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The green tea polyphenol (−)-epigallocatechin-3-gallate ameliorates experimental immune-mediated glomerulonephritis

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The unchecked overproduction of reactive oxygen and nitrogen species by inflammatory cells can cause tissue damage, intensify inflammation, promote apoptosis, and accelerate the progression of immune-mediated glomerulonephritis (GN). Here we tested whether the anti-inflammatory and antioxidant properties of the green tea polyphenol (−)-epigallocatechin-3-gallate (EGCG) favorably affect the development of immune-mediated GN. Pretreatment of 129/svJ mice with EGCG from 2 days before to 2 weeks after the induction of GN led to reduced proteinuria and serum creatinine, and marked improvement in renal histology when compared with vehicle-pretreated diseased mice. This pretreatment reduced oxidative stress, and normalized osteopontin, p65/nuclear factor-κB, inducible nitric oxide synthase, nitric oxide metabolites, p-Akt, phosphorylated extracellular signal-regulated kinases 1 and 2, p47phox, and myeloperoxidase, all of which were elevated in vehicle-pretreated diseased mice. Levels of glutathione peroxidase and peroxisome proliferator-activated receptor-γ (PPARγ), both reduced in the vehicle-pretreated diseased mice, were normalized. This renoprotective effect was reversed by concomitant administration of the PPARγ antagonist GW9662 throughout the EGCG pretreatment period. Importantly, mortality and renal dysfunction were significantly attenuated even when the polyphenol treatment was initiated 1 week after the onset of GN. Thus, EGCG reversed the progression of immune-mediated GN in mice by targeting redox and inflammatory pathways.

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KEYWORDS: anti-GBM disease; nitric oxide; oxidative stress; reactive oxygen species; renal pathology

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Anti-glomerular basement membrane antibody-induced glomerulonephritis (anti-GBM-GN) is pathologically and clinically the most severe form of immune-mediated GN. It is caused by circulating antibodies directed to the GBM components, non-collagenous-1 domain of the α3 or α5 chain of type IV collagen, leading to an inflammatory reaction in the glomerular capillaries.[1–4] The treatment of anti-GBM-GN aims to modulate the injury-causing immunologic process with high-dose corticosteroids, cytotoxic drugs, and plasmapheresis. However, end-stage renal disease develops in 40–70% of patients.[4,5]

Reactive oxygen species (ROS) are products of normal cellular metabolism that modulate physiological functions and affect innate immunity in infectious and noninfectious inflammation.[6] However, unchecked overproduction of ROS, reactive nitrogen species, and reactive chlorine species by inflammatory cells can cause further tissue damage, intensify inflammation, promote apoptosis, and accelerate progression of many diseases including anti-GBM-GN.[7,8] In physiologic condition, superoxide dismutase (SOD) catalyzes superoxide to molecular oxygen and hydrogen peroxide (H_2O_2). H_2O_2 is degraded to water and molecular oxygen by catalase or by glutathione peroxidase (GPx) in the presence of reduced glutathione. However, in the presence of electron donors such as iron, H_2O_2 is converted to hydroxyl radical, which is the most reactive and cytotoxic ROS. Additionally, myeloperoxidase (MPO), expressed in neutrophils and macrophages,

converts H_2O_2 to hypochlorous acid, a highly cytotoxic reactive chlorine species. Finally, H_2O_2 is an activator of nuclear factor-κB (NF-κB), a master regulator of pro-inflammatory cytokines, chemokines, and fibrogenic factors. Superoxide reacts with nitric oxide (NO) to produce peroxynitrite, a highly reactive nitrogen species. Excessive production and impaired capacity of the antioxidant system to inactivate superoxide and H_2O_2 play a central role in the pathogenesis of glomerular injury, hyperpermeability, and worsening of inflammatory glomerular disease.[9]

The green tea catechins, particularly (−)-epigallocatechin-3-gallate (EGCG), are potent anti-inflammatory and antioxidant agents shown to inhibit leukocyte chemotaxis, quench free radicals, chelate transition metals, and interrupt lipid peroxidation chain reaction.[10–13] EGCG is estimated to be 25 times more potent than vitamin E and 100 times more potent than vitamin C.[14] However, the effect of EGCG on immune-mediated renal injury has not been investigated. We tested the hypothesis that the anti-inflammatory and antioxidant properties of EGCG favorably affect the course of immune-mediated GN using a murine model of anti-GBM-GN. Here, we show that the EGCG administration significantly improves the laboratory and histopathological features of anti-GBM-GN.

RESULTS

EGCG attenuated the development of anti-GBM-GN

General data and biochemical/immunological measurements. At the end of the 2-week observation period, the vehicle-pretreated mice with anti-GBM-GN developed weight loss, renal insufficiency, and proteinuria in contrast to the normal controls. No mice died of disease during the course of the study. EGCG pretreatment led to significantly less proteinuria and normalized serum creatinine compared with the vehicle-pretreated mice (Table 1). The levels of immunoglobulin G (IgG) mouse anti-rabbit antibodies were comparable between the two groups of mice with anti-GBM-GN, indicating that the reduced renal disease in EGCG-pretreated mice was not caused by a decreased xenogenic immune response to the injected immunoglobulin (Figure 1).

Renal histology. The vehicle-pretreated mice showed moderate to severe renal injury characterized by crescent formation with marked intracapillary hypercellularity, obliterated capillary lumens, and thickened capillary walls (Table 1 and Figure 2). Tubular atrophy and dilation with hyaline casts and interstitial fibrosis were also noted. In comparison, the EGCG-pretreated mice exhibited milder renal injury with occasional crescent formation, and focal tubulointerstitial injury. No lesions were seen in the normal control group. In addition, both glomerular and interstitial

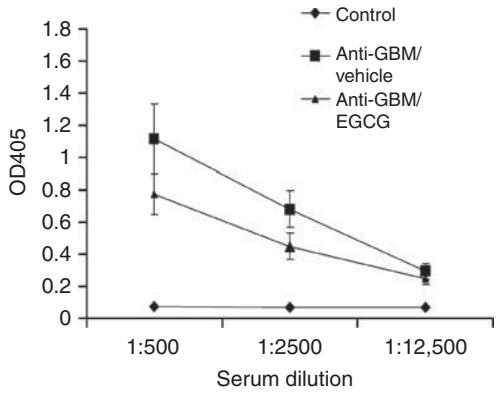

Figure 1 | Anti-rabbit immunoglobulin (Ig) response in (−)-epigallocatechin-3-gallate (EGCG)- or vehicle-pretreated mice with anti-glomerular basement membrane antibody-induced glomerulonephritis (anti-GBM-GN). Depicted are the serum levels of IgG mouse anti-rabbit antibodies (measured on day 14) assayed in serial dilutions. Values are expressed as mean ± s.e.m. $n = 7$ in each group.

