FcgammaRIV is a mouse IgE receptor that resembles macrophage FcepsilonRI in humans and promotes IgE-induced lung inflammation.

David A. Mancardi, Bruno Iannascoli, Sylviane Hoos, Patrick England, Marc Daëron, Pierre Bruhns

To cite this version:
David A. Mancardi, Bruno Iannascoli, Sylviane Hoos, Patrick England, Marc Daëron, et al.. FcgammaRIV is a mouse IgE receptor that resembles macrophage FcepsilonRI in humans and promotes IgE-induced lung inflammation.. Journal of Clinical Investigation, American Society for Clinical Investigation, 2008, 118 (11), pp.3738-50. <10.1172/JCI36452>. <pasteur-00363913>
FcγRIV is a mouse IgE receptor that resembles macrophage FcεRI in humans and promotes IgE-induced lung inflammation

David A. Mancardi,1,2 Bruno Iannascoli,1,2 Sylviane Hoos,3,4 Patrick England,3,4 Marc Daëron,1,2 and Pierre Bruhns1,2

1Institut Pasteur, Département d’Immunologie, Unité d’Allergologie Moléculaire et Cellulaire, Paris, France.
2INSERM, U760, Paris, France. 3Institut Pasteur, Département de Biologie Structurale et Chimie, Plateforme de Biophysique des Macromolécules et de leurs Interactions, Paris, France. 4CNRS, URA 2185, Paris, France.

Introduction
A novel murine receptor for the Fc portion of mAbs (FcR) was recently cloned on the basis of bioinformatics database search. This receptor is among the many FcR-like (FCRL) molecules identified in mammals, and it was first named murine FcRL3 (NCBI sequence BC027310) (1). FCRLs have no known ligand except murine FcRL3. As it was found to bind IgG, mouse FcRL3 was renamed mFcRγI.

mFcRγI binds mouse IgG2a and IgG2b with an intermediate affinity (equilibrium association constant \(K_a \approx 2.9 \times 10^5 \text{M}^{-1}\) and \(1.7 \times 10^7 \text{M}^{-1}\), respectively; ref. 2). Two main types of FcRs can be distinguished on the basis of their affinity for immunoglobulins. Monomeric immunoglobulins can bind to high-affinity (\(K_a \approx 10^8–10^{10} \text{M}^{-1}\)) but not to low-affinity (\(K_a \leq 10^6 \text{M}^{-1}\)) receptors. As a consequence, a proportion of high-affinity receptors are occupied in vivo, whereas low-affinity receptors remain free, even though they are exposed to high concentrations of circulating immunoglobulins in vivo (3). Immune complexes (ICs) bind to low-affinity receptors with a high avidity. They also bind to high-affinity receptors. Both types of receptors signal when they are aggregated at the cell surface by mAbs and multivalent antigen (Ag). Rather than on the affinity of receptors, signals generated upon FcR aggregation depend on functional motifs contained in the intracellular domains of FcR subunits engaged in receptor aggregates.

mFcRγI is an activating receptor (2). Like most activating FcRs, it associates with the common FcRγ subunit. FcRγ is a homodimer that contains 2 immunoreceptor tyrosine-based activation motifs (ITAM). The phosphorylation of FcR ITAMs by Src kinases initiates the constitution of an intracellular signaling complex, which activates an array of metabolic pathways leading to cell responses. FcRγ-dependent activation signals are amplified by FcRβ (4), another ITAM-containing subunit expressed in mast cells and basophils. mFcRγI does not associate with FcRβ.

In order for mFcRγI to be expressed at the cell membrane, it must associate with FcRγ (2). FcRγ indeed determines the membrane expression of multichain FcRs, i.e., mFcRγI, human and murine high-affinity receptors for IgE (FcεRI) and IgG (FcγRI), and human and murine low-affinity receptors for IgG (FcγRIIA) (3). FcRβ associates with multichain FcRs (6) expressed in mast cells and basophils (7). It is, however, mandatory for the expression of mFcεRI only (8, 9). FcεRI can therefore be expressed with FcRβ [hFcεRI(αεγ)] in mast cells and basophils or without in monococytes, macrophages, and neutrophils [hFcεRI(εγ)], especially in atopic individuals. hFcεRI may therefore be expressed in 2 forms depending on the cell type and on the species. mFcγRII is expressed in mouse monococytes, macrophages, and neutrophils. mFcγRIIIA was recently reported to bind mouse IgE of the b (IgEb) but not the a (IgEa) allotopy (10). We show here that mFcγRIIIA is a low-affinity receptor for IgE irrespective of the 2 known allotypes (11, 12). FcγRII promotes IgE-induced lung inflammation. These data lead us to propose a mouse model of IgE-induced lung inflammation in which cooperation exists between mast cells and mFcγRIIIA-expressing lung cells. We therefore suggest that a similar cooperation may occur between mast cells and mFcγRII-expressing lung cells in human allergic asthma.

Citation for this article: J Clin Invest. 118:3738–3750 (2008). doi:10.1172/JCI36452.
homology in extracellular domains, hFcyRIIIA was proposed to be the human homolog of mFcyRIV (1). We show that hFcyRIIIA has no detectable affinity for human IgE.

We also show that, in spite of having an intermediate affinity, mFcyRIV binds mouse IgG2a and IgG2b as monomers and functions as a high-affinity receptor. IgE ICs can, however, displace IgG2 from mFcyRIV. When aggregated by IgE ICs of both allotypes, mFcyRIV triggered Ca²⁺ responses in transfected cells and induced macrophage-like transformed cells and peritoneal macrophages to secrete TNF-α.

hFceRII(αγ) has not only an affinity for IgE but also the same quaternary structure and a similar tissue distribution as mFcyRIV. The engagement of hFceRII(αγ) was reported to activate monocytes (13) and neutrophils (14). We found that, when expressed by peritoneal macrophages from transgenic mice, hFceRII(αγ) triggered similar TNF-α responses as endogenous mFcyRIV upon aggregation by IgE ICs. The expression of hFceRII(αγ) is increased by monocytes, macrophages, and neutrophils of asthmatic patients. We found that mouse bronchoalveolar lavage (BAL) macrophages express neither mFceRI nor mCD23 but express mFcyRIV, and they secreted TNF-α upon stimulation with IgE ICs. Finally, using mice deficient for mFceRI/IIβ/IIA−/− mFceRI+/− mCD23−/− (quintuple-KO), that only expressed mFcyRIV, we demonstrate that the in vivo engagement of mFcyRIV by IgE ICs synergizes with mediators released by IgE-activated mast cells to induce lung inflammation.

On the basis of these results, we propose that mFcyRIV is a low-affinity IgE receptor expressed by a subset of myeloid cells, and that, rather than hFcyRIIIA, hFcyRIV(αγ) expressed by monocytes, macrophages, and neutrophils of atopic donors is the human equivalent of mFcyRIV. These properties endow mFcyRIV with what we believe to be novel physiopathological roles in murine models of allergic diseases and parasite infections.

