DNP (H1- ϵ -26) or 10 μ g IgE^b anti-DNP (SPE-7) dissolved in saline or the same volume of saline after intravenous injection of saline, anti-Fc ϵ RI, 2.4G2 or Fc γ RIV Fab antibody fragments (40 μ g). Cutaneous reactions were elicited 24 h later by the application of 25 μ l of 0.2% (wt/vol) DNFB (Sigma) in vehicle (acetone/olive oil (4:1)) in the right ear and the same volume of vehicle in the left ear. Ear thickness was measured with an engineering micrometer (Mitutoyo) at various intervals after challenge with DNFB and vehicle.

For the induction of macrophage-mediated cutaneous inflammation, sorted macrophages (CD11b⁺Gr-1⁻B220⁻) from C57BL/6, BALB/c and *Fcer1g*^{-/-} mice, which were sensitized with 30 μ l of 0.5% (wt/vol) DNFB at 6 d before purification, were purified by sorting of peritoneal lavage and spleen cells. Macrophages were stimulated with IFN- γ (100 U/ml) at 37 °C. After 48 h of incubation, macrophages were pretreated with 20 μ g/ml of Fc ϵ RI α and Fc ϵ RII Fab antibody fragments and were allowed to react with IgE^a anti-DNP or IgE^b anti-DNP or with IgE^a anti-TNP or IgE^b anti-TNP (50 μ g/ml) to allow binding of macrophages to the IgE-Fc γ RIV complexes. IgE-treated macrophages (1×10^6) were injected intradermally into the ears of C57BL/6, BALB/c or *Fcer1g*^{-/-} mice. After 30 min, ears were painted with 25 μ l of 0.2% (wt/vol) DNFB on the right ear and the same volume of vehicle on the left ear. Ear swelling was measured at the various intervals after challenge. Ear thickness was calculated as follows: $(R_t - L_t) - (R_0 - L_0)$, where R_t and L_t are the thicknesses of the DNFB-treated right ear and vehicle-treated left ear, respectively, at a specific time points after treatment, and R_0 and L_0 are the thicknesses of the right and left ears, respectively, at the beginning of the experiment (0 h).

Statistical analysis. Difference was compared with the two-tailed Student's *t*-test, and differences with a *P* value of less than 0.05 are considered statistically significant.

Accession codes. GenBank: hamster Fc γ RIV, EF158002; rat Fc γ RIV, EF158003.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

M.H. designed and did experiments and prepared the manuscript; R.S.D. assisted in experimental design and manuscript preparation; W.D.F. did the surface plasmon resonance analysis; S.N. and K.S. did the structural analysis; H.Y. and K.K. did the glycosylation analysis; R.P.S. contributed to the PCR analysis; and M.D.C. contributed to the study design, data interpretation and manuscript preparation.