Table 1 | General data and renal injuries in EGCG- and vehicle-pretreated mice with anti-GBM-GN and in normal controls

	Control	Anti-GBM/vehicle	Anti-GBM/EGCG
Body weight (g)	20.2 ± 0.4	17.0 ± 0.5**	19.1 ± 0.5##
Serum creatinine (mg/dl)	0.09 ± 0.01	0.15 ± 0.01**	0.10 ± 0.01##
Proteinuria (mg per 24 h)	0.78 ± 0.08	12.68 ± 1.45**	5.11 ± 1.01**,##
Glomerulonephritis score (0–4)	0 ± 0	3.5 ± 0.2**	2.0 ± 0.4*,#
Crescents (%)	0 ± 0	22.8 ± 4.2**	6.5 ± 3.3**,##
Tubulointerstitial injury score (0–5)	0 ± 0	2.4 ± 0.2**	1.2 ± 0.3**,##
Macrophage infiltration			
Glomeruli (cells per 50 glomerular cross-section)	1.4 ± 0.8	112.0 ± 20.6**	8.7 ± 3.8*,##
Interstitial (cells per 10 high-power field)	50.5 ± 7.2	520.3 ± 82.7**	132.3 ± 30.0*,##
Lymphocyte infiltration			
Glomeruli (cells per 50 glomerular cross-section)	1.9 ± 0.6	19.2 ± 8.4**	3.2 ± 0.8##
Interstitial (cells per 10 high power field)	3.8 ± 1.1	45.1 ± 10.2**	7.5 ± 3.6##

Abbreviations: Anti-GBM/EGCG, anti-glomerular basement membrane antibody-induced glomerulonephritis pretreated with (−)-epigallocatechin-3-gallate; anti-GBM/vehicle, anti-glomerular basement membrane antibody-induced glomerulonephritis pretreated with vehicle; GN, glomerulonephritis.
Values are mean ± s.e.m.; n=14 in each group.
*$P < 0.05$, **$P < 0.01$ vs control.
#$P < 0.05$, ##$P < 0.01$ vs anti-GBM/vehicle.

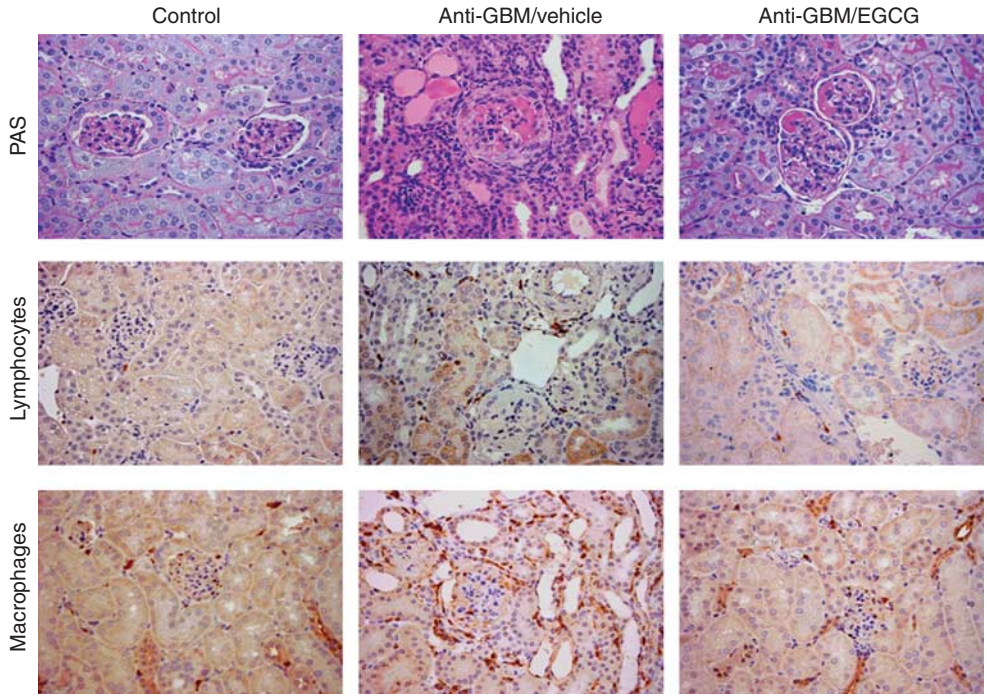

Figure 2 | Renal histology and immunohistochemical analysis of (−)-epigallocatechin-3-gallate (EGCG)- or vehicle-pretreated mice with anti-glomerular basement membrane antibody-induced glomerulonephritis (anti-GBM-GN). Kidneys from mice 2 weeks after induction of anti-GBM-GN with or without EGCG pretreatment and kidneys from normal control mice were evaluated by light microscopy. In the periodic acid-Schiff (PAS)-stained section, the kidneys in the vehicle-pretreated mice revealed severe glomerular hypercellularity with large crescent. There were also significant tubular atrophy and interstitial fibrosis with prominent interstitial inflammatory infiltrates (original magnification $\times 400$). Immunohistochemical analyses showed markedly increased interstitial infiltration of lymphocytes and macrophages in the vehicle-pretreated group (original magnification $\times 400$). In contrast, the EGCG-pretreated animals exhibited mild to moderate glomerular injury with minimal crescent formation and focal mild tubulointerstitial injury. No significant histopathological changes were observed in the normal control group.

infiltrations by macrophages and lymphocytes were significantly higher in the vehicle-pretreated mice compared with both the EGCG-pretreated mice and normal controls (Figure 2 and Table 1).

Markers of oxidative and nitrosative stress. Renal tissue and urine malondialdehyde (MDA) levels were modestly higher in the vehicle-pretreated mice than the normal control group. EGCG pretreatment normalized the MDA levels in both renal tissue and urine. Serum MDA levels were not significantly different among the three groups (Figure 3).

Renal tissue and urine H_2O_2 levels were significantly higher in the vehicle-pretreated mice than the normal control group, whereas serum H_2O_2 levels were not significantly different. Compared with the vehicle-pretreated mice, EGCG pretreatment significantly lowered H_2O_2 levels in renal tissue, urine, and serum (Figure 3).

Immunohistochemistry showed diffuse and strong nitrotyrosine staining in proximal and distal convoluted tubules (4+), glomerular cells (3+), and interlobular vascular endothelium (3+) of the vehicle-pretreated mice. The nitrotyrosine staining pattern and intensity were similar in the EGCG-pretreated mice, but the staining was weaker in the normal control mice. Similar results were obtained with western blot performed on kidney tissues. Renal tissues of the vehicle- and EGCG-pretreated mice had significantly higher nitrotyrosine than normal controls. Renal tissue nitrotyrosine abundance was not significantly different between the vehicle- and EGCG-pretreated mice (Figure 3).

NO pathway. Western blot showed similar levels of renal endothelial nitric oxide synthase (eNOS) expression in the three groups of mice (Figure 4). In contrast, inducible nitric oxide synthase (iNOS) protein content of renal tissue was significantly higher in the vehicle-pretreated animals than both the normal control and EGCG-pretreated groups. Total NO metabolite (nitrates + nitrites) content in kidney, urine, and serum was significantly higher in the vehicle-pretreated mice than the normal controls. EGCG pretreatment normalized the total NO metabolite levels in kidney, urine, and serum (Figure 4).