Figure 1
IgE ICs, but not monomeric IgE, bind to mFcyRIV. (A) Schematic representation of FLAG-tagged FcγR α chains associated or not with FcγRγ expressed by transfectants. Green boxes represent ITAMs; the gray box represents an immunoreceptor tyrosine-based inhibition motif; the black stripe represents the FLAG-tag. Histograms show the binding of anti-FLAG mAb (black line) to FLAG-tagged FcγR on CHO transfectants and the binding of anti-mFcyRI mAb (black line) to mFcyRI on BMMCs or the binding of the binding of IgE ICs. (B) Histograms show the binding of mouse IgE to FcγR-CHO and to BMMCs, using 50 µg/ml ultracentrifuged or nonultracentrifuged mouse IgE and 15 µg/ml FITC-conjugated F(ab′)2 anti-mouse Ig. Solid gray histograms represent the binding of FITC-conjugated F(ab′)2 anti-mouse Ig alone. The binding of SPE-7 to FcγRI was analyzed using 2 different lots of SPE-7 and each gave similar results. (C) Histograms show the binding of IgG2b ICs, IgE ICs, or IgE ICs to FcγR-CHO and to BMMCs as revealed by neutravidin staining. ICs were made using TNP-BSA-biotin and 15 µg/ml anti-TNP mAbs. Solid gray histograms show the binding of Ag alone as revealed by neutravidin staining. N.T., not tested. Data are representative of 2 (B) or 5 (C) independent experiments.

Results
mFcyRIV, but not hFcyRIIIA, is a low-affinity IgE receptor. The binding of murine IgE was investigated on CHO transfectants expressing similar levels of FLAG-tagged murine FcγRI (FcγR-CHO) (Figure 1A). High concentrations of 100,000 g ultracentrifuged monomeric IgE bound to mFceRI-expressing mouse BM-derived mast cells (BMMCs) used as positive controls, but not detectably to FcγRI-CHO (Figure 1B). Monomeric IgE of the 2 known allotypes, IgEα and IgEβ, displayed the same binding properties. Nonultracentrifuged preparations of the same IgE, however, bound to mFcyRIV and mFcyRIIIA but not to mFceRI or mFceRIIB, except SPE-7, which bound also to mFceRI. They bound in a manner similar to monomeric IgE binding to BMMCs (Figure 1B). Nonultracentri-
fuged IgE\(^b\) bound more efficiently to mFc\(\gamma\)RIV than nonultracentrifuged IgE\(^a\). These results indicate that nonultracentrifuged IgE preparations contained IgE aggregates that were removed upon ultracentrifugation, and that IgE aggregates but not monomeric IgE could bind to mFc\(\gamma\)RIV. Untreated and ultracentrifuged IgE solutions were analyzed for homogeneity and aggregate content by dynamic light scattering (Supplemental Figure 1). Both untreated but not ultracentrifuged IgE\(^a\) and IgE\(^b\) contained aggregates, but IgE\(^b\) contained these in higher proportion than IgE\(^a\). Noticeably, untreated IgE\(^b\) SPE-7 contained aggregates of bigger size than other IgE preparations. The higher proportion of aggregates among IgE\(^b\) than among IgE\(^a\) preparations may indeed explain their differential binding to mFc\(\gamma\)RIV.

As mFc\(\gamma\)RIV bound IgE aggregates, we investigated the binding of preformed IgE-Ag ICs and, as positive controls, of IgG2b ICs (2), to the same cells. IgG2b ICs bound comparably to mFc\(\gamma\)RI, mFc\(\gamma\)RIIB, mFc\(\gamma\)RIIIA, and mFc\(\gamma\)RIV. IgE ICs bound as efficiently as IgG2b ICs to mFc\(\gamma\)RIV, less efficiently to mFc\(\gamma\)RIIB and mFc\(\gamma\)RIIIA, and not detectably to mFc\(\gamma\)RI. mFc\(\gamma\)RIIB and mFc\(\gamma\)RIIIA were previously reported to behave as low-affinity IgE receptors (15). IgE ICs of the 2 allotypes bound comparably to mFc\(\gamma\)RIV (Figure 1C). These results suggest that mFc\(\gamma\)RIV has a low affinity for IgE.

To measure the affinity of mFc\(\gamma\)RIV for IgE, FLAG-tagged extracellular domains of the 4 murine Fc\(\gamma\)Rs and, as a positive control, of hFc\(\varepsilon\)RI were produced in HEK293T cells. These molecules were N-glycosylated as demonstrated by SDS-PAGE analysis before and after peptide:N–glycosidase F treatment (Figure 2A). They were covalently immobilized onto activated dextran surfaces and used for surface plasmon resonance (SPR) analysis. IgE bound to mFc\(\gamma\)RI, mFc\(\gamma\)RIIB, and mFc\(\gamma\)RIIIA but not to mFc\(\gamma\)RI. IgE bound to mFc\(\gamma\)RIV with a K\(_A\) of approximately 2.6 × 10\(^{-5}\) M\(^{-1}\) (Tables 1 and 2). IgE bound with a 10-fold lower K\(_A\) to mFc\(\gamma\)RIIB and mFc\(\gamma\)RIIIA. It dissociated much faster from Fc\(\gamma\)Rs than from hFc\(\varepsilon\)RI (Figure 2B). The association rate constant (k\(_{\text{on}}\)) of IgE for mFc\(\gamma\)RIV and for hFc\(\varepsilon\)RI were of similar magnitudes, but the dissociation rate constant (k\(_{\text{off}}\)) of IgE for mFc\(\gamma\)RIV was 250-fold higher (Table 1). As a consequence, IgE bound much more transiently to mFc\(\gamma\)RIV (t\(_{1/2}\) ≈ 5 seconds) than to hFc\(\varepsilon\)RI (t\(_{1/2}\) = 17 minutes). Comparable kinetic parameters were obtained for 3 IgE\(^a\) and 2 IgE\(^b\) (Table 2).
Since hFcγRIIIA has been proposed to be the homolog of mFcγRIV, we investigated the binding of human IgE on another set of CHO transfectants expressing similar levels of FLAG-tagged hFcγRIIIA or hFcγRIIB (Supplemental Figure 2A). The 2 known polymorphic variants of hFcγRIIIA (16) and the 3 known polymorphic variants of hFcγRIIB (17, 18) were included. BMMCs from hFcγRIIIs mice (19) were used as positive controls.

High concentrations of monomeric human IgE bound to hFcγRIIIs BMMCs but not to transfectants expressing hFcγRIIIA or hFcγRIIB (Supplemental Figure 2B). The same concentrations of the same IgE failed to bind to hFcγRIII+ CHO when in complex with F(ab′)2 anti-human F(ab′)2 (Supplemental Figure 2C). They also failed to bind to transfectants expressing hFcγRI, hFcγRIIIA (H131; or R131 variants; ref. 20), hFcγRIIB, or hFcγRIIC (data not shown). These complexes, however, bound more avidly than monomers to hFcγRIIIs BMMCs. Human IgE, therefore, do not bind to hFcγRIII. Supporting this observation, SPR analysis showed no measurable affinity of hFcγRIIIA (F176 or V174) or hFcγRIIB (NA1, NA2, or SH) for 3 human IgE (data not shown). These data altogether indicate that hFcγRIIIA is not the homolog of mFcγRIV as for their interactions with IgE.