COMPETING INTERESTS STATEMENT

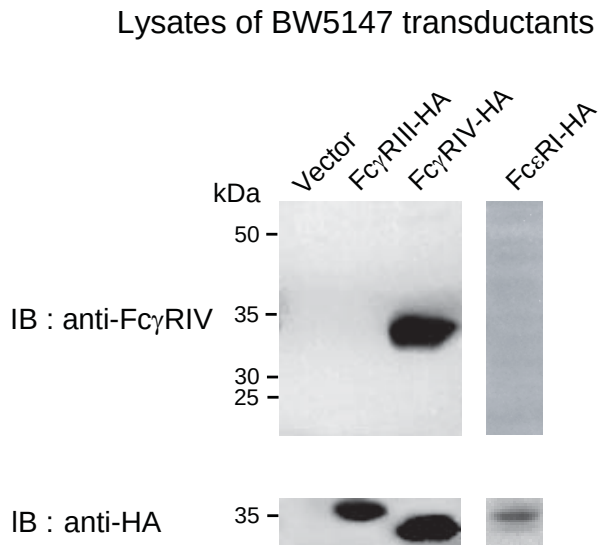
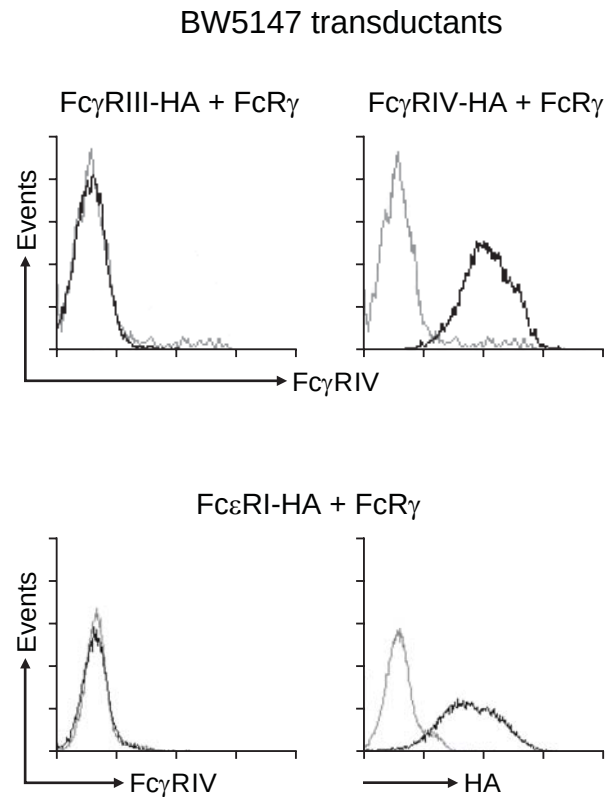
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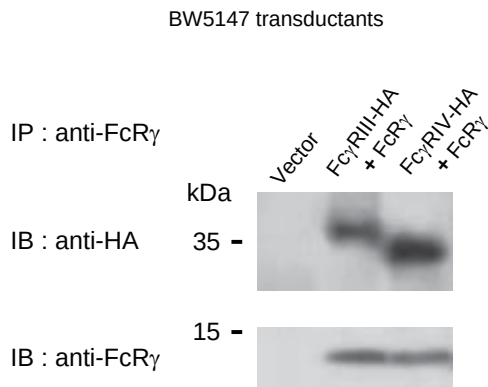
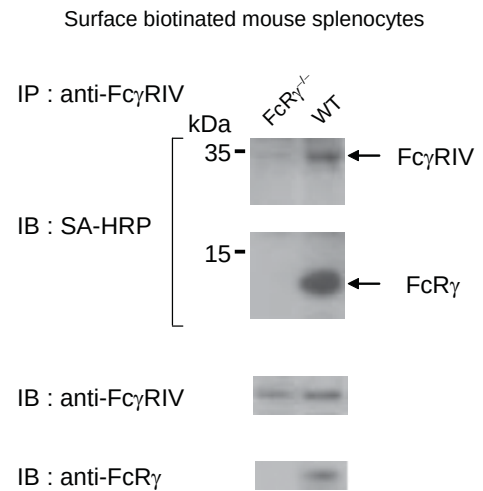
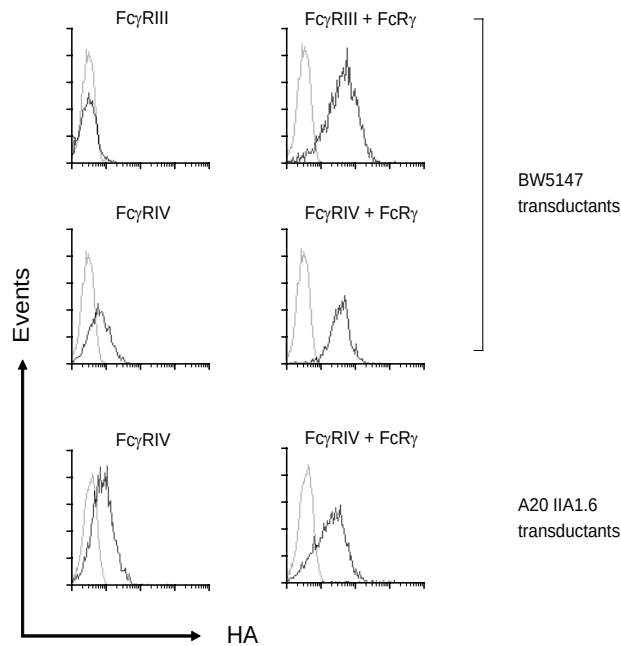
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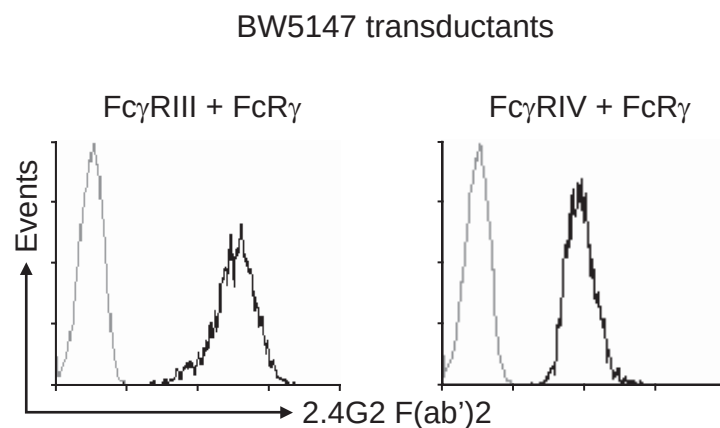
a**b**

