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Allergy-Associated FcRβ Is a Molecular Amplifier of IgE- and IgG-Mediated In Vivo Responses

David Dombrowicz,*§ Siqi Lin,* Véronique Flamand,* Anna T. Brini,* Beverly H. Koller,† and Jean-Pierre Kinet*‡
*Laboratory of Allergy and Immunology
Beth Israel Deaconess Medical Center, and
Department of Pathology
Harvard Medical School
Boston, Massachusetts 02215
†Pulmonary Medicine
University of North Carolina at Chapel Hill
Chapel Hill, North Carolina 27514

Summary

A role for the Fc receptor β chain (FcRβ) in the pathogenesis of allergy has been suggested by genetic studies. FcRβ is a subunit common to the high-affinity IgE (FcεRI) and low-affinity IgG (FcγRIII) receptors, both of which contribute to the initiation of allergic reactions. Current in vitro data suggest that FcRβ can function as either a positive or negative regulator, leaving a mechanistic explanation for its association with the development of atopy unclear. To address this controversy, we have generated novel mouse models relevant to human Fc receptor function. Analysis of FcεRI- and FcγRIII-dependent responses in these mice provides unequivocal genetic evidence that FcRβ functions as an amplifier of early and late mast cell responses and, remarkably, in vivo anaphylactic responses.

Introduction

A role of the Fc receptor β chain (FcRβ) gene in the pathogenesis of human allergic diseases has been suggested by two types of genetic studies. First, a genetic linkage was demonstrated between various atopic phenotypes and a region of human chromosome 11 encompassing the human (h) FcRβ gene (Cookson et al., 1989; Colee et al., 1993; Sandford et al., 1993; Shirakawa et al., 1994a). Second, an association was found between these atopic phenotypes and various polymorphisms located in the coding region of the FcRβ gene (Shirakawa et al., 1994b; Hill et al., 1995, 1996). However, whether and how FcRβ could significantly contribute to these allergic phenotypes remains unclear.

FcRβ participates in the function of receptors involved in initiating allergic reactions. FcRβ is a subunit of two related multisubunit receptor complexes, the high-affinity receptor for immunoglobulin E (IgE), FcεRI, (Kinet et al., 1988; Blank et al., 1989, Küster et al., 1992) and the low-affinity receptor for immunoglobulin G, FcγRIII (Kurosaki et al., 1992; Dombrowicz et al., 1997). Allergic reactions can be initiated through both of these receptors. Clustering of FcεRI-bound IgE by multivalent allergens results in cellular degranulation and the release of mediators of allergic reactions, including histamine, leukotrienes, and various cytokines (Costa et al., 1997). Similarly, clustering of FcγRIII with IgG-containing immune complexes activates mast cells and basophils to produce anaphylactic reactions very similar to those induced through FcεRI (Hazenbos et al., 1996; Dombrowicz et al., 1997; Miyajima et al., 1997). However, the level of impact of FcRβ on the function of these receptors is unknown. Thus far, FcRβ has only been consistently detected in cells of mast cell or basophil lineage, while in humans both FcεRI and FcγRIII are also found without FcRβ on many other cell types. Therefore, whether the contribution of the subset of receptors that contain FcRβ could significantly impact an individual's overall allergic response is an open question.

Current studies provide directly opposing conclusions as to how alterations of FcRβ function might translate into altered FcR-mediated responses. In vitro data comparing the signaling capacity of FcεRI$\alpha\beta\gamma_2$ and $\alpha\gamma_2$ receptor complexes show that $\alpha\beta\gamma_2$ complexes induce lyn-dependent tyrosine phosphorylation of FcRγ chains, activation of the syk tyrosine kinase, and calcium mobilization at a much higher level than their $\alpha\gamma_2$ counterparts (Scharenberg et al., 1995; Lin et al., 1996). In addition, mutational analysis indicates that the immunoreceptor tyrosine-based activation motifs (ITAM) (Cambier, 1995) of the FcRβ and FcRγ chains have different functions (Lin et al., 1996): the FcRγ ITAM does the actual signal transduction while the FcRβ ITAM does not itself signal but instead amplifies the FcRγ signal. However, the tyrosine-phosphorylated ITAM of FcRβ, but not that of FcRγ, binds to Src homology domain (SH2)–containing tyrosine phosphatases (SHP-1 and SHP-2) and SH2-containing inositol-5′-phosphatase (SHIP) in vitro, and SHP-2 can be coprecipitated with FcεRI after receptor engagement (Kimura et al., 1997a, 1997b). As SHP-1, SHP-2, and SHIP are phosphatases that participate in negative signaling by receptors containing an immunoreceptor tyrosine-based inhibitory motif (ITIM) (Scharenberg and Kinet, 1996), these data would suggest that FcRβ might have negative regulatory functions. The positive signaling role of FcRβ can be rationalized with its association with phosphatases by taking into account the different cellular contexts in which the respective data were produced, and by the fact that the lyn tyrosine kinase (Jouvin et al., 1994; Kimura et al., 1996; Lin et al., 1996) and SHIP/SHP-1 and SHP-2 phosphatases (Kimura et al., 1997a, 1997b) appear to compete for binding to the FcRβ ITAM. In this rationale, the respective amounts of lyn kinase/phosphatases in a particular context would play a major role in setting the level, positive or negative, at which FcRβ functions. This rationale, however, leaves open a critical question: what type of signaling function does an in vivo context produce for FcRβ?

Unequivocally addressing the function of FcRβ in vivo requires models in which FcRβ is present or absent from

‡To whom correspondence should be addressed (e-mail: jkinet@mercury.bidmc.harvard.edu).
§Present Address: Institut Pasteur de Lille, INSERM, U167 Lille, France.

Table 1. Structure and Cellular Distribution of FcεRI

	Mice	Humans	FcεRIαβγ_2 Mice	FcεRIαγ_2 Mice
Mast cells	$\alpha\beta\gamma_2$	$\alpha\beta\gamma_2/\alpha\gamma_2$	$\alpha\beta\gamma_2/\alpha\gamma_2$	$\alpha\gamma_2$
Basophils	$\alpha\beta\gamma_2$	$\alpha\beta\gamma_2/\alpha\gamma_2$	$\alpha\beta\gamma_2/\alpha\gamma_2$	$\alpha\gamma_2$
Monocytes	—	$\alpha\gamma_2$	$\alpha\gamma_2$	$\alpha\gamma_2$
Eosinophils	—	$\alpha\gamma_2$	$\alpha\gamma_2$	$\alpha\gamma_2$
Langerhans cells	—	$\alpha\gamma_2$	$\alpha\gamma_2$	$\alpha\gamma_2$
Dendritic cells	—	$\alpha\gamma_2$	?	?

its natural mast cell/basophil context and that otherwise reflect hFcR function. Since allergic reactions can be initiated by either FcεRI or FcγRIII, it is further desirable to study FcRβ function in both FcεRI and FcγRIII contexts. The construction of such models for FcεRI is complicated by two differences between mouse and human FcεRI systems. First, the structure of FcεRI varies according to the species. In mice, FcRβ is required for cell surface expression of FcεRI so that FcεRI is only expressed as $\alpha\beta\gamma_2$ tetramers on mast cells and basophils where both FcRβ and FcRγ are available (Ra et al., 1989), while in humans FcRβ is dispensable for FcεRI cell surface expression and FcεRI can be expressed as $\alpha\beta\gamma_2$ or $\alpha\gamma_2$ complexes (Miller et al., 1989; Küster et al., 1992). One consequence of the murine (m) requirement for FcRβ is that gene targeting of FcRβ in mice would result in no FcεRI expression, and would therefore not be a suitable approach for in vivo studies of how FcεRI functions in the absence of FcRβ. The second difference between the species is cell specificity of expression of FcεRI. In mice, FcεRI is exclusively expressed on mast cells and basophils. In contrast, in humans FcεRI expression is less restricted and is expressed in the mast cell and basophilic lineages as a mixture of $\alpha\beta\gamma_2$ or $\alpha\gamma_2$ complexes and as $\alpha\gamma_2$ complexes on other hematopoietic cells, such as monocytes (Maurer et al., 1994), dendritic cells (Maurer et al., 1996), Langerhans cells (Bieber et al., 1992; Wang et al., 1992), and eosinophils (Gounni et al., 1994) (Table 1). An important consequence of these differences in expression specificity is that the proportion of FcRβ-expressing FcεRI in humans is much smaller than in mice and, therefore, available mouse models do not accurately reflect hIgE/FcεRI physiology.

In this manuscript, we describe the construction and phenotypic characterization of mice with humanized expression of both FcεRI $\alpha\beta\gamma_2$ and $\alpha\gamma_2$ receptors or only FcεRI$\alpha\gamma_2$ receptors, along with a complementary set of mice that express both FcγRIII$\alpha\beta\gamma_2$ and $\alpha\gamma_2$ receptors or only FcγRIII$\alpha\gamma_2$ receptors. Using these mice, we performed both an in vitro analysis of the signaling role of FcRβ in its natural cellular context and an in vivo analysis of the function of FcRβ during FcεRI- or FcγRIII-mediated inflammatory responses. The results of these analyses demonstrate that FcRβ has the capacity to substantially affect the allergic responses of an individual animal, and that it achieves this effect by functioning as a net amplifier of in vivo responses in both FcεRI and FcγRIII contexts. This strongly supports the concept that the level of effector cell responsiveness determined by FcRβ can be an important determinant of human allergic responses.

