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Research Article

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Resistance of Fc Receptor-deficient Mice to Fatal Glomerulonephritis

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Abstract

Immune complex-mediated inflammation is a common mechanism of various autoimmune diseases. Glomerulonephritis (GN) is one of these diseases, and the main mechanism of the induction of GN has been unclear. We examined the contribution of Fc receptors in the induction of nephrotoxic GN by establishing and analyzing mice deficient in the Fc receptor γ chain (FcR γ). Whereas all wild-type mice died from severe glomerulonephritis with hypernitremia by administration of anti-glomerular basement membrane (GBM) antibodies, all FcR γ -deficient mice survived. Histologically, wild-type mice showed glomerular hypercellularity and thrombotic changes, whereas the renal tissue in FcR γ -deficient mice was almost intact. Deposition of anti-GBM antibody as well as complement components in the GBM were equally observed in both wild-type and knockout mice. These results demonstrate that the triggering of this type of glomerulonephritis is completely dependent on FcR⁺ cells. (*J. Clin. Invest.* 1998. 102:1229–1238.) **Key words:** Fc receptor • knockout mouse • glomerulonephritis • Masugi nephritis • anti-GBM antibody

Introduction

Deposition of autoreactive antibodies to self-organ causes immune complex (IC)¹-mediated inflammation and autoimmune diseases. Triggering of inflammation has been thought to be mediated mainly by complements and then various tissue-infiltrating cells. However, recent analysis of various Fc receptor

(FcR)-deficient (KO) mice has shown that FcR plays crucial roles in IC-triggered inflammations (1–5).

FcRs have important functions in the connection between humoral and cellular responses (6–8). In the mouse system, there are three types of Fc γ Rs: the high-affinity receptor Fc γ RI, capable of binding to monomeric IgG, and the two low-affinity receptors Fc γ RII and Fc γ RIII, which bind only to polymeric IgG. Whereas Fc γ RII is a single chain receptor, both Fc γ RI and Fc γ RIII are composed of multimeric subunits and require a homodimer of the γ subunit (FcR γ) for their assembly and cell surface expression as well as signal transduction through the Fc γ R. The FcR γ chain is also associated with the high-affinity FcR complex for IgE (Fc ϵ RI). FcRs are expressed on hematopoietic cell lineage, but different cell types exhibit different expression patterns of FcRs. All three Fc γ Rs are expressed on myeloid lineage cells including macrophages and neutrophils, whereas natural killer (NK) cells express only Fc γ RIII. By contrast, mast cells express both Fc ϵ RI and Fc γ R (Fc γ RII and Fc γ RIII).

Previous work by Takai et al. (1) demonstrated that FcR γ -KO mice showed no expression of Fc γ RI, Fc γ RIII, and Fc ϵ RI, and also that they failed to induce IgG-mediated endocytosis by macrophages and IgE-mediated anaphylaxis by mast cells. More importantly, it was shown that Arthus reaction induced by specific antigen (Ag)-antibody (Ab) IC, as a representative type III inflammation, was severely reduced in both FcR γ - and Fc γ RIII-KO mice (1, 3). FcR-KO mice also exhibited reduced auto-Ab-dependent experimental hemolytic anemia and thrombocytopenia, representative type II inflammatory responses (2). Since these inflammatory responses have been thought to be mediated primarily by complements, these results on experimental inflammatory type II and III responses suggested the possibility that a wide range of inflammatory diseases and related autoimmune diseases may be initiated by an FcR-dependent, rather than complement-dependent, mechanism. However, it is yet to be uncovered whether the development of long-termed organ diseases such as glomerulonephritis (GN) is also mediated by FcR in vivo.

GN is an inflammatory disease of the renal glomeruli characterized by damage to the glomerular basement membrane (GBM) and a wide array of disorders in kidney functions (9). It has been suggested that most cases of GN are induced by immune mechanisms. There are two representative types of immunologically induced experimental GN. One is the deposition of Ag-Ab ICs preformed in circulation or on the wall of glomerular capillaries, and the other is the emergence of auto-Abs against GBM (10) or other renal constituents such as renal tubular Ags. The latter GN can be induced by injecting anti-GBM Ab into rats, rabbits, and mice, and this is known as Masugi nephritis or nephrotoxic serum glomerulonephritis (NTGN) and serves as a model for human Goodpasture ne-

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1. *Abbreviations used in this paper:* BUN, blood urea nitrogen; DTH, delayed-type hypersensitivity; ES, embryonic stem; FcR, Fc receptor; GBM, glomerular basement membrane; GN, glomerulonephritis; HS, histological score; IC, immune complex; NK, natural killer; NTGN, nephrotoxic serum glomerulonephritis; ROMs, reactive oxygen metabolites; SRBC, sheep red blood cells; TK, thymidine kinase.

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phritis (11–16). On the other hand, there has been accumulating data that the development of GN is independent of Ab and dependent on T cell-mediated responses, particularly the delayed-type hypersensitivity (DTH) mechanism (17–20).

To clarify the function of IC deposition on FcR and FcR-expressing cells in the pathogenesis of GN, we established FcR-deficient mice by means of gene targeting of the FcR γ gene, and examined the induction and development of NTGN by anti-GBM Ab in these mice. Here we provide definitive evidence that the initial triggering of this type of GN is completely dependent on FcR.

Methods

Cloning of FcR γ gene and construction of targeting vector. A murine FcR γ genomic gene was isolated from a C57BL/6 genomic library with a mouse FcR γ cDNA probe (21). The 3.65-kb NcoI-MboI of genomic DNA fragment encompassing the 5' upstream of exon 1 and intron 1 was subcloned into pGEM3Z (Stratagene, La Jolla, CA). The NcoI site in intron 1 and the SmaI site in the vector were digested and the fragment was inserted into the HincII site of pMC1neopA (22). This construct was then digested with Sall and XhoI, and the resulting 4.5-kb fragment was ligated to the XhoI site of pIC19R/MC1-TK (22). The 1-kb short arm sequence spanning exons 3 and 4 was amplified with PCR by using C57BL/6 genomic DNA as a template. Primers used for amplification of intron 3 were 5'-ACGCGTCGACATCCAGGTCCGAAAGGCAGC-3' from exon 4 appended with Sall site and 5'-AATCGATGTTGAGACAGCATCTGCTTT-3' from exon 4 appended with ClaI site. The *neo* cassette was ligated in the opposite transcriptional direction as the FcR γ gene and replaced a 4-kb fragment including exon 2 (containing the starting ATG codon for FcR γ).