Supplementary Figure 1 Specificity of polyclonal antibodies to Fc γ RIV. **(a)** Mouse thymoma BW5147 cells were transduced with the HA-tagged Fc γ RIV, Fc γ RIII, or Fc ϵ RI with FcR γ cDNAs. Control BW5147 cells were transfected with the empty vector. The lysates of the transductants were separated by SDS-PAGE under reducing conditions, transferred onto membrane, blotted with the anti-Fc γ RIV, and visualized by chemiluminescence. **(b)** BW5147 cells, which express HA-tagged Fc γ RIV, Fc γ RIII, or Fc ϵ RI with FcR γ , were stained with anti-Fc γ RIV and analyzed by flow cytometry. Cell surface expression of Fc γ RIV, Fc γ RIII, and Fc ϵ RI were confirmed by flow cytometry using an anti-HA. Data were reproducible in three separate experiments.

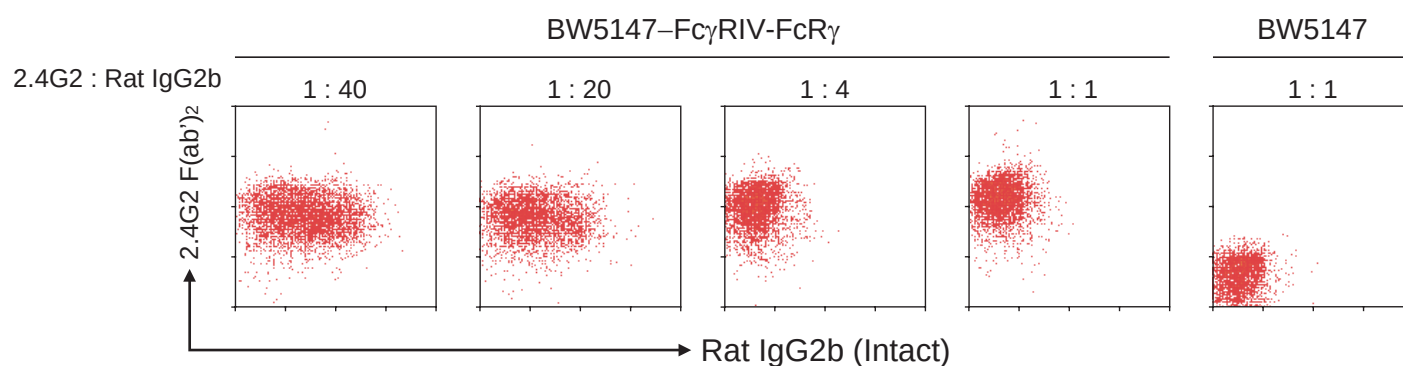
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Supplementary Figure 2 Association of Fc γ RIV with FcR γ and enhancement of Fc γ RIV surface expression by FcR γ . **(a)** Mouse thymoma BW5147 cells were transduced with HA-tagged Fc γ RIV or Fc γ RIII with FcR γ cDNAs. The cells were lysed and immunoprecipitated with an anti-FcR γ . The immunoprecipitates were separated in a 10% acrylamide gel under reducing conditions, transferred onto membranes, blotted with anti-HA (top) or anti-FcR γ (bottom), and visualized by chemiluminescence. **(b)** Assessment of Fc γ RIV expression in FcR γ -deficient versus normal mice. Splenocytes from FcR γ -deficient (Fc γ R1g^{-/-}) (left) or normal (WT) (right) mice were biotinylated and lysed in 1% NP-40, and subjected to immunoprecipitation with the anti-Fc γ RIV. The immunoprecipitates were resolved by SDS-PAGE, transferred onto membranes, blotted with streptavidin-HRP (top), anti-Fc γ RIV (middle) or anti-FcR γ (bottom), and visualized by chemiluminescence. **(c)** BW5147 (top and middle) or A20 IIA1.6 (bottom) cells transfected with Fc γ RIII (left top) or Fc γ RIV (left middle and bottom) and FcR γ . Viable cells were incubated with PE-labeled anti-HA and analyzed by flow cytometry. Results are representative of three separate experiments.