Results

Reconstitution of the Structural Characteristics and Cell-Specific Expression of hFcεRI in Transgenic Mice

To reconstitute FcεRI with both its human cell-specific distribution and its alternative structures, we took advantage of two characteristics of the hFcεRI system, one related to the promoter of the hFcεRIα gene and the other to the subunit assembly. First, a 2.9 kb fragment 5′ of the initiation codon from the hFcεRIα gene was active in a reporter gene assay in mouse monocytic cells and, as expected, in mast cells and basophils but not in fibroblasts or T cells (data not shown), suggesting that the 2.9 kb sequence contained the information necessary to achieve the cell-specific expression of hFcεRI. Based on these results, we anticipated that a transgenic mouse expressing the hFcεRIα gene including the 2.9 kb promoter sequence would reconstitute the cell-specific pattern of expression observed in humans. Second, as discussed above, the subunit assembly characteristics of human and murine FcεRI are different. Thus, it was further anticipated that a transgene of the complete hFcεRIα gene would result in assembly of hFcεRIα with endogenous mouse FcRβ and FcRγ in mast cells and basophils and in expression of a mixture of $\alpha\beta\gamma_2$ and $\alpha\gamma_2$ complexes on those cells and that in the other hFcεRIα-expressing hematopoietic cells where FcRγ but not FcRβ is expressed, such as monocytes, hFcεRIα chain would assemble with FcRγ and be expressed as $\alpha\gamma_2$ complexes.

We generated transgenic mice using the complete hFcεRIα gene under the control of the 2.9 kb promoter. The initial characterization of these mice was published recently (Dombrowicz et al., 1996) and included the demonstration that the hFcεRIα protein is expressed in bone marrow–derived cultured mast cells (BMMC), assembles with endogenous mouse FcRβ and FcRγ, and promotes surface expression of $\alpha\beta\gamma_2$ complexes. In addition, these mice were shown to undergo hFcεRI-mediated anaphylactic reactions, demonstrating the functionality of the reconstituted receptors. As a further characterization of these mice (hFcεRIα-transgenic, designated hFcεRIαTg), we have analyzed the tissue specificity of expression of the hFcεRIα transgene by measuring hFcεRIα expression on monocytes/macrophages, Langerhans cells, and eosinophils. Figure 1A shows that hIgE binds to the surface of BMMC and that this binding is inhibited by monoclonal anti-hFcεRIα (15–1). Because stainings are performed with hIgE, which does not bind to mFcεRI, only hFcεRIα are detected. No hIgE binding was observed on neutrophils obtained 2 hr after thioglycolate elicitation (Figure 1B) or on thymocytes and splenocytes

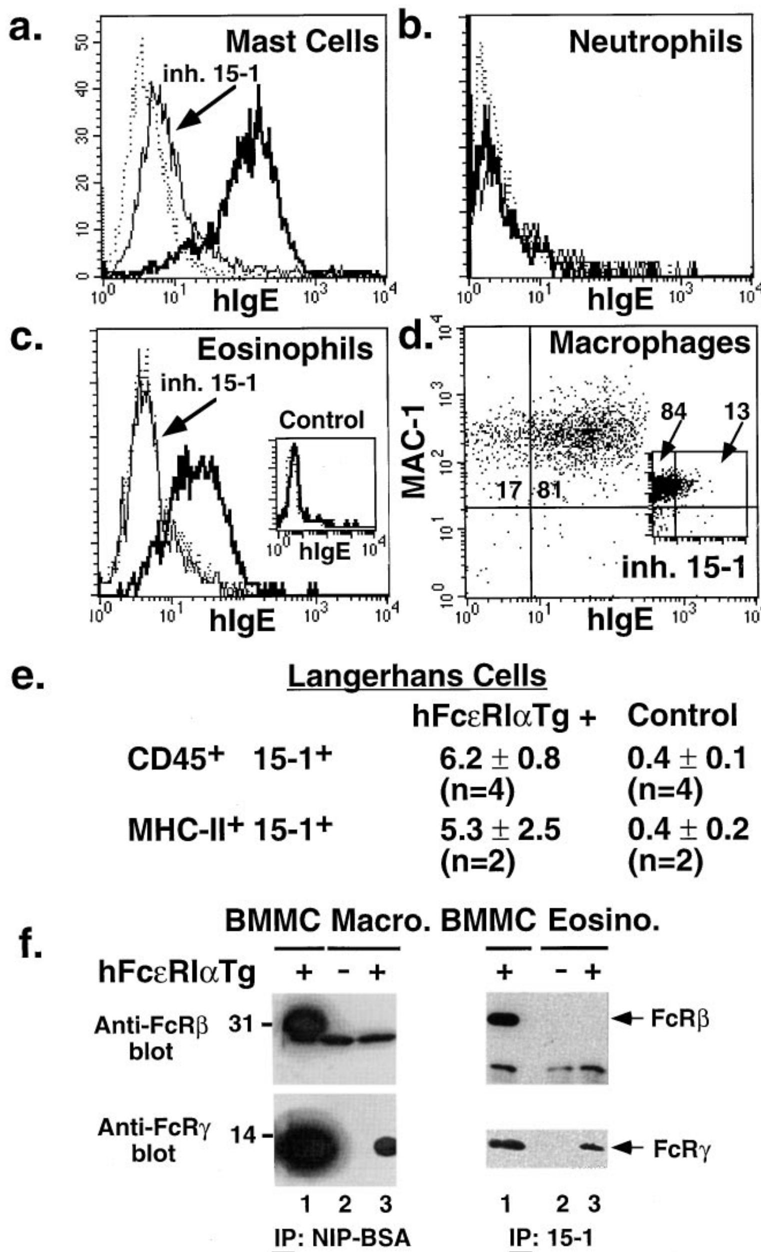

Figure 1. Tissue Distribution and Structure of hFcεRI in hFcεRIαTg Mice

(A–E) Flow cytometric analysis of surface expression of hFcεRI on BMMC (A), neutrophils (B), eosinophils (C), thioglycolate-elicited macrophages (D), and Langerhans cells (E) from hFcεRIαTg and FcεRIα$^{-/-}$ (control) (C, inset) animals. Cells were stained with biotinylated hIgE and SA-PE (A–D). Inhibition of hIgE binding by an excess of 15–1, thin line (A and C), or inset (D). Dotted line (A–C), unstained cells. (E) Double staining was performed with FITC-15–1 and PE-labeled anti-CD45 or anti-MHC-II (I-A^{b,d}). Values (percentages) are mean fluorescence ± standard error of the mean (SEM). (F) Subunit structure of hFcεRI at the surface of thioglycolate-elicited macrophages, eosinophils, and BMMC from hFcεRIαTg animals. Macrophages and eosinophils from FcεRIα$^{-/-}$ (hFcεRIαTg^{-}) mice were used as controls (lane 2). Macrophages and BMMC were incubated with hIgE and then lysed with digitonin. hIgE-receptor complexes were immunoprecipitated with NIP–BSA sepharose beads. Eosinophils and BMMC were incubated with 15–1, washed, and then lysed, and antibody-receptor complexes were immunoprecipitated with Gammabind Plus sepharose beads. The immunoprecipitated material was blotted with anti-FcRβ and anti-FcRγ antibodies after SDS–polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. Note that the bands migrating below the 31 kDa marker on the anti-FcRβ immunoblot correspond to the light chain of hIgE anti-NIP used in this experiment.

(data not shown). The hIgE binding capacity of eosinophils from the peritoneal cavity of mice infected with *Schistosoma mansoni* cercariae for 8–10 weeks (uninfected mice have very few eosinophils) is shown in Figure 1C. Binding of hIgE was also detected on bone marrow and circulating eosinophils (data not shown). This binding was inhibited by preincubation with an excess of 15–1 demonstrating the specificity of hFcεRI–hIgE interaction. The same population of peritoneal eosinophils obtained from nontransgenic animals was devoid of hIgE binding (Figure 1C, inset) and of mIgE binding (data not shown). Thioglycollate-elicited peritoneal macrophages also expressed hFcεRI (Figure 1D). Epidermal Langerhans cells isolated from trunk skin of hFcεRIαTg mice also expressed hFcεRI. Although murine Langerhans cells do not express a specific marker, they can be identified by a group of cell surface markers. Murine Langerhans cells express CD45 (leukocyte common antigen) and major histocompatibility complex class II (MHC-II) (I-A) but not T cell receptor (TCR) γδ. Langerhans cells and TCRαδ^{+} cells are the only two CD45^{+} cell populations found in normal epidermis (Elbe et al., 1989). We performed multicolor flow cytometric analysis using phycoerythrin (PE)-labeled anti-CD45, anti-I-A, anti-γδ, or anti-c-Kit antibodies and fluorescein isothiocyanate (FITC)–labeled 15–1. Double positive cells CD45^{+}15–1^{+} and MHC-II^{+}15–1^{+} were identified in transgenic animals but not in control mice (Figure 1E). The percentages of Langerhans cells (CD45^{+}MHC-II^{+}) recovered from the epidermis of both hFcεRIαTg and control mice were similar (data not shown). In hFcεRIαTg mice, 15–1^{+} cells were γδ^{-} and c-Kit^{-} (data not shown). This binding pattern indicates that cells identifiable as skin Langerhans cells from transgenic animals express