Generation of FcR γ -KO mice. BL6/III embryonic stem (ES) cells (23) were electroporated with 25 μ g of the ClaI-linearized target vector (24). Cells were selected in the presence of 150 μ g/ml of G418 (GIBCO BRL, Rockville, MD) and 2 mM of gancyclovir and surviving colonies were screened for homologous recombination by PCR with a pair of primers: a sense primer of *neo*^r gene (5'-GAACCTGCGTGCAATCCATCTTGTTCAT-3') and an antisense primer of the exon 5 sequence of FcR γ gene (5'-AATCGATGTGGTTTTCATGCTTCAGA-3'). Individual colonies from positive pools were analyzed by Southern blot. Of the 130 clones screened, we obtained two ES clones with specific homologous recombination. Chimera mice were generated by injecting 10–15 ES cells into BALB/c blastocysts and transferring into the uteri of pseudopregnant BDF1 mice. The chimeras were mated with C57BL/6 mice, and the chimeras derived from one of the ES clones exhibited germline transmission. FcR γ -KO mice were obtained by intercrossing the heterozygotes. The genotype was analyzed by Southern blot with a probe described in Fig. 1A.

RT-PCR analysis. cDNA was synthesized from 0.5 mg total RNA from spleen (24) with reverse transcriptase and random hexamers and used for PCR. Primers used were as follows: FcR γ , exon 3-specific primer (5'-AGTTCGACATCCAGGTCCGAAAGGCAGC-3') and exon 5-specific primer (5'-AATCGATGTGGTTTTCATGCTTCAGA-3'); β -actin, sense primer (5'-TGGAATCCTGTGGCATT-CATGAAAC-3') and antisense primer (5'-TAAAACGCAGCT-CAGTAACAGTCCG-3'). PCR was performed under the following conditions: 94°C for 1 min, 57°C for 1 min, and 72°C for 1.5 min with 27 cycles for β -actin or with 30 cycles for FcR γ .

Flow cytometric analysis. To prepare NK cells, splenocytes were treated with magnetic beads coupled with goat anti-mouse IgG Ab (Organon Teknica Co., Westchester, PA) to remove surface Ig (sIg)⁺ B cells, mixed with anti-CD4 mAb (GK1.5) and anti-CD8 mAb (53.6.7) and then incubated with beads coupled with goat anti-rat IgG Ab (Cappel, Aurora, OH). The resulting sIg⁻CD4⁻CD8⁻ cells

were used as NK-enriched cells (25). IL-2-activated NK cells expanding from this population in the presence of IL-2 for 5 d were stained with PE anti-NK1.1 mAb and FITC anti-Fc γ RIII/III mAb 2.4G2 (PharMingen, San Diego, CA) and analyzed by FACScan[®] with the Cell Quest program.

Phagocytosis analysis. Mice were injected intraperitoneally with 1 ml of 5% thioglycolate, and peritoneal exudate cells were harvested 3 d later. The cells were suspended in 10% RPMI medium in 24-well plates for 6 h at 37°C in a CO₂ incubator, and after the removal of the nonadherent cells, the adherent cells were used as a macrophage monolayer. Sheep red blood cells (SRBC) were surface biotinylated as described (26), and incubated with rabbit IgG antibiotin Ab (Rockland, Gilbertsville, PA). These opsonized SRBC were added to the macrophage monolayer and then incubated at 37°C for 90 min in a CO₂ incubator. The cells were washed with distilled water in order to lyse nonphagocytosed red cells hypotonically.

Preparation of rabbit anti-mouse GBM antiserum. Glomeruli were obtained from ddY mouse kidneys by sieving with No. 120 and No. 200 meshes and were disrupted by supersonic vibration. The final precipitate was digested by trypsin for 3 h in 0.1 M sodium citrate saline (pH 8.2). After centrifugation, the supernatant was dialyzed against distilled water, lyophilized, and used as the GBM Ag. Anti-GBM antisera were obtained by immunizing New Zealand White rabbits (Japan SLC Inc., Shizuoka, Japan) with 10 mg of the GBM Ag in CFA on the back four times weekly, and finally intravenously with 5 mg of the Ag without adjuvant 1 wk later. The sera obtained 7 d after the intravenous injection were used as rabbit anti-mouse GBM Ab. Immunofluorescent study revealed that the antiserum reacted in vivo and in vitro with GBM and tubular basement membrane of ddY and C57BL, but not with renal tubular epithelium (data not shown).

Induction of NTGN. For the induction of NTGN, two sets of 10 female and male C57BL/6 mice (8–12 wk of age; Japan SLC Inc.) were injected intraperitoneally with 250 μ g of rabbit IgG (Cappel) in CFA to generate anti-rabbit IgG Ab for induction of facilitating GN (16), followed by intravenous injection with 75 μ l of anti-GBM Ab 4 d later. The first set of mice served for renal histological examination. Half of the mice were killed 5 d and the other half 8–10 d after the intravenous injection of anti-GBM Ab. The second set was used to observe the natural course of survival for anti-GBM Ab-treated wild-type mice.

For the induction of NTGN in FcR γ -KO mice, 10 males and 10 females each of C57BL/6 and FcR γ -KO mice (total 40) were treated similarly and killed 14 d after the injection of nephrotoxic serum. Anti-GBM-treated C57BL/6 were killed just before death. Sera and renal tissue were obtained at time of killing.

Hematological examination and proteinuria measurement. The serum levels of creatinine, blood urea nitrogen (BUN), and total protein were determined by standard methods for blood chemistry: CRTN-ase-POD for creatinine, urease-GLDH for BUN, and Biuret for total protein. Urine was individually collected in a metabolic cage and the protein contents were measured by the Bradford method.

Complement titration. Since functional titration of mouse complement in the serum has been known to be extremely difficult, the titer of C3 as the representative of complements was measured by sandwich ELISA using two different goat anti-mouse C3 Abs, one of which was coupled with horseradish peroxidase (Cappel).

Histological examination. Blocks of renal tissue were fixed in 10% buffered formalin for routine histological examination. The severity of glomerular lesions was graded by the degree of glomerular changes (histological score, HS) as follows; 0, no or rare small foci of glomerular lesions; 1+, mild to moderate glomerular hypercellularity involving > 50% of glomeruli; 2+, severe glomerular hypercellularity and swelling involving almost all glomeruli; 3+, glomerular changes of 2+ and cellular crescent formation involving > 5% of glomeruli; 4+, thrombotic changes of glomerular tufts with deposition of fibrinoid material involving > 50% of glomeruli; 5+, diffuse thrombotic changes of glomeruli and marked dilatation of tubular lumina with epithelial flattening.

Immunofluorescent study. Small blocks of kidney obtained at autopsy were quickly frozen in acetone dry ice and cut into 4- μ m sections. The cryostat sections were stained with FITC-coupled IgG of goat anti-mouse IgG (Cappel) and goat anti-mouse complement C3 (Cappel), and FITC-coupled F(ab')₂ of goat anti-rabbit IgG (Leinco Technologies, Inc., St. Louis, MO).

Results

Generation of FcR γ -KO mice. A 15.7-kb genomic gene containing all five exons corresponding to the FcR γ cDNA and the flanking sequences was isolated from a C57BL/6 kidney library (Fig. 1 A) and all exon-intron boundaries were determined (data not shown). The targeting vector was constructed by replacing the fragment containing exon 2 with neomycin-resistance gene (*neo^r*) and attaching thymidine kinase gene (TK) from herpes simplex virus (21) at the 5' end, resulting in

the targeting vector containing 3.7 kb of 5' long and 1.0 kb of 3' short homologies (Fig. 1 A). The targeting construct was transfected into the BL6/III ES cell line (23). Two independent ES clones with a specific homologous recombination in one allele of the FcR γ gene were obtained and chimeric mice were produced by injecting these two ES clones into BALB/c blastocysts. Germline-transmitted mice were then obtained by mating these chimeras with C57BL/6 mice. These heterozygous mice were intercrossed to generate homozygotes for FcR γ mutation. Using the ES cell line with C57BL/6 origin, FcR γ -deficient mice with a genetic background of pure C57BL/6 were established without any backcross.