ROS-generating and antioxidant enzymes. Western blot analysis showed significantly higher expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase regulatory subunit p47phox in the vehicle-pretreated mice compared with both normal control and EGCG-pretreated mice, pointing to the increased ROS production in the vehicle-pretreated anti-GBM-GN mice (Figure 5). The expression of SOD-1 was modestly elevated in the vehicle-pretreated mice compared with the normal control mice

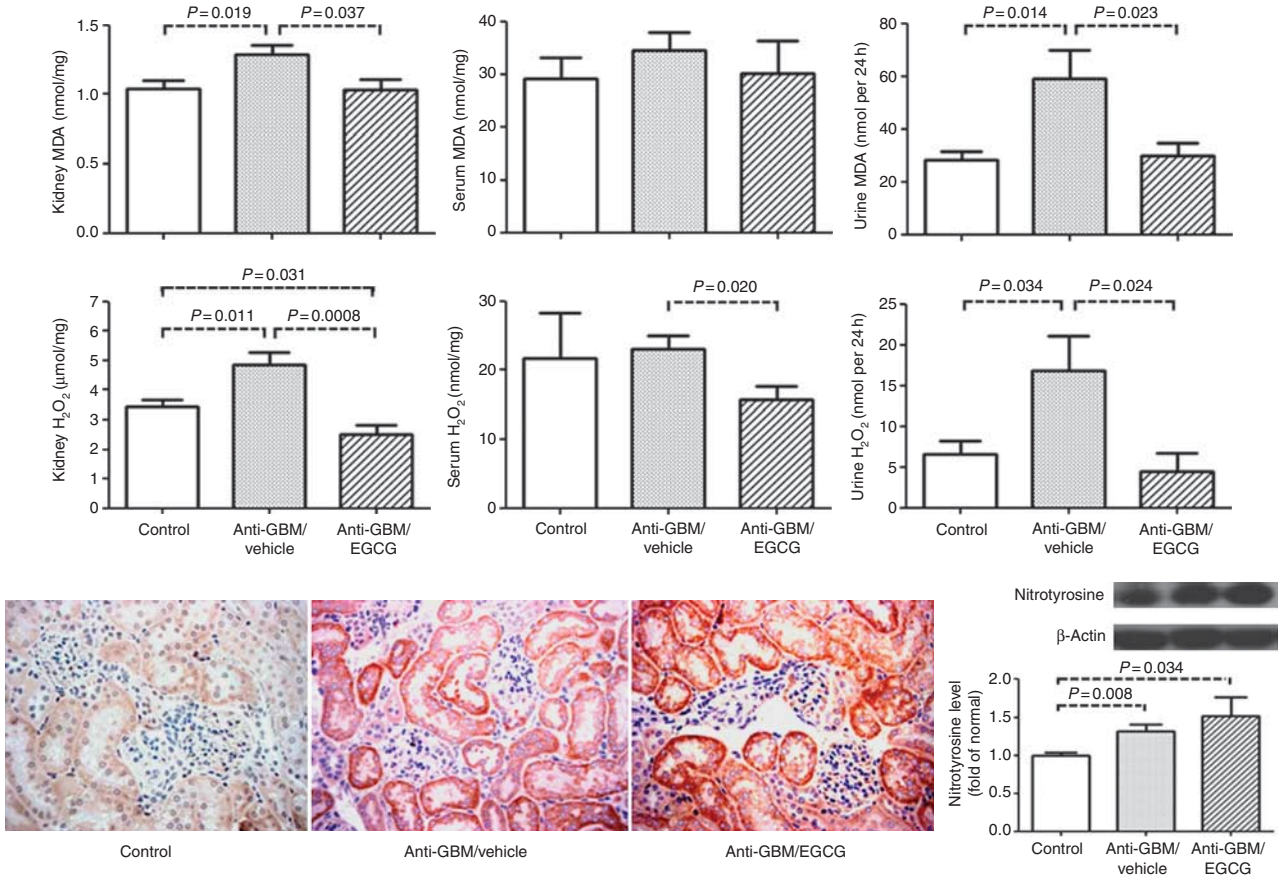

Figure 3 | Malondialdehyde (MDA), hydrogen peroxide (H_2O_2), and nitrotyrosine in mice with anti-glomerular basement membrane antibody-induced glomerulonephritis (anti-GBM-GN). Urine and renal tissue MDA levels were markedly elevated in mice with anti-GBM glomerulonephritis compared with the normal controls. Pretreatment with (−)-epigallocatechin-3-gallate (EGCG) normalized the urine and renal tissue MDA levels. Serum MDA levels were not significantly altered among the three groups. Urinary and renal tissue H_2O_2 levels were markedly elevated in mice with anti-GBM-GN compared with the normal control group. Serum H_2O_2 levels were not significantly different between the two groups. EGCG pretreatment was associated with a marked reduction of H_2O_2 levels in the urine, serum, and renal tissue. Immunohistochemistry showed diffuse and strong nitrotyrosine staining in both vehicle- and EGCG-pretreated animals, but the staining was much weaker in the normal control mice. Similar results were obtained with western blot analysis for nitrotyrosine performed on the kidney tissues. Values are expressed as mean ± s.e.m. $n = 7$ in each group.

(Figure 5). This phenomenon points to a biological response to the prevailing oxidative stress in the renal tissue. EGCG pretreatment further elevated the renal SOD-1 abundance (Figure 5). The renal tissue GPx1 expression was significantly lower in the vehicle-pretreated mice than both the normal control and EGCG-pretreated mice. Western blot showed significantly higher MPO in the vehicle-pretreated mice than both the normal control and EGCG-pretreated mice; the MPO levels in the latter two groups were comparable. There was no significant difference in the expressions of heme oxygenase-1 (HO-1) among the three groups (Figure 5). The renal tissue catalase activity was significantly lower in the vehicle-pretreated mice than both the normal control and EGCG-pretreated mice (Figure 5).

Molecules associated with inflammatory pathways. Immunohistochemistry showed diffuse and strong staining for osteopontin (OPN) in virtually all proximal and distal renal tubules (4+) of the vehicle-pretreated mice (Figure 6). The glomeruli showed positive staining for OPN in parietal epithelial cells (2+), scattered podocytes (3+), and infiltrating leukocytes (4+). Numerous interstitial macrophages were also strongly positive (4+). In the EGCG-pretreated mice, mild OPN expression was noted in occasional glomerular parietal epithelial cells (1+), distal tubules (1+), and interstitial infiltrating cells (1+). No significant glomerular or tubulointerstitial OPN staining was observed in the normal control mice. Western blot confirmed the immunohistochemical results, with significantly higher renal OPN expression in the vehicle-pretreated mice than both the EGCG-pretreated and normal control mice (Figure 6).

