Genetic Loci Modulate Macrophage Activity and Glomerular Damage in Experimental Glomerulonephritis

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ABSTRACT

The Wistar Kyoto (WKY) rat is uniquely susceptible to experimentally induced crescentic glomerulonephritis. Two major quantitative trait loci (QTLs) on chromosomes 13 (Crgn1) and 16 (Crgn2) with logarithm of odds >8, as well as five other loci (Crgn3 through 7), largely explain this genetic susceptibility. To understand further the effects of Crgn1 and Crgn2, we generated a double-congenic strain by introgressing these loci from glomerulonephritis-resistant Lewis rats onto the WKY genetic background. Induction of nephrotoxic nephritis in the double-congenic rats (WKY.LCrgn1,2) produced markedly fewer glomerular crescents, reduced macrophage infiltration, and decreased expression of glomerular TNF-α and inducible nitric oxide synthase expression compared with control animals. Bone marrow and kidney transplantation studies between parental and WKY.LCrgn1,2 strains, together with in vitro experiments, demonstrated that Crgn1 and Crgn2 contribute exclusively to circulating cell-related glomerular injury by regulating macrophage infiltration and activation. The residual genetic susceptibility to crescentic glomerulonephritis in WKY.LCrgn1,2 rats associated with macrophage activity (especially with enhanced metalloelastase expression) rather than macrophage infiltration. Taken together, these results demonstrate that a genetic influence on macrophage activation, rather than number, determines glomerular damage in immune-mediated glomerulonephritis.


Glomerulonephritis is a major cause of renal failure. Its most severe form is crescentic glomerulonephritis (Crgn), in which damage to glomerular capillaries leads to accumulation of inflammatory cells and proliferating epithelial cells in Bowman’s space. When untreated, Crgn rapidly progresses to irreversible renal scarring and end-stage renal failure.1 In rodents, there are marked strain differences in susceptibility to Crgn.2 To elucidate the genes that control susceptibility we have studied the model of nephrotoxic nephritis (NTN) in the WKY rat. NTN is induced by an intravenous injection of rabbit anti-rat glomerular basement membrane (GBM) antiserum. In the WKY rat strain, this leads to cellular crescents in almost all glomeruli by day 10, whereas Lewis (LEW) rats that share the same MHC haplotype (RT1-l) develop only mild glomerular hypercellularity with no crescents. The model is very reproducible, and the histology closely resembles that seen in human Crgn.3

In bone marrow (BM) and kidney transplant experiments, we previously showed that susceptibility to NTN in the WKY rat depends on both circulating and intrinsic renal factors.4 Indeed, macrophages...
from WKY rats show a number of differences compared with those from LEW rats, including enhanced Fc receptor–mediated functions such as antibody-dependent cytotoxicity.\(^5,6\) In addition, WKY glomerular mesangial cells showed increased monocyte chemotractant protein 1 (MCP-1) synthesis when compared with LEW ones.\(^4\) To determine the genes that are responsible for susceptibility to Crgn and for these cellular phenotypes, we carried out a genome-wide linkage analysis of an F2 population derived from WKY and LEW rats and identified seven Crgn quantitative trait loci (QTLs; Crgn1 through 7).\(^3\) Two major QTLs, each with highly significant logarithm of odds (LOD) scores >8, mapped on chromosomes 13 (Crgn1) and 16 (Crgn2).\(^3\) We have identified genes at each of these QTLs that control macrophage function in vitro. We showed that WKY rats lack an Fc receptor gene, Fcgr3-related sequence on chromosome 13, and that this is, in part, the cause of enhanced macrophage Fc receptor–mediated activity. At the chromosome 16 QTL, we identified the AP-1 transcription factor JunD and showed that there was increased synthesis of JunD in WKY compared with LEW macrophages, and this was responsible for enhanced oxygen burst activity and inducible nitric oxide synthase (iNOS) synthesis in WKY BM-derived macrophages (BMDMs).