The genotypes of wild-type (+/+), heterozygous (+/-), and homozygous (-/-) mice were identified by Southern blotting (Fig. 1 B). The null mutation of FcR γ gene was confirmed by the lack of FcR γ transcript by RT-PCR (Fig. 1 C) and Northern blotting (data not shown). FcR γ -KO mice were

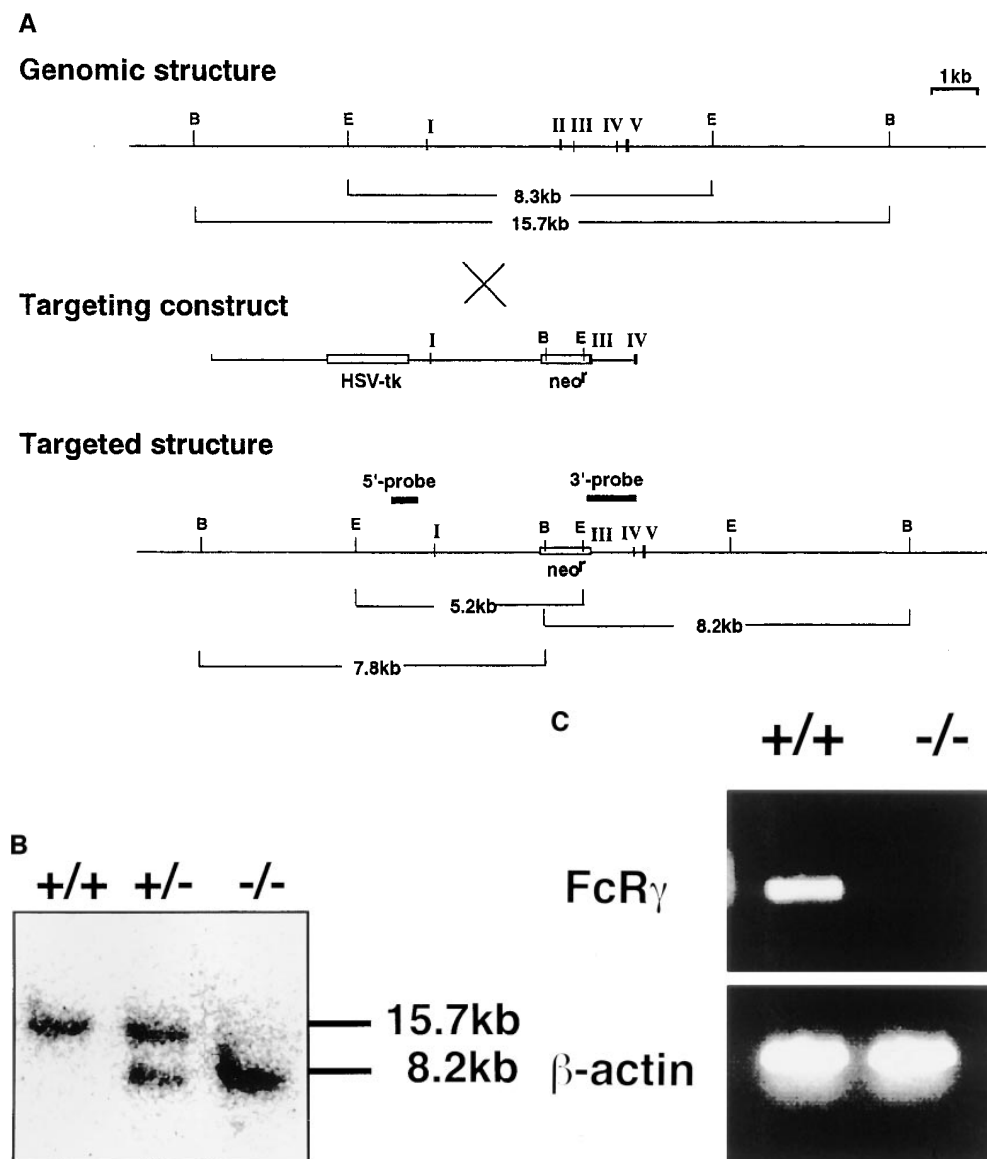


Figure 1. Disruption of FcR γ gene by gene targeting. (A) Genomic structure of mouse FcR γ gene and targeting vector. The FcR γ gene was isolated from a C57BL/6 genomic library. There is a polymorphic EcoRI site between C57BL/6 and 129-derived genomic FcR γ DNA. Exons are indicated by Roman numerals. The targeting vector consists of pGEM3Z plasmid, HSV-tk gene from pIC19R/MC1-TK, a 3.15-kb SalI-NcoI fragment spanning from 0.5 kb upstream of exon 1 to intron 1, *neo^r*, and a 1-kb fragment from exon 3 to exon 4. The probes used for hybridization were PCR products amplified with the primers of exon 4 and exon 5 and primers upstream of exon 1, and Southern blot using the former probe was shown in B. The bottom line indicates the predicted structure of the targeted locus. (B) Southern blot analysis of genomic DNA from the progeny of intercrosses of FcR γ heterozygous mutant mice. The genomic DNA from mouse tail was digested with BamHI and hybridized with the probe described above. The upper and lower bands correspond to the wild-type and targeted allele, respectively. (C) RT-PCR analysis of FcR γ transcript in FcR γ -/- mice. cDNAs prepared from spleen total RNA of wild-type (+/+) and FcR γ -deficient (-/-) mice were amplified by PCR with sets of primers from the sequences of exons 3 and 5. β -actin-specific primers were used as internal control.

born normally and were healthy, surviving as long as wild-type mice.

Lack of FcR expression and phagocytosis function in FcR γ -KO mice. As described by Takai et al. (1), FcR γ deficiency results in the loss of the surface expression of Fc γ RI, Fc γ RIII, and Fc ϵ RI, consistent with *in vitro* studies (27–29). We confirmed these results for our FcR γ ^{-/-} mice by analyzing NK cells first, as these cells lack Fc γ RII expression and exclusively express Fc γ RIII which can be stained with anti-Fc γ RIII/III mAb 2.4G2. Since the FcR expression on naive NK cells is relatively low and IL-2-stimulated NK cells express a higher level of FcR on the cell surface, we examined the expression of FcR on these NK cells. As shown in Fig. 2, we confirmed the lack of the Fc γ R expression on these activated NK cells from FcR γ ^{-/-} mice. Similarly, 2.4G2 staining of neutrophils and macrophages, which express Fc γ RI, Fc γ RII, and Fc γ RIII, showed decreases of the Fc γ R expression on the cell surface of these cells of FcR γ -KO mice although the changes on macrophages were very small (Fig. 2). The remaining staining of these cells, particularly macrophages, was due to Fc γ RII whose expression is independent of FcR γ .

