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FcγRI-Deficient Mice Show Multiple Alterations to Inflammatory and Immune Responses

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Summary

The inactivation of the mouse high-affinity IgG Fc receptor FcγRI resulted in a wide range of defects in antibody Fc-dependent functions. These studies showed the primary importance of FcγRI in endocytosis of monomeric IgG, kinetics, and extent of phagocytosis of immune complexes, in macrophage-based ADCC, and in immune complex-dependent antigen presentation to primed T cells. In the absence of FcγRI, antibody responses were elevated, implying the removal of a control point by the deletion of FcγRI. In addition, FcR-γ chain-deficient mice were found to express partially functional FcγRI. Thus, FcγRI is an early participant in Fc-dependent cell activation and in the development of immune responses.

Introduction

The receptors for immunoglobulins (Fc receptors, or FcR) play important roles in the activation and regulation of normal and pathological immunity. The IgG receptors (FcγR) comprise the largest family, encoding at least ten membrane proteins in humans and five in mice (Hulett and Hogarth, 1994). Virtually all of these belong to two low-affinity receptor groups, FcγRII and FcγRIII; however, both humans and mice also express a unique high-affinity receptor, FcγRI.

The investigation of Fc receptor function in vivo has depended heavily on "gene-knockout" mice and to a large extent on mice with a deletion of the common FcR-γ chain, shared by multiple activating Fc receptors (Ravetch and Bolland, 2001). While these mice have been useful, the simultaneous expression of multiple activating Fc receptors on cells, e.g., macrophages, dendritic cells, neutrophils, and mast cells, and their sharing of the common FcR-γ chain, has led to often inconclusive or ambiguous results, particularly with in vivo experiments. This is especially the case now that FcR-γ chain mice have been shown to express FcγRI (see Results), which until now has been widely assumed to be lacking in these mice (Hazenbos et al., 1996; Takai et al., 1996; Vora et al., 1997).

Quite apart from its unique biochemical and structural properties (Hulett et al., 1991; Sears et al., 1990), the mouse FcγRI has a number of unique properties that distinguish it from other IgG Fc receptors. It is the only high-affinity FcR binding IgG2a, a product of the adaptive Th1-type immune response, and it is also the receptor for the T-independent immunoglobulin IgG3, thereby linking the adaptive and innate immune systems (Gavin et al., 1998a). It is highly polymorphic with seven alleles defined in mice (Gavin et al., 2000) and highly responsive to IFN-γ (Hulett and Hogarth, 1994). Apart from receptor binding studies, relatively little is known of the biological functions of FcγRI in either normal or pathological immunity. The investigation of FcγRI function has depended on indirect or inferential studies using FcR-γ chain-deficient mice; thus, the contribution of FcγRI to Fc-dependent functions was not clear. Until now, mice specifically lacking FcγRI alone have not been available and there were no monoclonal antibodies specific for mouse FcγRI; thus, FcγRI remains the least characterized of the mouse FcR.

In this study of our FcγRI$^{-/-}$ mice, a number of surprising observations were made. It is obvious now that this receptor is crucial for some of the earliest events in immune responses, since receptor inactivation results in: (1) loss of uptake of immune complexes of the T cell-independent immunoglobulin IgG3, as well as a profound reduction in IgG2a uptake; (2) a 10-fold reduction in antibody-dependent killing of cells by macrophages; (3) impaired FcR-dependent delivery of antigen in immune complexes to antigen presentation pathways as ten times more antigen in immune complexes is required for stimulation of T cells by FcγRI$^{-/-}$ APC than by normal APC; (4) decreased immune complex-induced inflammation; and (5) enhancement of antibody responses.

Results

Disruption of the FcγRI Gene

A defective form of the FcγRI gene was constructed from genomic DNA in which the promoter and transcription initiation site, as well as the translation initiation codon ATG, were deleted. A neomycin resistance cassette was inserted into the second leader exon, resulting in disruption of this exon and the leader sequence, as well as generating a premature translation termination codon immediately downstream of exon 2 (Figure 1A). This DNA construct incorporated a unique HindIII site to be used to distinguish correctly integrated target DNA (8.3 kb) from the wild-type FcγRI (9.7 kb) genomic DNA. W9.5 ES cell clones positive for the targeted integration were microinjected into C57BL/6 blastocysts, and a founder chimera was backcrossed to 129/SvJ mice. Animals homozygous for the FcγRI null allele were identified by Southern hybridization analysis of HindIII-digested genomic DNA isolated from the progeny of the founder

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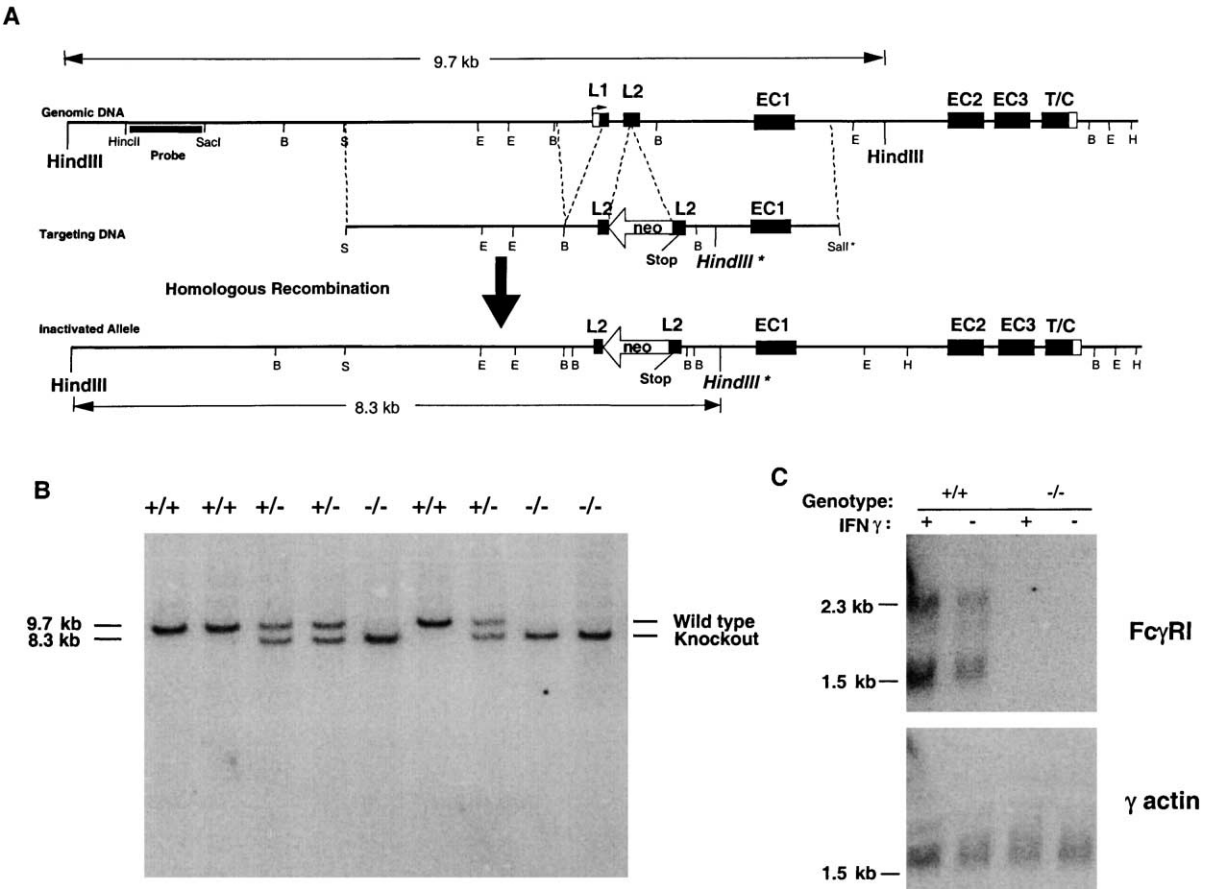

Figure 1. Inactivation of the Mouse FcγRI Gene

(A) Schematic representation of the mouse FcγRI gene and the inactivation strategy. The restriction map of the wild-type allele (upper) and inactivated allele (lower) are shown with exons represented as closed boxes. L1 and L2, leader region; EC1, EC2, and EC3, extracellular domains; T/C, transmembrane and cytoplasmic tail. The restrictions sites are as follows: B, BamHI; S, SalI; E, EcoRI; and H, HindIII.