Congenic strains have been widely used in the fine mapping of rodent QTLs in various studies using rat models, and they still constitute a powerful tool in QTL positional cloning.\(^7,8\) To examine further the effects of these genetic loci on chromosomes 13 and 16, we bred congenic rats. We have already reported the phenotypic effect of Crgn2 on NTN-related phenotypes and demonstrated that congenic rats in which LEW Crgn2 was introgressed into the WKY genetic background (WKY.LCrgn2) showed reduced glomerular crescents, fibrinoid necrosis, and macrophage infiltration.\(^8\) We have now investigated the interaction between the two major Crgn loci (Crgn1 and Crgn2) by generating a double-congenic rat strain in which both Crgn1 and Crgn2 from NTN-resistant WKY rats were introgressed into the genetic background of the WKY rat (WKY.LCrgn1L2). Our results show that Crgn1 and Crgn2 have an additive protective effect against glomerular crescent formation. We then used BM and kidney transplants to show that the major effects of these loci are on regulation of macrophage activation/infiltration rather than intrinsic renal cell function. We also showed that the residual susceptibility to Crgn in WKY.LCrgn1L2 is associated with differences in macrophage function compared with LEW and, in particular, that there is enhanced macrophage metalloelastase (matrix metalloproteinase 12 [MMP-12]) expression.

**RESULTS**

We previously generated a single-congenic strain for the chromosome 16 congenic interval (WKY.LCrgn2) and assessed Crgn susceptibility in this strain compared with WKY rats.\(^6\) Here, we analyzed the single effect sizes of Crgn1 and Crgn2 individually and investigated the combined effect of these two loci on Crgn susceptibility. A double-congenic strain for Crgn1 and Crgn2 was generated by introgressing the corresponding chromosome 13 and chromosome 16 segments from the donor LEW strain into the genetic background of the WKY strain (Figure 1). NTN was induced in WKY, WKY.LCrgn2, WKY.LCrgn1, and the double-congenic rats (WKY.LCrgn1L2; Figure 2). Introgression of LEW Crgn2 into the WKY recipients reduced glomerular crescents by 8%, and the single phenotypic effect of LEW Crgn1 corresponded to a reduction of 18%. Introgression of both LEW Crgn1 and Crgn2 into the WKY genetic background reduced glomerular crescents by 34%, demonstrating an additive effect of both Crgn loci on glomerular crescent formation (Figure 2, A and D). Macrophage infiltration, assessed by the measurement of ED–1–positive cells per glomerular cross-section, showed a significant reduction for WKY.LCrgn1L2 rats when compared with parental WKY strain (Figure 2, B and D). The interstitial macrophage numbers were not different between the WKY and all congenic strains (Supplemental Figure 1). Proteinuria was also measured in WKY and

![Figure 1](image-url) Genetic map showing the transferred chromosomal segments in congenic lines. Map distances are based on the SHRSP × BN genetic map (http://rgd.mcw.edu/) and are in cM. ■, Chromosomal region transferred from the donor LEW strain (designated as LL); □, chromosomal region transferred from the recipient WKY strain (designated as WW); △, recombination zone. Microsatellite markers D13Rat66 and D16Rat78 are underlined and represent the peak of linkage for Crgn1 and Crgn2, respectively.
Figure 2. NTN-related phenotypes and macrophage activation in single- and double-congenic lines (WKY, n = 6 rats; all congenics, n = 8 rats per strain). (A) Percentage of glomerular crescents in single-congenic (WKY.LCrgn1, WKY.LCrgn2) and double-congenic (WKY.LCrgn1,2) rats in comparison with parental WKY rats. (B) Macrophage infiltration assessed by quantitative measurement of percentage of ED-1+ area per glomerular cross-section (gcs). (C) Proteinuria levels measured in WKY and single- and double-congenic lines. (D) Histology showing a crescentic glomerulus in a WKY rat and a mildly hypercellular glomerulus in a WKY.LCrgn1,2 rat (hematoxylin and eosin). ED-1 immunohistochemistry demonstrates extensive glomerular monocyte and macrophage infiltration in WKY, whereas WKY.LCrgn1,2 rats display reduced staining. (E and F) Glomerular TNF-α (E) and iNOS (F) expression as assessed by qRT-PCR 10 days after injection of NTS (n = 6 rats per strain). (G) Macrophage activation was assessed in WKY, LEW, and congenic BMDMs by Fc receptor–mediated phagocytosis and oxidation (n = 5 rats per strain, without NTN induction). BMDMs are stimulated with Fc oxyBURST, and the WKY rat shows significantly more activation than all of the other strains at all time points (P < 0.001; error bars, SEM). (H) Sandwich ELISA for secretion of TNF-α in LPS-stimulated (100 ng/ml) parental and single- and double-congenic BMDMs (n = 6 rats per strain). **P < 0.01, *P < 0.05 versus WKY. Magnification, ×200 in D.

