

Fc γ Receptor Deficiency Attenuates Diabetic Nephropathy

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ABSTRACT

Among patients with diabetes, increased production of immunoglobulins against proteins modified by diabetes is associated with proteinuria and cardiovascular risk, suggesting that immune mechanisms may contribute to the development of diabetes complications, such as nephropathy. We investigated the contribution of IgG Fc γ receptors to diabetic renal injury in hyperglycemic, hypercholesterolemic mice. We used streptozotocin to induce diabetes in apolipoprotein E-deficient mice and in mice deficient in both apolipoprotein E and γ -chain, the common subunit of activating Fc γ receptors. After 15 weeks, the mice lacking Fc γ receptors had significantly less albuminuria and renal hypertrophy, despite similar degrees of hyperglycemia and hypercholesterolemia, immunoglobulin production, and glomerular immune deposits. Moreover, diabetic Fc γ receptor-deficient mice had less mesangial matrix expansion, inflammatory cell infiltration, and collagen and α -smooth muscle actin content in their kidneys. Accordingly, expression of genes involved in leukocyte infiltration, fibrosis, and oxidative stress was significantly reduced in diabetic kidneys and in mesangial cells cultured from Fc γ receptor-deficient mice. In summary, preventing the activation of Fc γ receptors alleviates renal hypertrophy, inflammation, and fibrosis in hypercholesterolemic mice with diabetes, suggesting that modulating Fc γ receptor signaling may be renoprotective in diabetic nephropathy.

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Complications affecting the macro- and microvasculature are the major causes of illness and death among diabetic patients.¹ Nephropathy, one of the microvascular complications of diabetes, is also an important macrovascular risk factor.² The diabetic kidney is exposed to a milieu of high glucose, oxidative stress, and advanced glycation end products, all of which contribute to nephropathy development by inducing glomerular cell activation, inflammatory infiltrate, and tubular epithelial-to-mesenchymal transition.^{3,4} Clinical observations and experimental animal studies have suggested that hyperlipidemia contributes to the progression of diabetic renal disease.^{1,5,6} Lipoprotein changes in diabetic nephropathy patients include LDL modification, mainly oxidation, glycation, and formation of advanced glycated end products.^{3,7} The

underlying pathologic mechanisms by which hyperlipidemia induces glomerulosclerosis include the glomerular infiltration of LDL and their oxidation by mesangial cells, thus favoring the release of mediators involved in leukocyte recruitment, mesangial proliferation, and matrix expansion.

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Accumulating evidence suggests that the immune system is involved in the pathophysiology of diabetes complications.³ Hypercholesterolemia itself triggers proinflammatory events through the activation of pathways associated with innate and adaptive immunity. Most forms of modified LDL are immunogenic and induce autoantibody production; these proinflammatory properties are greatly increased by the formation of LDL-containing immune complexes.⁸ This adaptive immune response to modified LDL has also been reported in mice.⁹ Patients with type 1 diabetes have elevated serum levels of antibodies, predominantly IgG against proteins modified by glycoxidation or lipoxidation, including oxidized LDL, malondialdehyde-modified LDL, and advanced glycation end products—modified LDL,^{10,11} even though these patient cohorts do not have the excessively increased hypercholesterolemia that is typical in patients with type 2 patients. Furthermore, LDL size, susceptibility to oxidation, and lipid fatty acid composition are critical in the formation of immune complexes in type 2 diabetic patients.⁸ Indeed, oxidized LDL-containing immune complexes represent a good marker to evaluate the effect of humoral immunity in diabetes complications.^{8,11,12}

At sites of injury, IgG immune complexes are recognized by infiltrating and resident cells through specific receptors for the Fc region (FcγRs).^{13,14} In mice, four different classes of FcγRs are described (FcγRI/CD64, FcγRIIb/CD32, FcγRIII/CD16, and FcγRIV), which differ by their distinct affinity, cellular distributions, and effector functions. FcγRs are expressed by leukocytes and tissue resident cells, including glomerular mesangial cells.^{15–17} Activating FcγRs (I, III, and IV) are associated with the immunoreceptor tyrosine-based activation motif—harboring common γ-chain, and upon ligand binding they stimulate phagocytosis, oxidative burst, and cytokine release, whereas inhibitory FcγRIIb nullifies cell activation.^{13,14} Altered FcγR expression has been found in patients and experimental models,^{18–21} and clinical studies have shown an association between different FcγR genotypes with diabetes and cardiovascular risk.^{22,23} In this study we examined the relevance of FcγRs to the pathogenesis of diabetic nephropathy in a model of type 1 diabetes accelerated by hypercholesterolemia. Our findings demonstrate that functional deficiency in activating FcγRs protects against glomerular and tubulointerstitial damage of diabetic nephropathy.

RESULTS

Evolution of the Diabetes Model

We compared the evolution of streptozotocin-induced diabetes in animals carrying a single genetic deficiency in apolipoprotein E (apoE mice²⁴) or the common γ-chain of activating FcγRs (γ mice^{25,26}) and a double deficiency in apoE and γ-chain (γapoE mice²¹). Wild-type (WT) mice were used as reference group. The blood glucose curves over the study period were very similar in all diabetic groups (Figure 1A), and the average blood glucose levels during the last 13

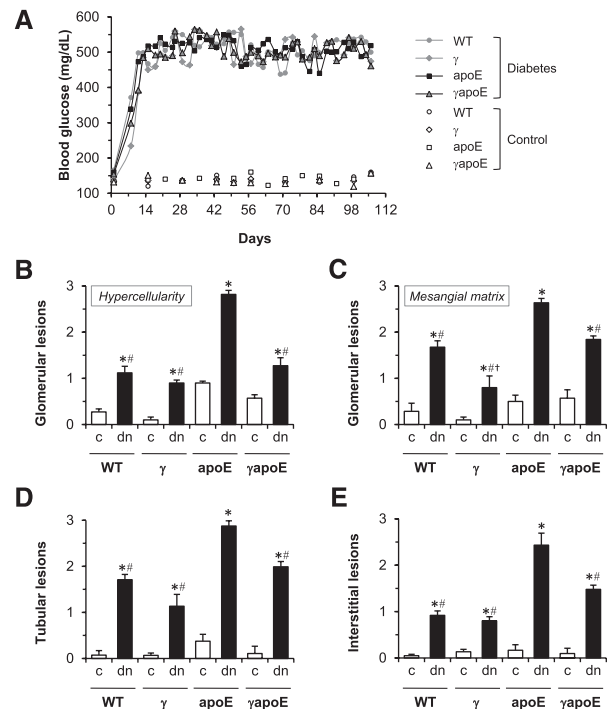


Figure 1. FcγR gene deficiency protects mice from diabetic renal injury. (A) Evolution of blood glucose in nondiabetic and diabetic mice from WT, γ, apoE, and γapoE groups. (B–E) The severity of morphologic renal damage was assessed using an arbitrary score (0–3) based on periodic acid-Schiff–stained kidney sections. Semiquantitative assessment of lesions in glomerular (B and C), tubular (D), and interstitial (E) compartments is shown. Bars represent the mean ± SEM of studied animals per group (controls: WT, *n*=7; γ, *n*=4; apoE, *n*=7; γapoE, *n*=7; diabetic: WT, *n*=12; γ, *n*=6; apoE, *n*=14; γapoE, *n*=13). c, control; dn, diabetic nephropathy. **P*<0.05 versus respective nondiabetic controls; #*P*<0.05 versus diabetic apoE mice; †*P*<0.05 versus diabetic WT mice.