Immunohistochemistry showed diffuse and strong staining for p65/NF-κB in nearly all proximal and distal renal tubules (4+) of the vehicle-pretreated anti-GBM-GN mice (Figure 6). Additionally, many glomerular infiltrating leukocytes and resident cells (3+) and interstitial macrophages (3+) were positive for p65/NF-κB. The staining was localized to both cytoplasm and nucleus, the latter signifying NF-κB activation. Conversely, in the EGCG-pretreated mice,

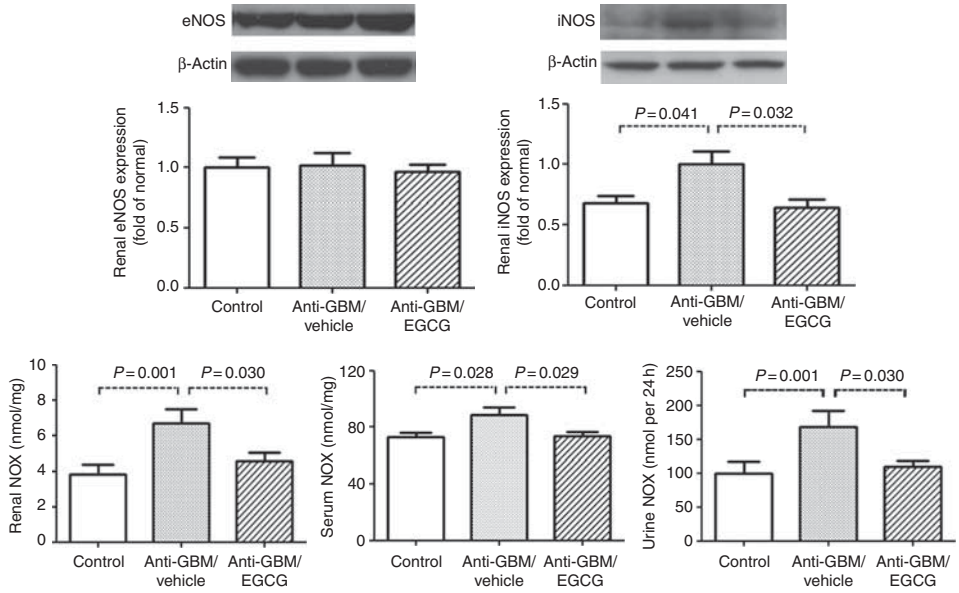

Figure 4 | Western blot analyses of endothelial and inducible nitric oxide synthase (eNOS and iNOS) and measurements of nitric oxide metabolites (NOX). Western blot showed similar levels of renal eNOS expression in the three groups. In contrast, iNOS protein content of renal tissue showed a significant rise in the vehicle-pretreated animals compared with both normal control and (−)-epigallocatechin-3-gallate (EGCG)-pretreated group. Total nitric oxide metabolites, NO2 + NO3 (NOX), in kidney, urine, and serum were significantly higher in the vehicle-pretreated mice compared with the normal control mice. EGCG pretreatment normalized the total NOX levels in kidney, urine, and serum. Values are expressed as mean ± s.e.m. $n = 7$ in each group.

mild focal p65/NF-κB was expressed in occasional glomerular and interstitial infiltrating leukocytes (1+) and scattered tubular epithelial cells (2+), with the reactivity primarily localized to cytoplasm. No significant glomerular or tubulointerstitial staining was seen in the normal control mice. Western blot confirmed the immunohistochemical results, with significantly higher renal p65/NF-κB expression in the vehicle-pretreated mice than both the EGCG-pretreated and normal control mice (Figure 6).

Peroxisome proliferator-activated receptor-γ (PPARγ) antagonist GW9662 co-administration reversed the renoprotective effect of EGCG. To examine whether PPARγ pathway is essential for the renoprotective effect of EGCG, a subgroup of EGCG-pretreated mice with anti-GBM-GN was concomitantly administered a selective PPARγ antagonist GW9662 (anti-GBM/EGCG + GW) following the same time schedule (day −2 to day 14). EGCG pretreatment led to significantly less proteinuria and normalized serum creatinine compared with both vehicle-pretreated and EGCG + GW-pretreated mice, the values in the latter two groups being comparable (Table 2). Both vehicle- and EGCG + GW-pretreated mice showed moderate to severe renal injury, whereas the EGCG-pretreated mice exhibited milder renal injury (Table 2).

Western blot analyses showed significantly lower expression of PPARγ and significantly higher expression of p-Akt and phosphorylated extracellular signal-regulated kinases 1 and 2 (p-ERK1/2) in the vehicle-pretreated mice compared with both the normal control and EGCG-pretreated mice (Figure 7). As expected, co-administration of GW9662 abolished the EGCG-induced upregulation of PPARγ. Interestingly, co-administration of GW9662 also abolished the EGCG-induced inactivation of p-Akt and p-ERK1/2.

EGCG initiated after the development of anti-GBM-GN ameliorated disease severity

To examine the therapeutic effect of EGCG, subgroups of anti-GBM antibody injected mice were allowed to develop severe nephritis before intervention. EGCG treatment was initiated on day 7 following anti-GBM administration and continued for 3 weeks (day 7 to day 28). On day 7, both groups of mice with anti-GBM-GN developed severe GN evidenced by severe proteinuria and azotemia. At the end of the 4-week observation period, the vehicle-treated group suffered a mortality rate of 42% compared with 10% in the EGCG-treated group. The vehicle-treated mice with anti-GBM-GN developed renal insufficiency and proteinuria in contrast to the normal control group on day 28. The EGCG-treated mice showed significantly less proteinuria and lower serum creatinine than the vehicle-treated mice (Table 3).

DISCUSSION

Our study shows that pretreating mice with EGCG 2 days before the induction of anti-GBM-GN leads to a dramatic and unequivocal improvement in the laboratory and histopathological parameters, evidenced by a significant reduction in proteinuria and serum creatinine, and improvement in histological findings including decreased glomerular crescents, less tubulointerstitial injury, and reduced glomerular and interstitial inflammatory cells. More importantly,