The lack of functional FcR was confirmed by the failure of phagocytosis by macrophages from these FcR γ -deficient mice. FcR-mediated phagocytosis was assessed by the ability of macrophages to internalize SRBC opsonized with IgG1. While IgG2a-opsonized SRBC were internalized by high-affinity Fc γ RI, IgG1 and 2b-opsonized SRBC were phagocytosed by low-affinity Fc γ RIII. As shown in Fig. 3, macrophages from wild-type mice internalized the IgG-opsonized SRBC. In contrast, macrophages from FcR γ -deficient mice failed to exhibit any phagocytosis of IgG-opsonized red cells. The observation that these macrophages from FcR γ ^{-/-} mice bind opsonized SRBC equally to those from wild-type mice due to the remaining Fc γ RII (data not shown) indicates that Fc γ RI/III is responsible for the internalization of opsonized SRBC, and FcR γ -deficient mice lost the ability of phagocytosis due to the lack of cell surface expression of Fc γ RI/III.

Fatal nephrotoxic serum nephritis was induced in wild-type C57BL/6 mice. Preimmunization of normal rabbit IgG after intravenous injection of anti-GBM Ab induced severe glomerular lesions in wild-type C57BL/6 mice. Female mice showed

lesions of greater severity than male mice. Fig. 4 A shows a representative renal histology of a female mouse 5 d after the administration of anti-GBM Ab, which demonstrates hypercellular changes of glomeruli and cellular crescent formation. At this stage, the main glomerular alteration was hypercellularity, and mild to moderate cellular crescent formation was observed in ~20% of glomeruli (Fig. 4 A). The hypercellularity was probably due to infiltration of monocytes and PMNs, and segmental proliferation of mesangial cells. About 10 d after the intravenous injection of antiserum, the condition of the mice became poor, showing emaciation, ascites, muscle weakness, and little mobility. All of these mice died within 24 h after showing such signs. The typical renal histology at this stage is proliferation of mesangial cells and variable thrombotic lesions of glomeruli. Fig. 4, B and C, shows severe thrombotic glomerular changes characterized by deposition of eosinophilic, amorphous, and fibrillar materials in glomerular tufts and dilatation of tubular lumina, and endothelial cells probably disappeared.

As shown in Fig. 5 A, electron microscopy of renal tissue of a female mouse 5 d after the anti-GBM Ab injection revealed that the hypercellular changes were mainly caused by intraglomerular accumulation of monocytes and PMNs. Peripheral interposition of mesangial cells and broad disappearance of epithelial foot processes were also observed. However, no epimembranous deposits, such as those observed in the IC-type GN, could be detected. The renal histological changes had further progressed 10 d after the anti-GBM Ab injection (Fig. 5 B). Glomerular endothelial cells had disappeared, and lumina were occluded by electron dense materials. These thrombotic materials were partially positive by fibrin staining method (data not shown). Epimembranous deposits could not be demonstrated at this stage.

Wild-type mice, particularly female mice, started to die as early as 7 d after injection of anti-GBM Ab, and all mice were dead within 12 d (Fig. 6). Since dramatic death from GN occurred in C57BL/6 mice but not in some other strains such as ddY mice in which GN was transient and recovered later (data not shown), the susceptibility to the lethality appears to be linked to their genetic background. It is noteworthy that female mice were more susceptible to this type of GN, demon-

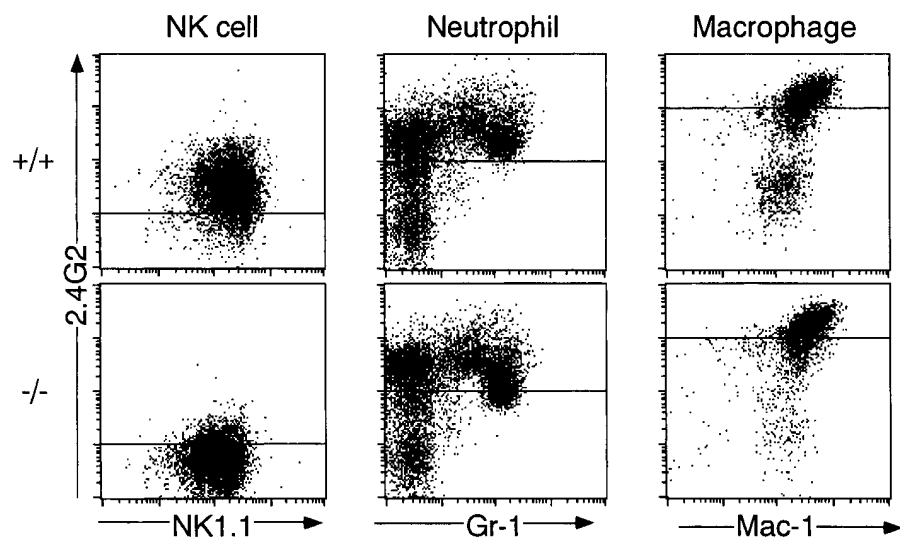


Figure 2. The expression of Fc γ R on the cell surface of NK cells, neutrophils, and macrophages from wild-type (+/+) and FcR γ -deficient (-/-) mice. Each population was prepared as described in Methods. These cells were stained with FITC-2.4G2 and PE-NK1.1 mAbs for NK cells, FITC-2.4G2 and PE-Mac-1 mAbs for macrophages, FITC-2.4G2 and PE-Gr-1 mAbs for neutrophils, and analyzed by FACScan[®].

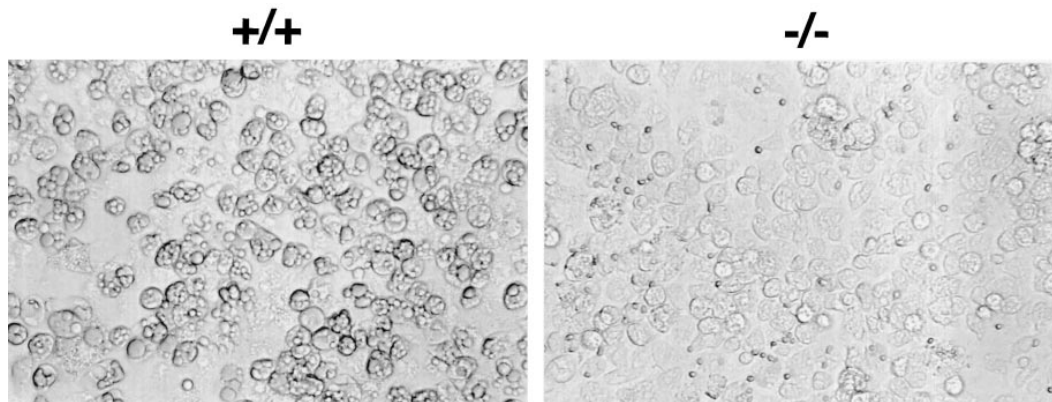


Figure 3. Inability of Fc γ RIII-mediated phagocytosis by macrophages from FcR γ -deficient mice. Surface-biotinylated SRBC were opsonized with antibiotin IgG and added onto the layer of macrophages which were isolated as the adherent cells from the peritoneal exudates of wild-type (+/+) and FcR γ -KO (-/-) mice. After 90 min of incubation at 37°C, non-phagocytosed SRBC were lysed hypotonically with water and micrographs were taken of the remaining cells ($\times 400$).

strated by the fact that with a half dose of anti-GBM Ab, only a few male mice died within 14 d, whereas all females died (data not shown).