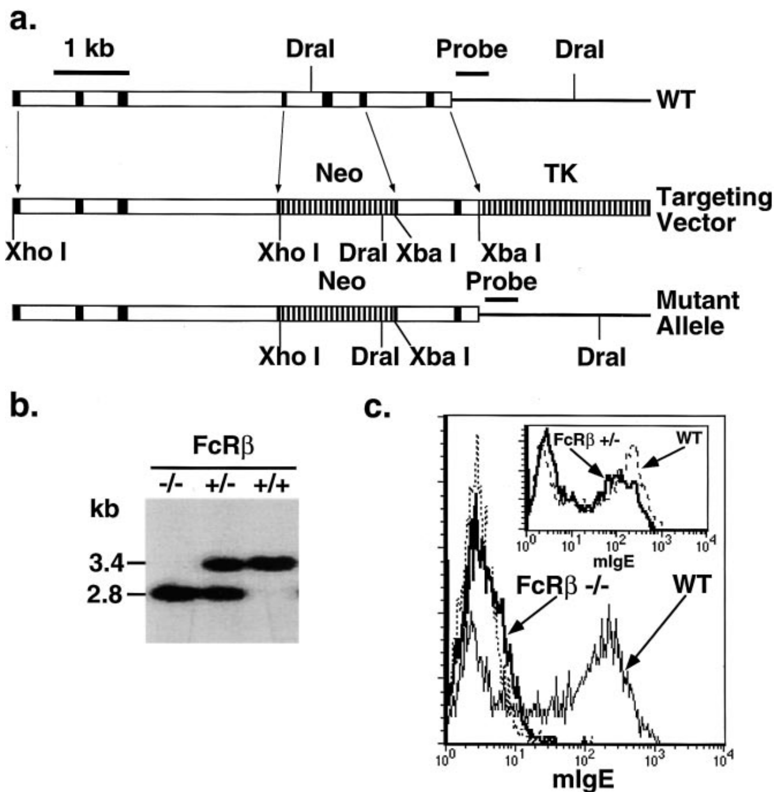

Figure 2. Gene Targeting of FcRβ

(A) Organization of the FcRβ gene. Map of the WT gene, the targeting construct, and the recombinant allele. Exons, closed boxes; introns, open boxes; *neo* and *tk* cassettes, striped boxes. Intron sizes were deduced from the human gene sequence (Küster et al., 1992) (GenBank accession number M89796). Locations of the cloning sites (XhoI and XbaI) and of the DraI restriction sites are indicated.
(B) Southern blot analysis of genomic DNA from $FcR\beta^{+/+}$, $FcR\beta^{+/-}$, and $FcR\beta^{-/-}$ mice. DNA was digested with DraI. The probe used is shown in (A).
(C) FcεRI expression on BMMC from $FcR\beta^{+/+}$, $FcR\beta^{+/-}$, and $FcR\beta^{-/-}$ mice. BMMC were stained with FITC-labeled mIgE and analyzed by flow cytometry.

FcεRI. Taken together, the above data indicate that the hFcεRIαTg mice have a cell expression pattern of hFcεRIα that is indistinguishable from that seen in humans.

We next assessed the subunit structure of FcεRI found in various cell types from these mice. hIgE (anti-NIP [4-hydroxy-3-nitrophenyl-acetyl]) was bound to thioglycolate-elicited peritoneal macrophages and BMMC from hFcεRIαTg and control animals. After washing, the cells were lysed and the surface hFcεRI receptors were immunoprecipitated. The precipitates were then immunoblotted with monoclonal anti-FcRβ (JRK) or polyclonal anti-FcRγ antibodies, both of which react with the corresponding murine subunits. mFcRβ and mFcRγ were immunoprecipitated with hIgE in BMMC from hFcεRIαTg mice (Figure 1F, left, lane 1). By contrast, in macrophages from hFcεRIαTg mice, only FcRγ was associated with hFcεRIα (Figure 1F, left, lane 3). The amount of FcRγ associated with hFcεRIα was clearly less abundant in macrophages than in BMMC despite the higher number of macrophages used in this experiment. Two factors contributed to this result. The first is the lower number of FcεRI receptors expressed on macrophages when compared to mast cells. The second is the lower detergent stability of the $\alpha\gamma_2$ receptor complex resulting in dissociation of the $FcR\gamma_2$ homodimer from the hFcεRIα chain, as previously described (Lin et al., 1996). Analysis of eosinophil FcεRI subunit structure was conducted in a similar way. Eosinophils and control BMMC were first saturated with 15–1, washed, and lysed, and the surface receptors were immunoprecipitated. As in macrophages, mFcRγ but not mFcRβ was found associated with hFcεRIα on eosinophils from hFcεRIαTg mice (Figure 1F, lane 3). In nontransgenic mice, neither FcRβ nor FcRγ was detected in macrophages and eosinophils (Fig 1F, lane 2). Thus, these data demonstrate that the hFcεRIα transgene not only recapitulates the appropriate cell specificity of expression of hFcεRI but also the appropriate cell type/structure pattern observed in human cells (Table 1).

Generation of FcεRI$\alpha\beta\gamma_2$ and FcεRI$\alpha\gamma_2$ Mice

To analyze the function of FcRβ in a physiological context, we chose to compare hFcεRIαTg mice to corresponding mice with the same hFcεRIα cell distribution but expressing only $\alpha\gamma_2$ receptors. We reasoned that a targeted disruption of the mFcRβ gene in these hFcεRIαTg mice should result in FcεRI expressed only as $\alpha\gamma_2$ complexes. We first inactivated one mFcRβ gene allele in embryonic stem (ES) cells and proceeded to generate mice homozygous for this targeted mutation (Figures 2A and 2B). Surface expression of mFcεRI on BMMC was similar in $FcR\beta^{+/-}$ and wild-type (WT) mice (Figure 2C, inset) but as expected from previous studies (Ra et al., 1989) was abolished in $FcR\beta^{-/-}$ mice (Figure 2C), demonstrating the requirement of FcRβ for surface expression of mFcεRIα. $FcR\beta^{-/-}$ mice were then crossed with hFcεRIαTg mice. Progeny of the second generation thus contained animals expressing the hFcεRIα transgene in the $FcR\beta^{-/-}$ or $FcR\beta^{+/-}$ background. For the purpose of clarity, hFcεRIαTg/$FcR\beta^{+/-}$ and hFcεRIαTg/$FcR\beta^{-/-}$ mice will be referred to as FcεRI$\alpha\beta\gamma_2$ and FcεRI$\alpha\gamma_2$, respectively.

A prerequisite for the analysis of the FcRβ function

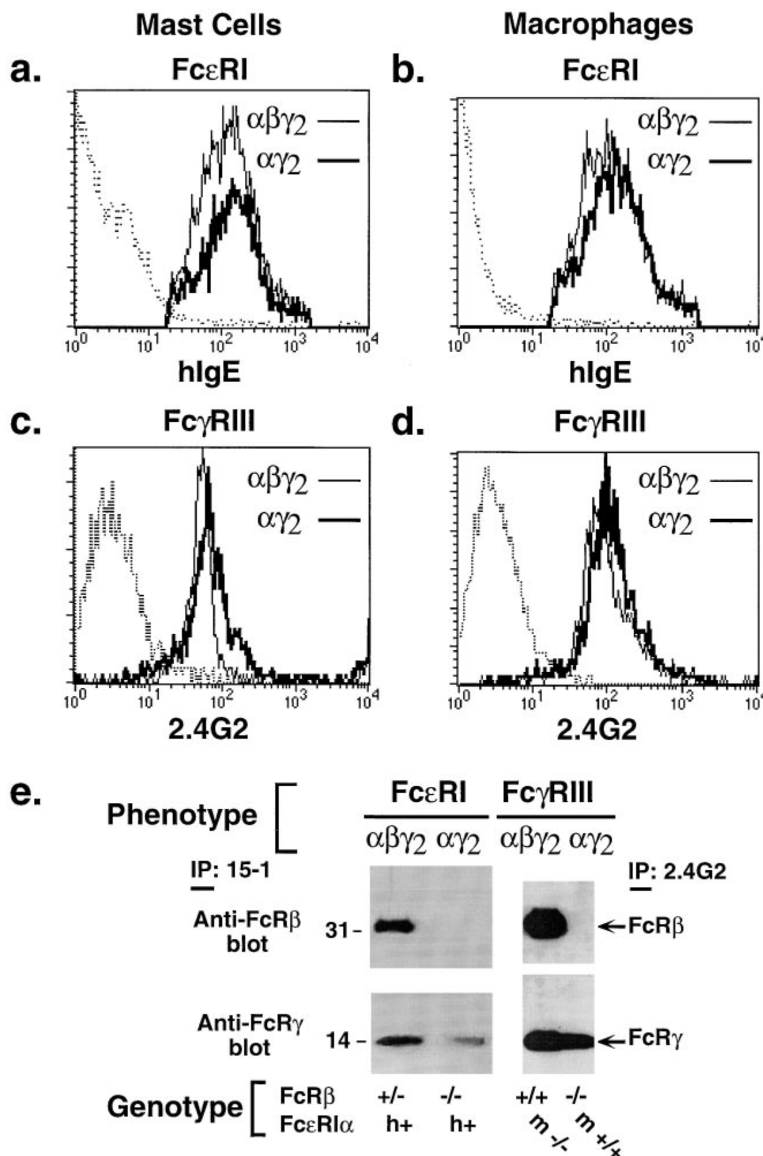

Figure 3. Expression and Structure of FcεRI and FcγRIII on Peritoneal Cells and BMMC

(A–D) FcεRI and FcγRIII expression on peritoneal mast cells and macrophages from $\alpha\beta\gamma_2$ and $\alpha\gamma_2$ mice. Flow cytometry analysis on gated c-Kit$^+$ (A and C) and MAC-1$^+$ (B and D) cells after hIgE and 2.4G2 staining. Dotted lines, unstained cells.