IgE ICs displace monomeric IgG from mFcγRIV. mFcγRIV was described as having an intermediate affinity for mouse IgG2a and IgG2b. Although this affinity is at least 10-fold higher than that of murine low-affinity FcγR (2), IgE ICs bound similarly to mFcγRIV, to mFcγRII, and to mFcγRIIIA (Figure 1C). This affinity is 5-times lower than that of high-affinity mFcγR (3), and we wondered whether monomeric IgG would bind to mFcγRIV. The same CHO transfectants that were used in Figure 1 were incubated with 100,000 g ultracentrifuged monomeric IgG1, IgG2a, or IgG2b. As expected, neither IgG1 nor IgG2b bound to mFcγRI, mFcγRII, or mFcγRIIIA, whereas IgG2a bound to mFcγRII but not to mFcγRIIIA or mFcγRIIIA. Both IgG2a and IgG2b, but not IgG1, bound to mFcγRIV (Figure 3A). mFcγRIV, therefore, binds monomeric IgG2a and IgG2b, even though it has an intermediate affinity.

To investigate whether, when bound onto mFcγRIV and crosslinked, monomeric IgG2 can activate cells, we generated quintuple-KO mice. Such mice express mFcγRIV as their sole FcR. Thio- glycolate-elicted peritoneal macrophages from these mice secreted comparable amounts of TNF-α upon challenge with F(ab′)2 goat anti-mouse (GaM) when preincubated with monomeric IgG2a or IgG2b but not when preincubated with monomeric IgEa or IgEb (Figure 3B). mFcγRIV, therefore, functions as an activating high-affinity receptor for IgG2a and IgG2b.

mFcγRIV being also a low-affinity receptor for IgE, we wondered whether IgE ICs could bind to mFcγRIV in the presence of IgG. The binding of IgE ICs was reduced when mixed with a saturating concentration of IgG2a (Supplemental Figure 3A). IgG2a bound to mFcγRIV under these conditions, albeit slightly less than in the absence of IgE ICs (Supplemental Figure 3B). Likewise, the binding of IgE ICs was reduced but remained detectable when mFcγRIV CHO were incubated with IgE ICs diluted 1:2 in normal mouse serum (Figure 3C). IgEa and IgEb behaved similarly in every condition. Therefore, IgE ICs and noncomplexed IgG2a compete with each other for binding to mFcγRIV.

We next investigated whether IgE ICs could bind to mFcγRIV when saturated by IgG. We found that IgE ICs could displace previously bound IgG2a (Supplemental Figure 3C) and bind to mFcγRIV (Supplemental Figure 3D). Likewise, we found that IgE ICs bound comparably to mFcγRIV CHO, whether these were preincubated with normal mouse serum diluted 1:2 or not (Figure 3D). IgE of both allotypes behaved similarly. These data imply that IgG2a can dissociate from mFcγRIV. IgG2a and IgG2b indeed dissociated rapidly from mFcγRIV at 37°C (3 s t1/2 ≤ 10 minutes) (Figure 3E). This observation is in keeping with the fast dissociation rates measured from SPR analysis (koff [IgG2a] = 0.0631±0.0145 s−1 and koff [IgG2b] = 0.117±0.011 s−1; Figure 3F).

Table 1  Kinetic parameters of soluble FcR ectodomains for IgEα (C48-2)A

<table>
<thead>
<tr>
<th></th>
<th>koff (M−1s−1)</th>
<th>kass (s−1)</th>
<th>t1/2 (s)</th>
<th>Ks (M−1)</th>
<th>Kd (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mFcγRI</td>
<td>−/−</td>
<td>−/−</td>
<td>−/−</td>
<td>−/−</td>
<td>−/−</td>
</tr>
<tr>
<td>mFcγRIIB</td>
<td>n.m.</td>
<td>n.m.</td>
<td>n.m.</td>
<td>2.0×104</td>
<td>5.0×10−5</td>
</tr>
<tr>
<td>mFcγRIIA</td>
<td>n.m.</td>
<td>n.m.</td>
<td>n.m.</td>
<td>2.0×104</td>
<td>5.1×10−5</td>
</tr>
<tr>
<td>mFcγRIIV</td>
<td>2.60×104</td>
<td>0.1000</td>
<td>3</td>
<td>2.6×104</td>
<td>4.2×10−6</td>
</tr>
<tr>
<td>hFcγRI</td>
<td>1.75×104</td>
<td>0.0003</td>
<td>1.000</td>
<td>5.9×10−7</td>
<td>1.7×10−8</td>
</tr>
</tbody>
</table>

AData generated by injecting soluble monomeric IgEa (C48-2) and IgEb (Supplemental Figure 2A) indicated in Figure 2B onto immobilized FcR ectodomains; koff, equilibrium dissociation constant; kass, dissociation rate constant; kass, association rate constant; n.m., not measurable.

Table 2  Kinetic parameters of mFcγRIV ectodomains for mouse IgEa and IgEbA

<table>
<thead>
<tr>
<th></th>
<th>koff (M−1s−1)</th>
<th>kass (s−1)</th>
<th>t1/2 (s)</th>
<th>Ks (M−1)</th>
<th>Kd (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgEa</td>
<td>4.4×10−6</td>
<td>0.180</td>
<td>1.7</td>
<td>2.4×10−3</td>
<td>4.1×10−6</td>
</tr>
<tr>
<td>IgEb</td>
<td>3.9×10−6</td>
<td>0.065</td>
<td>4.6</td>
<td>6.0×10−7</td>
<td>1.7×10−4</td>
</tr>
<tr>
<td>IgEα</td>
<td>2.6×10−6</td>
<td>0.180</td>
<td>1.5</td>
<td>1.4×10−7</td>
<td>7.1×10−6</td>
</tr>
<tr>
<td>IgEb</td>
<td>1.0×10−6</td>
<td>0.050</td>
<td>6.0</td>
<td>2.0×10−4</td>
<td>4.9×10−6</td>
</tr>
<tr>
<td>IgEα</td>
<td>3.3×10−6</td>
<td>0.044</td>
<td>6.8</td>
<td>7.5×10−6</td>
<td>1.3×10−5</td>
</tr>
</tbody>
</table>

AData generated by injecting soluble monomeric IgEa and IgEb onto immobilized mFcγRIV ectodomains.
response to IgE ICs or to IgE ICs (Figure 4E). IgE IC–induced TNF-α secretion was abrogated by preincubating cells with 9G8 or mFcγRIV-blocking mAb 9E9 (Figure 4F and confirmed by anti–TNF-α ELISA in Supplemental Figure 4C).