These results showed that fatal GN was induced in wild-

type C57BL/6 by preimmunization with rabbit Ig and subsequent intravenous administration of rabbit anti-GBM Ab.

FcR γ -deficient mice did not develop NTGN. To examine the contribution of FcR to the initiation of GN, we compared the

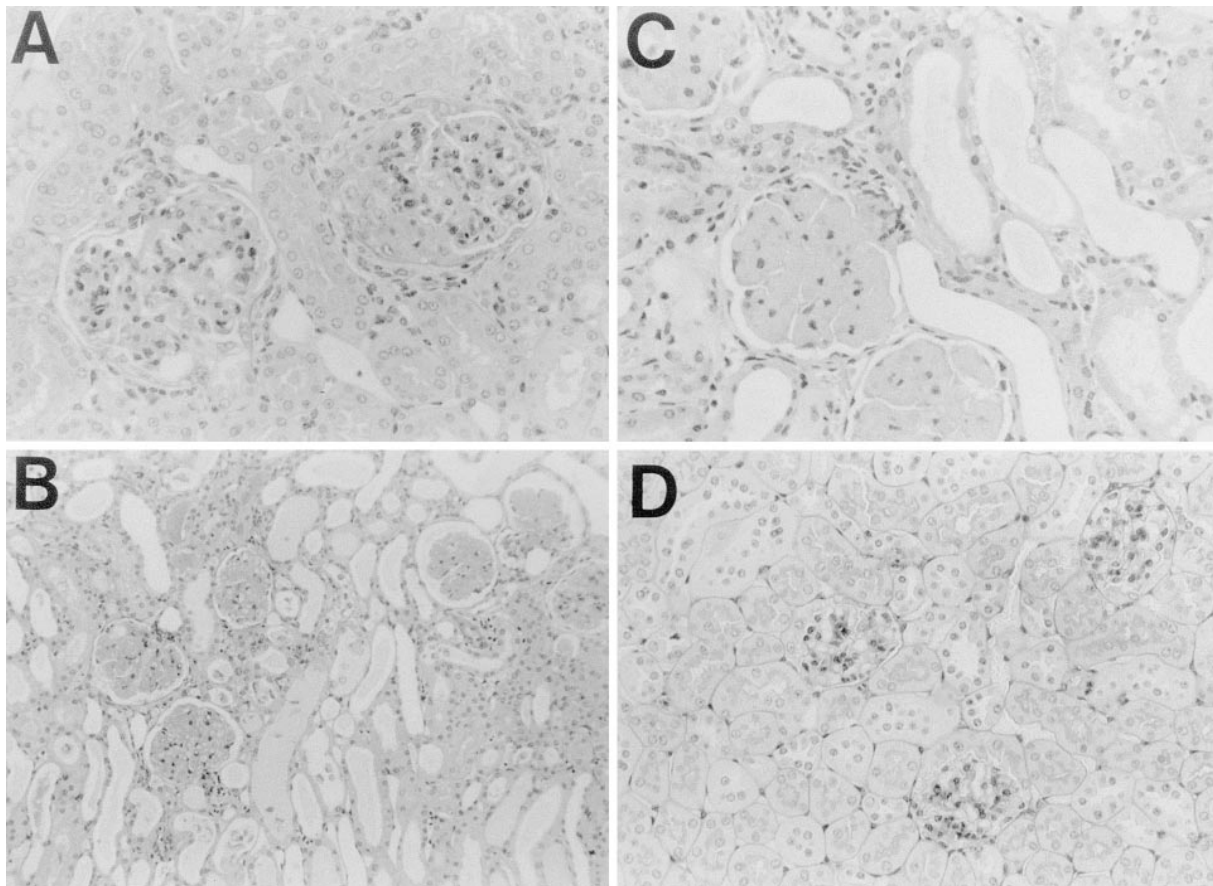


Figure 4. Renal histology of representative wild-type C57BL/6 mice 5 d (A) and 10 d (B and C) and FcR γ -deficient mice 14 d (D) after injection of anti-GBM Ab. Mice were immunized intraperitoneally with 250 μ g rabbit IgG 4 d before the injection of anti-GBM Ab, and killed to examine glomerular changes at early stage (A) and at late stage just before mice died (B–D). Renal sections were subjected to PAS staining. B and C showed the same histology with different magnifications. A, C, and D, $\times 400$; B, $\times 100$.