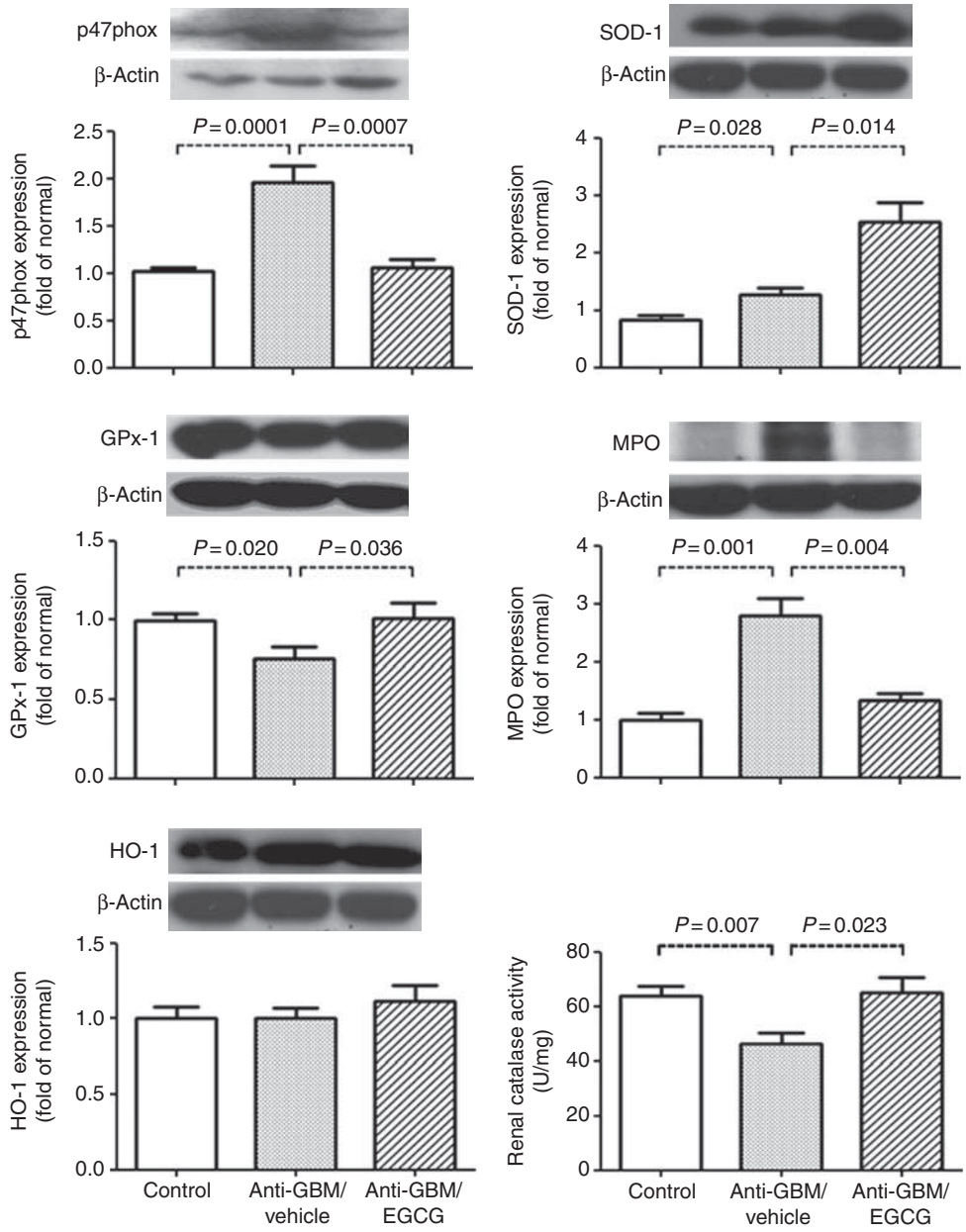

Figure 5 | Reactive oxygen species (ROS)-generating and antioxidant enzymes in mice with anti-glomerular basement membrane antibody-induced glomerulonephritis (anti-GBM-GN). Western blot showed significantly higher expressions of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunit p47phox in vehicle-pretreated mice with anti-GBM-GN compared with both the normal control and (−)-epigallocatechin-3-gallate (EGCG)-pretreated mice. The expression of superoxide dismutase-1 (SOD-1) was significantly higher in the EGCG-pretreated mice compared with the vehicle-pretreated mice, in which the SOD-1 expression was modestly elevated compared with the normal controls. The renal tissue glutathione peroxidase (GPx) expression was significantly reduced in vehicle-pretreated mice and was restored by EGCG supplementation. The expression of myeloperoxidase (MPO) was significantly elevated in the vehicle-pretreated mice and was normalized by EGCG pretreatment. There was no significant difference in the expressions of heme oxygenase-1 (HO-1) among the three groups of mice. The renal catalase activity was significantly lower in the vehicle-pretreated mice compared with both the normal control and EGCG-pretreated mice. Values are expressed as mean ± s.e.m. $n = 7$ in each group.

administration of EGCG to mice with full-blown anti-GBM-GN also ameliorates renal injury and reduces mortality rate.

Our data also show that reduced renal disease in EGCG-pretreated mice was not related to a less severe systemic immune response to the administered rabbit immunoglobulin because the levels of IgG mouse anti-rabbit antibodies were comparable between the vehicle- and EGCG-pretreated mice with anti-GBM-GN. We further demonstrate that glomerular disease in the mouse model of anti-GBM-GN is accompanied by oxidative damage as shown by high renal and urine levels of oxidative stress markers MDA and H_2O_2. Pretreatment with EGCG significantly reduced the renal and

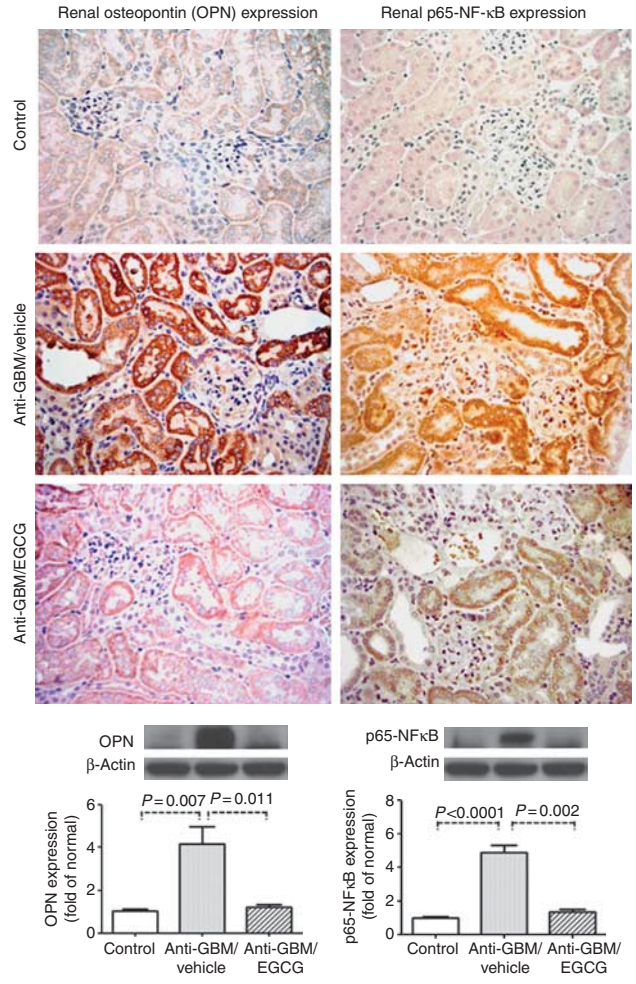

Figure 6 | Western blot analysis and immunolocalization of osteopontin and p65/nuclear factor-κB (NF-κB) in renal tissues from mice with anti-glomerular basement membrane antibody-induced glomerulonephritis (anti-GBM-GN) and normal controls. Immunohistochemical analysis showed marked upregulation of osteopontin and p65/NF-κB in the vehicle-pretreated animals compared with the (−)-epigallocatechin-3-gallate (EGCG)-pretreated and normal control groups. The vehicle-pretreated animals showed diffuse osteopontin and p65/NF-κB expressions in renal tubules, resident glomerular cells, and infiltrating leukocyte in glomeruli and interstitium. The above findings were confirmed by western blot analyses. For western blot analyses, values are expressed as mean ± s.e.m. $n = 7$ in each group.