weeks of study did not reach statistical significance (Table 1). Furthermore, similar levels of glycated hemoglobin A1c (Table 1) confirmed the equivalent hyperglycemia. Diabetes increased serum creatinine and urea levels and the urinary albumin-to-creatinine ratio (Table 1), indicating abnormal GFR in mice.²⁷ This effect was enhanced in apoE mice, which also exhibit severe hypercholesterolemia. Interestingly, diabetic γapoE mice, despite having apoE deficiency and high cholesterol, experienced an improvement in the biochemical measures compared with diabetic apoE mice; however, the levels remained elevated with respect to nondiabetic γapoE controls. Moreover, except for cholesterol levels, the clinical and biochemical characteristics of diabetic γapoE did not differ significantly from those in diabetic WT and single γ mice.

Functional Deficiency in Activating FcγRs Protects Mice from Diabetic Renal Injury

Histologic scoring of periodic acid-Schiff–stained kidney sections revealed that diabetes caused moderate to severe renal damage in apoE mice (Figure 1, B–E and Supplemental Figure 1).

Table 1. Functional and structural data from diabetic mice and nondiabetic controls

Mice	WT		γ		apoE		γ apoE	
	Control (n=7)	Diabetes (n=12)	Control (n=4)	Diabetes (n=6)	Control (n=7)	Diabetes (n=14)	Control (n=7)	Diabetes (n=13)
Blood glucose (mg/dl)	137 \pm 6	512 \pm 12 ^a	139 \pm 2	507 \pm 9 ^a	141 \pm 5	511 \pm 6 ^a	136 \pm 4	508 \pm 8 ^a
Hemoglobin A1c (μ g/dl)	ND	13.9 \pm 0.7	ND	ND	ND	14.1 \pm 0.9	ND	13.8 \pm 0.9
Change in body weight (g)	4.3 \pm 0.5	-1.7 \pm 0.7 ^b	-4.8 \pm 0.6	-0.5 \pm 0.4 ^b	9.0 \pm 1.6 ^c	-4.6 \pm 0.8	4.6 \pm 0.4	-2.2 \pm 0.7 ^b
Kidney-to-body weight ratio (mg/g)	14.0 \pm 0.3	16.4 \pm 0.6 ^{a,b}	13.8 \pm 0.1	16.1 \pm 0.8 ^{a,b}	14.4 \pm 0.7	19.8 \pm 0.7 ^a	14.3 \pm 0.2	16.1 \pm 0.7 ^{a,b}
Cholesterol (mg/dl)	65 \pm 12	73 \pm 8	74 \pm 8	92 \pm 4	259 \pm 19 ^c	762 \pm 76 ^a	250 \pm 22 ^c	729 \pm 64 ^{a,d}
Serum creatinine (mg/dl)	0.22 \pm 0.02	0.38 \pm 0.03 ^{a,b}	0.22 \pm 0.02	0.33 \pm 0.02 ^{a,b}	0.31 \pm 0.03	0.59 \pm 0.05 ^a	0.27 \pm 0.04	0.41 \pm 0.03 ^{a,b}
BUN (mg/dl)	27.1 \pm 1.1	37.3 \pm 2.7 ^{a,b}	ND	ND	23.4 \pm 2.6	52.2 \pm 3.7 ^a	27.6 \pm 1.5	35.2 \pm 2.3 ^{a,b}
Urinary albumin-to-creatinine ratio (μ g/ μ mol)	15.4 \pm 5.4	32.5 \pm 6.7 ^{a,b}	11.51 \pm 2.30	30.63 \pm 3.83 ^a	26.2 \pm 2.0 ^c	81.3 \pm 14.5 ^a	23.4 \pm 1.3 ^c	36.6 \pm 6.1 ^{a,b}
Glomerular volume (μ m ³ \times 10 ⁵)	1.39 \pm 0.11	1.83 \pm 0.08 ^{a,b}	1.36 \pm 0.13	1.70 \pm 0.16 ^b	1.98 \pm 0.08	3.36 \pm 0.23 ^a	1.85 \pm 0.11	2.23 \pm 0.14 ^b

Data are expressed as the mean \pm SD. ND, not determined.

^a P <0.05 versus respective nondiabetic controls.

^b P <0.05 versus diabetic apoE mice.

^c P <0.05 versus nondiabetic WT mice.

^d P <0.05 versus diabetic WT mice.

By contrast, γ apoE mice were partially protected from the development of diabetic renal injury and exhibited a significant decrease in renal histologic changes, including glomerular

hypercellularity (mesangial proliferation and infiltrating cells; Figure 1B) and mesangial matrix expansion (Figure 1C); tubular atrophy, dilation, and epithelial glycogen deposits (Figure 1D); and interstitial fibrosis and infiltrate (Figure 1E). Accordingly, diabetic γ apoE mice had significantly less fractional kidney weight and glomerular volume (measures of kidney hypertrophy) than did diabetic apoE mice (Table 1). Furthermore, comparisons of WT and γ mice showed that single Fc γ R deficiency decreased measures of diabetic renal injury, but this trend did not reach statistical significance except in mesangial matrix expansion (Figure 1, B–E and Table 1).