(B) Southern hybridization analysis of HindIII-digested genomic DNA isolated from the progeny of the founder mouse was probed with a 1.8 kb HincII/SacI fragment derived from the 5′ end of the FcγRI gene. Wild-type (+/+) mouse DNA had a hybridizing band of 9.7 kb, the receptor-deficient mice (−/−) had a hybridizing band of 8.3 kb, and heterozygous animals (+/−) had both.

(C) Northern blots of total RNA extracted from bone marrow-derived macrophages (BMM) of normal (+/+) or receptor-deficient (−/−) mice. BMM were also cultured for 24 hr in the presence or absence of IFN-γ. The blot was probed with FcγRI cDNA, then reprobed with γ actin cDNA to ensure equal loading of mRNA in each lane.

(Figure 1B). FcγRI $^{-/-}$ mice were fertile and showed no developmental abnormalities. Expression of FcγRI mRNA and protein was examined in bone marrow-derived macrophages (BMM) from these mice. Northern blots showed the 1.5 and 2.3 kb mRNA species in normal but not in FcγRI-deficient BMM, even following treatment with IFN-γ, a potent inducer of receptor expression (Figure 1C).

FcγRI protein was absent from the surface of FcγRI-deficient BMM, as these cells bound neither the specific anti-FcγRI monoclonal antibody (X54-5/7.1) nor two different monomeric ligands (mouse IgG2a and human IgG), whereas binding to normal BMM was as expected. Inactivation of the FcγRI gene did not affect the expression of two other macrophage cell surface markers, detected by mAb F4/80 (specific for macrophages) and 2.4G2 (Figure 2). The 2.4G2 mAb recognizes a shared epitope of FcγRII and FcγRIII, and expression was identical in BMM from both normal and FcγRI-deficient mice. Thus, these animals clearly lack FcγRI as (1) they have no FcγRI mRNA even in the presence of IFN-γ; (2) their macrophages are unable to bind the ligands of FcγRI; and (3) there is no binding of an anti-FcγRI antibody to these macrophages.

Loss of Endocytosis by FcγRI-Deficient BMM

The capacity of BMM to internalize IgG was investigated by first pulsing BMM with monomeric mouse IgG2a followed by subsequent crosslinking by goat anti-mouse IgG and internalization tracked using fluorescence microscopy (Figure 3). As expected, normal BMM bound IgG2a showing ring staining on the outer cell membrane in the absence of crosslinking antibody. Following the addition of goat anti-mouse Ig, the receptor-bound IgG2a was internalized, and after 20 min appeared in intracellular vesicles (Figure 3B). Identical results were obtained using the BMM treated with the monoclonal anti-FcγRI antibody rather than IgG (data not shown).

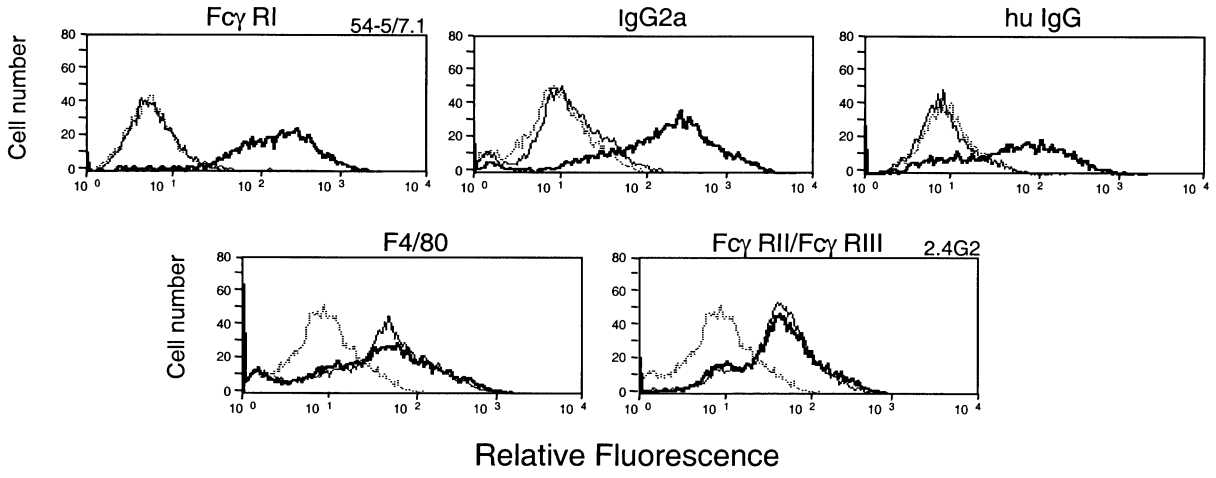

Figure 2. Expression of FcR by Normal and FcγRI-Deficient BMM

Flow cytofluorometric analysis of FcγRI and other cell surface markers of BMM from wild-type (dark lines) and FcγRI-deficient (light lines) mice. Expression of FcγRI was tested using a specific anti-FcγRI mAb, X54-5/7.1 (IgG1) or FcγRI ligands, mouse IgG2a, and human IgG. Expression of the macrophage-specific marker was detected with F4/80 mAb. The low-affinity receptors, FcγRII and FcγRIII, were detected using the mAb 2.4G2. Background binding of the appropriate FITC-labeled anti-IgG conjugates (anti-mouse, -rat, or -human) Fab′$_2$ fragments (dotted lines).

However, BMM lacking FcγRI failed to bind monomeric IgG2a (Figures 2 and 3C) and did not internalize IgG (Figure 3D), indicating that the uptake of monomeric IgG by macrophages is mediated only by FcγRI.