urine levels of MDA and H_2O_2 in mice with anti-GBM-GN. A main source of ROS in inflammatory conditions is NADPH oxidase, which is abundantly expressed in neutrophils and macrophages and responsible for single-electron reduction of oxygen to superoxide anion. We found a significant upregulation of the $p47^{phox}$ regulatory subunit of NADPH oxidase in the vehicle-pretreated mice and its reversal with EGCG pretreatment, paralleling the changes in the intensity of inflammatory infiltration found on histological examination. In addition to reducing the enzyme abundance, EGCG may inhibit NADPH oxidase activity.[15,16]

Oxidative stress in immune-mediated GN can also be caused by impaired capacity of antioxidant system to inactivate ROS. Despite severe oxidative stress and inflammation, SOD was only slightly upregulated, whereas catalase and GPx were downregulated in the vehicle-pretreated mice compared with the normal control mice. The normalization of renal catalase and GPx levels would be consistent with the reduced H_2O_2 levels in EGCG-pretreated mice. Additionally, EGCG pretreatment was associated with marked upregulation of SOD, as previously shown.[17] MPO is abundantly expressed in the neutrophils and macrophages and converts H_2O_2 to highly cytotoxic hypochlorous acid. The markedly elevated MPO seen in the vehicle-treated mice and consequent production of hypochlorous acid must contribute to renal injury in this model of immune-mediated GN.

EGCG supplementation has been shown to significantly attenuate cisplatin-induced renal injury in rats by activating HO-1.[18] However, our data showed no significant changes of renal HO-1 expression among the three groups of mice, suggesting that HO-1 does not play a major role in anti-GBM-GN, and EGCG pretreatment does not significantly affect HO-1 in this disease model.

Although EGCG pretreatment significantly lowered NO metabolites in kidney, urine, and serum in mice with anti-GBM-GN, it did not reduce the kidney tissue abundance of nitrotyrosine, an indicator of nitrosative stress.[19] This might be because of the limited duration of the study, as disappearance of the preformed nitrotyrosine would depend on the turnover of proteins with nitrated tyrosine residues. The similar expression of eNOS in all three groups suggests

Table 2 | Peroxisome proliferator-activated receptor-γ (PPARγ) modulates the renoprotective effect of EGCG

	Control	Anti-GBM/vehicle	Anti-GBM/EGCG	Anti-GBM/EGCG+GW
Body weight (g)	19.2 ± 0.5	16.7 ± 0.6*	19.3 ± 0.3$^{\#}$	18.2 ± 0.7
Serum creatinine (mg/dl)	0.073 ± 0.004	0.138 ± 0.018**	0.079 ± 0.007$^{\#\#}$	0.131 ± 0.024**,†
Proteinuria (mg per 24 h)	0.69 ± 0.13	12.45 ± 2.51**	5.66 ± 1.9$^{\#}$,**	11.89 ± 1.10**,†
Glomerulonephritis score (0–4)	0 ± 0	3.5 ± 0.2**	1.8 ± 0.6$^{\#}$,*	3.6 ± 0.3**,†
Crescents (%)	0 ± 0	25.0 ± 5.8**	2.6 ± 1.9$^{\#\#}$,*	43.5 ± 11.9**,†
Tubulointerstitial injury score (0–5)	0 ± 0	2.4 ± 0.2**	0.7 ± 0.4$^{\#}$,*	2.7 ± 0.4**,†

Abbreviations: Anti-GBM/EGCG, anti-glomerular basement membrane antibody-induced glomerulonephritis pretreated with (−) epigallocatechin-3-gallate; anti-GBM/EGCG+GW, anti-glomerular basement membrane antibody-induced glomerulonephritis pretreated concomitantly with (−) epigallocatechin-3-gallate and PPARγ antagonist GW9662; anti-GBM/Vehicle, anti-glomerular basement membrane antibody-induced glomerulonephritis pretreated with vehicle.
Values are mean ± s.e.m.; n=6 or 7 in each group.
*$P < 0.05$, **$P < 0.01$ vs normal control.
$^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs anti-GBM/vehicle.
$^{\dagger}P < 0.05$ vs anti-GBM/EGCG.

that the increased levels of NO metabolites in the vehicle-treated mice with anti-GBM-GN is unrelated to eNOS activity and must be because of the observed upregulation of iNOS. Similarly, the reduction in the level of NO metabolites in the EGCG-pretreated mice appears to be because of downregulation of iNOS. Accordingly, increased NO production and the associated nitrosative stress in anti-GBM-GN is because of the induction of iNOS in inflammatory and resident cells.[20] This is supported by parallel reductions of iNOS and inflammatory cells in kidneys of the EGCG-pretreated mice. In addition, direct removal of peroxynitrite and other reactive nitrogen species by EGCG may have contributed to the reduction of tissue and urinary NO metabolites.[21]

NF-κB plays a critical regulatory role in inflammation and immune responses and its activation has been implicated in experimental GN.[22–24] In the vehicle-pretreated mice, there was diffuse and strong p65/NF-κB expression in renal cortex with the immunoreactivity localized both in cytoplasm and nucleus, the latter signifying NF-κB activation. Conversely, in the EGCG-pretreated mice, there was mild focal p65/NF-κB expression primarily in the cytoplasm. These results are

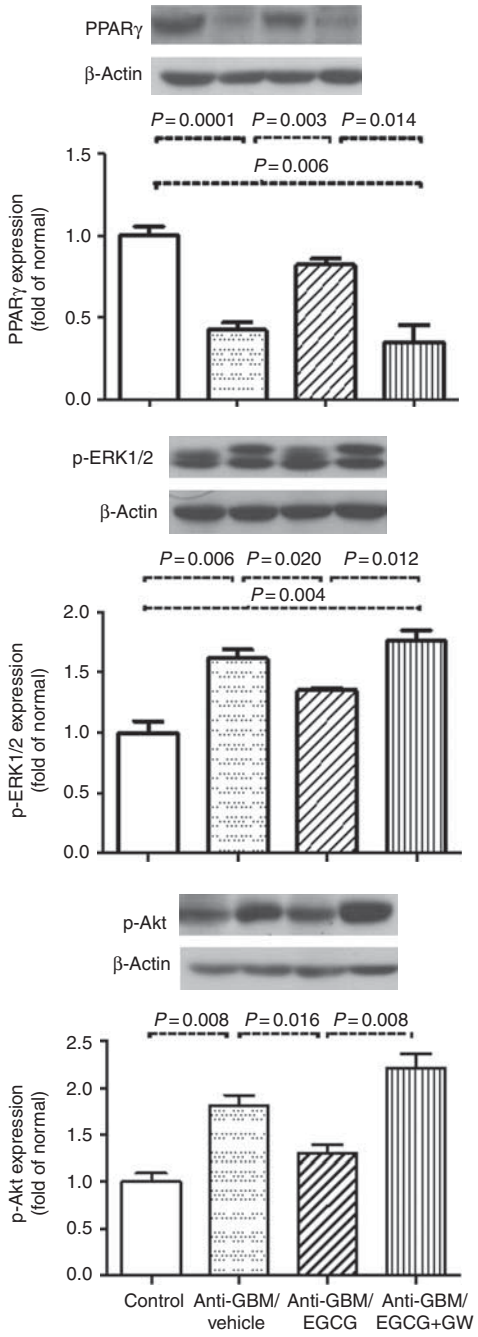

Table 3 | EGCG administration after the development of anti-GBM-GN ameliorates disease severity