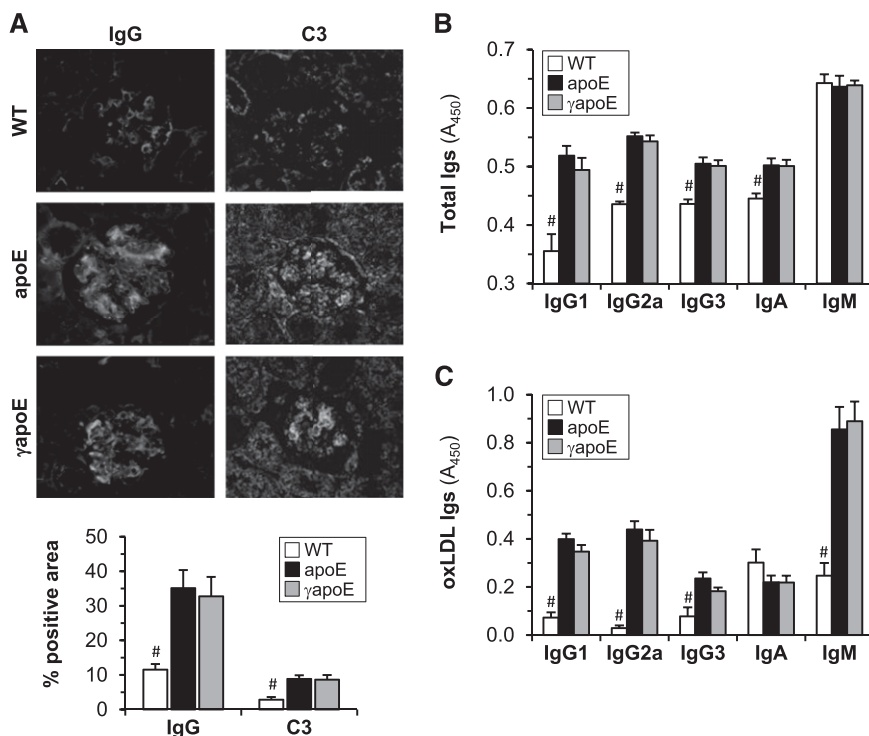


Figure 2. Immune findings in diabetic mice. (A) Representative micrographs of IgG and C3 immunodetection in diabetic mice (WT, apoE, and γ apoE groups) and quantification (lower panel). Original magnification \times 400. (B, C) ELISA assay for total (B) and oxidized LDL (oxLDL)-specific (C) immunoglobulins in serum samples from diabetic mice. Bars represent the mean \pm SEM of studied animals per group (WT, n =12; apoE, n =4; γ apoE, n =13). # P <0.05 versus diabetic apoE mice.

Immune Findings in Diabetic Mice

Immunofluorescence analysis identified IgG and C3 deposits in the glomeruli of diabetic apoE and γ apoE mice (Figure 2A), without significant differences in the quantity and location. Diabetes also induced the production of total immunoglobulins, an effect markedly evident in hypercholesterolemic mice (Figure 2B). Determination of the specific oxidized LDL isotypes revealed the predominance of IgG1, IgG2a, IgG3, and IgM in sera from diabetic apoE and γ apoE mice compared with WT mice (Figure 2C). However, relative ratios in the apoE and γ apoE groups (IgM/IgG: 0.85 \pm 0.01 versus 0.89 \pm 0.03; IgG2/IgG1: 1.10 \pm 0.09 versus 1.13 \pm 0.03) did not reach statistical significance, thus indicating similar IgM to IgG conversion and no apparent Th1/Th2 bias, respectively.

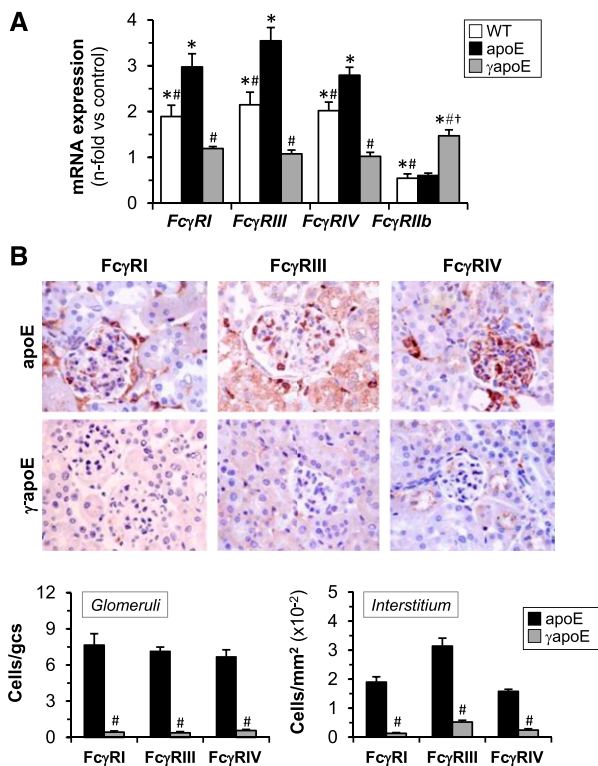


Figure 3. Renal expression of FcγRs in diabetic mice. (A) Real-time PCR analysis of activating FcγRs (types I, III, and IV) and inhibitory FcγRIIb in renal samples. Results are normalized by 18S expression and expressed as fold increases versus control. (B) Representative micrographs (original magnification, ×400) of activating FcγR immunodetection in diabetic mice (apoE and γapoE groups) with quantification of positive cells in glomeruli and interstitium (lower panel). Bars represent the mean ± SEM of $n=12$ –14 animals per group. gcs, glomerular cross-section. * $P<0.05$ versus respective nondiabetic controls; # $P<0.05$ versus diabetic apoE mice; † $P<0.05$ versus diabetic WT mice.

Because relative FcγR expression modulates the amplitude of the inflammatory response, we examined the expression of the FcγR family members in diabetic kidneys. Real-time PCR analysis revealed increased mRNA levels of activating (I, III, and IV) but not inhibitory (IIb) receptors in diabetic apoE mice, thus resulting in a net activating profile (Figure 3A). Immunostaining confirmed the localization of activating FcγR isoforms in apoE kidneys, mainly in infiltrating leukocytes and also in glomerular mesangial cells (Figure 3B). By contrast, γapoE mice showed unaffected FcγR gene expression by diabetes, except the inhibitory FcγRIIb (Figure 3A). Quantification of positive cells in glomeruli and interstitium revealed significant differences between apoE and γapoE groups (Figure 3B).

Lack of Activating FcγRs Attenuates Inflammation in Diabetic Kidneys

Diabetes was associated with an increase in renal infiltration of macrophages (CD68) and T lymphocytes (CD3), broadly

distributed in glomeruli and interstitium of diabetic apoE mice (Figure 4A). FcγR deficiency significantly reduced the leukocyte accumulation in both compartments (Figure 4A and Supplemental Figure 2). Furthermore, the gene and protein expressions of mediators regulating leukocyte infiltration, such as intercellular adhesion molecule-1, chemokines (CCL5 and CCL2), and chemokine receptor (CCR2), were ameliorated in diabetic γapoE compared with apoE mice (Figure 4, B–D).

Activating FcγRs Mediate Renal Fibrosis in Diabetic Mice

Analysis of collagen distribution by picrosirius red staining indicated that FcγR deficiency attenuated the intraglomerular and tubulointerstitial fibrosis associated with diabetes (Figure 5A and Supplemental Figure 3). Moreover, immunostaining for α-smooth muscle actin (α-SMA, a marker of myofibroblast-type cells) was increased in diabetic kidneys, mainly in apoE mice. Cells expressing α-SMA were detected primarily in the renal interstitium and to a lesser extent in glomeruli, and were significantly reduced in γapoE mice (Figure 5B and Supplemental Figure 3). Accordingly, the renal expression of extracellular matrix proteins (collagen I and fibronectin) and profibrotic factors (TGF-β and connective tissue growth factor [CTGF]) was significantly attenuated in diabetic γapoE mice (Figure 5, C and D). In addition, a significant improvement of tubulointerstitial damage was indicated by reduced gene expression of kidney injury molecule-1²⁸ in diabetic γapoE compared with apoE mice (Figure 5C).