Mice Deficient in the FcR-γ Chain Express Functional FcγRI

During the initial characterization of our FcγRI-deficient mice, we also analyzed cells of the FcR-γ chain-deficient mice for control purposes. The FcR-γ chain is shared by a number of Fc and other receptors (Ernst et al., 1993; Ra et al., 1989), and mice deficient in FcR-γ chain show a loss of FcεRI and FcγRIII expression and, despite no direct measurement, have consistently been reported to lack FcγRI (Hazenbos et al., 1996; Takai et al., 1994, 1996). However, using anti-FcγRI monoclonal antibody (X54-5/7.1) and immunofluorescence analysis of BMM from FcR-γ chain-deficient mice unexpectedly revealed significant levels of FcγRI expression, at about 1/5 the level of the receptor expressed on normal macrophages. Most importantly, the receptor was functional and bound monomeric IgG2a (Figure 4A). The bound IgG2a was internalized as for FcγRI in normal BMM (compare Figures 3 and 4B), although the intensity of fluorescence was lower, being consistent with the reduced receptor expression. Receptor internalization

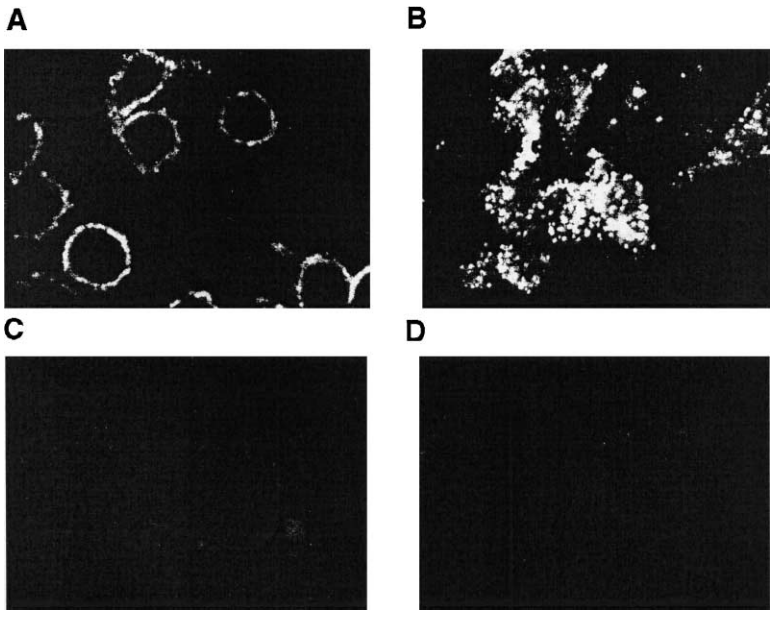

Figure 3. Endocytosis of Mouse IgG2a by BMM

Normal (A and B) and FcγRI-deficient (C and D) BMM were incubated with monomeric IgG2a antibody at 4°C for 60 min and then incubated at 37°C for 20 min in the absence (A and C) and the presence (B and D) of cross-linking Fab′$_2$ fragments of goat anti-mouse IgG. Following incubation, the cells were fixed, permeabilized, and incubated with FITC-conjugated anti-goat IgG for 30 min and viewed by fluorescence microscopy (original magnification ×400).

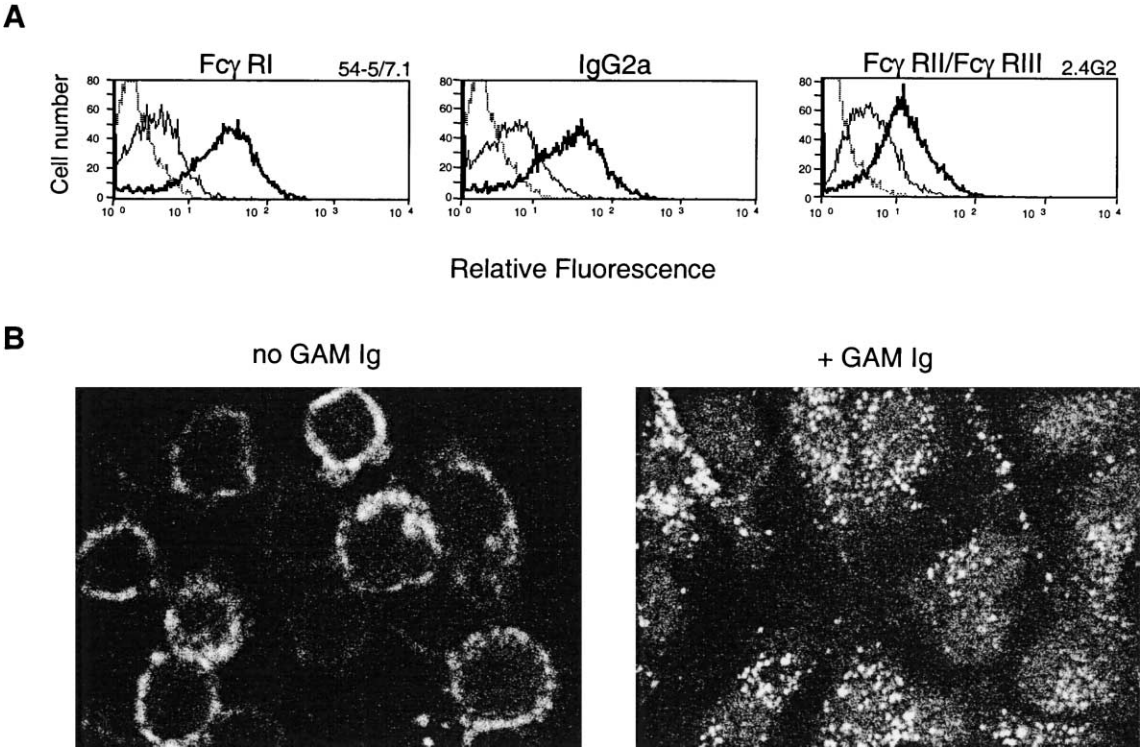

Figure 4. Expression of FcγRI by FcR-γ Chain-Deficient BMM

(A) Flow cytometry of FcR expression by BMM derived from normal (dark lines) and FcR-γ chain-deficient mice (light lines) was detected with the anti-FcγRI mAb X54-5/7.1, mouse IgG2a, or mAb 2.4G2. Background binding of FITC-labeled anti-IgG conjugates (anti-mouse or -rat) Fab'_2 fragments is shown as dotted lines.

(B) Fluorescence micrographs of the endocytosis of monomeric IgG2a via FcγRI from FcR-γ chain-deficient BMM. The cells were incubated with IgG2a antibody at 4°C for 60 min, then incubated at 37°C for 20 min in the absence (no GAM Ig) or the presence (+ GAM Ig) of crosslinking Fab'_2 fragments of goat anti-mouse IgG. Following incubation, the cells were fixed, permeabilized, and incubated with FITC-conjugated anti-goat IgG for 30 min (original magnification ×400).

was also shown using Fab'_2 fragments of the anti-FcγRI antibody (data not shown). Thus, these experiments show that the high-affinity FcγRI is still expressed and at least partly functional, with endocytosis able to occur in BMM in the absence of the FcR-γ chain.