Human FcεRI(αγ) is a functional equivalent of mouse FcγRIV. In spite of having a high affinity and being expressed on mast cells and basophils, hFcεRI shares with mFcγRIV similar structures and tissue distributions: both can be expressed by monocytes, macrophages, and neutrophils, and they associate with FcRγ, but not FcRβ, in these cells (3). To investigate whether hFcεRI and mFcγRIV could respond similarly to IgE IC, we used hFcεRIΔ mice. As described previously (21), hFcεRI were expressed on the same cells in these mice as in atopic patients. They were detected on blood GR1+ Mac1+ polynuclear cells and GR1− Mac1− cells (Figure 5A). They were also detected and with a higher expression on thioglycolate-elicited peritoneal macrophages from transgenic but not from WT mice as described. mFcγRIV was expressed similarly in WT and in hFcεRIΔ macrophages. IgE ICs induced WT macrophages to

Figure 3
mFcγRIV is a high-affinity receptor for IgG2a/2b, but IgE ICs can bind to mFcγRIV in the presence of high IgG concentrations. (A) Histograms show the binding of monomeric mouse IgG1, IgG2a, or IgG2b (27–35) (10 μg/ml 100,000 g ultracentrifuged) to FcγR+ CHO, revealed by FITC-conjugated F(ab′)2 GaM. (B) Thioglycolate-elicited peritoneal macrophages from quintuple-KO mice were incubated with indicated concentrations of monomeric mouse Ig (μg/ml) and assayed for TNF-α secretion following incubation with F(ab′)2 GaM. Curves represent the percentage of cytotoxicity as a function of supernatant dilution. (C and D) Histograms show the binding of IgE ICs (C38-2a) or IgE ICs (C48-2b) when diluted 1:2 in normal mouse serum or in PBS to mFcγRIV+ CHO or (D) when mFcγRIV+ CHO were preincubated with normal mouse serum diluted 1:2. Solid gray histograms show binding of Ag alone. IC binding was revealed by neutravidin staining. (E) mFcγRIV+ CHO were preincubated for 1 hour at 4°C with saturating concentrations of indicated IgG. Curves represent the percentage of IgG2a (open diamonds) or IgG2b (27–35) (filled diamonds) bound to these cells after an incubation at 37°C for increasing time periods. Insets show corresponding histograms at 0 (bold black line), 10 (dark gray line), 30 (black line), and 60 (light gray line) minutes. Solid gray histograms show binding of secondary Abs alone. a-, anti-

(F) SPR sensograms resulting from the injection of IgG2a or IgG2b (C48-4) onto immobilized mFcγRIV ectodomains. Data are representative of 2 (A–D and F) or 3 (E) experiments that gave similar results.
mFCγRIβ engagement by IgE ICs induces cell activation in transformed cells. (A) The histogram represents the binding of 9G8 to mFCγRIβ-DT40. Transfectants were loaded with Fluo-3, and the intracellular Ca²⁺ concentration was monitored, following triggering (arrows) by Ag alone (black line), IgE alone (thin lines), or IgE ICs (thick lines). Curves represent the relative intracellular Ca²⁺ concentration as a function of time. Two different lots of SPE-7 were assayed and gave similar results. (B) TNF-α secreted by MH-S cells, induced by LPS or by 1 μg/ml 9G8 and the indicated concentrations (μg/ml) of goat anti-hamster F(ab’)2 (GaH), was titrated in supernatants. (C) Histograms represent the binding of indicated mAbs on IFNγ-treated or untreated MH-S cells. Solid gray histograms represent the binding of isotype controls. (D and E) TNF-α secreted by IFNγ-treated 2.4G2 F(ab’)2–saturated MH-S cells, induced by the following reagents, was titrated in supernatants: (D) IgG2b ICs (Gork) or IgE ICs (C48-2b) and (E) IgG1 ICs (C38-2a) or IgE ICs (C48-2b). (F) IFNγ-treated MH-S cells were saturated with both 2.4G2 F(ab’)2 and Polymyxin B–treated 9G8, 9E9, or irrelevant hamster IgG (Iso.). TNF-α secreted by these cells induced by IgE ICs (C48-2b) was titrated in supernatants. Curves represent the percentage of cytotoxicity as a function of MH-S supernatant dilution. All Ig concentrations are indicated in μg/ml. Data are representative of 3 (A–C) or 2 (D–F) experiments. Mean ± SD of triplicates in TNF-α bioassays are represented (B, D, and E).
mFcγRIIB/IIIA, as expected, nor mCD23, and they bound IgE ICs of either allotype. The binding of IgE ICs was abrogated by 9G8 or by 9E9. BAL cells secreted TNF-α when challenged with IgE ICs but not when challenged with monomeric IgE (Figure 6B). WT mouse 2.4G2 F(ab′)2-blocked BAL cells also secreted TNF-α in response to polymyxin-treated IgE ICs, excluding a possible LPS contamination as being responsible for TNF-α secretion (Figure 6C). IgE of the 2 allotypes behaved similarly (Figure 6D).

To determine whether bronchoalveolar macrophage mFcγRIIV-dependent IgE-induced signals can induce an inflammatory reaction in vivo, we first used mice lacking mFcεRI and mCD23, the 2 classical IgE receptors. When instilled intranasally (i.n.), IgE ICs induced no significant infiltration of polymorphonuclear cells in the bronchoalveolar space. We hypothesized that mast cell activation is required for initiating the inflammatory response in this model, as it has been described in an autoantibody-dependent arthritis model (23). As the mast cells in the mFcεRI−/−mCD23−/− mice lack the high-affinity receptor for IgE, they cannot be activated by IgE ICs. Therefore, we chose to substitute in vivo airway mast cell activation by i.n. instillation of supernatant of WT BMMCs activated in vitro. Supernatant from high numbers of IgE-sensitized BMMCs challenged with Ag induced a dose-dependent alveolar infiltration, starting at a dose equivalent to 5 × 10⁶ stimulated BMMCs (Figure 7A, 2 left panels). IgE ICs, which failed to induce a significant inflammation when administered alone, induced a robust influx of Mac1+ Gr1+ polynuclear cells in bronchial alveoli when administered to mFcεRI−/−mCD23−/− mice 1 day after an i.n. instillation of a dose of IgE-stimulated BMMC supernatant, which did not induce a detectable inflammation (2.5 × 10⁶ stimulated BMMCs, inducing 1% infiltration, equivalent to the infiltration induced by unstimulated BMMC controls) (Figure 7A, 2 right panels). Because mFcεRI−/−mCD23−/− mice express mFcγRIIV only, and as negative controls, in FcRγ−/− mice, which express no activating FcR, IgE ICs induced a similar weak infiltration in quintuple-KO and FcRγ−/− mice. They induced a marked Mac1+ Gr1+ polynuclear infiltration in quin-
tuple-KO (Figure 7B) but not in FcRγ−/− mice (Figure 7C) when administered after a dose of IgE-stimulated BMMC supernatant, which induced no detectable infiltration. No significant increase in lymphocyte populations in the BALs from quintuple-KO mice could be observed, either by flow cytometry (BMMC supernatant, 0.10% ± 0.03%; IgE-ICs, 0.09% ± 0.06%; BMMC supernatant plus IgE-ICs, 0.17% ± 0.10%; variations not significant, Student’s t test) or by cytospin analysis (data not shown). Mediators released by IgE-sensitized mast cells upon engagement of FcεRI by Ag can, therefore, enable bronchoalveolar macrophages to induce lung infiltration, upon engagement of FcyRIV by IgE ICs.