In Vitro Effects of Activating FcγR Deficiency

To gain insight into the molecular mechanisms of immune responses in the diabetic kidney, the direct effect of FcγR activation in renal cells was further analyzed in primary mesangial cells from apoE and γapoE mice. Incubation of apoE cells with soluble IgG aggregates (as a model for immune complexes) increased the expression of inflammatory genes (intercellular adhesion molecule-1, CCL5, CCL2, and CCR2), extracellular matrix proteins (collagen I and fibronectin), and profibrotic factors (TGF-β and CTGF), as determined by real-time PCR (Figure 6A) and Western blot (Figure 6C). We also tested the capacity of potential causative immune complexes prepared by mixing oxidized LDL with mouse anti-oxidized LDL (IgG1 and IgG2 subclasses). Similar to soluble IgG aggregates, oxidized LDL-immune complexes markedly stimulated inflammatory gene expression in a concentration- and time-dependent manner in apoE cells (Figure 6, B and D). In contrast, γapoE mesangial cells failed to respond to both soluble IgG aggregates and oxidized LDL-immune complexes and showed reduced expression of inflammatory and fibrotic genes (Figure 6, A–D).

Effects of FcγR Deficiency on Oxidative Stress

Intracellular superoxide generation was measured with the sensitive dye dihydroethidium. As shown in Figure 7A, the intense red fluorescence found in kidneys from diabetic

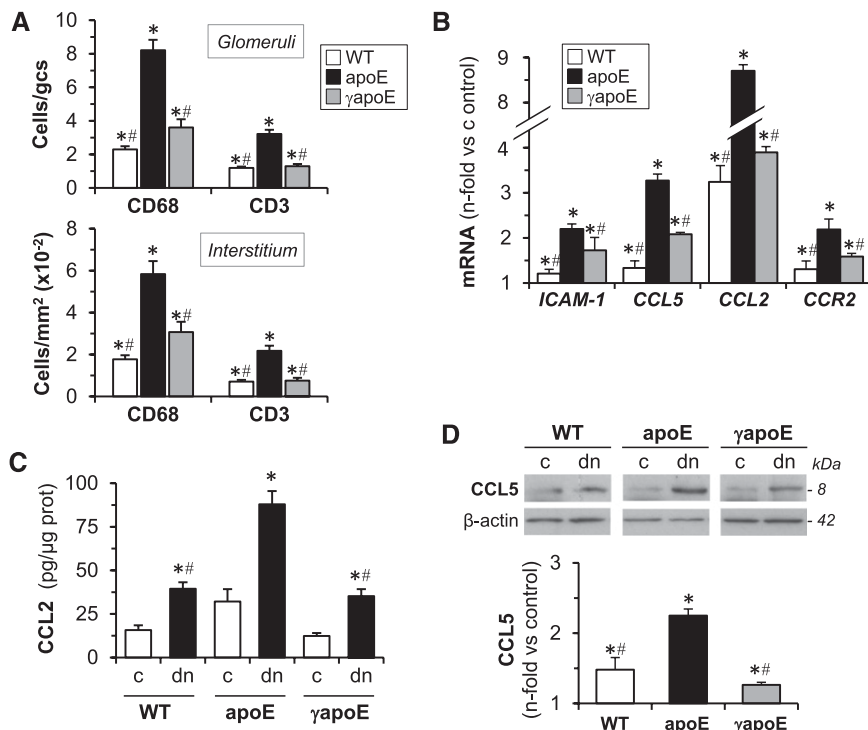


Figure 4. Renal inflammatory findings in diabetic mice. (A) Infiltrating macrophages (CD68) and T lymphocytes (CD3) in renal samples were analyzed by immunoperoxidase. Summary of the quantification of CD68⁺ and CD3⁺ cells in glomeruli (upper panel) and interstitium (lower panel). (B) Real-time PCR analysis of inflammatory genes in renal cortex from diabetic mice. Values were normalized by 18S expression and expressed as fold increases versus respective nondiabetic controls. (C) The concentration of CCL2 in renal cortex was measured by ELISA. (D) Representative immunoblots for CCL5 protein in control and diabetic mice. Summary of densitometric analysis is expressed as fold increase versus control group. Bars represent the mean \pm SEM of total number of animals per group. c, control; dn, diabetic nephropathy; gcs, glomerular cross-section; ICAM-1, intercellular adhesion molecule-1. * $P < 0.05$ versus respective nondiabetic controls; # $P < 0.05$ versus diabetic apoE mice.

apoE was significantly attenuated in γ apoE mice. *In vitro*, oxidized LDL-immune complexes increased the intracellular superoxide generation in apoE mesangial cells through a mechanism involving NADPH-oxidase activation, as determined by lucigenin chemiluminescence assay in cells preincubated with specific inhibitors (Figure 7B). The oxidative response to oxidized LDL-immune complexes was markedly attenuated in cells from γ apoE mice. Consistent with this, the membranous translocation of NADPH oxidase subunits (p67^{phox} and p47^{phox}) observed after immune complex treatment in apoE cells was reduced in γ apoE cells (Figure 7C).

DISCUSSION

Although diabetic nephropathy has been traditionally considered a nonimmune disease, accumulating evidence indicates the prominent role of immunoinflammatory mechanisms in its development and progression.³ We investigated the

immune mechanisms activated by hyperlipidemia that contribute to nephropathy acceleration in diabetic mice. Our results demonstrate that gene deficiency in the common γ -chain required for expression and functionality of activating Fc γ R protects mice from diabetic renal injury. Lack of activating Fc γ R in diabetic hypercholesterolemic mice improved renal function and attenuated renal inflammation, hypertrophy, fibrosis, and oxidative stress.

Studies in genetically modified mice have demonstrated the relevant role of Fc γ R in the pathogenesis of immunoinflammatory diseases.^{13,14} Thus, knockout mice for single and multiple activating Fc γ R are less susceptible to the development of GN and atherosclerosis,^{19,21,26} whereas lack of inhibitory Fc γ RIIb gene aggravates the disease. Fc γ R deficiency also protects from pancreatic injury in an autoimmune diabetes model.²⁰ In line with these studies, to our knowledge our study is the first to describe the involvement of activating Fc γ R-mediated responses in the diabetic kidney.

We used the diabetic apoE knockout mouse as the experimental model because it combines the effects of hyperglycemia and hyperlipidemia, two clinically important risk factors for nephropathy.²⁹ The apoE mouse shows little evidence of albuminuria and renal lesions but develops accelerated atherosclerosis and nephropathy when made diabetic with streptozotocin injection.^{5,6} Consistent with this, our diabetic

apoE mice exhibited albuminuria and a decline in renal function that exceed those in diabetic WT mice. Interestingly, these measures were attenuated in diabetic γ apoE; consequently, diabetic complications progressed slowly in γ apoE compared with equally diabetic apoE mice, even though both strains share the apoE deficiency. By contrast, there were no significant changes in hyperglycemia and hypercholesterolemia between apoE and γ apoE mice, which argues against a direct effect of Fc γ R deficiency on the metabolic severity of diabetes. Therefore, it is conceivable that Fc γ R deficiency affects development of both nephropathy and atherosclerosis associated with diabetes through similar mechanisms, including lack of cell responses to immune complexes containing proteins modified by the interaction between diabetes and hypercholesterolemia.