Selective Impairment of Phagocytosis in FcγRI$^{-/-}$ BMM

Major differences in the phagocytosis of immune complexes of IgG2a and IgG3 but not IgG1 by FcγRI-deficient BMM were apparent (Figure 5). (1) IgG2a: The kinetics and extent of uptake of IgG2a complexes was profoundly impaired in FcγRI-deficient BMM but was not entirely absent (Figure 5A). Normal macrophages rapidly phagocytosed IgG2a-opsonized SRBC (IgG2a-EA) at a rate of 400 SRBC per 100 BMM in 20 min to a maximum of 1000 per 100 BMM by 40 min, which was unaltered at 60 min (Figure 5A). This was more extensive and rapid than IgG1-EA phagocytosis via FcγRIII, where after 60 min, only 300 SRBC were ingested (compare Figures 5A and 5C). However, in the absence of FcγRI, BMM were able to ingest IgG2a-EA but at a greatly reduced rate, ingesting fewer than 400 SRBC per 100 BMM in 60 min, thus showing that FcγRI provides a mechanism for the rapid uptake of complexes. It is clear that the slower but significant uptake of IgG2a-EA in the absence of FcγRI suggests the involvement of FcγRIII. This is consistent with the rate of uptake being equivalent to IgG1-EA uptake via FcγRIII (Figure 5C). Thus, on normal macrophages, endocytosis of soluble IgG or phagocytosis of particulate IgG occurs via different mechanisms, and despite the continued expression of FcγRI in FcR-γ chain-deficient mice, phagocytosis of IgG2a-EA could not occur, indicating the importance of this ITAM-dependent pathway in phagocytosis. This is consistent with previous reports on the importance of the FcR-γ chain in phagocytosis (Takai et al., 1996). (2) IgG3: The kinetics of uptake of IgG3-EA by normal BMM was slower than that of IgG2a-EA, but the loss of IgG3 phagocytosis was more dramatic than that of IgG2a-EA (Figure 5B). Uptake of IgG3-EA, a T cell-independent immunoglobulin, defined as a ligand for FcγRI (Gavin et al., 1998a), was zero at all time points in the FcγRI-deficient BMM, clearly confirming that FcγRI is the only IgG3 receptor and that FcγRII/FcγRIII has no involvement in IgG3 binding. These results also provide useful controls and indicate that BMM did not "spontaneously" engulf antibody-coated SRBC. (3) IgG1: As expected, the phagocytosis of IgG1-EA by BMM from FcγRI$^{-/-}$ mice was indistinguishable from normal BMM since this IgG subclass does not bind to FcγRI (Figure 5C). However, it should be noted that the rate of uptake

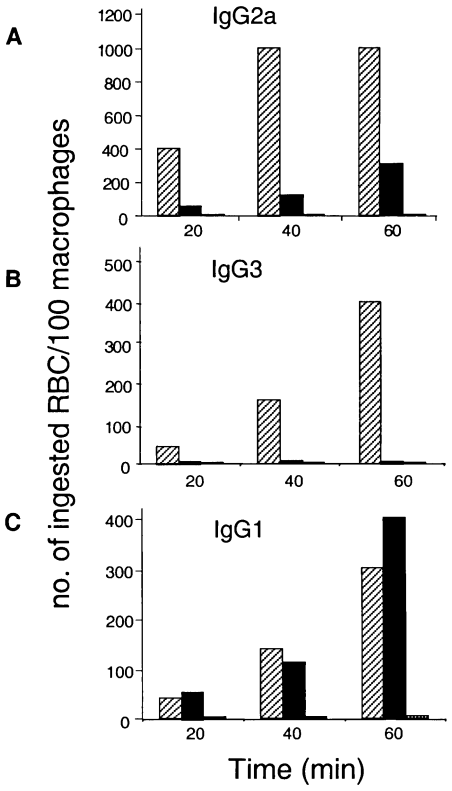

Figure 5. Kinetics and Specificity of Uptake of Immune Complexes by BMM

Ingestion of erythrocytes sensitized with mouse IgG2a (A), IgG3 (B), or IgG1 (C) by BMM from FcγRI$^{+/+}$ (hatched), FcγRI$^{-/-}$ (black), and FcR-γ chain$^{-/-}$ (gray) mice. The number of erythrocytes ingested per 100 BMM represented the uptake of complexes after 20, 40, and 60 min incubation. There was no uptake (<5 erythrocytes/100 BMM) in the absence of sensitizing antibody. Results are expressed as the mean of three experiments; standard errors of the mean were <10%.

was considerably slower than that of IgG2a-EA as mentioned above. This result also indicates that there is no "global" defect in phagocytic pathways in the FcγRI-deficient BMM. No phagocytosis above background levels was seen with BMM from the FcR-γ chain$^{-/-}$ mice following a 1 hr incubation with IgG1-EA.

Reduced Antibody-Dependent Cell-Mediated Cytotoxicity by FcγRI$^{-/-}$ BMM

FcγR play a major role in the killing of antibody-coated target cells (Fanger et al., 1989). This function has been largely attributed to FcγRIII (Takai et al., 1994) and analysis principally focused on NK cells (Hazenbos et al., 1996). However, macrophages participate in antibody-dependent cell-mediated cytotoxicity (ADCC) and express both FcγRI and FcγRIII, but the specific roles of these receptors in ADCC have not been defined. BMM expressing both FcγRI and FcγRIII in wild-type mice were potent effector cells (Figure 6); however, BMM lacking FcγRI but expressing unchanged levels of FcγRIII (see Figure 2) were considerably less efficient, with the loss of FcγRI resulting in a reduction of between 30%–60% in antibody-dependent cell-mediated lysis of SRBC (Figure 6A). Thus, ADCC in the absence of FcγRI but through FcγRIII requires more antibody to achieve the same level of killing, indicating a major role for FcγRI in macrophage ADCC. The differences in the quantity of antibody required to achieve killing probably reflect differences in the affinity of the high- and low-affinity receptors for antibody.

Antigen Presentation Is Impaired in FcγRI$^{-/-}$ BMM

The capacity of cells to present antigen following its uptake in an immune complex was severely impaired in cells lacking FcγRI. Normal and FcγRI-deficient BMM were pulsed with human Ig (huIg) and their capacity to stimulate CD4^{+} T cells isolated from huIg primed mice determined (Figure 6B). At low antigen concentration (10 μg/ml huIg), the FcγRI-deficient BMM were unable to induce T cell proliferation (1000 cpm) compared with normal BMM where near-maximum stimulation (14,000 cpm) was achieved (Figure 6B). Furthermore, ten times more antigen (100 μg/ml compared to 10 μg/ml) was required by the FcγRI-deficient BMM in order to stimulate T cell proliferation equivalent to that obtained by normal BMM. The differences in the affinity between FcγRI and FcγRIII are likely to account for the difference in immune complex-dependent antigen presentation between normal and FcγRI-deficient BMM. Thus, at low immune complex (antigen) concentration and in the absence of FcγRI, macrophages expressing FcγRII and FcγRIII were unable to bind and present antigen efficiently. Clearly then, FcγRI is required for optimal antigen presentation through this pathway.

FcγRI$^{-/-}$ Mice Have Reduced Inflammatory Responses

FcR have a major role in immune complex-induced vasculitis and inflammation, and it has recently been reported that the use of soluble FcR proteins and/or inactivation of low-affinity FcR genes can reduce inflammation (Hazenbos et al., 1996; Ierino et al., 1993; Sylvestre and Ravetch, 1996). We examined the inflammatory response in vivo in the FcγRI$^{-/-}$ mice using a reverse passive Arthus model (Figure 6C). The induction of inflammation, measured by footpad swelling after local deposition of antibody in the presence of high levels of circulating antigen, indicated that the loss of FcγRI results in alterations to both the kinetics and the magnitude of the Arthus reaction but not its complete ablation. The Arthus reaction in the wild-type mice peaked at approximately 3–5 hr with a steady decline over the ensuing 20 to 24 hr. This was typical of the kinetics seen in a number of experiments. By contrast, the Arthus reaction measured in the FcγRI$^{-/-}$ mice was slower to develop and, while it peaked at 5 hr, showed a maximum increase in swelling that was 70% of that observed in the wild-type mice. Moreover, the inflammation resolved more rapidly, returning to background by approximately 8 hr (Figure 6C). This reaction was specific, as no inflammation was detected in the absence of ovalbumin nor in the absence of ovalbumin-specific IgG. It is clear therefore that the high-affinity IgG receptor also plays a role in the generation of immune complex-induced inflammation; the presence of FcγRII and FcγRIII (the