(E) Subunit structure of FcεRI and FcγRIII expressed on the surface of BMMC. FcεRI structure was analyzed as described in Figure 1F (right), and FcγRIII structure was analyzed similarly except that 2.4G2 was substituted for 15–1 prior to immunoprecipitation with protein A sepharose-bound anti-rat IgG.

by comparing the two types of mice is to insure that hFcεRI expression levels are equivalent on cells freshly isolated from each strain. These levels were measured after staining freshly isolated peritoneal mast cells and macrophages from both types of mice with hIgE. Both FcεRI$\alpha\beta\gamma_2$ and FcεRI$\alpha\gamma_2$ mice bind identical amounts of hIgE, indicating that they express identical amounts of surface hFcεRI on their peritoneal mast cells and macrophages (Figures 3A and 3B). Note that mast cells of FcεRI$\alpha\beta\gamma_2$ mice still express mFcεRI while the FcεRI$\alpha\gamma_2$ mice do not, because mFcεRIα is not expressed on the cell surface in the absence of FcRβ. In hFcεRIαTg mice in the FcεRI$\alpha^{-/-}$ background (Dombrowicz et al., 1996), mast cells do not express mFcεRI, and these mice express higher levels of hFcεRI on their mast cells when compared to the corresponding FcR$\beta^{-/-}$ mice (data not shown). Thus, it seems that hFcεRIα associates with endogenous FcRβ and FcRγ more efficiently than it does with FcRγ alone. For the purpose of the desired comparison of FcεRI function in the presence or absence of FcRβ, it is therefore fortuitous that the presence of endogenous mFcεRIα as a competitor compensates for the presence of FcRβ as a facilitator of FcεRI surface expression.

A second prerequisite for the analysis of the FcRβ function is to confirm that FcεRI$\alpha\beta\gamma_2$ mice expressed tetrameric FcεRI and that FcεRI$\alpha\gamma_2$ mice expressed trimeric FcεRI. Primary cultures of BMMC were generated and equal numbers of BMMC from each strain were incubated with 15–1, washed, and lysed. The surface receptors bound to 15–1 were then immunoprecipitated and analyzed by Western immunoblotting for the presence of FcRβ and FcRγ subunits (Figure 3E, left). As predicted, the FcεRI$\alpha\beta\gamma_2$ mice have detectable FcRβ and FcRγ in anti-hFcεRIα immunoprecipitates, while FcεRI$\alpha\gamma_2$ mice only have FcRγ and no FcRβ.

These results demonstrate that the mast cells of FcεRI$\alpha\beta\gamma_2$ and FcεRI$\alpha\gamma_2$ mice bind hIgE equivalently. In addition, these mice express FcεRI on the same cell types as in humans. They therefore represent an in vivo model system with a human pattern of FcεRI expression in which the effect of FcRβ on FcεRI-mediated inflammatory reactions can be studied.

Generation of FcγRIII$\alpha\beta\gamma_2$ and FcγRIII$\alpha\gamma_2$ Mice

A second focus of this work was to examine whether FcRβ might affect immune complex–mediated allergic responses, since it is able to associate with FcγRIII as well as FcεRI. Therefore, we chose to create murine models in which FcγR-mediated responses could be compared in the presence or absence of FcRβ. As with FcεRI, the difference in complexity between mouse and human FcγR systems was important to consider prior to chosing a strategy for construction of these models. Two types of low-affinity receptors, FcγRII and FcγRIII, bind IgG-containing complexes in mice, and both are involved in the initiation of inflammatory responses mediated by these immune complexes. While FcγRII in mice mediate solely inhibitory signals through its ITIM, in humans FcγRII can mediate either inhibitory (FcγRIIB) or activating signals (FcγRIIA) depending on the presence of an ITIM or an ITAM in its cytoplasmic tail (Cambier et al., 1995; Daeron et al., 1997). However, when FcγRII and FcγRIII are viewed together as a low-affinity IgG receptor class, the overall distribution of inhibitory and activating subtypes is conserved such that distinct effector cell types in the two species respond to immune complexes in very similar ways. Unlike FcεRI, both mouse and human FcγRIIIα can be expressed as either $\alpha\gamma_2$ or $\alpha\beta\gamma_2$ receptors depending on whether FcRβ is present. Therefore, a full FcγRIII$\alpha\gamma_2$ phenotype is generated simply via elimination of FcRβ expression, and FcR$\beta^{-/-}$ mice can be used as a model of solely FcγRIII$\alpha\gamma_2$-initiated responses. However, these mice show an up-regulation of FcγRIII surface expression relative to WT mice, presumably because mFcεRIα, which requires FcRβ for surface expression, is then no longer available to compete with FcγRIIIα for available FcRγ chains (Dombrowicz et al., 1997). To compensate for this, we chose to use mFcεRI$\alpha^{-/-}$ mice as our primary comparative model of FcγRIII$\alpha\beta\gamma_2$-mediated responses because mFcεRIα is then also no longer available to compete with mFcγRIIIα for FcRβ, and these mice have an up-regulation of FcγRIII surface expression that is similar to that of the FcR$\beta^{-/-}$ mice (see results below). Note that our analyses below also included WT mice in parallel for comparison purposes although their lower level of FcγRIII expression precludes unambiguous interpretation of their responses. As above, for the purpose of clarity the FcεRI$\alpha^{-/-}$ mice (which express mFcγRIII$\alpha\beta\gamma_2$ receptors) and the FcR$\beta^{-/-}$ mice (which express FcγRIII$\alpha\beta_2$ receptors) will be referred to as FcγRIII$\alpha\beta\gamma_2$ and FcγRIII$\alpha\gamma_2$ mice, respectively.

Documentation of receptor expression and composition are also prerequisites for experimental analysis of the FcγRIII mice. Analysis of the surface expression of FcγR on peritoneal mast cells and macrophages from the FcγRIII$\alpha\beta\gamma_2$ and FcγRIII$\alpha\gamma_2$ mice is shown in Figures 3C and 3D. The 2.4G2 antibody recognizes both FcγRII and FcγRIII, so that this analysis can only give an approximate idea of the uniformity of FcγRIII expression. However, studies of FcR$\gamma^{-/-}$ mice in which surface expression of FcγRIII is abolished suggest that between 50%–80% of 2.4G2 staining is due to FcγRIII (Takai et al., 1994). As can be seen, surface FcγR expression is quite similar in the two strains, implying that FcγRIII expression is also quite similar. Analysis of peritoneal mast cells and macrophages from WT mice in parallel with the above showed that 2.4G2 staining was weaker than on either of the above (data not shown).

The subunit composition of the FcγR was characterized on 2.4G2 immunoprecipitates of BMMC from both FcγRIII$\alpha\beta\gamma_2$ and FcγRIII$\alpha\gamma_2$ strains of mice (Figure 3E, right). As illustrated, 2.4G2 immunoprecipitates from FcγRIII$\alpha\beta\gamma_2$ mice contain both FcRβ and FcRγ, while those from FcγRIII$\alpha\gamma_2$ mice contain only FcRγ. The lower levels of FcRγ seen in the lane for the FcγRIII$\alpha\gamma_2$ strain is probably due to the lower detergent stability of the FcγRIII$\alpha\gamma_2$ complexes, as expected based on what is seen for analogous FcεRI complexes. Since 2.4G2 does not discriminate between mouse FcγRIII and FcγRII, the association between FcRβ and FcγRIII was confirmed by immunoprecipitation with antibodies specific for the FcγRIII and FcγRII cytoplasmic tails followed by Western immunoblotting for FcRβ, which revealed an association only between FcRβ and FcγRIII (Dombrowicz et al., 1997). Taken together, the results demonstrate that these mice are appropriate models in which to analyze the role of FcRβ in the regulation of in vivo allergic reactions initiated through FcγRIII.