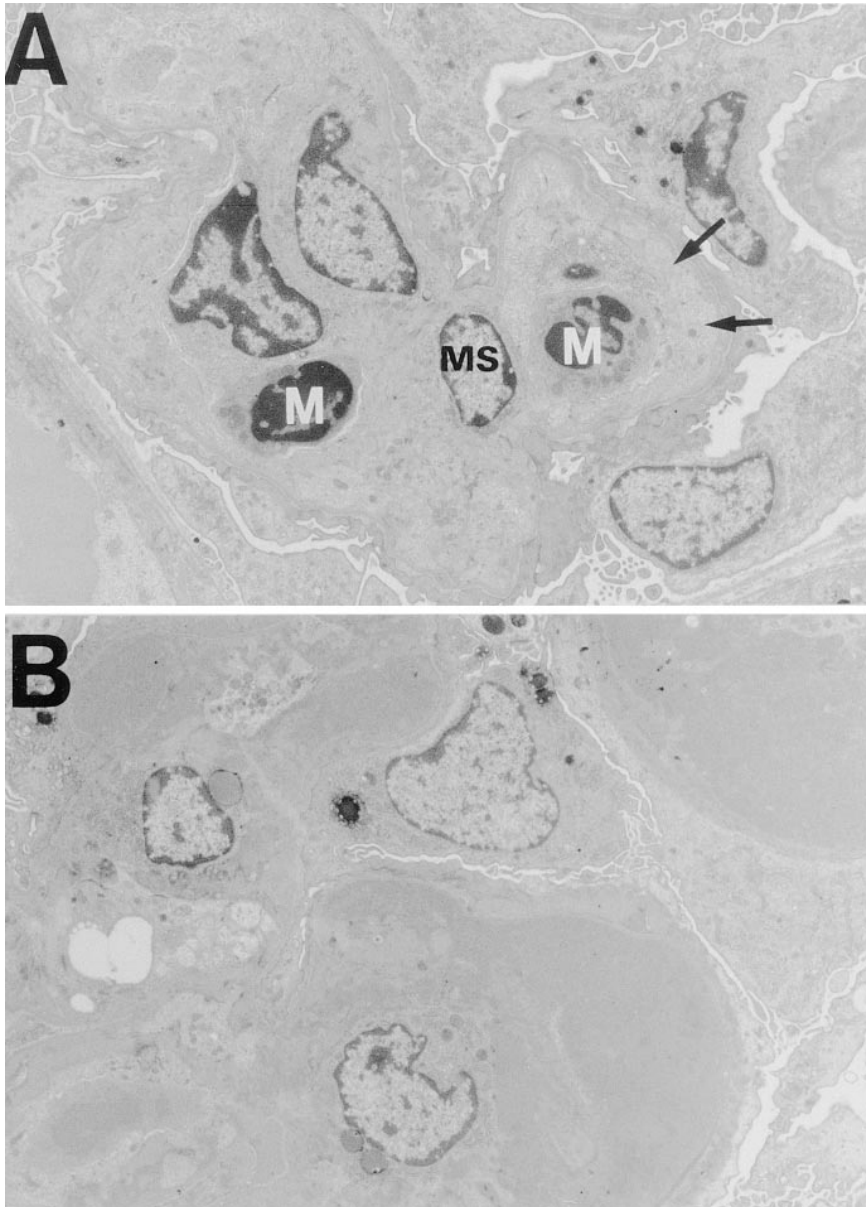


Figure 5. Electron microscopic analysis of glomeruli of wild-type C57BL/6 mice 5 d (A) and 10 d (B) after injection of anti-GBM Ab. Mice were treated as described in the legend to Fig. 3. (A) Accumulation of monocytes (M) and intracapillary obstruction by these cells are seen. Peripheral interposition of mesangial cells (arrows) and broad fusion of epithelial foot processes are also seen. MS, Mesangial cells ($\times 5,400$). (B) Glomerular epithelial cells have disappeared, and lumina are occluded by electron dense materials ($\times 4,800$).

susceptibility of FcR $\gamma^{-/-}$ mice to NTGN with that of wild-type mice. The condition of all wild-type mice became poor, showing emaciation, ascites, muscle weakness, and moribundity from 7 to 12 d after the anti-GBM Ab administration as described above. By contrast, all FcR $\gamma^{-/-}$ mice survived and were quite healthy (Fig. 6).

The kidney function of these mice was examined by hematological analysis of sera. As shown in Table I, whereas wild-type mice showed marked increases in the serum levels of BUN and creatinine, indicating development of renal failure, in FcR $\gamma^{-/-}$ mice these levels stayed within normal range. Renal histological examination of wild-type mice revealed that all mice developed severe GN with tubular changes (HS = 4.00 ± 1.09), comprising glomerular hypercellularity and variable thrombotic obliteration of glomerular capillary lumina. The histological changes of female mice (HS = 4.70 ± 0.64) were worse than those of male mice (HS = 3.50 ± 1.12). In contrast, as shown in Fig. 4 D, the renal morphology of FcR $\gamma^{-/-}$

mice was almost normal (HS = 0.45 ± 0.59), and no indication of inflammatory response or tissue damage except weak glomerular hypercellularity was observed. When again analyzed 2–3 wk later, the renal morphology had not changed (data not shown). These results demonstrated that NTGN was not triggered and did not develop in the absence of FcR.

To eliminate the possibility that FcR γ -deficient mice have defective immune responses against rabbit Ig, we examined DTH and the Ab response against rabbit Ig as cellular and humoral immunity, respectively. There were no significant differences between wild-type and FcR γ -deficient mice for both DTH response as measured by footpad swelling and the serum titer of anti-rabbit Ig Ab after immunization with rabbit Ig (data not shown).

Since proteinuria is induced even without renal tissue damage, it was also checked periodically after anti-GBM Ab administration (Table II). While wild-type mice soon developed severe proteinuria, FcR $\gamma^{-/-}$ mice showed an initial delay, in-

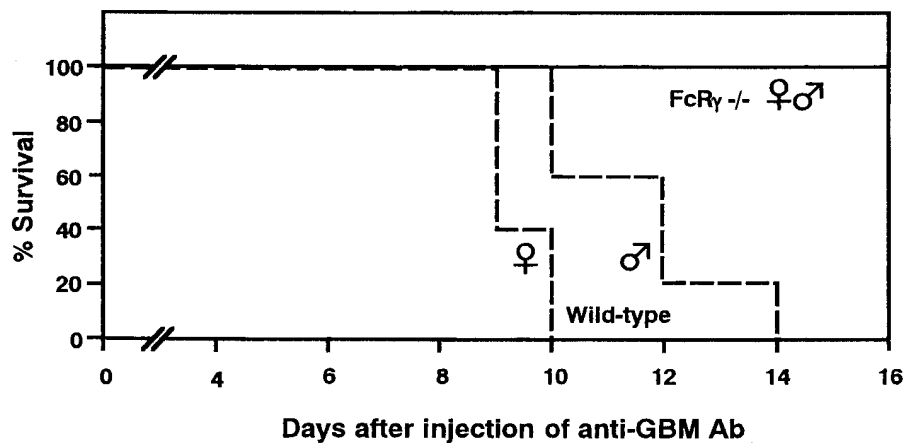


Figure 6. Survival curves of wild-type (broken line) and FcR γ -deficient (solid line) mice after anti-GBM injection. 75 μ l of anti-GBM Ab was administered intravenously and the survival rate was measured. All FcR γ -/- mice survived even after 16 d and none died. Each group consisted of 8–10 mice.

creasing later to finally reach similar levels as the wild-type mice. Wild-type mice exhibited anuria and eventually died, whereas the KO mice recovered from proteinuria. These results demonstrated that acute proteinuria is induced in both wild-type and FcR γ -KO mice but that the development of GN with tissue damage is induced only in wild-type mice.

Deposition of anti-GBM Ab and complement to GBM is not sufficient for initiation of GN. By our protocol with preimmunization of rabbit Ig, mice may already have raised anti-rabbit Ig Ab when anti-GBM Ab was administered, and may form ICs with anti-GBM Ab in blood, which might cause the failure to react directly on GBM. To exclude this possibility, we analyzed the presence of anti-GBM Ab in the renal area 12 h after the injection of anti-GBM Ab (Fig. 7, A and E). GBMs in both wild-type and FcR γ -/- mice were indistinguishably stained with FITC anti-rabbit Ig Ab, demonstrating that the failure of GN development in FcR γ -/- mice was not due to prevention of specific binding of anti-GBM Ab to GBM by IC formation at the initial stage. In addition, we found that GBM was already stained weakly with FITC anti-mouse Ig Ab which detects mouse anti-rabbit Ig Ab (data not shown), indicating that autologous Ab started to react with rabbit anti-

GBM Ab as early as at 12 h. GBM was stained in linear, not granular fashion, demonstrating that anti-GBM Ab reacted along GBM but was not deposited as IC. It is noteworthy that a couple of degranulated PMNs per one slice of glomerulus were observed in wild-type mice but not at all in FcR γ -/- mice in the renal tissues of this stage (data not shown).