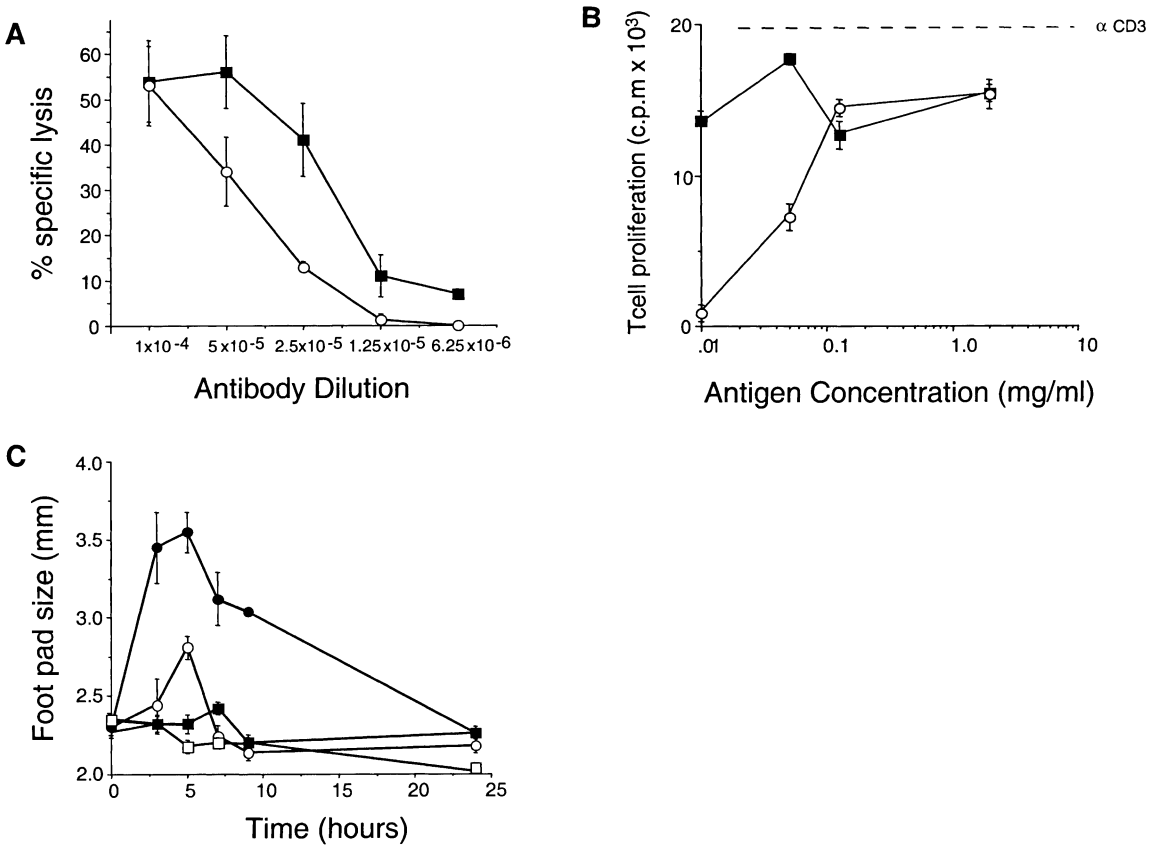

Figure 6. Antibody-Dependent Cell-Mediated Cytotoxicity, Antigen Presentation by BMM, and Arthus Reaction In Vivo

(A) ADCC of ^{51}Cr-labeled SRBC by BMM from FcγRI$^{+/+}$ (closed squares) and FcγRI$^{-/-}$ (open circles) mice at a range of concentrations of anti-erythrocyte antibody and an E:T ratio of 1:1. Results are expressed as a percentage of specific lysis in four individual experiments ±SEM.

(B) FcγRI-deficient mice showed impaired antigen presentation. Irradiated spleen APCs from FcγRI$^{-/-}$ (open circles) and FcγRI$^{+/+}$ (closed squares) mice were cultured with antigen-specific T cells and incubated with a range of antigen (human IgG) concentrations. T cell proliferation was measured by ^{3}H-thymidine uptake. The level of proliferation induced by anti-CD3 mAb alone is indicated by the dashed line. Results are expressed as the mean of three individual experiments ±SEM.

(C) FcγRI-deficient mice have impaired inflammation. Immune complex-induced inflammation was determined in the footpad using a reverse passive Arthus reaction induced by OVA/anti-OVA complexes. The extent of inflammation was determined by measurement of swelling in the footpad of FcγRI$^{+/+}$ (closed circles) and FcγRI$^{-/-}$ (open circles) mice. Specificity of the Arthus reaction was determined by injection of nonimmune IgG in the presence of antigen in FcγRI$^{+/+}$ (closed squares) and FcγRI$^{-/-}$ (open squares) mice. Results are expressed as the mean of three individual experiments ±SEM. There were three mice per experimental group.

low-affinity immune complex receptors) could not compensate for the loss of FcγRI.

Increased IgG Responses in FcγRI$^{-/-}$ Mice

Antibody production was investigated in both wild-type and FcγRI-deficient mice using NP-KLH as antigen. Mice were immunized, then NP-specific IgG1, IgG2a, IgG2b, and IgG3 were determined for individual mice 14 days after immunization (Figure 7A). Compared to wild-type mice, FcγRI$^{-/-}$ mice showed 2- to 5-fold increases in the levels of IgG subclasses following immunization. This was most noticeable for IgG2b, IgG3, and IgG1; IgG2a was less marked although the difference was statistically significant ($p < 0.05$). Thus, the lack of FcγRI resulted in a generalized increase in IgG levels. The number of antibody-forming cells induced following immunization was determined in a modified hemolytic plaque-forming cell assay. Spleens of FcγRI$^{-/-}$ mice immunized with SRBC showed a 2- to 3-fold increase in antibody-forming cells for both IgM and IgG compared to those of normal mice (Figure 7B). Antibody half-life in the FcγRI$^{-/-}$ mice was not different from FcγRI$^{+/+}$ mice (data not shown). Thus, FcγRI appears to play a key role in the modulation of antibody responses in vivo, and deletion results in an increase in the number of antibody-forming cells.

Discussion

The inactivation of the gene encoding the unique high-affinity Fc receptor—FcγRI—resulted in a wide range of defects in antibody Fc-dependent effector cell function, as well as elevated antibody production in vivo. The studies presented herein showed that FcγRI is far more effective than other FcγR in initiating endocytosis of soluble IgG, phagocytosis of immune complexes, antibody-dependent killing of cells, and the delivery of immune complexes to antigen presentation pathways. In