Discussion
mFcγRIV shares several properties with hFcγRIIIA (hCD16A) and, for this reason, it was first named CD16-2 (1). mFcγRIV was found to have an intermediate affinity for mouse IgG2a (K\text{A} = 2.9 \times 10^{-7} \text{M}^{-1}) and IgG2b (K\text{A} = 1.7 \times 10^{-7} \text{M}^{-1}) (2). hFcγRIIIA was also found to have an intermediate affinity for human IgG (K\text{A} = 2.5 \times 10^{-7} \text{M}^{-1}) (24, 25). The higher affinity of hFcγRIIIA than that of hFcγRIIB was proposed to result from the association of hFcγRIIIA with the FcRγ subunit (26). This association confers on mFcγRIV and hFcγRIIIA their cell-activating properties. The high amino acid sequence homology (64.9%) of the extracellular domains of mFcγRIV and hFcγRIIIA may also account for their similar intermediate affinities for IgG. These common features, altogether, support the current view that hFcγRIIIA is a human equivalent of murine FcγRIIIB. Our data challenge this view. We show here that mFcγRIV is an IgE receptor. IgE ICs, but not monomeric IgE, bound to mFcγRIV. This was observed on mFcγRIV-expressing CHO, transformed macrophagic cells, BM-derived cultured macrophages (data not shown), and freshly isolated ex vivo BAL macrophages. IgE\text{a} and IgE\text{b} bound to all 4 cell types similarly. When measured by SPR analysis, the mean affinity of mFcγRIV for 5 different IgE was K\text{A} = 4 \times 10^{-8} \text{M}^{-1}. IgE\text{a} and IgE\text{b} bound with the same affinities. These data, altogether, identify mFcγRIV as a low-affinity receptor for IgE, irrespective of IgE allotypes. Inter-

Figure 6
mFcγRIV engagement by IgE ICs induces TNF-α secretion by BAL macrophages. (A) Density plots and histograms represent the binding of indicated mAbs, 9E9 anti-mFcγRIV mAb, or isotype controls alone (solid gray histogram, top row) to BAL cells from mFcγRIIB+/mFcγRIIIA− mice, or the binding of IgE\text{a} ICs (C38-2\text{a}) or IgE\text{b} ICs (C48-2\text{b}) or Ag alone (TNP-BSA-biotin) (solid gray histogram, bottom row). Numbers in the dot plots represent the percentage of cells in the upper-left quadrant. IC binding was revealed by neutravidin staining. IgE IC binding was assayed in the presence or in the absence of 10 μg/ml 9G8, 9E9, or irrelevant hamster IgG. (B–D) TNF-α secretion by BAL macrophages, harvested from indicated mice and induced by the following reagents, was titrated in supernatants: (B) IgE\text{a} ICs (C48-2\text{a}) or (C) Polymyxin B–treated (PMB-treated) IgE\text{a} ICs (C48-2\text{a}) or (D) IgE\text{b} ICs (C48-2\text{b}) or IgE\text{b} ICs (C48-2\text{b}). The effect of (open circles) Polymyxin B treatment on (filled circles) 1 ng/ml LPS-induced TNF-α secretion is shown in inset. Curves represent the percentage of cytotoxicity as a function of supernatant dilution. Ig concentrations are indicated in μg/ml. Data are representative of 2 (A, B, and D) independent experiments that gave similar results.
Interestingly, 2 amino acids (K<sub>117</sub> and E<sub>132</sub>) (27), which are involved in the formation of 2 salt bridges between the extracellular domains of hFc<sub>ε</sub>RI and the Fc portion of human IgE and which are critical for the binding of IgE, are conserved in mFc<sub>γ</sub>RIV. mFc<sub>γ</sub>RIV was recently reported to bind mouse IgE<sub>b</sub> but not IgE<sub>a</sub> (10). We found the same preference for IgE<sub>b</sub> when using the same commercially available IgE under the same conditions, i.e., when not ultracentrifuged and at the same high concentrations. Binding of IgE of both allotypes, however, was abrogated when aggregates were removed by ultracentrifugation. The 2 IgE<sub>b</sub> anti-DNP, SPE-7 and C48-2, were reported to spontaneously aggregate and to activate mast cells in the absence of Ag (28). Aggregates present in higher amounts in IgE<sub>b</sub> preparations could account for the preference of mFc<sub>γ</sub>RIV for nonultracentrifuged IgE<sub>b</sub> observed by Hirano et al. (10) and by us. In contrast with mFc<sub>γ</sub>RIV, hFc<sub>γ</sub>RIIIA is not an IgE receptor. hFc<sub>γ</sub>RIIIA had no measurable affinity for human IgE when studied by SPR, and human IgE failed to bind to hFc<sub>γ</sub>RIIIA CHO, whether as monomers or as F(ab')<sub>2</sub>-anti-human F(ab')<sub>2</sub> complexes. hFc<sub>γ</sub>RIIIA and hFc<sub>γ</sub>RIIB gave similar results, irrespective of known polymorphisms.

We also found that IgG2a and IgG2b monomers bind to mFc<sub>γ</sub>RIV and that mFc<sub>γ</sub>RIV-bound IgG2 triggers cytokine secretion when crosslinked on ex vivo macrophages. mFc<sub>γ</sub>RIV therefore fulfills the criteria defining high-affinity receptors. If so, one expects mFc<sub>γ</sub>RIV to be occupied by IgG in vivo. mFc<sub>γ</sub>RIV may therefore not be available for IgE ICs, especially as the concentration of IgG is much higher than that of IgE in vivo. However, we found that IgG2a/2b rapidly dissociated from mFc<sub>γ</sub>RIV at 37°C and that IgE ICs replaced IgG2a when added to IgG-saturated mFc<sub>γ</sub>RIV CHO. This finding is of critical importance as it provides a possible biological significance to mFc<sub>γ</sub>RIV as a low-affinity receptor for IgE.