single- and double-congenic rats, and we confirmed our previous finding that introgression of LEW Crgn2 on a WKY genetic background did not significantly reduce proteinuria levels; however, introgression of Crgn1 led to reduced proteinuria levels in both WKY.LCrgn1 and WKY.LCrgn1,2 rats (Figure 2C). Because infiltration of macrophages into the glomeruli is the main source of iNOS9,10 and TNF-α,11,12 we then investigated iNOS and TNF-α expression in the glomeruli extracted 10 days after NTN induction, as a measure of macrophage activation within the inflamed glomeruli. The results showed that WKY.LCrgn1,2 animals had significantly reduced glomerular TNF-α (Figure 2E) and iNOS expression (Figure 2F) compared with parental WKY rats and single-congenic rats 10 days after NTN induction, confirming the combined protective effect of Crgn1 and Crgn2 on glomerular inflammation. To examine the effect of these loci on macrophage function, we studied macrophage activation in primary parental and congenic macrophages by Fc receptor–mediated phagocytosis and oxidation (Fc oxyBURST assay; Figure 2G). These results showed that when LEW Crgn1 and/or Crgn2 is introgressed to the WKY genetic background, the Fc receptor–mediated macrophage activation is similar to that observed in LEW BMDMs (Figure 2G). Furthermore, BMDMs from single- and double-congenic animals secrete reduced levels of TNF-α after LPS stimulation when compared with WKY rats (Figure 2H). We additionally assessed macrophage activation by measuring mRNA levels of IL-6 and iNOS after LPS stimulation. Quantitative real-time PCR (qRT-PCR) results confirmed increased BMDM iNOS expression in the WKY macrophages when compared with LEW strain6 and showed that both Crgn1 and Crgn2 control macrophage activation, because WKY. L.Crgn1, WKY.L.Crgn2, and WKY.LCrgn1,2 animals had similar BMDM iNOS (Supplemental Figure 2a) and IL-6 (Supplemental Figure 2b) expression as the NTN-resistant LEW rats.
To assess the relative effects of these loci on circulating cells and on intrinsic renal cells, we carried out BM and kidney transplant experiments. We previously showed that in WKY rats that were given isologous WKY BM, glomerular crescent formation in NTN was similar to that in unmanipulated WKY rats and that no crescents were seen in LEW rats given isologous BM.\(^4\) When BM was transferred from WKY.\(L^{Crgn1,2}\) to WKY rats (WKY.\(L^{Crgn1,2}\) \(\rightarrow\) WKY), followed by induction of NTN, the rats showed similar glomerular crescent formation to WKY.\(L^{Crgn1,2}\) animals that did not receive a transplant (\(P = 0.28\)), suggesting that \(Crgn1\) and \(Crgn2\) exert their effect primarily through effects on BM-derived cells and not intrinsic renal cells (Figure 3A). In keeping with this, NTN induction in WKY.\(L^{Crgn1,2}\) kidneys transplanted to WKY rats led to as many crescents in the transplanted kidney as in the native WKY kidney (Figure 3B), demonstrating that there is no effect of these loci on intrinsic renal susceptibility to crescent formation. We also tested the effect of \(Crgn1\) and \(Crgn2\) on mesangial cell MCP-1 production by ELISA (Figure 3C) and qRT-PCR (Figure 3D). Although we confirmed the previously shown MCP-1 production differences between inbred WKY and LEW rats, these results demonstrate that neither \(Crgn1\) nor \(Crgn2\) contributes to mesangial cell MCP-1 production (Figure 3, C and D), suggesting that these loci do not affect intrinsic renal cell function.