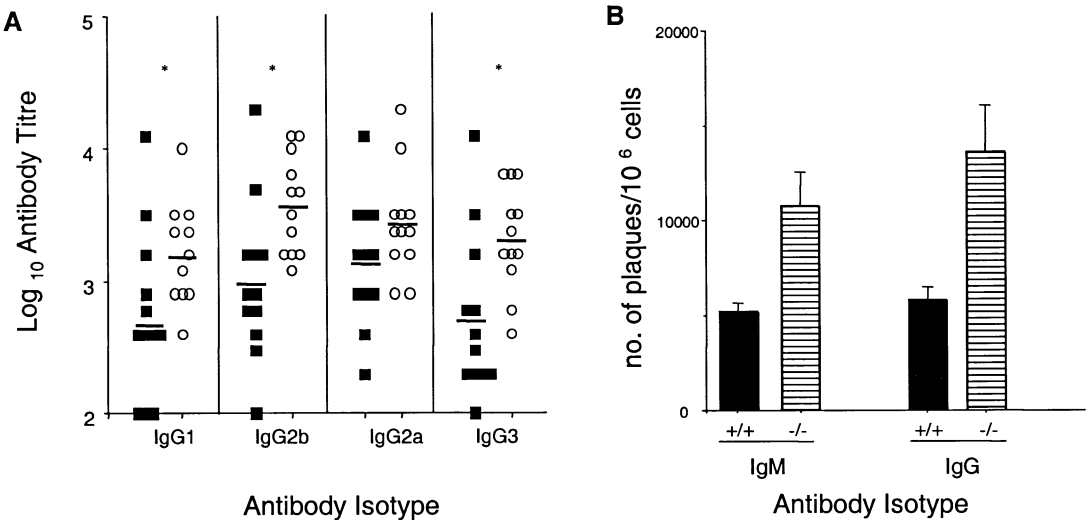

Figure 7. FcγRI-Deficient Mice Have an Increased Antibody Production

(A) FcγRI$^{-/-}$ (open circles) and FcγRI$^{+/+}$ (closed squares) mice were immunized with 100 μg of NP-KLH i.p., and serum Ig titres were determined at day 14 postimmunization. Symbols represent antibody titre from individual mice. The mean titre of each experimental group of 12 mice is represented as a bar; * indicates $p < 0.05$.

(B) Plaque assays were performed on FcγRI$^{-/-}$ (hatched) and FcγRI$^{+/+}$ (black) mice immunized i.v. with SRBC. The number of plaques forming cells per 10^6 spleen cells = 52/3 × number of plaques per chamber/5. Results were expressed as the mean of three experiments ± SEM.

addition, in vivo inflammation was reduced and, interestingly, antibody production was increased 2- to 5-fold over normal mice.

Defining the role of the least studied Fc receptor, the high-affinity IgG receptor FcγRI, in antibody-dependent effector functions and in immune responses in vivo has been confounded by the lack of mice deficient in only FcγRI. Indeed, to date no direct analysis of FcγRI has been possible. Much of the current "understanding" has been inferred largely from indirect analysis of FcR-γ chain-deficient mice (Sylvestre and Ravetch, 1996; Takai et al., 1994, 1996; Vora et al., 1997) and to some extent from other Fc receptor-deficient mice (Hazenbos et al., 1996). One of the major difficulties with such analyses is the widespread distribution of FcR-γ chain and its promiscuous interactions with multiple receptors—FcγRI, FcγRIII, FcεRI, and the CD3 complex. Attempts to minimize the ambiguity in interpretation of data from such mice include the use of different IgG subclasses, especially IgG2a, as specific probes for different receptors. This has only confounded the problem, as it is clear that receptor specificity is not as strict as often assumed; i.e., IgG2a binds in complexes to FcγRI, FcγRII, and FcγRIII (see Figure 5A) (Hogarth et al., 1985; Hulett et al., 1991) .

Thus, the studies shown herein show (1) the widely held view that FcR-γ chain-deficient mice lack FcγRI (Hazenbos et al., 1996; Takai et al., 1994, 1996) is clearly not correct, the presence of the receptor confirmed both by ligand binding studies and the use of specific monoclonal anti-receptor antibodies; (2) although some FcγRI functions (phagocytosis) are impaired due to lack of the FcR-γ chain, endocytosis of IgG remains intact, which was also confirmed by the use of receptor-specific monoclonal antibodies. Clearly, this has implications for FcR-dependent antigen presentation and other in vivo functions, especially given the role of immune complexes in modifying antibody responses in vivo and in particular as the delivery of immune complexes to the class II antigen presentation pathway occurs in the absence of functional FcR-γ chain, albeit with reduced efficiency (van Vugt et al., 1999).

The analysis of mice deficient only in FcγRI has enabled us to understand the independent role of this receptor in antibody-dependent systems, and there are a number of key observations arising from the studies presented herein.

The uptake of small or soluble immune complexes or IgG requires different cell-based mechanisms to those involved in phagocytosis. The uptake of soluble IgG is clearly mediated by FcγRI, not the low-affinity receptors, presumably by virtue of the differences in affinity between FcγRI and FcγRII, or FcγRIII. The process of endocytosis does not require FcR-γ chain as discussed above and is clearly independent of the ITAM/tyrosine kinase pathway necessary for processes such as phagocytosis (see Figures 3 and 4) (Takai et al., 1994).

The phagocytic uptake and destruction of particulate immune complexes is an important mechanism of resistance to infection and is an important role of FcR generally. The comparison of normal and FcγRI-deficient BMM showed that FcγRI is the major IgG2a receptor and provides the most rapid and extensive uptake of immune complexes. FcγRI is clearly more efficient than the low-affinity receptors even for highly opsonized SRBC, which efficiently engage the FcγRII and FcγRIII. IgG2a binding and phagocytosis still occur in the absence of FcγRI, presumably through FcγRIII where the kinetics of uptake of IgG2a and IgG1 complexes is almost identical (Figure 5, note difference in scales). An interesting practical observation is that IgG2a is often used as a specific probe for FcγRI, especially in the

analysis of other FcR-deficient mice, and although FcγRII/FcγRIII do not detectably bind monomeric IgG2a, they clearly bind IgG2a complexes (Figure 5) (Hulett and Hogarth, 1994; Hulett et al., 1991; Unkeless and Eisen, 1975).

On macrophages, both FcγRI and FcγRIII are expressed and active in ADCC (Figures 2 and 6A), but FcγRI is more effective at lower antibody concentration and presumably is more important earlier in humoral responses when the concentration of circulating antibodies is low, with FcγRIII becoming increasingly important as antibody concentrations rise. To date, the analysis of ADCC has focused on FcγRIII and NK cells (Hazenbos et al., 1996; Takai et al., 1994; Vora et al., 1997) using FcR-γ chain and/or FcγRIII-deficient animals, and little analysis of macrophage ADCC has been performed. The studies of others using FcR-γ chain-deficient mice show a complete ablation of ADCC (Clynes et al., 1998), which, taken together with the analysis herein, is consistent with FcγRI and FcγRIII being involved in macrophage ADCC, but clearly these receptors would be engaged at different times in a developing immune response.

The uptake of small complexes by FcγRI-deficient BMM for antigen presentation to primed T cells was studied herein. These macrophages express all the other fully functional Fc receptors, including the FcR-γ chain, in their normal cellular environment unlike studies using transfected cells that never normally express these receptors. The data (Figure 6B) show clear evidence that at lower antibody concentrations, FcγRI is extremely efficient in uptake of immune complexes for subsequent antigen presentation. Indeed, the capture of antigen in immune complexes and its subsequent presentation is reduced by 70% by deletion of FcγRI. Over ten times more antigen (human IgG) is required to achieve the same level of stimulation when complexes are taken up via the low-affinity receptors in the absence of FcγRI; i.e., other FcγR inefficiently deliver antigen in immune complexes to antigen presenting pathways.