Apart from biochemical improvement, Fc γ R deficiency also affected early glomerular changes in the diabetic kidney, as evidenced by reduced hypertrophy, mesangial matrix expansion, and glomerulosclerosis in γ apoE mice. This finding

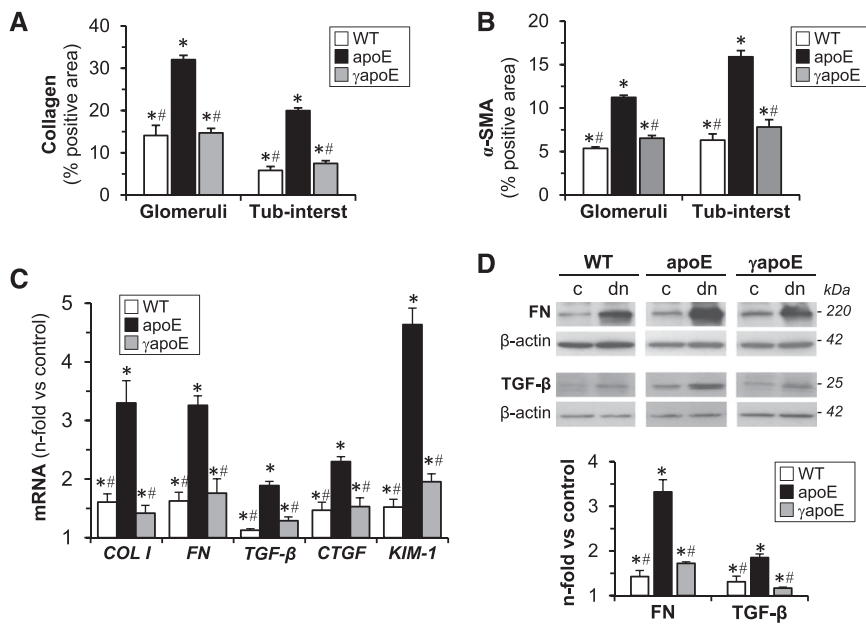


Figure 5. Lack of activating FcγRs reduces renal fibrosis in diabetic mice. (A) Quantitative analysis of collagen (picrosirius red staining)-positive areas in glomeruli and tubulointerstitial (Tub-interst) compartments of diabetic mice. (B) Quantification of α-SMA immunostaining in both compartments. (C) Real-time PCR analysis of extracellular matrix proteins (COL I, collagen I; FN, fibronectin), profibrotic factors (TGF-β and CTGF), and tubular injury marker (KIM-1) in the renal cortex. Values were normalized by 18S expression and expressed as fold increases versus respective nondiabetic controls. (D) Representative immunoblots for fibronectin and TGF-β in the renal cortex and summary of quantitative analyses in control and diabetic mice. Bars represent the mean ± SEM of total number of animals per group. c, control; dn, diabetic nephropathy. **P*<0.05 versus respective nondiabetic controls; #*P*<0.05 versus diabetic apoE mice.

indicates that activating FcγRs are important in the exacerbation of diabetic nephropathy due to hyperlipidemia. However, a more general role of FcγR in diabetic complications should not be discarded. In fact, there was a trend toward improved renal function (*i.e.*, mesangial matrix expansion) in single γ-chain-deficient mice under normolipidemic conditions. This result is consistent with the protective effect against pancreatic injury in the autoimmune diabetes model previously reported.²⁰

Infiltrating leukocytes constitute a significant proportion of the hypercellular glomerulus and participate in the pathogenesis of diabetic nephropathy either by direct interaction with renal cells or through the release of cytokines and growth factors involved in cell proliferation and matrix production.^{4,30} In our study, lack of activating FcγRs decreased the number of infiltrating macrophages and T cells, and the expression of α-SMA, a marker of mesangial dedifferentiation/activation and epithelial-myofibroblast transdifferentiation in progressive renal diseases.³¹ Diabetic γapoE mice were also protected from the development of tubular atrophy and interstitial fibrosis, hallmarks of ESRD. These observations, in conjunction with a reduced expression of inflammatory genes,

extracellular matrix proteins, and profibrotic factors, suggest that activating FcγRs contribute to inflammation and fibrosis, the major mechanisms for diabetic renal disease.

In diabetic patients, increased levels of IgG immune complexes containing oxidized, malondialdehyde-modified, and advanced glycation end product-modified LDL have been associated with the development of albuminuria and retinopathy.^{8,10–12,32} Animal model studies have demonstrated that glomerular levels of IgG increase with the duration of diabetes and correlate with albuminuria and renal inflammatory infiltrate.^{33–35} Our study reveals glomerular immune deposits and high levels of total and oxidized LDL-specific antibodies in diabetic hypercholesterolemic mice, without differences between apoE and γapoE mice. Further analysis of IgM/IgG and Th1/Th2 ratios did not substantiate changes in lymphocyte responses. Therefore, FcγR deficiency may alter the renal cell responses to immune complexes without affecting the synthesis and glomerular deposition of IgG in hyperglycemic, hypercholesterolemic mice. Because of functional and biochemical alterations, the glomeruli of diabetic patients seem to be more susceptible to immune-mediated injury. In fact, glomerular IgG deposits have been found in renal biopsy specimens

from diabetic patients,^{36,37} thus suggesting that immune complexes containing proteins modified by diabetes may induce renal injury. Our findings support this concept and demonstrate that immune response contributes to the evolution of diabetic nephropathy in hypercholesterolemic mice through the activation of FcγR present in both infiltrating and resident cells.

To further substantiate the involvement of FcγRs on intrinsic glomerular cells, we used a homologous *in vitro* system of apoE mesangial cells stimulated with standard immune complexes (soluble IgG aggregates) and potential causative immune complexes containing oxidized LDL.⁸ Besides confirming previous reports on FcγRs and renal injury,^{15–17,38} our results demonstrate that mesangial FcγR activation by oxidized LDL-immune complexes are of pathologic importance in the acceleration of diabetic renal disease.