The BM transplant experiments also shed light on the role of \(Crgn\) genes outside of \(Crgn1\) and \(Crgn2\). We compared crescent formation in WKY rats that received a transplant of BM from LEW rats or from double-congenic WKY.\(L^{Crgn1,2}\) rats. We found that, although WKY.\(L^{Crgn1,2}\) \(\rightarrow\) WKY rats developed a significantly increased number of glomerular crescents when compared with LEW \(\rightarrow\) WKY rats (\(P < 0.01\); Figure 3A), glomerular macrophage numbers were not different (\(P = 0.32\)) in the two groups (Figure 4A). This suggests that there is a difference in the phenotype of the macrophages that leads to more glomerular injury even though the numbers of macrophages are similar. MMP-12 is predominantly expressed in mature tissue macrophages\(^{13}\) and is a major factor for glomerular injury in anti-GBM nephritis.\(^{14,15}\)

On the basis of these findings, we hypothesized that MMP-12 expression differences in parental and congenic primary macrophages could partly explain the observed macrophage infiltr-
A strong positive correlation (Figure 3A), and this was confirmed by Figure 3B. We measured MMP-12 expression in glomeruli from three groups of BM-transplanted rats after NTN induction: LEW → WKY, WKY.LCrgn1,2 → WKY, and LEW → WKY.LCrgn1,2 (Figure 5A). The MMP-12 expression profile was found to mirror the severity of glomerular crescent formation in the three groups (Figure 3A), and this was confirmed by a strong positive correlation ($R = 0.61, P < 0.001$) between MMP-12 expression and percentage of glomerular crescents (Figure 5B). We also studied MMP-9 because this was previously reported for its capacity to degrade constituents of GBM such as type IV collagen and to play a protective role in anti-GBM nephritis model in the mouse. Although WKY and WKY.LCrgn1,2 BMDMs express relatively increased MMP-9 when compared with LEW, we found that glomerular MMP-9 expression did not correlate with the glomerular crescent formation in the transplant groups (Supplemental Figure 3).

It was previously reported that CD8$^+$ cells infiltrate the glomerulus at an early stage in the course of NTN, and our previous studies showed that they represent a subset of ED-1$^+$ macrophages. We therefore asked whether there was a difference in the infiltration of CD8$^+$ cells in WKY.LCrgn1,2 → WKY kidneys compared with LEW → WKY. We found similar numbers of CD8$^+$ cells in the WKY.LCrgn1,2 → WKY and LEW → WKY glomeruli after NTN induction (Supplemental Figure 4), and CD8 infiltration was markedly reduced compared with ED-1$^+$ cells 10 days after the nephrotoxic serum (NTS) injection.

**DISCUSSION**

Our previous work identified seven QTLs that control susceptibility to Crgn in the WKY rat. The QTLs with the highest LOD scores were on chromosomes 13 and 16, and we have designated these Crgn1 and Crgn2. We have now generated congenic rats in which we introgressed these loci from the resistant LEW strain into the WKY genetic background and also generated a double-congenic strain, WKY.LCrgn1,2. This has allowed us to assess the magnitude of the effect of these QTLs on Crgn susceptibility, to examine the effect on cellular phenotypes, and to elucidate the possible role of the other QTLs (Crgn3 through 7). Our previous studies showed a modest protective effect of Crgn2 on NTN-related phenotypes, confirming the previously established linkage of Crgn2 on chromosome 16. This work has provided direct evidence of additive protective effects of Crgn1 and Crgn2 on glomerular crescent formation. In the double-congenic rats, we found a reduction of 34% in crescent formation compared with WKY rats.

We previously showed that susceptibility to Crgn in the WKY rat was associated with differences in the phenotypes of macrophages and of glomerular mesangial cells. Our results in the congenic rats suggest that Crgn1 and Crgn2 exert their effects predominantly on BM-derived cells and specifically on macrophages. Thus, when WKY rats were given BM transplants from double-congenic rats, they developed similar numbers of crescents in NTN to double-congenic animals, whereas kidneys from double-congenic animals transplanted into WKY rats developed the same number of crescents in NTN as the native kidneys.