Figure 7
mFc<sub>γ</sub>RIV engagement by IgE ICs in vivo promotes lung infiltration. (A) mFc<sub>ε</sub>R<sup>II</sup>–/–mCD23<sup>–/–</sup> mice were instilled i.n. with IgE<sub>a</sub> anti-OVA (2C6<sub>a</sub>), Ag (OVA), preformed IgE<sub>a</sub> ICs (2C6<sub>a</sub>-OVA) (n = 7), or LPS (n = 2) (first panel), or with cell-free supernatants corresponding to indicated numbers of unstimulated (Unst.) or activated WT BMMCs (cell equivalent [eq.] × 10<sup>6</sup>) (n = 2) (second panel) on day 0. Bold is used to indicate the dose used in B and C. mFc<sub>ε</sub>R<sup>II</sup>–/–mCD23<sup>–/–</sup> mice were instilled i.n. with the same cell-free supernatants (sup.) corresponding to 2.5 × 10<sup>6</sup> activated BMMCs on day 0 (a dose that does not induce a significant infiltration [1%] compared with that induced by supernatant from unstimulated BMMCs) and with preformed IgE<sub>a</sub> ICs on day 1 as indicated. Bar graphs represent the percentage of Mac1<sup>+</sup> Gr1<sup>+</sup> in BAL from these mice 24 hours after i.n. instillation (3 left panels) or the percentage of polynuclear cells in cytospins of BAL (right panel) on day 3. (B and C) Quintuple-KO (n = 5) or FcR<sub>γ</sub>–/– (n = 3) mice were instilled i.n. with the same cell-free supernatants as in A, corresponding to 2.5 × 10<sup>6</sup> activated BMMCs on day 0, and with preformed IgE<sub>a</sub> ICs (2C6<sub>a</sub>-OVA) on day 1. A representative density plot of BAL cells from these mice on day 2 for each experimental condition is shown. Bar graphs represent the percentage of Mac1<sup>+</sup> Gr1<sup>+</sup> in BAL or the percentage of polynuclear cells in cytospins of BAL. Error bars represent mean ± SD (A–C) and significant differences between sample means are indicated (***P < 0.001; **P ≤ 0.01; *P < 0.05; n.s., P > 0.05; Student's t test).
The ITAM-containing FcRγ subunit provides mFcγRIV with cell-activating properties. IgE ICs triggered an increase in the intracellular Ca²⁺ concentration in mFcγRIV+ DT40 cells. IgEα and IgEβ induced similar Ca²⁺ responses. As expected, the anti-DNP IgEα SPE-7 also triggered Ca²⁺ responses in the absence of Ag, supporting the interpretation that it spontaneously aggregates. Noticeably, SPE-7 contained qualitatively bigger aggregates than other IgEs tested by dynamic light scattering. We also observed that, differing from all other IgE tested, SPE-7 bound to mFcγRI. SPE-7 has been reported previously to exhibit Ag multiplicity, binding other molecules than DNP and IgE-binding molecules, to preexist in 2 different conformations, and to exist as 4 different conformations in the presence of Ag (29). It has also been reported that SPE-7 activates mast cells in the absence of Ag (28, 30), but that monovalent hapten abolishes these properties (31), suggesting that SPE-7 auto-aggregates due to a low affinity for its own structure and/or recognizes other cell surface components (32) (reviewed in ref. 33). Altogether, these data obtained by others strongly support our data that SPE-7 contains high–molecular weight aggregates, induces activation in the absence of Ag, and binds other molecules than DNP and IgE-binding molecules, including mFcγRI. Data obtained with this mAb could be questionable, especially when used at high local concentrations in vitro and in vivo: a local injection may considerably affect the probability of high local concentrations of SPE-7 aggregates compared with a systemic (intravenous) injection and therefore induce or not induce cross binding to other molecules.

In addition to triggering calcium responses, IgE ICs triggered TNF-α secretion by a macrophage cell line, MH-S, and by ex vivo macrophages. IgEα and IgEβ ICs induced the secretion of similar amounts of TNF-α. The TNF-α secretion was due to mFcγRIV, as responses of both cell types were not affected by 2.4G2 F(ab′)2, while responses of MH-S were abolished by 9G8. ICs made of IgG2 ICs and IgE ICs induced TNF-α secretions of similar intensities, indicating that they bound to mFcγRIV with similar avidities, although mFcγRIV has markedly different affinities for IgG2 and IgE. IgE ICs can therefore activate macrophages via mFcγRIV. IgE ICs may form when IgE is synthesized locally. A local IgE production was described in the upper respiratory tract of atopic patients and in the bronchial mucosa of asthmatic patients (34). Antibody class-switching to IgE, and therefore local IgE production, is enhanced locally in the nasal mucosa in grass pollen-allergic subjects suffering from rhinitis during the pollinic season (35). They can also form in helmhnt infections, in which specific IgE and parasite Ag are present in high concentrations.

Human macrophages do not express FcRγRIV. They can, however, express another IgE receptor, hFcγRI. Although hFcγRI has a high affinity whereas mFcγRIV has a low affinity for IgE, both can be engaged by IgE ICs. Moreover, hFcγRI is expressed without FcRβ in macrophages and neutrophils and especially in atopic patients (13, 22). Mouse macrophage/neutrophil FcRγIV and human macrophage/neutrophil FcRγRI(αγ) therefore have the same quaternary structure: an IgE-binding subunit and the same ITAM-containing FcRγ subunit. hFcγRI expressed in macrophages from transgenic mice triggered TNF-α to a similar extent as mFcγRIV when engaged by IgE ICs. mFcγRIV and hFcγRI have therefore similar functional properties. hFcγRI aggregation was described to trigger Ca²⁺ responses and cytokine secretion in monocytes from atopic patients (13) and in neutrophils from asthmatic patients (14). As hFcγRI(αγ) is overexpressed on monocytes, neutrophils, and eosinophils in atopic patients and particularly on alveolar macrophages (22), we examined freshly isolated BAL cells for mFcγRIV expression and function. BAL macrophages from WT, mFcγRIIB/IIIA−/−, and mFcγRIIB/IIIA−/− mice expressed mFcγRIV, but neither mFcγRII (mCD23) nor mFcγRI. Binding of IgE ICs to these cells was mFcγRIV dependent, as binding was abolished by 9G8 or 9E9. IgE ICs, but not monomeric IgE, induced these BAL cells to secrete TNF-α. Comparable results were obtained with BAL cells from mFcγRIIB/IIIA−/− mice. IgEα ICs and IgEβ ICs gave similar results. These IgE-induced, mFcγRIV-dependent biological responses of alveolar macrophages are of potentially high physiological relevance. TNF-α, which induces bronchial hyperresponsiveness (36), airway inflammation by neutrophils and eosinophils (37), and activates airway smooth muscle (38), was indeed recognized as playing a major role in asthma-associated remodeling and pulmonary inflammation, especially in asthma refractory to corticosteroid therapy (39).