	Control (*n*=8)	Anti-GBM/ vehicle (*n*=12)	Anti-GBM/ EGCG (*n*=10)
Body weight (g)			
Baseline	19.8 ± 0.3	20.0 ± 0.5	19.6 ± 0.4
Day 28	20.0 ± 0.2	19.4 ± 0.5	19.0 ± 0.5
Serum creatinine (mg/dl)			
Baseline	0.073 ± 0.004	0.071 ± 0.003	0.074 ± 0.004
Day 7	0.077 ± 0.005	0.181 ± 0.033**	0.153 ± 0.058**
Day 28	0.086 ± 0.007	0.274 ± 0.044**	0.151 ± 0.043*,#
Proteinuria (mg per 24 h)			
Baseline	0.61 ± 0.07	0.73 ± 0.13	0.64 ± 0.12
Day 7	0.74 ± 0.12	14.47 ± 2.39**	13.28 ± 2.73**
Day 28	0.67 ± 0.14	13.06 ± 2.15**	6.62 ± 1.53*,#
Number of death/ mortality rate	0/0%	5/42%**	1/10%*,#

Abbreviations: Anti-GBM/EGCG, (−) epigallocatechin-3-gallate treatment started 7 days after the induction of anti-glomerular basement membrane glomerulonephritis; anti-GBM/vehicle, vehicle started 7 days after induction of anti-glomerular basement membrane glomerulonephritis; GN, glomerulonephritis.
Values are mean ± s.e.m.
*$P<0.05$, **$P<0.01$ vs normal control.
#$P<0.05$ vs anti-GBM/vehicle.

Figure 7 | Western blot analyses of peroxisome proliferator-activated receptor-γ (PPARγ), phosphorylated extracellular signal-regulated kinases 1 and 2 (p-ERK1/2), and p-Akt in renal tissues from mice with anti-glomerular basement membrane antibody-induced glomerulonephritis (anti-GBM-GN) and normal control. To examine whether PPARγ pathway is essential for the renoprotective effect of (−)-epigallocatechin-3-gallate (EGCG), a subgroup of EGCG-pretreated mice with anti-GBM-GN was concomitantly administered with a selective PPARγ antagonist GW9662 following the same time schedule (day −2 to day 14). Western blot analyses showed significantly lower expression of PPARγ in the vehicle-pretreated mice compared with both the normal control and EGCG-pretreated mice. Co-administration of GW9662 abolished the EGCG-induced upregulation of PPARγ. Western blot analyses showed significantly higher expression of p-Akt and ERK1/2 in the vehicle-pretreated mice compared with both the normal control mice and EGCG-pretreated mice. Interestingly, co-administration of GW9662 abolished the EGCG-induced inactivation of p-Akt and p-ERK1/2. Values are expressed as mean ± s.e.m. $n=7$ in each group.

consistent with the reported inhibitory effects of EGCG on NF-κB, such as in a rat model of diabetic nephropathy.[25] Normally, translocation of NF-κB to the nucleus is prevented by binding to its inhibitor, IκB. Phosphorylation of IκB by IκB kinase causes ubiquitination and degradation of IκB and translocation of NF-κB to the nucleus.[26] This process is triggered by ROS and inflammatory cytokines, accounting for the observed changes in NF-κB distribution in our vehicle- and EGCG-pretreated animals. In addition, EGCG may decrease IκB kinase activity, thereby blocking nuclear translocation of NF-κB and expression of its downstream gene products including iNOS.[27]

OPN is a secreted phosphoprotein and chemokine that promotes formation and progression of crescentic GN in human and animal models.[28–31] Our vehicle-pretreated mice showed diffuse and strong OPN expression, whereas mild and focal OPN expression was noted in the EGCG-pretreated mice. As expression of this chemokine is induced by inflammation, our observations reflect florid inflammation in the vehicle-pretreated mice and its attenuation with EGCG pretreatment.

PPARγ belongs to a family of nuclear hormone receptors with protective role in kidney diseases.[32] PPARγ agonists attenuate nephropathy in experimental models of anti-GBM-GN,[33] lupus erythematosus, type 2 diabetes, and nondiabetic glomerulosclerosis, and decrease proteinuria in patients with type 2 diabetes and nondiabetic renal disease.[34–38] Our study shows that PPARγ expression is downregulated in mice with anti-GBM-GN and is restored by EGCG pretreatment. Furthermore, concomitant administration of EGCG with a selective PPARγ antagonist GW9662 prevented the EGCG-induced upregulation of PPARγ and reversed the renoprotective effect of EGCG, pointing to the essential role of PPARγ pathway in mediating the anti-inflammatory capacity of EGCG. It has been shown that EGCG inhibits the mitogen-activated protein kinase pathways and that pretreatment with EGCG prevents ultraviolet B-induced H_2O_2 production and inhibits ultraviolet B-induced phosphorylation of ERK1/2, c-Jun N-terminal kinase, and p38 in cultured human epidermal keratinocytes.[39] Therefore, restoration of renal PPARγ expression by EGCG may be related to suppression of ERK1/2 activation, which is consistent with the observed reduction of p-ERK1/2 in EGCG-pretreated mice. Additionally, inhibition of mitogen-activated protein kinases by EGCG could inhibit the phosphatidylinositol 3-kinase/Akt pathway,[26] which is consistent with the observed reduction of p-Akt in the EGCG-pretreated mice. This is also supported by a recent study showing attenuation of inflammation in MRL/lpr mouse mesangial cells via inhibition of the Akt pathway by EGCG.[40]

In summary, our data suggest that EGCG ameliorates laboratory and histological abnormalities in this mouse model of immune-mediated GN. These salutary effects of EGCG are likely mediated by its anti-inflammatory and antioxidative properties. These observations demonstrate the potential utility of EGCG as a therapeutic agent for the treatment of immune-mediated GN and other immune-mediated diseases.

MATERIALS AND METHODS

Animal model and experimental design

Anti-GBM serum was generated by Lampire Laboratories (Pipersville, PA).[41,42] Preimmune rabbit serum was used as negative control. All studies were reviewed and approved by the institutional review committee at UT Southwestern. Anti-GBM-GN was induced in male 129/svJ mice (Jackson Laboratories, Bar Harbor, ME).[41–44] Briefly, 40 8-week-old mice were presensitized on day −5 (5 days before inducing anti-GBM-GN) with rabbit IgG (120 μg per mouse) in complete Freund's adjuvant (Sigma, St Louis, MO). On day 0, the mice received anti-GBM serum; 120 μg of total IgG in a 100 μl volume was administered intravenously. This dosing regimen was sufficient to induce proteinuria but not mortality. All mice were maintained in a specific pathogen-free colony.

Anti-GBM antibody injected mice were randomly assigned to either the vehicle or EGCG-pretreated (Sigma-Aldrich, St Louis, MO) group. The latter group ($n = 14$) received 50 mg/kg/day[45] of EGCG orally initiated on day −2 (2 days before inducing anti-GBM-GN) and continued until killing on day 15. Normal saline was given to the vehicle group ($n = 14$) with the same protocol. A group ($n = 14$) without anti-GBM antibody or EGCG administration was included as normal control. On day −5 (baseline) and on day 14, sera and 24-h urine samples were collected from all mice using metabolic cages, with free access to drinking water. All animals were killed on day 15. The kidneys were processed for further analyses described below.