To further investigate whether the failure of GN development in FcR γ -/- mice was due to insufficient binding of anti-GBM Ab on GBM at the later stage when wild-type mice developed GN, we performed immunofluorescence staining with FITC anti-rabbit Ig Ab for anti-GBM Ab (Fig. 7, B and F) and with FITC anti-mouse Ig Ab (Fig. 7, C and G). GBMs in both wild-type and FcR γ -/- mice were specifically stained with both Abs in a linear fashion, demonstrating that anti-GBM Ab and mouse anti-rabbit Ig Ab bound to GBM in wild-type and FcR γ -/- mice. This indicated that the failure of GN development in FcR γ -/- mice was not due to insufficient binding of anti-GBM Ab or secondary anti-mouse Ig Ab.

We then investigated whether there was any difference in complement binding in the renal lesion of normal and FcR γ -/- mice after injection of anti-GBM Ab in order to determine the contribution of complements in the induction of this disease. For this purpose, we stained renal sections with anti-C3 Ab. Specific binding of C3 was observed in GBM in a linear fashion in both wild-type and FcR γ -/- mice (Fig. 7, D and H). In addition to the deposition of C3 on the renal tissue, we measured the serum level of C3 by ELISA as the representative of the serum complement titer since functional titration of mouse complement by cytotoxicity is extremely difficult. The relative amounts of C3 in wild-type and FcR γ -KO mice ($n = 5$) were

Table I. Blood Chemistry of Mice Injected with Anti-GBM Ab

Mouse	n	Anti-GBM Ab injection	Creatinine	BUN	Total protein
			mg/dl	mg/dl	g/dl
FcR γ +/+					
Female	10	+	0.529 \pm 0.26	259.0 \pm 127	3.15 \pm 0.76
Male	10	+	0.359 \pm 0.20	63.6 \pm 16.7	3.16 \pm 0.63
Female	10	-	0.143 \pm 0.03	23.4 \pm 4.30	4.39 \pm 0.26
Male	10	-	0.138 \pm 0.04	24.8 \pm 3.13	4.57 \pm 0.22
FcR γ -/-					
Female	9	+	0.150 \pm 0.06	24.8 \pm 14.9	5.10 \pm 0.87
Male	10	+	0.153 \pm 0.03	25.6 \pm 2.12	4.85 \pm 0.33
Female and male	7	-	0.138 \pm 0.05	21.0 \pm 4.70	5.13 \pm 0.72

Serum was collected from female FcR γ +/+ mice just before death, and 8 d after anti-GBM injection from other mice. Three sets of identical experiments were performed. Each value was measured as described in Methods and expressed as mean \pm SD. n indicates the number of mice tested.

Table II. Proteinuria Induced by Anti-GBM Ab Administration

Mouse	Days after anti-GBM Ab treatment			
	0	4	8	12
FcR γ +/+	3.7 \pm 0.7	20.5 \pm 27.8	56.6 \pm 52.1	77.2 \pm 67.9
FcR γ -/-	3.1 \pm 0.8	8.9 \pm 10.4	71.3 \pm 25.9	77.8 \pm 30.1

Urine was collected from individual mice ($n = 5$, male) at the indicated times after anti-GBM Ab administration (50 μ l in this experiment). The numbers indicate mean \pm SD of protein level (mg/d).

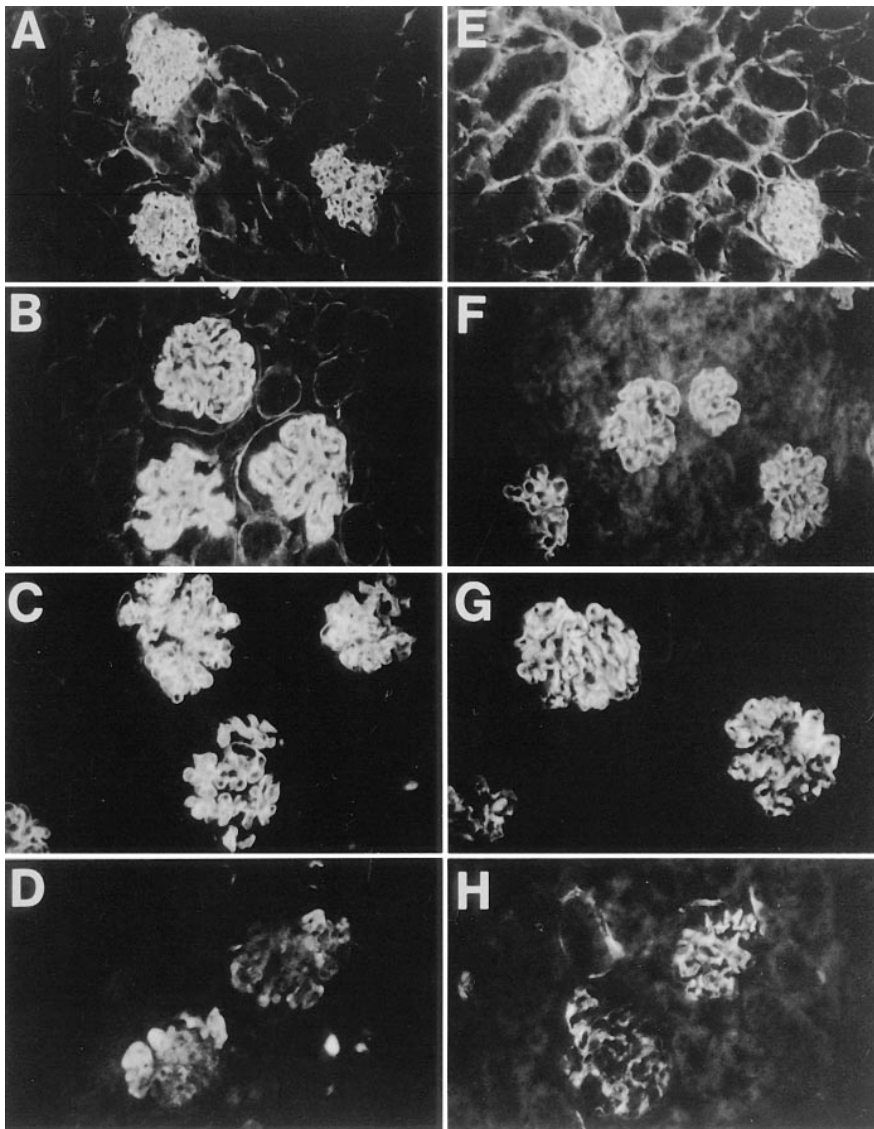


Figure 7. Immunofluorescence staining of GBM of wild-type (A–D) and FcR γ -deficient (E–H) mice after administration of anti-GBM Ab ($\times 400$). (A and E) Staining of the kidney at the initial phase of anti-GBM nephritis. Mice were injected with anti-GBM Ab and killed 12 h later. The kidney was stained with FITC anti-rabbit Ig Ab. (B and F) Staining of heterologous Ab in GBM at the time of induced GN. Mice were killed 8 d after anti-GBM Ab injection and the renal section was stained with FITC anti-rabbit Ig Ab. (C and G) Staining of autologous Ab in GBM at the time of induced GN (8 d after anti-GBM Ab injection). The renal section was stained with FITC anti-mouse Ig Ab. (D and H) Deposition of C3 on GBM from both wild-type and FcR γ ^{-/-} mice. The same renal sections as B and F and C and G were stained with FITC anti-C3 Ab.