These results prompted us to examine the role of mFcγRIV in a murine model of passive lung inflammation. Surprisingly, while IgE ICs did induce a weak influx of polymuclear cells in BAL (8%–10%) when instilled i.n. in mFcγRII/−/mCD23−/− mice, they did induce an almost undetectable influx (2.5%) when instilled i.n. in quintuple-KO mice. Therefore, IgE-ICs may induce some infiltration in BAL by triggering mFcγRIIA on mast cells. Lung inflammation involves multiple cell types. Among these, mast cells are well known as the initiators of IgE-induced allergic reactions. We therefore “primed” mice by instilling i.n. supernatants of IgE-sensitized mast cells challenged with Ag in vitro. Mast cell supernatants, collected 30 minutes after challenge, contained granular and lipid mediators but low levels of cytokines (e.g., 92 ± 6 pg/ml TNF-α). These supernatants induced a dose-dependent lung infiltration of polymuclear cells in mFcγRII/−/− mice, and supernatants from high numbers of activated mast cells were required for inducing a significant infiltration. When administered i.n. at a dose which induced no detectable inflammation, supernatants from activated mast cells enabled IgE ICs to induce a marked lung infiltration not only in mFcγRII−/− and mCD23-deficient mice, but also in quintuple-KO mice, which express mFcγRIV only, but not in FcγRI-deficient mice, which express no activating FcR. The absence of inflammation observed in FcγRI-deficient mice rules out the participation of IgE-binding molecules that are not associated with the FcRγ chain, e.g., galectin-3 (40). The use of quintuple-KO mice excluded the contributions not only of the 2 main IgE receptors (mFcγRII and mCD23) but also those of the 2 “minor” IgE receptors (mFcγRIIB and mFcγRIIA) to the inflammatory process we observed. It leaves mFcγRIV as the sole candidate among all known FcRs. mFcγRIV-expressing cells may therefore synergize with mFcγRI-expressing mast cells to generate an IgE-induced lung inflammation. Whether unidentified products in mast cell supernatant enhanced the responsiveness of mFcγRIV-expressing macrophages to IgE ICs, whether vasoactive mediators initiated the extravasation of polymuclear cells into tissues, or whether secreted proteases facilitated their migration by degrading the extracellular matrix is unknown. The responsible mediators in supernatants from activated BMIMCs may not be mast cell specific; other cells could, however, possibly cooperate with macrophages in vivo.

In conclusion, we demonstrate here that mFcγRIV is a low-affinity receptor for IgE, irrespective of allotypes. It can therefore operate in all mouse strains. mFcγRIV has the same quaternary structure, the same tissue distribution, the same ligands, and the same functional properties as human FcγRI(αγ). Mice may therefore
be a better model for IgE-dependent inflammation, allergies, and parasite infections than previously thought, macrophage FcεRI playing, in murine models, the role played by macrophage FcεRI in patients. The cooperation between mast cells and mFcεRI-expressing lung cells proposed here in a mouse model of IgE-induced lung inflammation, indeed suggests that a similar cooperation may occur between mast cells and hFcεRI-expressing lung cells in allergic asthma.

Methods

Cells and cDNAs. CHO-K1, HEK293T, DT-40, and MH-S cells were from the ATCC and cultured as recommended by the manufacturer. BMMCs were obtained from mouse BM cells cultured in OptiMEM (Invitrogen) in 10% FCS, supplemented with 5% X63-IL-3-conditioned medium for 4 weeks (≥ 90% mast cells).

cDNAs coding for mouse FcεRI, FcεRIIB1 (Ly17.2 haplotype), FcεRIIIA (H haplotype; ref. 41), and FcRγ were previously cloned in the laboratory. cDNA from C57BL/6 and 129/Sv spleen cells were used to clone mFcεRI and mFcεRIIB1 (Ly17.1), respectively. Both mFcεRIIB1 haplotypes gave identical results throughout this study; only results obtained with Ly17.2 haplotype are presented. Human FcεRI and FcεRIIIA(R113) cDNAs were from J. Van de Winkel and J. Leusen (University Medical Center Utrecht, Utrecht, The Netherlands). hFcεRIIB1 (NA1, NA2, or SH) cDNAs were provided by S. Santoso and U. Sachs (Institute for Clinical Immunology and Transfusion Medicine, Giessen, Germany). cDNA from human blood cells were used to clone hFcεRIIA(V160), and hFcεRIIIA(F190) by site directed mutagenesis. A cDNA sequence coding for a FLAG tag was inserted immediately 3′ of the signal sequence cleavage site in all FcR cDNAs. Resulting constructs were cloned into pNT (neomycin30). cDNAs corresponding to EC domains of all FcRs were cloned into p3XFLAG-CMV-14 (Sigma-Aldrich). Stable transfectants were obtained and sorted to equivalent surface expression by flow cytometry on a MoFlo (Dako) or FACSaria (Becton Dickinson).

Mice. mFcεRIIB1/IIIA−/− and mFcεRIIB1/IIIA−/− C57BL/6 (N5B6) mice (42) were provided by S. Verbeek (Leiden University Medical Center, Leiden, The Netherlands) and backcrossed to generation N8. hFcεRIIB1−/− (N6B6) and mFcεRIIB1-deficient (43) (N5B6) mice were provided by J.-P. Kinet (Harvard Institutes of Medicine, Boston, Massachusetts, USA) and backcrossed to N12 B6. mCd23-deficient (44) (N12B6) mice were provided by M. Lamers (Max-Planck-Institute for Immunobiology, Freiburg, Germany). Quintuple-KO (N6B6) mice were obtained by intercrosses. WT C57BL/6J and 129/Sv were purchased from Charles River Laboratories, and FcRγ−/− mice (N12B6) were purchased from The Jackson Laboratories. BALS were purchased from ketamin-syalazin anesthetized mice, using 6 washes of 1 ml normal saline solution, and cells were purified by adherence. Peritoneal macrophages were harvested from mice injected i.p. with 2 ml thioglycollate (Bio-Rad) 4–5 days prior to harvest and purified by adherence. All mouse protocols were approved by the Animal Care and Use Committees of Comité régional d'éthique en matière d'expérimentation animale, Ile-de-France, France.

mAbs and reagents. Mouse IgEs, anti-2,4,6-trinitrophenyl hapten (anti-TNP) mlgE3 (C38-2b, C48-2a, 15.3.2) and anti-dansyl mlgE3 (27-74), were purchased from BD Biosciences — Pharmingen; anti-DNP mlgE3 (SPE-7%) was purchased from Sigma-Aldrich; mouse IgE2 anti-OVA (2C6) were provided by L. Kobzik (Harvard School of Public Health, Boston, Massachusetts, USA) (45); and the mouse IgE2 anti-DNP mAb 26828-I was used as culture supernatant. All these IgEs were purified from culture supernatants of hybridomas by the manufacturers or in house and cannot therefore contain another allotype of IgE. Several batches of each commercial IgE used were used and gave identical results.