Enhanced oxidative stress has been implicated in early and late events of diabetic nephropathy.³⁹ The multicomponent phagocyte-like NADPH oxidase is a major source of superoxide production in many nonphagocytic cells, including mesangial and tubular cells, and its activity can be upregulated by hyperglycemia, hyperlipidemia, and immune stimulation.^{40–43}

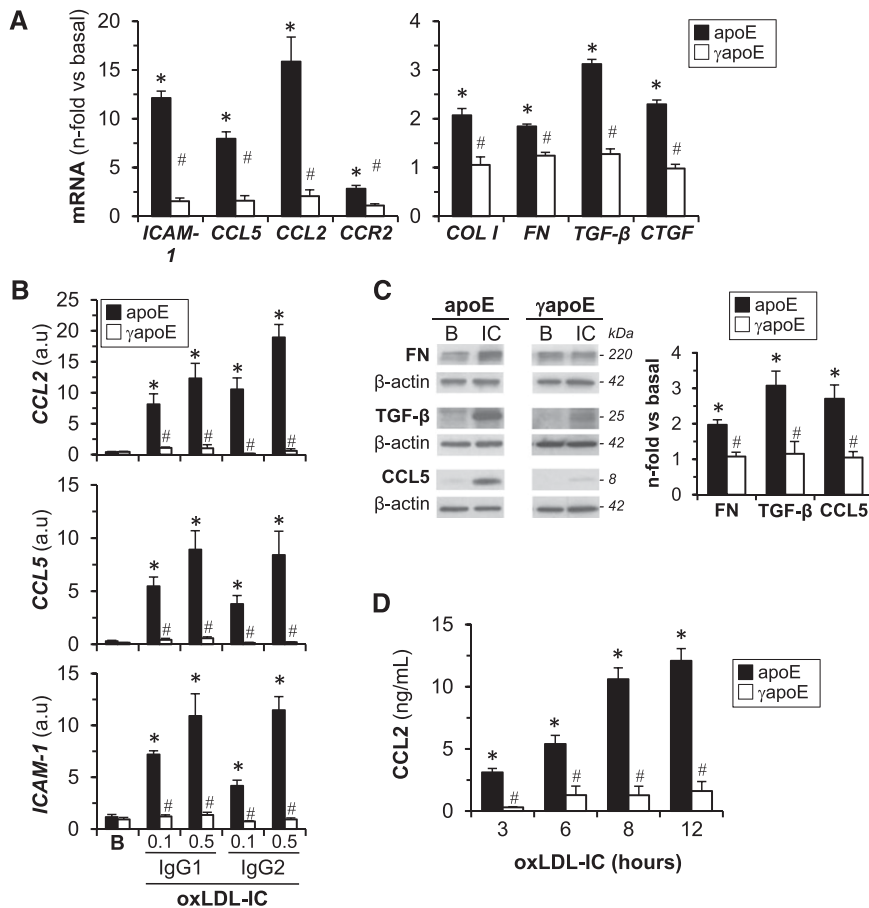


Figure 6. FcγR-mediated responses in mesangial cell cultures. (A and B) Quiescent cells from apoE and γapoE mice were stimulated for 6 hours with soluble IgG aggregates (150 μg/ml) (A) or different concentrations of oxidized LDL (oxLDL)-immune complex (IC) (IgG1 and IgG2 isotypes) (B). The mRNA expression of the indicated genes was measured by real-time PCR. Values were normalized by 18S endogenous control and expressed as fold increases versus basal conditions or arbitrary units (a.u.). (C) Representative immunoblots of fibronectin, TGF-β, and CCL5 production after 8 hours of stimulation. Data from densitometric analysis are corrected by loading control (β-actin) and expressed as n-fold versus basal conditions. (D) Time course of mesangial CCL2 protein expression (ELISA) under oxidized LDL-immune complex stimulation. Values are mean ± SEM of 3–6 independent experiments. ICAM-1, intercellular adhesion molecule-1. **P* < 0.05 versus basal; #*P* < 0.05 versus apoE cells.

We believe this study is the first to demonstrate that FcγR engagement in mesangial cells activates assembly of NADPH oxidase subunits (p47^{phox} and p67^{phox}) and superoxide generation. Furthermore, FcγR deficiency impaired superoxide production *in vivo*, thus suggesting that renal oxidative stress depends on the FcγR activation in the diabetic kidney.

The coordinate expression of activating and inhibitory FcγRs ensures the homeostasis of immunoinflammatory responses.^{13, 14} At sites of inflammation, combinations of pro- and anti-inflammatory mediators alter the FcγR balance toward activation or inhibition, respectively.⁴⁴ We found a predominance of activating versus inhibitory FcγRs in diabetic apoE mice, and a clear localization in both infiltrating and glomerular mesangial cells. The shift in relative ratio toward activating

FcγR was absent in γapoE mice, which is consistent with the reported role that the γ-chain plays in expression and functionality of activating FcγRs.^{13,14,19,20,26} Interestingly, the inhibitory FcγRIIb was upregulated by diabetes in γapoE mice. The most plausible function of FcγRIIb in renal inflammation in the absence of activating FcγRs is the efficient removal of immune complexes from the kidney. Thus, it is very likely that activating FcγR is required for initiating immune complex-mediated inflammation in the diabetic kidney, but limited in their response by inhibitory FcγRIIb, as previously reported in immune diseases.^{19,21,45}

In summary, this study underlines that activating FcγRs are crucial in development and progression of diabetic nephropathy in hypercholesterolemic mice by controlling renal inflammation, fibrosis, and oxidative stress. These observations suggest that FcγRs represent a novel target in the therapeutic interventions for nephropathy in diabetic patients with dyslipidemia, high levels of circulating immune complexes, and rapid course. We therefore propose that strategies to modulate the FcγR balance and effector functions could determine the net functional effect and consequently retard the progression of diabetic nephropathy. Indeed, blockade of activating FcγRs and overexpression of inhibitory FcγRIIb on effector cells are among the underlying anti-inflammatory mechanisms of intravenous immunoglobulins and Fc fragments in immune-mediated diseases.^{14,46} Although such approach was recently reported to attenuate autoimmune diabetes in mice,⁴⁷ future studies are needed to confirm their clinical benefit in diabetic patients.

CONCISE METHODS

Diabetes Model

Type 1 diabetes was induced in male apoE (*n* = 14), γ (*n* = 6), γapoE (*n* = 13), and WT (*n* = 12) mice (C57BL/6 background) at 10 weeks of age by intraperitoneal streptozotocin injection (125 mg/kg body weight in 10mM citrate, pH 4.5) once a day for 2 consecutive days. Control mice (*n* = 4–7 per group) received citrate. Animals without water restriction were maintained on standard diet and were monitored every 2–3 days for blood glucose. Diabetes was defined as blood glucose levels of >350 mg/dl. Mice with glucose levels >500 mg/dl received intermittent low dosages of insulin (1–1.5 IU subcutaneously) to prevent death. After 15 weeks of streptozotocin injection, mice were anesthetized and saline perfused. Dissected kidneys were