Role of FcRβ in Early Signaling Mediated through FcεRI

As discussed above, data on FcRβ function in the FcεRI context suggest directly opposing functions for FcRβ as either a positive or negative regulator of signaling. As a first step to address this apparent discrepancy, we examined how the presence of FcRβ affected signaling in its natural mast cell context by comparing FcεRI signaling in normal mast cells obtained from FcεRI$\alpha\beta\gamma_2$ and FcεRI$\alpha\gamma_2$ mice, focusing on the role of FcRβ for the generation of early intracellular signals mediated through FcεRI. BMMC were stimulated in such a way that the number of receptors engaged with hIgE and antigen on cells from FcεRI$\alpha\gamma_2$ animals was equal or greater than the ones on cells from corresponding FcεRI$\alpha\beta\gamma_2$ mice (Figure 4A). After stimulation with hIgE and its cognate antigen, cells were lysed and tyrosine phosphorylation of the FcRγ chain and Syk was analyzed by anti-FcRγ and anti-Syk immunoprecipitation, and the respective immunoprecipitates were immunoblotted with an anti-phosphotyrosine antibody. The tyrosine phosphorylation state of Syk is known to closely parallel its activation state. Engagement of FcεRI$\alpha\beta\gamma_2$ induces much greater FcRγ and syk tyrosine phosphorylation than does engagement of FcεRI$\alpha\gamma_2$ (Figure 4B). We then compared calcium mobilization in BMMC derived from these mice (Figure 5A). Calcium mobilization in the BMMC that expressed FcεRI$\alpha\beta\gamma_2$ was enhanced relative to that in the BMMC expressing FcεRI$\alpha\gamma_2$. Both of these results paralleled observations in the reconstitution and cultured cell

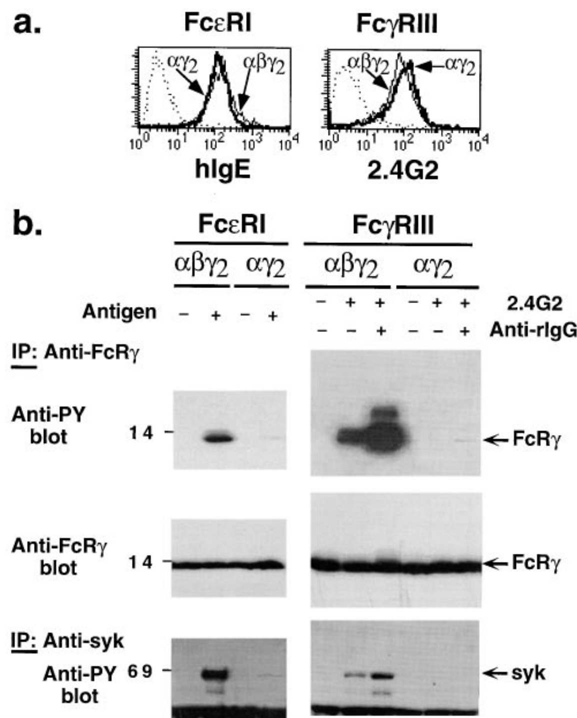

Figure 4. FcRβ Amplifies Phosphorylation of FcεRI and FcγRIII on BMMC

(A) Flow cytometric analysis of FcεRI and FcγRIII ligand occupancy at the time of BMMC stimulation. BMMC from FcεRI $\alpha\beta\gamma_2$ and $\alpha\gamma_2$ mice were incubated with 50 ng/ml per 10^6 cells hIgE; BMMC from FcγRIII$\alpha\beta\gamma_2$ and $\alpha\gamma_2$ mice were incubated with 100 ng/ml per 10^6 cells and 1 μg/ml per 10^6 cells of the 2.4G2 antibody, respectively, to insure that the number of receptors engaged on cells from $\alpha\gamma_2$ mice was equal or higher than the ones from $\alpha\beta\gamma_2$ animals.

(B) FcRβ amplifies FcRγ and Syk tyrosine phosphorylation in BMMC through FcεRI and FcγRIII. BMMC were incubated with hIgE and triggered or not with antigen; BMMC were triggered with 2.4G2 alone or not and further stimulated or not by cross-linking with anti-rat IgG. Lysates were immunoprecipitated with anti-FcRγ or anti-Syk antibodies, and the products were analyzed by immunoblotting with anti-phosphotyrosine antibodies.

models. Estimation of the extent of enhancement seen for each of these early parameters suggests that the FcRβ gain in these systems is around 5- to 10-fold, nearly identical to our previous results from transfected cultured cell models. These results substantiate the FcRβ amplifier concept for early signaling events mediated by FcεRI in a natural mast cell context.

Role of FcRβ in Early Signaling Mediated through FcγRIII

Although previous studies have not detected differences in signaling between FcγRIII$\alpha\beta\gamma_2$ versus $\alpha\gamma_2$, the substantial homology between FcεRIα and FcγRIIIα and the clear amplifying effect of FcRβ on FcεRIα signaling led us to reexamine the effect of FcRβ on FcγRIII early signaling using BMMC from the FcγRIII$\alpha\beta\gamma_2$ and FcγRIII$\alpha\gamma_2$ mice. FcγRIII-dependent signaling was analyzed by comparing FcRγ tyrosine phosphorylation and Syk tyrosine phosphorylation induced through FcγRIII cross-linking with the rat monoclonal antibody 2.4G2 or 2.4G2 plus a secondary cross-linker (Figure 4B). In these experiments, the number of receptors engaged with 2.4G2 on BMMC from FcγRIII$\alpha\gamma_2$ mice was equal or greater than the one on cells from FcγRIII$\alpha\beta\gamma_2$ animals (Figure 4A). Since both inhibitory (FcγRII) and stimulatory (FcγRIII) FcγR are expressed on mast cells (Alber et al., 1992; Daeron et al., 1997), and 2.4G2 binds to both FcγRII and FcγRIII, this antibody provides a stimulus that is somewhat analogous to that induced by immune complexes that engage both receptors. Tyrosine phosphorylation of FcRγ and Syk was detected in FcγRIII$\alpha\beta\gamma_2$ BMMC when stimulated with 2.4G2 alone, while neither tyrosine-phosphorylated FcRγ nor syk was detectable after stimulation of FcγRIII$\alpha\gamma_2$ BMMC (Figure 4B). If a secondary cross-linker was added, the FcRγ/syk tyrosine phosphorylation signals were further enhanced in the FcγRIII$\alpha\beta\gamma_2$ BMMC and became detectable in the FcγRIII$\alpha\gamma_2$ BMMC. Consistent with these results, no calcium mobilization was observed in FcγRIII$\alpha\gamma_2$ BMMC stimulated with low (data not shown) or high doses (1 μg/10^6 cells) of 2.4G2 antibodies. In contrast, FcγRIII$\alpha\beta\gamma_2$ cells exhibited a strong calcium response even with a low-dose stimulus (10 ng/10^6 cells). However, subsequent addition of anti-rat IgG was able to induce calcium mobilization in FcγRIII$\alpha\gamma_2$ BMMC, although the flux was still weaker than in FcγRIII$\alpha\beta\gamma_2$ cells despite prior depletion of calcium stores by 2.4G2 alone (Figure 5B). The latter results demonstrate that stimuli producing small FcγRIII clusters (dimers with 2.4G2) are sufficient to trigger FcγRIII$\alpha\beta\gamma_2$ but not FcγRIII$\alpha\gamma_2$ BMMC. Conversely, in the absence of FcRβ larger clusters (obtained after secondary cross-linking) were required to induce signaling. Therefore, these data indicate that FcRβ is able to amplify signaling through FcγRIII.

FcRβ Function in Late Effector Responses

We next investigated whether the differences observed in these early intracellular signaling events (occurring within 1–5 min after the initial stimulus) could be translated into meaningful differences in mast cell degranulation and cytokine release, two responses that are observed after long periods of stimulation (several minutes to 6 hr) and are directly relevant to the pathogenesis of allergic diseases. Degranulation of BMMC expressing FcεRI$\alpha\gamma_2$ or $\alpha\beta\gamma_2$ was induced by cross-linking receptor-bound IgE with multivalent antigen. An antigen dose-response experiment (Figure 5C) demonstrates that FcRβ's presence amplifies the cell degranulation response through FcεRI with a gain factor of 3- to 5-fold. Similar results were obtained when BMMC were triggered using 15–1 with further cross-linking (Figure 5D). However, cross-linking with 15–1 alone did not induce a measurable response. We also analyzed the degranulation response of BMMC after stimulation with 2.4G2. Initiation of degranulation responses of BMMC via FcγRIII followed the same general pattern as via FcεRI. Very little degranulation was seen after FcγRIII$\alpha\gamma_2$ engagement with 2.4G2 alone, while identically stimulated FcγRIII$\alpha\beta\gamma_2$ cells released 35% (maximum) of their total content (Figure 5E). Analysis of the effect of FcRβ on cytokine release was performed by measuring interleukin (IL)-6 production after cell triggering via FcεRI or