A subgroup of EGCG-pretreated mice with anti-GBM-GN was concomitantly administered a selective PPARγ antagonist GW9662 (Sigma) following the same schedule (day −2 to day 14).

To explore the therapeutic effect of EGCG, 22 anti-GBM antibody-injected mice were allowed to develop full-blown nephritis (for 7 days) before intervention. In order to produce more severe GN, the mice received a higher dose of anti-GBM serum (140 μg of total IgG per mouse). On day 7 following anti-GBM administration, the mice were randomized into either EGCG- or vehicle-treated group. The former group ($n = 10$) received 50 mg/kg/day of EGCG orally (gavage) for 3 weeks (day 7 to day 28) until killing on day 29. Normal saline was given to the vehicle group ($n = 12$) with the same protocol. A group ($n = 8$) without anti-GBM antibody or EGCG administration was included as normal control. On days 7 and 28, sera and 24-h urine samples were collected from all mice. All animals were killed on day 29.

Measurement of urine protein and serum creatinine

All urine samples were centrifuged at 14,000 r.p.m. for 5 min. Clear supernatant was used for urine protein assay (Coomassie Plus protein assay kit, Cat. no. 23236; Pierce, Rockford, IL). The serum creatinine levels were measured as previously described.[46]

Measurement of systemic immune response to injected anti-GBM antibodies

Mouse antibodies to rabbit immunoglobulin were assayed by enzyme-linked immunosorbent assay as described previously.[42] Briefly, purified rabbit Ig (Sigma) was coated onto Immulon I plates (Dynatech, Chantilly, CA) and then blocked. Serially diluted mouse sera were added to the plates and any bound mouse anti-rabbit Ig was detected using alkaline-phosphatase-conjugated goat

anti-mouse IgG (Roche, Branchburg, NJ) that did not crossreactive with rabbit Ig and P-nitrophenyl phosphate substrate (Sigma). Color development was measured spectrophotometrically at 405 nm. All assays were run in duplicate, and samples were reanalyzed when standard errors $>10\%$ were found.

Measurement of markers of oxidative stress and antioxidant system

Total concentration of NO metabolites in serum, urine, and renal cortical tissue was determined with the QuantiChrom Nitric Oxide Assay Kit (BioAssay Systems, Hayward, CA).[42] Renal tissue samples were first rinsed with phosphate-buffered saline (pH 7.4, containing 0.1 mg/ml heparin) three times to remove red blood cells and clots.

Catalase activity in renal tissue was measured with the catalase assay kit (ab83464; Abcam, Cambridge, MA) according to the manufacturer's protocol. Renal tissue was homogenized in 200 µl cold assay buffer and centrifuged for 10 min at 14,000 r.p.m. Clear supernatant was used for the assay.

H_2O_2 was determined in urine and renal tissue with peroxide assay kit of BioAssay Systems that utilizes the chromogenic Fe^{3+}-xylenol orange reaction, in which a purple complex is formed when Fe^{2+} is oxidized to Fe^{3+} by H_2O_2. The intensity of the color (at 540–610 nm) is an accurate measure of the H_2O_2 level.

Lipoperoxides in serum, urine, and renal tissue were determined by measurement of malondialdehyde-thiobarbituric acid with a TBARS assay kit (Cat. no. 10009055; Cayman Chemical Company, Ann Arbor, MI), according to the manufacturer's protocol.

Renal histopathology and immunohistochemistry

Next, 3 µm sections of formalin-fixed and paraffin-embedded kidney tissues were cut and stained with hematoxylin and eosin and periodic acid-Schiff reaction. These sections were examined in a blinded fashion. The GN was graded on 0–4 scale and the tubulointerstitial injury was graded on 0–5 scale, as detailed previously.[42,47]

The numbers of intrarenal leukocytes within the glomeruli and interstitium were counted by staining formalin-fixed and paraffin-embedded tissue sections with antibodies to lymphocytes (CD3, Cat. no. MCA1477, 1:500; AbD Serotec, Raleigh, NC) and macrophages (Iba1, Cat. no. 019-19741, 1:500; Wako Chemicals, Richmond, VA). The expression and localization of p65/NF-κB (Cat. no. SC-109, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA), OPN (Cat. no. SC-21742, 1:200; Santa Cruz Biotechnology), and nitrotyrosine (Cat. no. SC-101358, 1:200; Santa Cruz) were also evaluated. Standard avidin-biotin complex method was used for immunohistochemical staining, which was estimated from 0 to 3 as described previously.[47]

Western blot

Western blotting was performed as previously described.[48] Briefly, 40 µg total protein was size-fractionated and transferred onto nitrocellulose membrane (Hybond-ECL; Amersham, Buckinghamshire, UK) at 350 mA for 2.5 h using the Mini Trans-Blot system (Bio-Rad, Hercules, CA). The membrane was prehybridized in 10 ml blocking buffer (1 × Tris buffered saline, 0.1% Tween-20, and 5% nonfat milk powder) for 1 h and then hybridized overnight at 4 °C with the following primary antibodies (diluted with the same buffer): anti-eNOS (Cat. no. 610296, 1:5000; BD Biosciences, San Jose, CA), anti-iNOS (Cat. no. ab3523, 1:2000; Abcam), anti-nitrotyrosine (1:2000), anti-p65/NF-κB (1:2000), anti-phosphorylated Akt (Ser 473, 1:1000; Cell Signaling, Danvers, MA), anti-phosphorylated ERK1/2 (Thr202/Tyr204, 1:1000, Cat. no. 9101), anti-HO-1 (Cat. no. ab13243, 1:5000; Abcam), anti-PPARγ (Cat. no. ab19481, 1:1000; Abcam), anti-NADPH oxidase p47phox (Cat. no. SC-14015, 1:500; Santa Cruz), anti-SOD-1 (Cat. no. sc-271014, 1:1000; Santa Cruz), anti-MPO (Cat. no. ab45977, 1:1000; Abcam), or anti-GPx1 (Cat. no. ab22604, 1:5000; Abcam). The membrane was washed for 30 min in a shaking bath (the wash buffer, Tris-buffered saline and Tween-20, was changed three times every 10 min) and then incubated for 1 h with block buffer plus anti-mouse or anti-rabbit IgG tagged with horseradish peroxidase at a final titer of 1:5000. The washes were repeated before the membrane was developed with a light emitting nonradioactive ECL reagent (SuperSignal West Dura kit; Thermo Scientific, Rockford, IL) and subjected to autoluminography for 5 min. The band intensities were measured using Scion Image (WinB403; http://rsb.info.nih.gov/nih-image/).

Statistical analyses

Data are presented as mean ± s.e.m. Analysis of variance, Fisher's exact test, and Student's *t*-test were used in statistical evaluation as appropriate. The *P*-values of $\leqslant 0.05$ were considered significant.

DISCLOSURE

All the authors declared no competing interests.

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