Autoimmune or other pathological immune responses often produce immune complexes that initiate destructive inflammatory responses. The cutaneous reverse passive Arthus reaction is an in vivo model for immune complex disease, featuring local tissue destruction by antibody-mediated inflammatory responses, with macrophages and neutrophils recruited to the site of inflammation. The independent role of FcγRI has not been reported previously, but our data indicate that the Arthus reaction in the FcγRI$^{-/-}$ mice was less severe than in normal mice. The high-affinity IgG receptor therefore plays a role, possibly through efficient capture of antibody at inflammatory sites, in enhancing this immune complex-induced inflammation; however, the presence of the low-affinity receptors FcγRII and FcγRIII could not fully compensate for the loss of FcγRI. This is consistent with previous studies defining a major role of FcR in this response first indicated by the use of soluble recombinant FcγR that inhibited the Arthus reaction (Ierino et al., 1993). Subsequent studies with complement-deficient mice and other FcR knockout mice have shown that the response is largely complement independent but mast cell dependent and downregulated in FcR-γ chain and FcγRIII-deficient mice (Sylvestre and Ravetch, 1996; Hazenbos et al., 1996; Takai et al., 1994), with FcγRIIb (Takai et al., 1996) acting as a regulator of the response.

Immune complexes are well-known to influence immune responses (Heyman, 2000). Immunization of mice lacking FcγRI resulted in 2- to 5-fold increases in primary antibody responses in vivo, which was reflected in an increase in the numbers of antibody-producing cells (Figure 7). These data indicate that FcγRI has a role in the regulation of antibody responses and suggest that in the absence of FcγRI, a control point is bypassed either by default or as an active process. Such a role was suggested by the 30-year-old work of Parish and coworkers (Liew and Parish, 1972) who showed that cytophilic IgG, which by definition bound to FcγRI, could modulate humoral and cellular responses. There are also a series of additional observations that are consistent with a regulatory role for FcγRI in antibody responses. Indeed, exaggerated antibody responses and elevated levels of circulating Ig have been observed in other mouse strains with deletions of the cytoplasmic tail of FcγRI but with intact FcR-γ chain. These mouse strains include Biozzi "high responder" mice bred for enhanced antibody production and the NOD mice, which have abnormal levels of IgG1 and IgG2b, but IgG2a is less affected, similar to the findings in the FcγRI$^{-/-}$ mice herein (Gavin et al., 1998b; Luan et al., 1996; Regnault et al., 1999). The deletions in the cytoplasmic tail of FcγRI have been analyzed in transfection models and shown to affect the intracellular handling of immune complexes bound by FcγRI and subsequent antigen presentation, independent of the FcR-γ chain (Gavin et al., 1998b; van Vugt et al., 1999). It is interesting then that analysis by others of FcR-γ chain-deficient mice (Clynes et al., 1998; Vora et al., 1997), which we now know to express lower quantities of FcγRI, showed no consistant increase in antibody production. However, there were suggestive although inconclusive changes in antibody responses in the study by Vora et al. (1997); these may relate to the reduced but not absent expression of FcγRI.

The mechanism by which FcγRI participates in the development of antibody responses is not clear; however, FcγRI has several unique properties that enable early participation in immune responses. It is the low-affinity receptor for the T cell-independent immunoglobulin IgG3 and the high-affinity receptor for IgG2a, the archetypal antibody product of the Th-1 response, and it does not bind IgG1, the archetypal antibody product of the Th-2 response, which is preferentially bound by the low-affinity Fc receptors. The switch from IgM to IgG3 can occur without T cell help and requires IFN-γ provided by the innate immune system. The production of IFN-γ would also be expected to simultaneously upregulate FcγRI (Hulett and Hogarth, 1994). As the adaptive immune response develops, especially the IFN-γ-dependent Th-1 response, IgG2a, the high-affinity ligand, is produced, thereby linking ligand production to receptor upregulation and the innate and adaptive immune responses. In a Th-2 response, the IgG1 produced by the adaptive immune system would not engage FcγRI and presumably would have little effect on responses until the concentrations of immune complexes rise sufficiently to engage the low-affinity receptors. Thus, the early engagement of FcγRI at the inter-

face of innate and adaptive immunity, when IgG2a concentrations are low, may well provide a control point for Th-1 type responses. In such a model, the loss of FcγRI may result in the continued development of antibody responses, particularly as other immune complex-dependent regulatory pathways such as the coengagement of activating ITAM-dependent FcR with the negative regulatory FcγRIIb (Ravetch and Bolland, 2001; Takai et al., 1994) would not efficiently occur until antibody concentrations in immune complexes had risen to levels that would effectively engage FcγRIII. The timing of the coengagement of the negative regulating FcγRIIb with the activating receptors may well be critical in controlling activities such as antigen presentation and therefore the overall response.

The enhanced antibody production in vivo (Figure 7) but reduced antigen presentation in vitro (Figure 6C) by BMM appears paradoxical. However, it should be noted that primed T cells were used in the antigen presentation experiments whereas the primary antibody response was examined in vivo. Clearly, the stimulation of primed T cells is more readily achieved by immune complex uptake via FcγRI than via the low-affinity receptors. However, in naive mice, FcγRI may be involved early in the development of the immune response, as well as having a later role in enhancing stimulation of primed T cells.

Finally, it is clear that FcγRI is of primary importance in many Fc-dependent effector cell functions, and while the basis for the elevated antibody responses it still uncertain, it is clear in vivo that FcγRI receptor inactivation or mutation is correlated with altered antibody responses.

Experimental Procedures

Engineering of the FcγRI Null Allele and Its Detection

A genomic clone of mouse FcγRI gene was isolated from λ Fix II genomic library of 129/J DNA. A defective form of the FcγRI gene was constructed by deleting the promoter and also disrupting the coding region. First, a 400 bp segment of the 5′ end of the gene was deleted using splice overlap extension PCR. This deletion removed part of the promoter and the transcription initiation site, as well as the translation initiation ATG. Secondly, a neomycin resistance gene was inserted into the second leader exon, resulting in the disruption of this exon and the leader sequence, as well as the generation of a frame shift mutation. This induced a premature termination codon immediately downstream of exon 2 (Figure 1A). A unique HindIII site was used to distinguish correct integration of the targeting DNA (8.3 kb) from the wild-type FcγRI (9.7 kb) genomic DNA (Figure 1B). Following electroporation of this DNA into W9.5 embryonic stem (ES) cells, and selection, 480 clones were screened by genomic Southern blots (Barnett and Kontgen, 2001; Kontgen and Stewart, 1993) and probed with a 1.8 kb HincII/SacI fragment derived from DNA 5′ of the FcγRI gene. Four clones that had undergone homologous recombination were identified, from which two were microinjected into C57BL/6 blastocysts.

Mice

The founder chimeric mouse was crossed to 129/SvJ mice, and genomic DNA from subsequent progeny was isolated and screened by Southern hybridization for the presence of the FcγRI null allele. Homozygous animals contained either the wild-type "+" 9.7 kb allele or the 8.3 kb "−" inactivated allele (+/+ or −/−, respectively). Heterozygous animals are shown in figures as "+/−." FcR-γ chain-deficient mice (FcR-γ chain$^{-/-}$) were obtained from Dr. J. Ravetch (Takai et al., 1994).

Bone Marrow-Derived Macrophages

Bone marrow-derived (BMM) stem cells were obtained from the femurs of mice and cultured in RPMI 1640 (CSL Biosciences, Australia) and 30% L cell conditioned medium (LCM) for 4 days. Nonadherent cells were then harvested and cultured for a further 4 days until confluent. For some assays, BMM were cultured for a further 24 hr in the presence or absence of 100 U IFN-γ (Genzyme, USA) (Gavin et al., 1996).