47.2 \pm 10.6 and 34.0 \pm 15.6, respectively, indicating that the complement titer was not significantly different between wild-type and FcR γ -KO mice. The weak staining of C3 in wild-type mice was probably due to the severe damage of glomeruli. These results clearly demonstrated that the deposition of IC and complements per se did not trigger any significant inflammatory response in evoking GN.

Discussion

NTGN induced by anti-GBM Ab represents an experimental model for human Goodpasture's syndrome (14). Patients suffering from this type of GN show rapidly progressive renal lesions and severe hemorrhagic pulmonary involvement. Although NTGN has been analyzed extensively and the target Ag was found to be the NC1 domain of type IV collagen (30), the molecular basis of this disease has been largely undetermined. The recent progress in gene targeting has provided new approaches for dissecting the complicated mechanisms of the induction of diseases. In this study, we established FcR γ -defi-

cient mice in order to analyze the function of FcR in NTGN. Since FcR γ ^{-/-} mice lack the cell surface expression of Fc γ RI, Fc γ RIII, and Fc ϵ RI on various cells, we used these mice as FcR-deficient mice to investigate the function of FcR in the induction of nephritis. To this end, we provide definitive evidence that the induction of NTGN by anti-GBM Ab is completely dependent on FcR but is independent of complements.

In the classical model of anti-GBM nephritis (11–13, 15, 31), the intravenous injection of heterologous anti-GBM Ab induces glomerular inflammation, which is initiated by binding of anti-GBM Ab to the GBM and followed by activation of complements with accumulation of PMNs and monocytes (32). Deposition of fibrin, necrosis of glomerular capillaries, and crescent formation may be seen in severe cases. The thrombotic change may become accelerated, resulting in diffuse obliteration of glomerular tufts, which has been categorized as a specific type of NTGN (11, 15). This early reaction of the so-called heterologous phase is accompanied by proteinuria. After 5–7 d, the second phase develops as a consequence of the binding of autologous Ab to the heterologous Igs already deposited along the GBM. When rabbit Ig is immunized before

anti-GBM Ab injection, the autologous phase develops much faster and stronger (16). In our experimental model, severe NTGN could be thus induced in C57BL/6 mice. Although a mouse model with anti-GBM disease has been established recently even by immunizing with a single collagen Ag (20), this study is the first case of fatal NTGN in the mouse. It is of particular interest that the lethality from this nephritis is influenced by the genetic background of the mice (20), because other mice such as ddY recovered easily from this acute disease. It was surprising to find that all FcR γ ^{-/-} mice remained healthy and survived even after the application of an accelerated type of lethal nephritis. In wild-type C57BL/6 mice, male mice were more resistant to lethal nephritis and females died earlier. Histological analysis as well as blood chemistry confirmed the correlation between the lethality and severity of nephritis; all female mice eventually developed severe thrombotic changes, obliterating glomerular tufts. Therefore, we concluded that the wild-type mice died from GN induced by anti-GBM Ab.

Binding of anti-GBM Ab to GBM was observed equally in wild-type mice and FcR γ ^{-/-} mice from the initial phase (several hours) to the subsequent nephritic stage (several days). Autologous Ab (mouse anti-rabbit Igs) bound to GBM was equally confirmed in both groups during the course of the experiments. Furthermore, we showed that not only anti-GBM Ab but also complements (C3) bound to GBM in a linear fashion equally in wild-type and FcR γ ^{-/-} mice. The fact that FcR γ ^{-/-} mice survived and did not develop any significant signs of nephritis, in spite of the equivalent binding of heterologous and autologous Abs as well as complements to wild-type mice, clearly indicates that the glomerular deposition of these Abs and complements was not sufficient to trigger GN. In contrast, proteinuria was induced in both wild-type and KO mice, suggesting that the deposition of IC induced transient proteinuria independently of further induction of GN as suggested previously (33). Collectively, these results indicate that FcR⁺ cells are required to initiate inflammatory processes and tissue damage. The complement-independent and PMN-dependent induction of nephritis has been demonstrated by using technically limited systems in which complements were depleted by cobra venom factor treatment (34) and PMNs were depleted by irradiation (28, 34), or by the use of beige mice (35). Our results are consistent with these earlier reports and provide more unequivocal evidence for the use of FcR-deficient mice. Recent studies suggest that anti-GBM GN is induced in T cell-dependent and Ab-independent fashion (17–20). Our present data demonstrate that IC and FcR are required for GN induction, and hence T cell-mediated response may not necessarily be required for the induction, but rather may play a role in the later phase.

By using similar FcR-deficient mice, Sylvestre et al. (4) demonstrated recently that Arthus reaction induced by specific Ag-Ab complex was complement-independent but FcR-dependent. The same authors also showed, by transferring mast cells from FcR^{-/-} mice into mast cell-deficient *w/w* mutant mice, that mast cells are primarily responsible for inducing Arthus reaction (34). Provided that the FcR-dependent mechanism for the induction of nephritis would be similar to that for the induction of Arthus reaction, FcR⁺ cells inducing anti-GBM nephritis might also be mast cells. However, there have been several reports with indirect evidence to demonstrate that PMNs were crucial for inducing anti-GBM nephritis (28,

32, 35). Together with our finding that PMNs appeared in glomeruli at the early stage of our model, the FcR⁺ cells used to trigger GN may be PMNs. Currently, this question is being addressed by experiments using our system, in which various FcR⁺ cells will be transferred into anti-GBM Ab-injected FcR γ ^{-/-} mice.

There are several different types of GN including NTGN and Heymann nephritis, as well as IC-induced nephritis including serum sickness and SLE. The former has now been found to be completely FcR-dependent and complement-independent in this study. A recent study by Clynes et al. demonstrated that spontaneously occurring GN in NZB/NZW mice was also diminished in FcR-deficient mice (36). It still needs to be determined whether FcR-dependent induction can also be extended to other types of GN.

Both Arthus reaction and NTGN were induced in an FcR-dependent fashion, but the FcR⁺ cells inducing the diseases might be different. It is known that PMNs induce reactive oxygen metabolites (ROMs) upon stimulation through Fc γ receptors (36, 37). In addition, ROMs have been identified as mediators in the pathogenesis of GN and have been shown to play a role in the cellular injury (36–39). Therefore, it may be speculated that soluble Ag-Ab complex can trigger mast cells to secrete various mediators for the induction of Arthus reaction, whereas auto-Ab-like anti-GBM Ab activates PMNs to produce ROMs to initiate inflammatory diseases.

It becomes more likely that this mechanism observed in NTGN, the FcR-dependent induction of diseases, can be widely applied to many other auto-Ab-dependent inflammatory and autoimmune diseases.

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