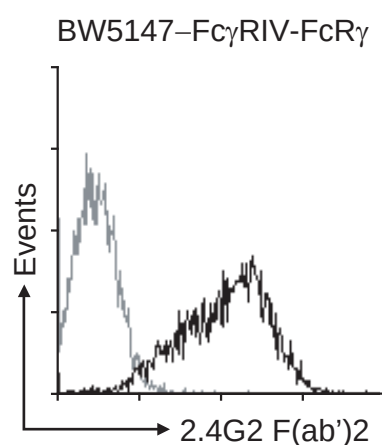
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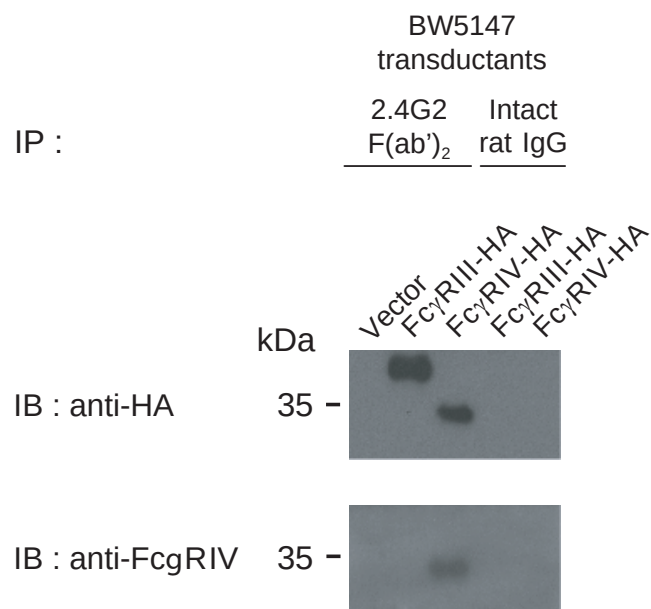
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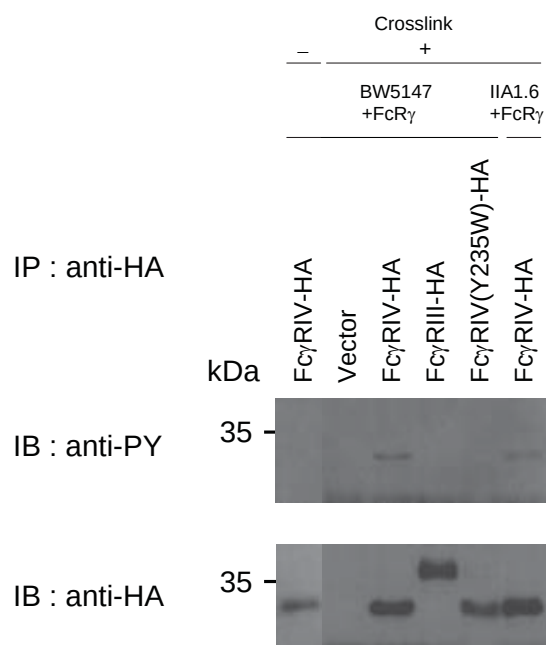