Northern Blot Analysis

Total RNA was extracted from BMM using RNAzol (GIBCO, USA) according to the manufacturer's protocol. 10 μg of RNA was electrophoresed on a formaldehyde-agarose (1%) gel and transferred to Hybond N+ membrane (Amersham, Life Sciences, USA) and probed with ^{32}P-labeled 1.3 kb FcγRI cDNA fragment. The membrane was washed twice in 0.2 × SSC, 1% SDS at 65°C for 30 min and exposed overnight to Kodak XAR5 film (Kodak, Eastman, USA). The relative amounts of RNA were checked by reprobing with γ actin cDNA to ensure equal loading of RNA in each lane.

Flow Cytofluorometric Analysis

Flow cytometric analysis of BMM was carried out using a Facscalibur flow cytometer (Becton-Dickinson). Expression of FcγRI was tested using a specific anti-FcγRI mAb, X54-5/7.1 (IgG1) (P.S.T., unpublished data) or FcγRI ligands, mouse (anti-TNP mAb) IgG2a, and human IgG (pooled IgG) (Novartis, Switzerland). Macrophages were detected with the specific antibody F4/80, and the low-affinity IgG receptors, FcγRII and FcγRIII, were detected using the 2.4G2 antibody (Unkeless, 1979). Expression of all markers was compared to background binding of the appropriate FITC-labeled anti-IgG conjugates (anti-mouse, -rat, or -human) Fab′$_2$ fragments.

Endocytosis Assay

FcγRI-mediated internalization of IgG was examined using a modification of a previously described method (Harrison et al., 1994). BMM were isolated from FcγRI$^{+/+}$, FcγRI$^{-/-}$, and FcR-γ chain$^{-/-}$ mice and allowed to attach in eight-well chamber slides (NUNC, Sweden). The cells were then incubated with 10 μg/ml of anti-FcγRI (X54-5-7.1) Fab′$_2$ or 10 μg/ml of whole IgG2a for 60 min at 4°C, washed in 0.5%BSA in PBS, and then incubated in the presence or absence of 50 μg/ml crosslinking antibody (goat anti-mouse IgG, Fab specific) (Silenus, Australia). The slides were returned to 4°C or warmed to 37°C for 20 min. The cells were washed, fixed in 3% paraformaldehyde in 80 mM K_2PIPES for 5 min followed by 3% paraformaldehyde in 100 mM $Na_2B_4O_7$ for 10 min, and permeabilized in 0.1% Triton-X 100 (BDH chemicals, Australia) for 10 min. The internalized IgG was detected with FITC-conjugated anti-mouse or anti-goat antibody (Silenus, Australia) following incubation for 30 min at 37°C, then visualized using confocal microscopy (Optiscan, Australia).

Phagocytosis Assay

Immune complexes of IgG-sensitized sheep erythrocytes (EA) using IgG known isotype were generated as follows. SRBC were sensitized by incubation with 2,4,6 trinitrobenzene sulphonic acid (TNBS) (Hulett et al., 1991), then sensitized with monoclonal anti-TNP antibodies, IgG3, IgG2a, or IgG1. Phagocytosis assays were performed using monolayers of BMM which were incubated with 2% EA for various times from 10 to 60 min at 37°C. The monolayers were then washed to remove unattached EA, and surface-bound EA were lysed by brief hypertonic shock (PBS, 1 mM NaCl [pH 2.5]), then fixed in 0.5% glutaraldehyde. Ingested EA were visualized using light microscopy. The uptake of complexes was represented by the number of EA ingested per 100 BMM at each time point. There was no uptake (<5 EA/100 BMM) in the absence of sensitizing antibody. Results are expressed as the mean of three individual experiments.

Antibody-Dependent Cellular Cytotoxicity

Cytotoxicity assays were carried out in 96-well V-bottomed tissue culture plates with 2 × 10^5 target cells (SRBC) labeled with 100 μCi ^{51}Cr for 60 min at 37°C in medium (RPMI 1640). The target cells were washed several times and incubated for 10 min at 37°C with rabbit anti-SRBC antibody at various concentrations. The cells were mixed with effector cells (BMM) at various effector-to-target (E:T)

ratios. The release of ^{51}Cr was measured after a 4 hr incubation at 37°C. Values for maximal release were obtained by the addition of 1 M HCl. Specific cytoxicity was calculated according to Hogarth et al. (1985) and expressed as the mean of quadruplicate samples ±SEM.

Proliferation Assays

Antigen presentation assays were performed according to a modification of the method described by (Romball and Weigle, 1993). Mice were injected in both hind footpads with 200 μg of huIg (Novartis, Switzerland) emulsified in CFA. The inguinal and popliteal lymph nodes were harvested aseptically at 9 days postimmunization and single-cell suspensions prepared. CD4-enriched T cells were prepared by the removal of plastic adherent cells by incubation for 2 hr at 37°C, followed by depletion of B cells and $CD8^+$ T cells using B220 and CD8-coated magnetic beads (Dynal, Australia) resulting in a >95% pure $CD4^+$ T cell population. 100 μl of T cells was cultured (2×10^6 cells/ml) in flat-bottomed 96-well plates with irradiated adherent spleen cells from unimmunized $Fc\gamma RI^{+/+}$ or $Fc\gamma RI^{-/-}$ mice as APC (3×10^6 cells/ml). Quadruplicate wells were set up with a range of antigen concentrations from 0.01 to 2 mg/ml of huIg or 1 μg/ml anti-CD3 and pulsed 5 days later with 1 μCi/ well of ^3H-thymadine. Uptake was measured at 6–8 hr later by scintillation counter (Packard Top Count, Packard Instruments, USA).

Reverse Passive Arthus Reaction

Type III hypersensitivity responses were investigated using a reverse passive Arthus reaction as described by (Ierino et al., 1993). Inflammatory responses were assayed using mice injected i.v. with 40 μg of ovalbumin (Sigma, USA). Five minutes later, the mice were injected with 50 μg of either anti-ovalbumin or nonimmune IgG into the footpad. The swelling induced was then measured at regular time intervals for 24 hr using dial gauge microcallipers (Ierino et al., 1993).

Antibody Response

8- to 12-week-old mice were immunized i.p. with NP-KLH (100 μg/mouse). The mice were bled at day 0, 7, 14, and 21, and levels of anti-NP isotypes were quantitated by ELISA using isotype-specific antibodies. The 96-well plates (Nunc, Denmark) were coated with 20 μg/ml $NP^{13}BSA$ in PBS overnight at 4°C; the plates were blocked with 1% BSA for 2 hr at room temperature and then washed in PBS/0.05%Tween 20. Serum was added, serially diluted, and incubated for 1 hr at 37°C. Plates were washed, and biotin-conjugated isotype-specific antibodies (Pharmingen, USA) were added and incubated for 1 hr at room temperature; the plates were washed and incubated with streptavidin-HRP for 1 hr at room temperature, washed, and detected with 0.05% ABTS (Boehringer Mannheim, Germany).

Plaque-Forming Cell Assay

Plaque-forming cell (PFC) assays were performed as described previously (Sandrin et al., 1984). 8- to 10-week-old mice were immunized with 0.2 ml of a 2% suspension of SRBC in normal saline. Seven days after immunization, the spleen cells were prepared into a single-cell suspension. The red blood cells were lysed in 0.83% NH_4Cl and the leukocytes resuspended at 5×10^6 cells/ml. 500 μl of leukocytes was mixed with 50 μl of a 50% suspension of SBRC, 50 μl of guinea pig complement, and 50 μl of medium (direct-PFC) or 50 μl of rabbit anti-mouse Ig (for indirect-IgG PFC) and then incubated at 37°C for 1.5 hr in slide chambers. The plaques were counted per chamber (1/2 slide).

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