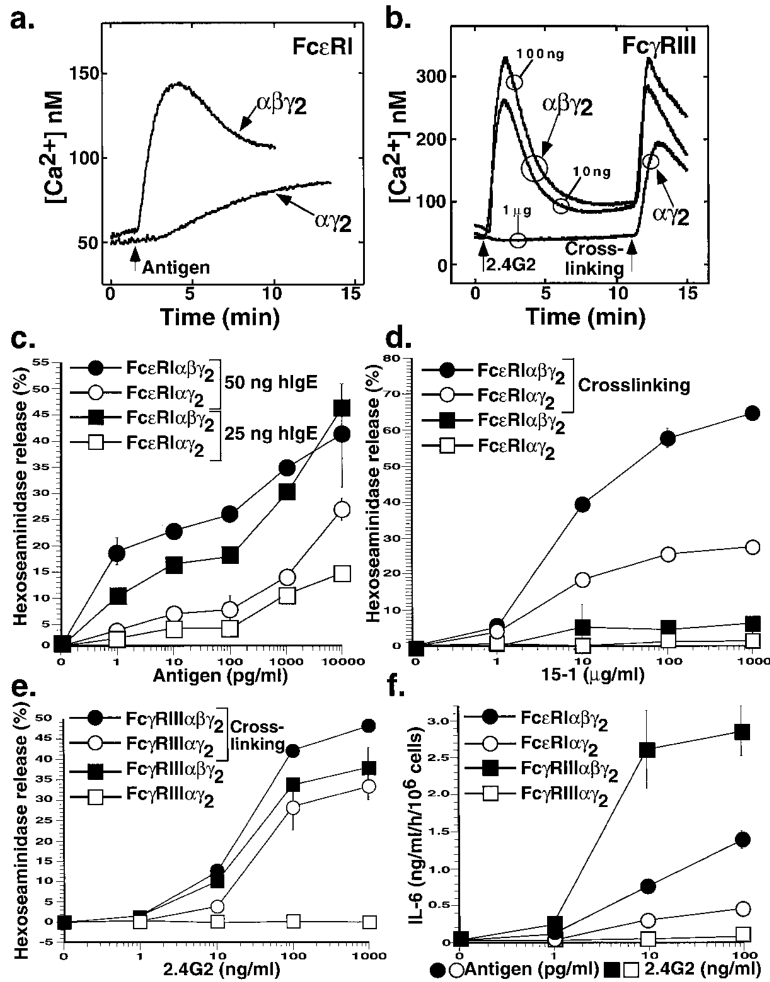

Figure 5. FcRβ Amplifies BMMC Calcium Mobilization, Degranulation, and IL-6 Release through FcεRI and FcγRIII

(A) FcεRI-induced calcium mobilization. BMMC from FcεRIαβγ_2 and αγ_2 mice were loaded with Fura-2 and 50 ng/ml per 10^6 cells anti-NIP hIgE before being stimulated with NIP–BSA (12 ng/ml per 10^6 cells).

(B) FcγRIII-induced calcium mobilization. Fura-2–loaded BMMC from FcγRIIIαβγ_2 and αγ_2 mice were stimulated with 10 or 100 ng or 1 mg/ml per 10^6 cells of 2.4G2 and further cross-linked with 10 μg/ml of anti-rat IgG.

(C and D) FcεRI-induced degranulation as measured by β-hexoseaminidase release. (C) BMMC from FcεRIαβγ_2 and αγ_2 mice were incubated with 25 or 50 ng/ml per 10^6 cells hIgE and stimulated with NIP–BSA antigen. (D) BMMC from FcεRIαβγ_2 and αγ_2 mice were triggered with increasing doses of anti-hFcεRIα (15.1) antibody and further cross-linked or not with 10 μg/ml of anti-mouse IgG F(ab′)$_2$ fragments. Values are means ± SEM; when not visible, error bars are within the symbols.

(E) FcγRIII-induced degranulation as measured by β-hexoseaminidase release. BMMC from FcγRIIIαβγ_2 and αγ_2 mice were stimulated with increasing doses of 2.4G2 antibody and further cross-linked or not with 10 μg/ml anti-rat IgG. Values are means ± SEM; when not visible, error bars are within the symbols.

(F) IL-6 production; BMMC from FcεRIαβγ_2 and αγ_2 mice were loaded with 50 ng/ml per 10^6 cells anti-NIP hIgE and then stimulated with NIP–BSA; BMMC from FcγRIIIαβγ_2 and αγ_2 mice were triggered with 1, 10, and 100 ng/ml per 10^6 cells of 2.4G2 antibody. Culture medium samples were removed after 6 hr stimulation. Values are means ± SEM; when not visible, error bars are within the symbols.

FcγRIII (Figure 5F). IL-6 production in αβγ_2 BMMC was higher than in αγ_2 BMMC after stimulation with either FcεRI or FcγRIII. Thus, FcRβ is not only able to amplify early intracellular signaling events in mast cells but also later effector responses. In addition, the amplification effect of FcRβ on these effector responses is of sufficient magnitude that its presence produces large responses to stimuli that would otherwise produce little or no response (as in the case of degranulation and cytokine production induced by stimulation with 2.4G2 alone).

Role of FcRβ in IgE-Mediated Allergic Responses

We have demonstrated that FcRβ amplifies IgE- and IgG-mediated early and late mast cell responses in vitro. The next step was to determine whether the amplifying function observed in vitro would translate into measurable differences in in vivo responses. Since FcRβ expression is restricted to mast cells and basophils and the majority of the FcεRI receptors in the humanized mouse model are, as in humans, in the form of αγ_2 complexes, it was not a priori obvious that the FcRβ amplification effect would have a significant impact on in vivo responses in these mice. In addition, the broadened expression range of hFcεRI produces a large number of nonmast cell-expressing FcεRIαγ_2, raising the possibility that it might be difficult to adequately load mast cell FcεRI to produce significant anaphylactic responses. Therefore, to verify that our IgE sensitization protocol would produce loading of both mast cells and nonmast cells, we first examined in situ hIgE binding on peritoneal mast cells and macrophages 24 hr after the injection of 100 μg of anti-NIP hIgE. The data presented in Figures 3A and 3B show that the peritoneal mast cells and macrophages of FcεRIαβγ_2 and FcεRIαγ_2 mice express similar levels of hFcεRI as assessed by exposure ex vivo to a saturating amount of ligand. After in situ exposure to hIgE, there is detectable bound hIgE that is equal in both the FcεRIαβγ_2 and FcεRIαγ_2 mice, although the level of receptor occupancy is much lower than saturation (compare Figure 6A with the level of cell fluorescence produced when the receptors are saturated [Figures 3A and 3B]). When using this injection protocol followed by administration of antigen to promote systemic anaphylaxis, only small anaphylactic responses were elicited (as measured by rectal temperature drop) (data not shown), and we were unable to detect any difference between the FcεRIαβγ_2 and FcεRIαγ_2 mice. Since the in situ data indicated that at best only one tenth of hFcεRI receptors were bound with hIgE using this protocol, we inferred that the overall IgE binding capacity of these mice is such that 100 μg of IgE is not adequate to produce a high enough receptor saturation level to produce a robust in vivo anaphylactic response.

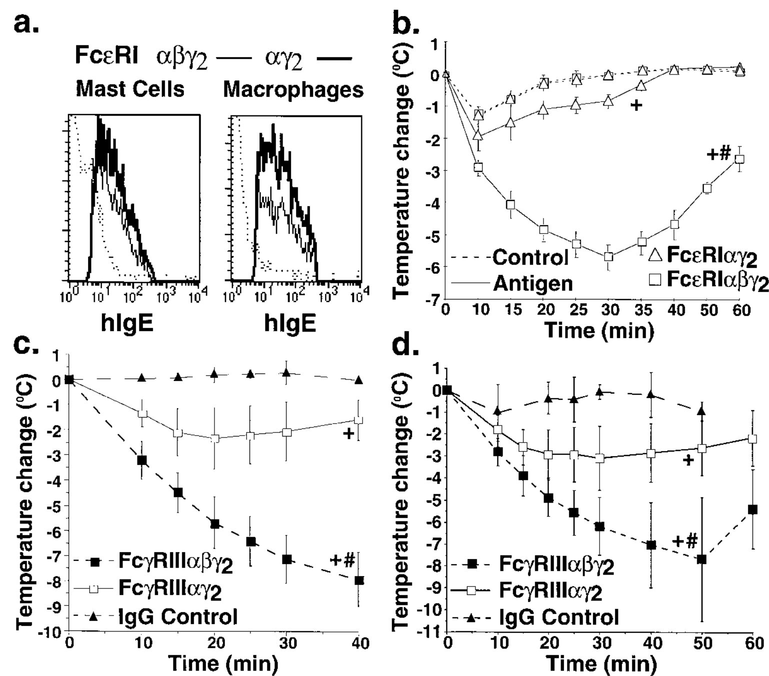

Figure 6. FcRβ Amplifies Systemic Anaphylaxis through FcεRI and FcγRIII

(A) FcεRI ligand occupancy on peritoneal mast cells and macrophages 24 hr after intravenous injection of 100 μg hIgE. Flow cytometric analysis of gated c-Kit$^+$ (left) and MAC-1$^+$ (right) cells showed that receptor engagement was similar in FcεRI $\alpha\beta\gamma_2$ and $\alpha\gamma_2$ mice. Dotted line, unstained cells.

(B) Rectal temperature changes in FcεRI$\alpha\beta\gamma_2$ and $\alpha\gamma_2$ mice (n = 4) after 3 intravenous injections at 12 hr intervals of 50 ng of anti-NIP hIgE and intravenous challenge, 4 hr after the last injection, with 1 mg of NIP–BSA. Controls (FcεRI$\alpha\beta\gamma_2$, n = 3; FcεRI$\alpha\gamma_2$, n = 2) received NIP–BSA only. Data show mean values + SEM; $p < 0.001$ by ANOVA versus data from control mice (+) or versus FcεRI$\alpha\gamma_2$ mice (#).