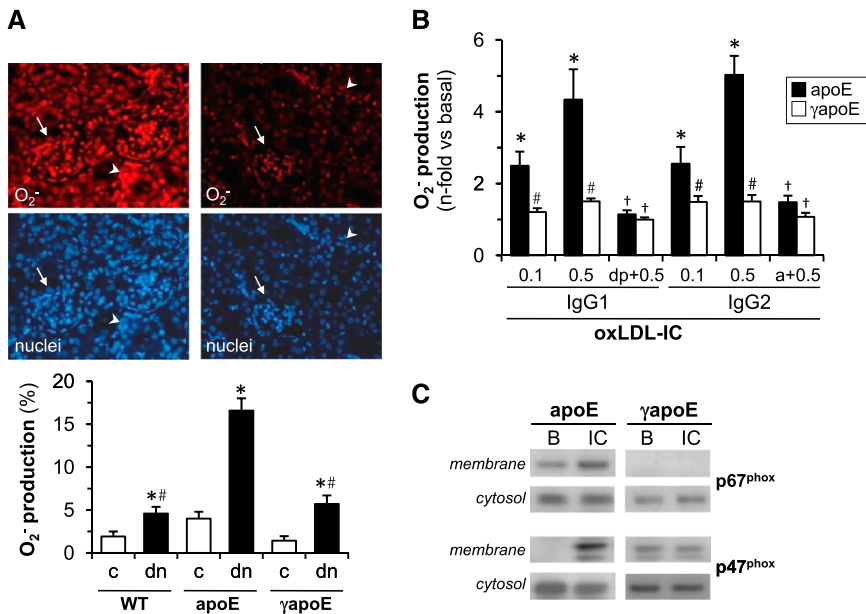


Figure 7. Activating FcγRs induce oxidative stress. (A) *In situ* detection of superoxide production in kidney sections. Representative micrographs of oxidized dihydroethidium (red) and nuclear staining (DAPI, blue) in diabetic apoE and γapoE mice (magnification ×200). Arrows and arrowheads indicate positive cells in glomeruli and tubulointerstitium, respectively. Graphical analysis of each condition measured as percentage of red fluorescence versus total cell nuclei. Bars represent the mean ± SEM of total number of animals per group. (B) NADPH oxidase-dependent superoxide production in mesangial cells stimulated for 60 minutes with different concentrations of oxidized LDL (oxLDL)-immune complex (IC) (IgG1 and IgG2) was detected by lucigenin assay. Cells were preincubated with apocynin (a) and diphenyleneiodonium (dpi) before stimulation. Values are mean ± SEM of three independent experiments. (C) Translocation of NADPH oxidase subunits in mesangial cells. Representative immunoblots (n=3) of p47^{phox} and p67^{phox} in the membrane and cytosolic fractions 60 minutes after immune complex stimulation. c, control; dn, diabetic nephropathy. ICAM-1, intercellular adhesion molecule-1; *P<0.05 versus respective (A) nondiabetic controls or (B and C) basal conditions; #P<0.05 versus (A) diabetic apoE mice or (B and C) apoE cells; †P<0.05 versus stimulated cells.

(0–3 scale) according to the extent of glomerular changes (hypertrophy, hypercellularity, and mesangial expansion; 30 glomeruli per sample), tubular lesions (atrophy and degeneration; 20 fields at ×40 per sample), and interstitial damage (fibrosis and infiltration; 20 fields at ×40 per sample). Glomerular area was quantified by a computerized image analysis system on periodic acid-Schiff-stained sections and used to calculate glomerular volume. Renal fibrosis was examined by picrosirius red staining. Glomerular immune deposits were detected by direct immunofluorescence with antimouse IgG and C3 antibodies. Macrophages (CD68), T lymphocytes (CD3), FcγR isoforms (I, III, and IV), and α-SMA were assessed by indirect immunoperoxidase. Positive staining (20 fields at ×20 magnification) were quantified using Image Pro-Plus analysis software, and positive area was expressed as percentage of total area (arterial staining was excluded in α-SMA measurement). CD68-, CD3-, and FcγR-positive cells were expressed as number per glomerular cross-section and interstitial area measured (mm²).

Cell Cultures

Mesangial cells from apoE and γapoE mice were cultured in RPMI 1640 containing 10% FCS, 2 mM glutamine, and antibiotics.⁴⁸ Quiescent cells were pretreated with the NADPH oxidase inhibitors (10⁻⁴ M apocynin and 10⁻⁵ M diphenyleneiodonium) before immune complex stimulation. Soluble immune complexes (150 μg/ml) were obtained by heat aggregation (63°C, 30 minutes) of mouse IgG. Oxidized LDL-immune complexes were prepared by mixing human oxidized LDL (1 mg/ml)

with mouse IgG1 and IgG2 at 1:0.1 and 1:0.5 ratio of oxidized LDL to IgG.

mRNA and Protein Expression

Total RNA from cells and tissues was extracted and target gene expression was analyzed by real-time PCR and normalized to housekeeping 18S transcripts. Total proteins were homogenized in buffer containing 1% Triton X-100 and 0.5% NP-40 and protease inhibitors. Cytosol and membrane fractions were separated using a compartment protein extraction kit. Conditioned media were collected for fibronectin expression. Proteins (25 μg) were electrophoresed, transferred, and then immunoblotted with antibodies against CCL2, fibronectin, TGF-β, NADPH oxidase (p47^{phox} and p67^{phox}), and β-actin (loading control). CCL2 levels were determined by ELISA.

Oxidative Stress

Intracellular superoxide in renal sections was assessed by fluorescence microscopy with the superoxide-sensitive fluorescent dye (2 μM).

snap-frozen (expression studies) or stored in 4% paraformaldehyde (histology). All studies were performed according to the protocols approved by the Institutional Animal Care and Use Committee. Plasma cholesterol and urea were determined by autoanalyzer. Creatinine levels were determined by creatininase enzymatic method. Glycated hemoglobin A1c, albuminuria, and serum immunoglobulins (G1, G2a, G3, A, and M) were tested by ELISA. Albumin-to-creatinine ratio was calculated as an alternative marker for 24-hour urinary albumin excretion. Specific anti-oxidized LDL immunoglobulins were measured in plates coated with oxidized LDL and native LDL (1 mg/ml, kindly provided by Dr. J.A. Moreno, IIS-FJD, Madrid). Values were calculated by subtracting the mean absorbance of native LDL plate from that of oxidized LDL.

Renal Morphology and Immunohistochemistry

Paraffin-embedded kidney sections (3 μm) were stained with periodic acid-Schiff, and renal lesions were semiquantitatively graded

Superoxide production was expressed as the number of positive cells versus total cells (nuclear DAPI staining) as described elsewhere.⁴⁹ NADPH-dependent oxidase activity in cell homogenates was measured by chemiluminescence using 5 μ M lucigenin and 100 μ M NADPH, and data were expressed as relative units per μ g protein.

Statistical Analyses

Data are presented as mean \pm SEM. GraphPad Prism program was used for statistical analyses. Differences across groups were tested by one-way ANOVA, followed by *post hoc* Bonferroni pairwise comparison. *P* values <0.05 were considered to represent a statistical significant difference.

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DISCLOSURES

None.