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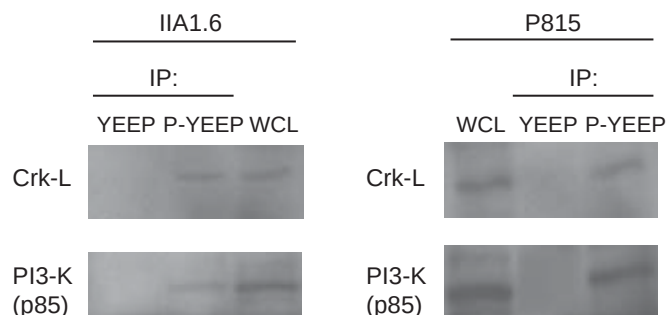


Supplementary Figure 3 The 2.4G2 monoclonal antibody recognizes both Fc γ RIII and Fc γ RIV. (a) BW5147 cells transduced with Fc γ RIII-FcR γ or Fc γ RIV-FcR γ were stained with 2.4G2 F(ab')₂ fragments (2.5 μ g/ml) and detected by flow cytometry. (b) BW5147 or Fc γ RIV-FcR γ -BW5147 cells were stained with 2.4G2 F(ab')₂ fragments and intact rat IgG2b in the indicated ratio (2.5-100 μ g/ml) and detected by flow cytometry. (c) Fc γ RIV-FcR γ -BW5147 cells were stained with 2.4G2 F(ab')₂ fragments (2.5 μ g/ml) after intact rat IgG2b pretreatment (200 μ g/ml). (d) Fc γ RIII-FcR γ -, Fc γ RIV-FcR γ -, or empty vector-transduced BW5147 cells were lysed and immunoprecipitated with 2.4G2 F(ab')₂ fragments or intact rat IgG2b. Immunoprecipitates were separated by SDS-PAGE, transferred to the membrane, and blotted with anti-HA or anti-Fc γ RIV. Data are representative of three independent experiments.