(C) Rectal temperature changes in FcγRIII$\alpha\beta\gamma_2$ (n = 9) and FcγRIII $\alpha\gamma_2$ (n = 7) mice after intravenous injection of 100 μg 2.4G2. Control animals (n = 3) received an equal amount of nonspecific rat IgG. Values are means ± SD; $p < 0.005$ by ANOVA versus control mice (+) or versus FcγRIII$\alpha\gamma_2$ (#).

(D) Rectal temperature changes in FcγRIII$\alpha\beta\gamma_2$ (n = 23) and FcγRIII$\alpha\gamma_2$ (n = 9) mice after iv injection of 400 μg anti-DNP IgG$_1$ and challenge with 1 mg DNP-HSA 24 hr later. Control animals (n = 15) received an equal amount of nonspecific mouse IgG and were challenged as described. Values are means ± SD; $p < 0.005$ by ANOVA versus control mice (+) or versus FcγRIII$\alpha\gamma_2$ (#).

(C and D) Note that the FcγRIII$\alpha\beta\gamma_2$ and control temperature curves were from a large contemporaneously generated data set that included control, WT, and FcRγ$^{-/-}$ together with FcγRIII$\alpha\beta\gamma_2$ (Dombrowicz et al., 1997), and FcγRIII$\alpha\gamma_2$ (unpublished data); the data on FcγRIII$\alpha\beta\gamma_2$ mice and control that have been previously published (Dombrowicz et al., 1997) are therefore shown with a dotted line.

Based on the above data, we designed a new protocol of three consecutive injections of IgE with the intention of producing a higher receptor saturation level prior to antigenic challenge. FcεRI$\alpha\beta\gamma_2$ and FcεRI$\alpha\gamma_2$ mice were injected three times at 12 hr intervals with 50 μg of anti-NIP hIgE before being challenged intravenously with the corresponding antigen (NIP–BSA [bovine serum albumin]). In these conditions, FcεRI$\alpha\beta\gamma_2$ mice displayed a strong temperature drop (5.7°C) after 30 min. In contrast, FcεRI$\alpha\gamma_2$ mice had a much weaker response (2.0°C drop after 10 min) and completely recovered after 40 min (Figure 6B), a response reminiscent of the one described above for both types of mice after a single 100 μg loading with IgE. Taken together these data demonstrate that FcRβ displays an amplifier effect on IgE-mediated responses in vivo with a gain of about 5-fold and suggest that the manifestation of this amplifier effect in an in vivo response depends at least in part on an adequate level of saturation of mast cell FcεRI$\alpha\beta\gamma_2$ receptors.

Role of FcRβ in IgG-Mediated Allergic Responses In Vivo

To determine whether FcRβ could also play a role in FcγRIII-mediated systemic anaphylactic responses, the FcγRIII$\alpha\beta\gamma_2$ and FcγRIII$\alpha\gamma_2$ mice were injected either with 2.4G2 antibodies or with IgG-containing immune complexes and monitored for anaphylactic responses by body temperature measurements. We verified that the in situ loading of mast cells and macrophages was equivalent or lower in FcγRIII$\alpha\beta\gamma_2$ animals compared to FcγRIII$\alpha\gamma_2$ mice (data not shown). A single intravenous injection with 100 μg of 2.4G2 results in a maximal drop in body temperature of 8°C in FcγRIII$\alpha\beta\gamma_2$ mice relative to control values (Figure 6C). By comparison, the temperature drop induced by 2.4G2 injection in the FcγRIII$\alpha\gamma_2$ mice was reduced to only 2°C relative to control values. Preformed immune complexes composed of anti-DNP (dinitrophenyl) IgG$_1$ and the corresponding antigen (DNP-HSA [human serum albumin]) duplicated almost perfectly the results obtained with the 2.4G2 stimulus in FcγRIII$\alpha\beta\gamma_2$ and $\alpha\gamma_2$ mice (Figure 6D). Analysis of the level of amplification indicates that FcRβ provides a gain factor of around 3. In addition, we performed a parallel analysis of WT mice, which express a lower level of surface FcγRIII (a subset of which contains FcRβ) than do the FcγRIII$\alpha\gamma_2$ mice. While respondingly less robustly than the FcγRIII$\alpha\beta\gamma_2$ mice, the WT mice showed a temperature drop (approximately 4 degrees) (data not shown) that was statistically significantly larger than the FcγRIII$\alpha\gamma_2$ mice in spite of the lower level of FcγRIII expressed in the WT mice, implying that the FcRβ amplification effect is responsible for a substantial proportion of the anaphylactic responses under normal conditions. Therefore, the ability of FcRβ to amplify mast cell effector responses in vitro translates into a marked enhancement in anaphylactic responses mediated via either FcεRI or FcγRIII.

Discussion

In this paper, we have generated mice with distinct FcεRI and FcγRIII structural phenotypes and used them to compare cellular and whole animal responses mediated by the two receptors functioning with or without FcRβ. The data presented are unequivocal genetic evidence that FcRβ has the capacity to substantially modulate

an individual animal's allergic response, and that this capacity is due to its function as a net amplifier of effector cell responses.

As discussed in the introduction, FcRβ has been suggested to play a role in the development of atopy. However, the idea that effector cell responses could vary significantly enough among different individuals to have an important impact on the development of allergic responses has been very controversial. In fact, while atopy is widely regarded as a multifactorial disease, most models of atopy focus on the dysregulation of cellular (e.g., T helper type 1 or T helper type 2) and humoral responses that are responsible for the initial production of high levels of allergen-specific IgE. However, our data directly demonstrate the importance of FcRβ in the manifestation of allergic responses: animals that lack FcRβ have such substantially reduced responses that no degree of dysregulation of humoral responses would be able to produce a sufficient level of sensitization to allow them to manifest as atopic. The large effect that FcRβ has on allergic responses in both FcεRI and FcγRIII receptor contexts is a formal demonstration of the capacity of FcRβ to contribute to allergic responses. In addition, we present biochemical data, in vitro cell activation data, and in vivo inflammatory response data that show that FcRβ functions as a net amplifier of FcεRI and FcγRIII effector cell responses. Together, these demonstrations strongly support the concept that atopy-associated isoforms of FcRβ may contribute to the manifestation of atopy through some modulation of FcRβ's ability to promote effector responses in vivo and provide a strong rationale for proceeding with the investigation of the functional effect of FcRβ polymorphisms on the gain provided by FcRβ.

Given that FcRβ is able to have an important influence on the manifestation of atopy via its role in setting the level of effector cell responses, what types of polymorphisms would one logically expect to find? Obvious possibilities that would result in a gain of function include polymorphisms in the promoter or regulatory regions resulting in increasing FcRβ expression, polymorphisms in the transmembrane domains capable of increasing assembly and the proportion of $\alpha\beta\gamma_2$ complexes expressed on effector cells, and polymorphisms around the ITAM that could stabilize association with kinases or destabilize association with phosphatases. However, polymorphisms that would be dominant in the nonallergic population should also be considered. For example, it is conceivable that alleles of inactive or less active FcRβ (for example ITAM-less or ITAM-mutant FcRβ) have been overlooked in nonallergics. When a particular polymorphism has shown a potential functional effect in vitro, the animal model developed and used here should be adaptable for testing the function of polymorphisms in vivo.

Our analyses of these mice also provide an intriguing insight into hFcR biology. The large differences in magnitude of response between the FcγRIII$\alpha\gamma_2$ and FcγRIII$\alpha\beta\gamma_2$ mice after exposure to the weak 2.4G2 stimulus demonstrates that the FcRβ amplifier may be particularly important for immune responses to weak stimuli, such as might be encountered in the earliest stages of inflammatory responses to invading pathogens. It is tempting to speculate that enhanced IgE- or IgG/FcεRI immune complex-mediated activation of mast cells and basophils in response to invading pathogens (Zhang et al., 1992; Echtenacher et al., 1996; Malaviya et al., 1996) via increased expression or qualitatively more active FcRβ has provided a selective advantage that has driven the accumulation of atopy-associated forms of FcRβ in modern human populations.

In summary, we have created and characterized two novel murine models that are relevant to hFcR function. Using these mice as a model system, we have shown that FcRβ functions in its natural context as a net amplifier of FcεRI- and FcγRIII-mediated mast cell activation and in vivo responses and have formally demonstrated the importance of the level of effector cell responsiveness in the manifestation of an allergic response. This information provides an important conceptual basis for understanding how polymorphic forms of FcRβ might contribute to or retard the development of human atopy.

Experimental Procedures

Targeting of the FcRβ Gene

The mouse FcRβ gene was isolated from a 129λ DASH library (a gift from P. Love, National Institutes of Health [NIH], Bethesda, MD). Targeted disruption of the gene was achieved by replacement of exons IV–VI (corresponding to base pairs [bp] 344–577 of the mFcRβ cDNA sequence) (Ra et al., 1989) with a *neo* cassette. A 4 kb fragment encompassing exons I–IV (bp 38–343 of the mouse FcRβ cDNA sequence) and a 1.4 kb fragment encompassing exons VI–VII (bp 578–1007) were amplified by polymerase chain reaction using appropriate primers and cloned respectively into the XhoI and XbaI of the pJNS2 vector (Dombrowicz et al., 1993). The construct was linearized by NotI and electroporated into D3 or E14 TG2a ES cells. ES cells were grown and selected as previously described (Koller and Smithies, 1989). Genomic DNA was extracted from clonal ES cells, digested with DraI, and hybridized by Southern blot to a 440 bp fragment located 16 bp downstream of exon VII. The hybridizing bands were 3.4 kb for the WT and 2.8 kb for the disrupted allele. Two of 109 G418-resistant, ganciclovir-sensitive clones were found positive for homologous recombination.