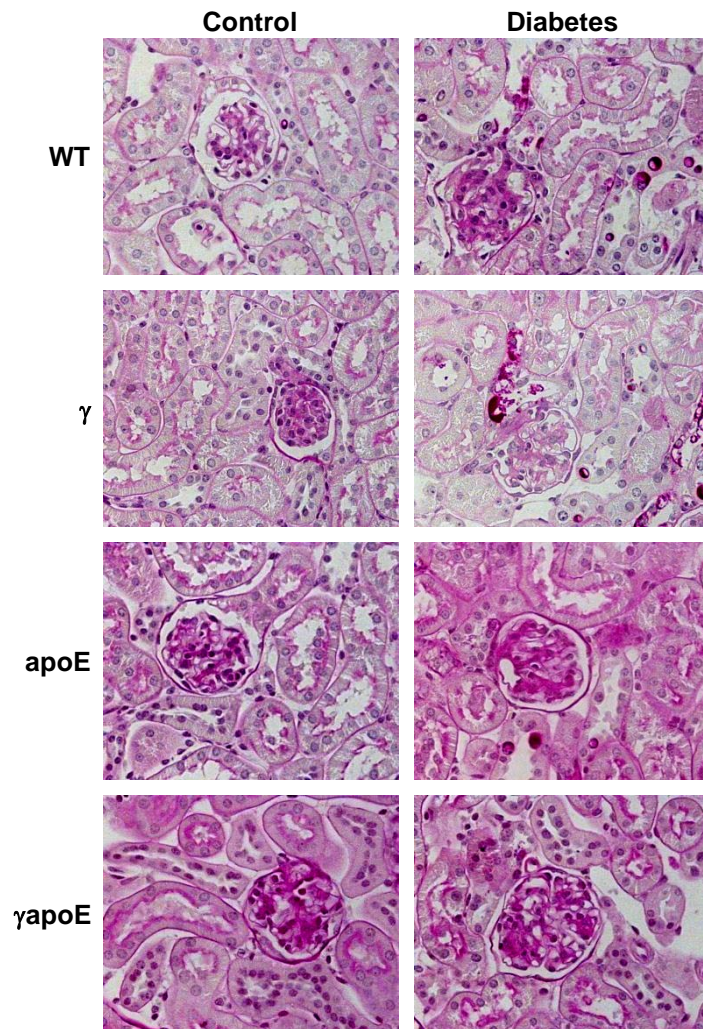
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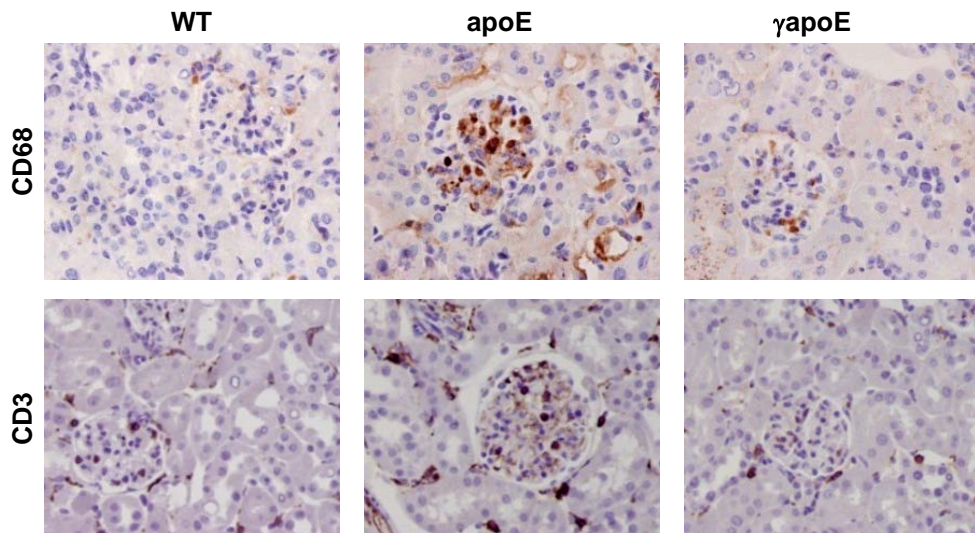
This article contains supplemental material online at <http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2011080822/-DCSupplemental>.

Supplemental Figure S1 (Lopez-Parra et al.)



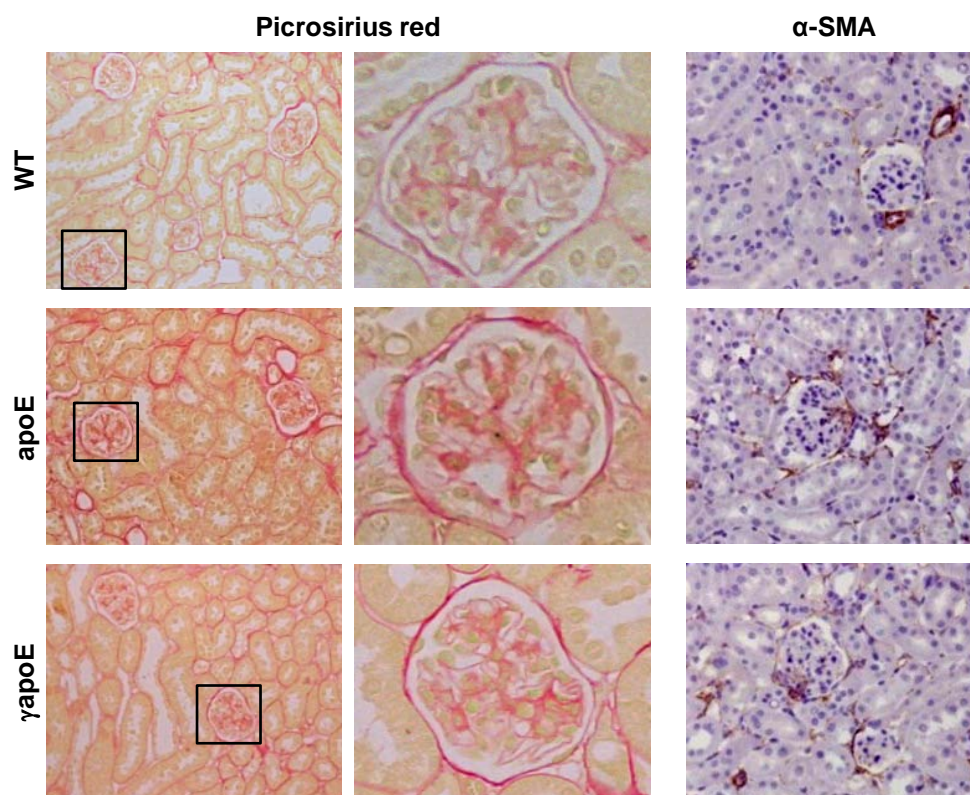
Representative micrographs of PAS staining in renal sections of nondiabetic and diabetic mice from WT, γ , apoE, and γ apoE groups. Original magnification x400.

Supplemental Figure S2 (Lopez-Parra et al.)



Representative micrographs showing positive immunostaining for macrophages (CD68) and T lymphocytes (CD3) in renal samples from diabetic WT, apoE and γ apoE groups as specified. Original magnification x400.

Supplemental Figure S3 (Lopez-Parra et al.)



Representative micrographs of picrosirius red staining and α -smooth muscle actin (α -SMA) immunoperoxidase in renal sections from diabetic mice. Original magnification x200 (glomeruli at high-magnification view).