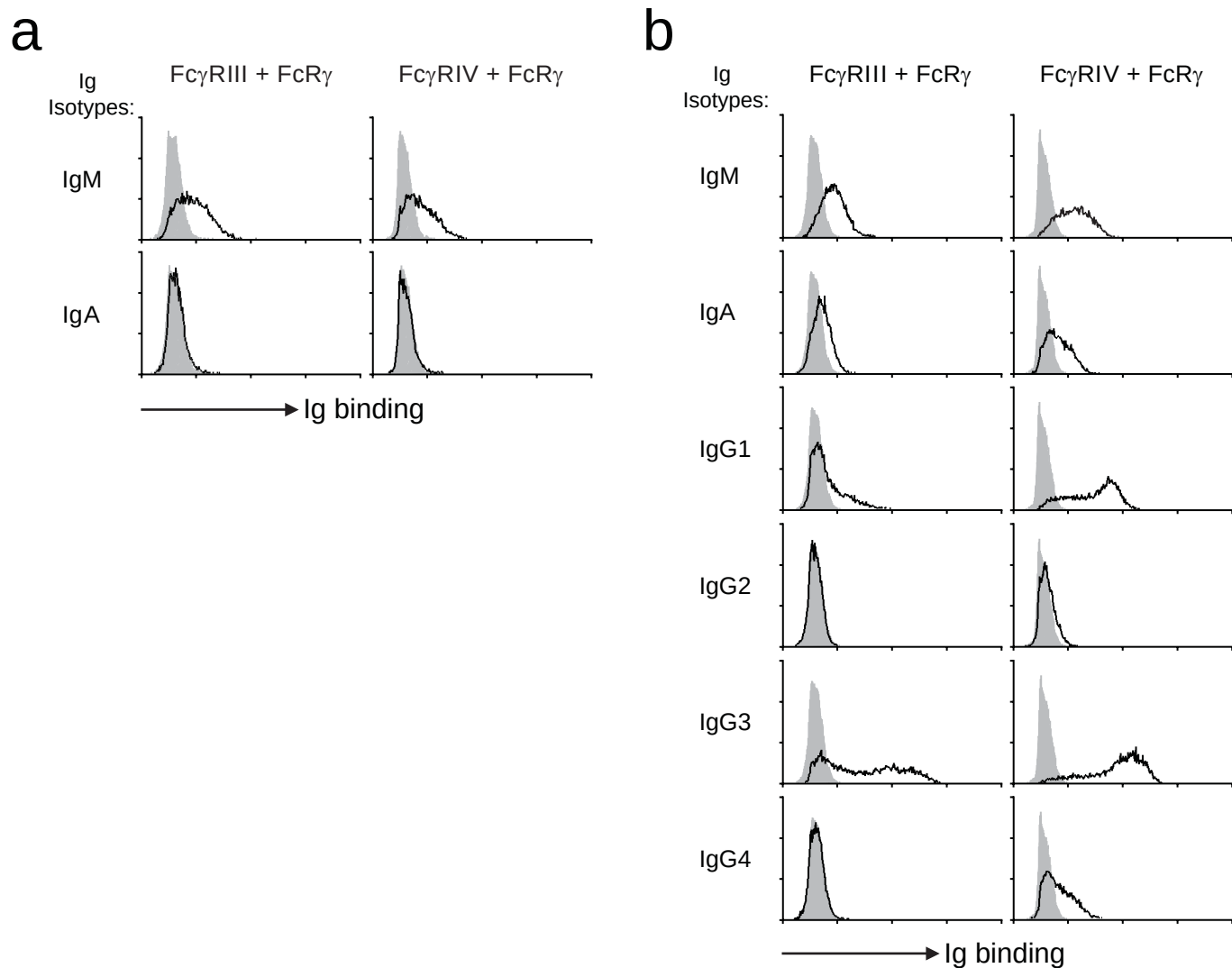
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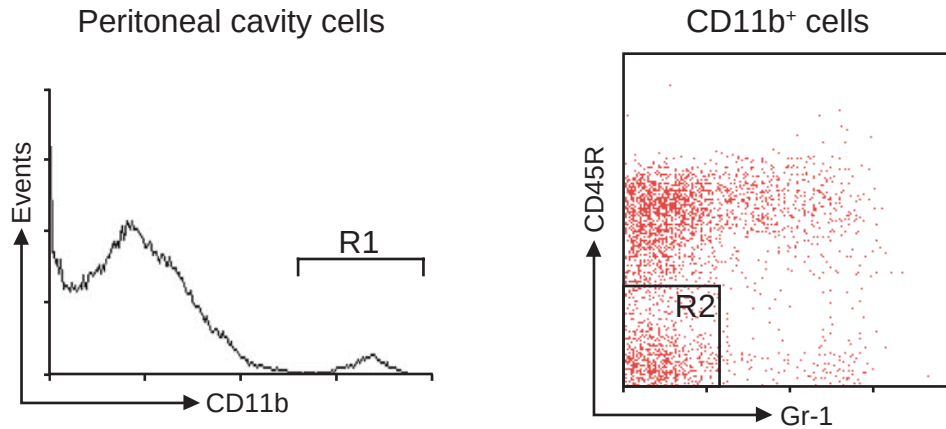
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Supplementary Figure 4 Fc γ RIV phosphorylation and association with Crk-L and PI3K . **(a)** BW5147 cells were transduced with the empty vector, HA-tagged Fc γ RIV, Fc γ RIII, or Fc γ RIV (Y235W) with FcR γ cDNAs. A20 IIA1.6 cells were transduced with HA-tagged Fc γ RIV and FcR γ cDNAs. The cells were cultured in the medium without serum for 12 h and stimulated with 2.4G2 followed by treatment with anti-rat Ig. Stimulated cells were lysed and immunoprecipitated with an anti-HA. The immunoprecipitates were separated in a 12% acrylamide gel under reducing conditions, transferred onto membranes, blotted with anti-phosphotyrosine (top) or anti-HA (bottom), and visualized by chemiluminescence. **(b)** Lysates of A20 IIA1.6 and P815 mastcytoma cell were incubated with biotinylated phosphopeptides corresponding to the YEPP motif of the Fc γ RIV α chain and mixed with streptavidin beads. The precipitates were separated by SDS-gel electrophoresis, transferred onto membrane, blotted with anti-Crk-L or PI3K, and visualized by chemiluminescence. All data are representative of three independent experiments.