Animals

Chimeric, heterozygous (+/−), and homozygous (−/−) animals for the disrupted FcRβ allele were generated as previously described (Koller and Smithies, 1989); FcRβ$^{-/-}$ animals were on a BALB/c F_2 background. FcεRIα$^{-/-}$ animals have been previously described (Dombrowicz et al., 1993) and were on a BALB/c F_6 background. FcRβ$^{-/-}$ animals were compared to age-matched FcεRIα$^{-/-}$ for all relevant experiments. FcRβ$^{-/-}$ were also crossed with the previously described transgenic mice for the hFcεRIα gene (hFcεRIαTg) (Dombrowicz et al., 1996). Progeny of the second generation thus contained hFcεRIαTg/FcRβ$^{-/-}$ or hFcεRIαTg/FcRβ$^{+/-}$ animals. Littermates were used in all appropriate experiments. hFcεRIαTg mice used for these crosses were in a BALB/c WT background, so that hFcεRIαTg/FcRβ$^{+/-}$ animals were expressing both human and murine FcεRI on their mast cells. Animals used for the study of hFcεRI cellular distribution were on a FcεRIα$^{-/-}$ background and thus were expressing only hFcεRI.

Cells

Peritoneal Cells

Peritoneal cells were obtained by flushing the peritoneal cavity with 2 × 10 ml of ice-cold Hanks' balanced salt solution containing 1% fetal calf serum and 10 IU/ml heparin. Cells were collected on ice and the clumps removed by filtration on nylon filters. Thioglycollate-elicited macrophages were obtained 72 hr after intraperitoneal injection with 1 ml of 5% thioglycolate. After overnight cultures, adherent cells were used for flow cytometry analysis. For Western blotting experiments, peritoneal cells were incubated at 10^6 cells/ml with 10

mg/ml collagen-coated dextran beads (Cytodex 3, Pharmacia) for 8 hr and adherent macrophages harvested by collagenase treatment (500 μg/ml).

Neutrophils

Neutrophils were collected 2 hr after thioglycolate injection as non-adherent cells.

Eosinophils

Eosinophils were obtained from mice (6–12 weeks old) that had been infected with 80 cercariae of *S. mansoni* and sacrificed 8 weeks after infection (Williams et al., 1993). Peritoneal, bone marrow, and blood eosinophils were harvested and cultured overnight in complete RPMI containing 2.5 ng/ml murine IL-5 for removal of adhering macrophages. Nonadherent cells were collected for analysis. Percentage of eosinophils was determined after staining with Phloxine B (Unopette, Becton Dickinson). Eosinophils represented 55% and 45% of the peritoneal cells from transgenic and control animals, respectively.

Langerhans Cells

Epidermal cell suspensions were obtained as described (Schuler and Steinmann, 1985; Borkowski et al., 1996).

BMMC

BMMC were obtained as previously described (Dombrowicz et al., 1993, 1996).

Flow Cytometry

Flow cytometric analyses were performed on 3×10^5 cells as follows. All of the incubation steps were on ice for 30 min in 100 μl of phosphate-buffered saline (PBS) with 0.1% BSA and 0.05% sodium azide. Unless specified otherwise, all of the antibody final concentrations were 10 μg/ml. hFcεRI expression was analyzed using three different methods after saturation of FcγRII/III receptors with an excess of unlabeled 2.4G2, a rat IgG2bκ (Unkeless, 1979). A chimeric hIgE molecule (Schwarzbaum et al., 1989), composed of an Fc portion of hIgE and of an anti-NIP F(ab) portion of mIgE and referred to as hIgE for the purpose of clarity, was used. After washing, hIgE was revealed with a biotinylated anti-mouse $\lambda1^+\lambda2$ light chains (Pharmingen) and streptavidin-phycoerythrin (SA-PE) (1:2500). Alternatively, hFcεRI was detected with biotinylated hIgE followed by SA-PE or with FITC-labeled anti-FcεRIα (15–1) (Wang et al., 1992). FcγRIII expression was examined using FITC-labeled 2.4G2 (Pharmingen). Neutrophils and eosinophils were identified on the basis of their forward and side scatters. After saturation of FcγRI with normal mouse IgG and of FcεRII with an anti-CD23 (Pharmingen), peritoneal cells (mast cells and macrophages) were identified by additional staining with PE- or FITC-labeled anti-c-Kit (Pharmingen) and anti-MAC-1 antibodies (Boehringer), respectively. Langerhans cells were identified by staining with either PE-labeled anti-CD45, anti-MHC-II (I-$A^{b,d}$), anti-TCRγδ T cells, or anti-c-Kit antibodies (Pharmingen). Langerhans cells are characterized by their $CD45^+$, MHC-II $(I\text{-}A)^+$, $TCR\gamma\delta^-$ phenotype. Dead cells were excluded by propidium iodide.

Subunit Analysis of FcεRI and FcγRIII Complexes

Western blot experiments on BMMC and eosinophils were performed as previously described (Dombrowicz et al., 1996, 1997). Cells were saturated with 10 μg/ml 15–1 or 2.4G2 for 2 hr at 4°C, washed, and lysed in 1% digitonin. Surface-expressed receptors were immunoprecipitated with Gamma Bind Plus sepharose beads (Pharmacia) or with rabbit anti-rat IgG bound to protein-A sepharose (Pharmacia). After SDS–polyacrylamide gel electrophoresis and electrotransfer on polyvinylidene difluoride membrane, immunoprecipitates were immunoblotted with anti-FcRβ (JRK) (Rivera et al., 1988) and anti-FcRγ (934) (Letourneur et al., 1991) antibodies. Macrophages were loaded with 10 μg/ml hIgE at 5×10^6 cells/ml. Cells were lysed at 5×10^7 cells/ml in 1% digitonin. Lysates were immunoprecipitated with 20 μl of NIP–BSA agarose beads for 2 hr at 4°C. All of the subsequent steps were performed as described above.

Receptor Phosphorylation and Calcium Mobilization

To determine the amount of hIgE required for engagement of equal numbers of receptors on BMMC from $Fc\epsilon RI\alpha\beta\gamma_2$ and $\alpha\gamma_2$ mice, overnight incubation of cells with different doses of hIgE ranging from 4–100 ng/ml per 10^6 cells was performed. Flow cytometry analysis of hIgE binding was performed as described above. A dose of 50 ng/ml per 10^6 cells resulted in a similar loading of cells from both $Fc\epsilon RI\alpha\beta\gamma_2$ and $\alpha\gamma_2$ animals. Likewise, to determine the dose of 2.4G2 necessary for engagement of the same number of receptors on BMMC from $Fc\gamma RIII\alpha\beta\gamma_2$ and $\alpha\gamma_2$ mice, cells were incubated for 20 min at 37°C with 2.4G2 doses ranging from 10 ng to 1 μg/ml per 10^6 cells. Binding of 2.4G2 was revealed using FITC-labeled anti-rat IgG. Similar and almost saturating loading was achieved with 100 ng and 1 μg/ml per 10^6 cells of 2.4G2 for cells from $Fc\gamma RIII\alpha\beta\gamma_2$ and $\alpha\gamma_2$ mice, respectively.

Experiments of phosphorylation and calcium mobilization were performed as previously described, except that FcεRI was triggered with 12 ng/ml NIP–BSA (Dombrowicz et al., 1996; Lin et al., 1996).

Degranulation

Cellular degranulation was measured by the release of β-hexoseaminidase as described before, with minor modifications (Hirasawa et al., 1995). Cells were incubated overnight with 50 ng/ml per 10^6 cells hIgE or with various doses of anti-FcεRIα (15–1) for 2 hr, washed twice, and stimulated with NIP–BSA or with 10 μg/ml goat anti-mouse IgG $F(ab')_2$ for 20 min. Stimulation with 2.4G2 antibodies was performed for 25 min and, when appropriate, 10 μg/ml anti-rat IgG was added without prior washing, after 10 min incubation.

IL-6 Production

IL-6 production was measured by enzyme-linked immunosorbent assay (Endogen) on 50 μl of culture medium according to the manufacturer's instructions, after 6 hr stimulation with 50 ng/ml per 10^6 cells hIgE and 1, 10, and 100 pg/ml per 10^6 cells of NIP–BSA or with 1, 10, and 100 ng/ml per 10^6 cells 2.4G2.

Anaphylaxis

Anaphylactic reactions were induced and measured as before (Dombrowicz et al., 1997) except that hIgE-induced systemic anaphylaxis was achieved by 3 iv injections of 50 μg of anti-NIP hIgE in 200 μl of PBS at 12 hr intervals followed 4 hr later by an iv challenge with 1 mg of NIP–BSA in 200 μl of PBS.

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