FITC-conjugated rat anti-mlgG2a, FITC-conjugated anti-Mac1, FITC-conjugated anti-hFcεRI, PE-conjugated anti-IgA, PE-conjugated anti-mCD23, PE-conjugated anti-Gr1, and hamster IgG were purchased from Pharmingen; unlabeled, HRP- or FITC-labeled anti-FLAG (M2) and LPS were purchased from Sigma-Aldrich; unlabeled and FITC-labeled F(ab′)2, GaM were purchased from Jackson ImmunoResearch Laboratories Inc.; PE-conjugated neutralvidin was purchased from Molecular Probes; and unlabeled and FITC-labeled F(ab′)2, goat anti-hamster (GaH) were purchased from Serotec. Hamster anti-mFcεRI mAb 9G8 and 9E9 were provided by J.V. Ravetch (The Rockefeller University, New York, New York, USA); mlgG1 anti-TNP (D10), mlgG2b anti-TNP (GORK), and mlgG2a anti-SRBC (12-6-22) mAbs were purchased by B. Heyman (Uppsala Universitet, Uppsala, Sweden); and human myeloma IgE PS was provided by T.F. Huff (Virginia Commonwealth University, Richmond, VA, USA). Recombinate murine IFN-γ was purchased from Peprotech, and low-endotoxin lysozyme-free OVA was purchased from MP Biomedicals. BSA was trinitrophenylated using trinitrobenzene sulfonic acid to 5 or 16 moles of TNP per mole of BSA.

Monomeric Ig binding assays. Aggregates in stock solutions were removed by an 18-hour ultracentrifugation at 100,000 g, and 2 × 106 cells were incubated with monomeric Ig at indicated concentrations for 1 hour at 4°C. Cell-bound Ig was detected using 15 μg/ml F(ab′)2, FITC-conjugated GaM or PE-conjugated F(ab′)2, anti-human F(ab′)2-specific Abs for 30 minutes at 4°C.

IC Ig binding assays. Unless otherwise specified, mouse ICs were preformed by incubating 10 μg/ml TNPpC–BSA-biotin with 30 μg/ml anti-TNP mAbs for 1 hour at 37°C, and 2 × 105 cells were incubated with ICs for 2 hours at 4°C. ICs bound to cells were detected using PE-conjugated neutralvidin at 2 μg/ml for 30 minutes at 4°C.

Serum or monomeric IgG2a versus IgE IC competition assay. A total of 2 × 106 cells were incubated successively with serum diluted 1:2 or with 30 μg/ml mlgG2a (12-6-22), washed, and incubated with IgE ICs for 2 hours at 4°C; or incubated simultaneously with IgE ICs diluted 1:2 in normal mouse serum; or incubated with both mlgG2a and IgE ICs for 2 hours at 4°C. Bound mlgG2a was revealed using 5 μg/ml FITC-conjugated F(ab′)2, anti-mlgG2a, and bound IgE ICs were revealed using PE-conjugated neutralvidin for 30 minutes at 4°C. Saturation of mFcεRIl by mlgG2a or mlgG2b was obtained from the concentration of 30 μg/ml and up (data not shown).

Dynamic light scattering. Untreated or ultracentrifuged IgE solutions at 300 μg/ml in PBS were analyzed immediately after ultracentrifugation for homogeneity and presence of aggregates of high molecular weight, using a DynaPro MS800 dynamic light scattering instrument (Wyatt). Triplicates of 20 measurements at 25°C were averaged with acquisition periods of 10 seconds. During the illumination, the photons scattered by proteins were collected at a 90-degree angle on a 10-second acquisition period and were fit with the analysis software Dynamics (Wyatt). Intensity fluctuations of the scattered light, resulting from Brownian motion of particles, were analyzed with an autocorrelator to fit an exponential decay function, then measuring a translational diffusion coefficient D. For polydisperse particles, the autocorrelation function was fit as the sum of contributions from the various size particles using the regularization analysis algorithm. D is converted to a hydrodynamics radius Rh through the Stokes-Einstein equation (Rh = kT/6πηD, where η is the solvent viscosity, kT is the Boltzmann’s constant, and T is the temperature).

Apparent molecular weights for a spherical particle were deduced from histograms of distribution of percent intensity versus radius to identify the peak containing monomeric IgE.

Measurement of intracellular free calcium concentration. Mobilization of intracellular free calcium concentration was determined as described previously (46).

Secretion of TNF-α in MH-S and BAL macrophages. For 4 hours, 2.4 × 106 MH-S cells or 1.2 × 107 alveolar macrophages were incubated with indicated
reagents at 37°C. If indicated, preincubations were performed for 1 hour at 4°C: mFcRRIIB/mFcRRIIA were blocked by 20 μg/ml 2.4G2 F(ab′)2, or mFcRIV was blocked by 10 μg/ml 9G8 or 9E9. If indicated, reagents were preincubated 1 hour at 37°C in the presence of 2 μg/ml Polyoxymyxin B.

Secretion of TNF-α in peritoneal macrophages. All plates were coated in sodium carbonate, with or without the presence of 100 μg/ml TNPα-BSA containing 2 μg/ml Polyoxymyxin B, saturated with 1 mg/ml BSA, and incubated with 30 μg/ml IgEa anti-TNP (C48-2) or without for 1 hour at 37°C. Cells were preincubated in the presence of 20 μg/ml 2.4G2 F(ab′)2, added to wells, and incubated for 3 hours at 37°C. For peritoneal macrophages from quintuple-KO mice, cells were incubated for 1 hour with monomeric Ig as indicated, washed 3 times, and incubated with 15 μg/ml F(ab′)2; GaM for 3 hours at 37°C. If indicated, mice were injected intravenously with 20 μg Hamster anti-mFcRIV mAb 9E9 or irrelevant Hamster IgG 1 day before macrophage harvest.

Serial dilutions of cell-free supernatants were harvested and titrated for TNF-α by a cytotoxic assay on L929 cells that could be abrogated by anti-TNF-α mAbs as described previously (47). Additionally, TNF-α was titrated using an anti–TNF-α ELISA kit (R&D Systems).

Production of soluble FcR ectodomains tagged with 3xFLAG fusion protein. cDNA constructs coding for soluble FcR ectodomains tagged with a 3xFLAG peptide were transfected by a standard calcium chloride technique into HEK293T cells. Fusion proteins from 96-hour supernatants were purified lysis to 780 RU. Regeneration was performed using 10 mM NaOH. Aggregation of soluble immunoglobulin G was separated from monomer Ig using Ultrafree-CL PTMK Ultracel-PL 300-kDa Cut-off spin columns (Millipore). (As an internal control of the experiment, 100% of mouse IgM anti-TNP was retained on the column.) A range of Ig concentrations was injected into flow cells at a flow rate of 20 μl/min, with a contact and dissociation time of 150 seconds and 300–500 seconds, respectively. Binding response was recorded as RU (1 RU = 1 pg/mm2) continuously, background binding was automatically subtracted, and equilibrium dissociation time of 150 seconds and 300–500 seconds, respectively.

12. Shinkai, Y., Nakachi, H., Honjo, T., and Okumura, K. 1988. Mouse immunoglobulin allelotypes: multiple differences between the nuclear acid sequenc-
es of the IgEα and IgEβ allelomes. Immunogenetics. 27:288–292.
globulin E (Fc epsilon RI): role in asthma. PASEJB 15:940–949.