Supplementary Figure 5 Comparative analysis of the mouse and human Ig isotype-binding specificities for Fc γ RIII and Fc γ RIV. Fc γ RIV or Fc γ RIII was expressed on the cell surface of BW5147 cells by co-transduction with FcR γ . Transduced cells incubated with mouse IgM and IgA (**a**) or human IgM, IgA, IgG1, IgG2, IgG3, and IgG4 (**b**) were analyzed by flow cytometry and the results displayed as bi-exponential histograms. Data are representative of three separate experiments.



Supplementary Figure 6 The peritoneal cavity cells from C57BL/6, BALB/c, *Fcgr3*^{-/-}, or *Fcer1g*^{-/-} mice were stained with anti-CD11b, anti-Gr-1, and anti-B220/CD45R. CD11b⁺Gr-1⁻CD45R⁻ macrophages (R2) were isolated by sorting (>98% purity). The cells from all mice showed similar profiles and C57BL/6 cells are shown as representatives. All data are representative of three independent experiments.

Supplementary Table 1

Comparison of Fc γ RIII and Fc γ RIV Expression by Different Cell Lines^a

<u>Cell Line</u>	<u>FcγRIII</u>	<u>FcγRIV</u>
Pro-B (Raw8.1)	+	—
Pre-B (70Z/3)	—	—
Immature B (WEHI-231)	—	—
B (A20)	—	—
B (X16C8.5)	—	—
T (EL4)	—	—
T (BW5147)	—	—
Immature DC (JAWSII)	++	++
NK (L NK5E6)	++	—
NKT (NKT)	+	—
NKT (2C12)	+	—
Fibroblast (NIH3T3)	—	—
Macrophage (IC-21)	++	++
Macrophage (PU5-1.8)	+	+
Macrophage (P388D)	+	+
Macrophage (RAW264.7)	+	+
Myeloid (WEHI-3)	++	+
Neutrophil (MRPO)	++	+

^a The relative expression of Fc γ RIV and Fc γ RIII in different cell types was evaluated by quantitative real-time PCR analysis. *Actb* expression was used to control the mRNA amounts in each sample.

Supplementary Table 2

Ig binding affinities of Fc γ RIII and Fc γ RIV^a

	Fc γ RIII	Fc γ RIV
	K _A (M ⁻¹)	K _A (M ⁻¹)
Mouse		
IgG1	2.8 x 10 ⁵	—
IgG2a	7.2 x 10 ⁵	3.0 x 10 ⁷
IgG2b	1.2 x 10 ⁶	2.7 x 10 ⁷
IgG3	—	ND
IgM	—	—
IgA	—	—
IgE ^a	—	—
IgE ^b	—	6.8 x 10 ⁵
Human		
IgG1	ND	6.0 x 10 ⁶
IgG3	ND	1.0 x 10 ⁷

^a Surface plasmon resonance analysis was performed to measure affinity constants of Fc γ RIV and Fc γ RIII. Fc γ RIV or Fc γ RIII was attached to the chip and mouse and human Igs were injected through flow cells.

—, undetectable binding; ND, not determined