There is a clear effect of Crgn1 and Crgn2 on macrophage phenotype, because both loci regulate glomerular macrophage infiltration and control IL-6 and iNOS expression in...
BM-derived macrophages. In contrast, neither Crgn1 nor Crgn2 controls the enhanced mesangial cell MCP-1 synthesis seen in WKY rats. These findings are entirely consistent with our positional cloning studies focusing on Crgn1 and Crgn2. As we previously showed, Crgn1 includes the gene coding for the α subunit of the activatory Fcγ receptor, Fcγ3. We showed that most laboratory rat strains express two forms of Fcγ3 but that in the WKY rat, a newly identified paralogue, Fcγ3-related sequence (Fcγ3-rs), was absent. We then established an inhibitory role for Fcγ3-rs in macrophage activation, because COS7 cells co-transfected with Fcγ3 and Fcγ3-rs showed 70% inhibition of Fcγ3-mediated phagocytosis. This suggests that the deletion of Fcγ3-rs from the WKY genome explains partly the uncontrolled activation in the macrophages of this strain. The locus on chromosome 16 (Crgn2) contains the AP-1 transcription factor Jund, which is highly expressed in the macrophages (but not in mesangial cells) of the NTN-susceptible WKY rat. We also established an important role for Jund in macrophage activation, because knockdown of Jund expression levels by small interfering RNA in WKY BM-derived macrophages reduces Fc receptor–mediated macrophage activation. Together these data indicate that Fcγ3 in Crgn1 and Jund in Crgn2 are susceptibility genes for Crgn and act by regulating macrophage activation.

Although we have shown that Crgn1 and Crgn2 control susceptibility to Crgn through effects on macrophage infiltration and activation, the double-congenic (WKY.LCrgn1,2) strain still showed significant glomerular crescent formation, indicating effects of other loci. In our linkage analysis, we identified five other QTLs, Crgn through 7. This study provides insights into the role of these QTLs. In our BM transplant experiments, we found that WKY rats given LEW BM developed 12% glomerular crescents in NTN, whereas those that received a transplant of WKY.LCrgn1,2 BM developed 52% crescents; however, the numbers of infiltrating glomerular macrophages were very similar. This suggests that the macrophages from the double-congenic animals have a more proinflammatory phenotype. We investigated this by focusing on macrophage activation molecules previously reported to be involved in GBM degradation. MMPs, particularly MMP-12, was previously described to cause glomerular injury in the WKY anti-GBM nephritis model. Unlike MMP-12, MMP-9 showed a protective effect in the accelerated model of crescentic nephritis in the mouse as MMP-9 knockout mice showed exacerbated nephritis with increased crescent formation and fibrin deposits compared with wild-type controls. We showed that MMP-9 and MMP-12 expressions were significantly increased in WKY.LCrgn1,2 and WKY BMMDMs when compared with LEW, demonstrating that they are under the control of genes outside of Crgn1 and Crgn2; however, only MMP-12 expression correlated with the percentage of crescents in WKY glomeruli after transplantation with either LEW or WKY.LCrgn1,2 BM and after NTN induction. Because MMP-12 is mainly produced by macrophages infiltrating the glomerulus in anti-GBM nephritis, our results suggest that macrophage activation status, partly explained by increased MMP-12, contributes to the susceptibility to glomerular injury encoded by Crgn3 through 7. We also investigated whether infiltration of CD8+ cells was the cause of relatively more glomerular crescents in WKY.LCrgn1,2 and WKY BM transplanted to WKY; WKY.LCrgn1,2→WKY, double congeneric BM transplanted to WKY; LEW→WKY.LCrgn1,2, LEW BM transplanted to double congeneric.
different genetic loci. In summary, WKY macrophages show enhanced antibody-dependent cytotoxicity and Fc receptor-mediated phagocytosis controlled by Crgn1, enhanced cytokine and iNOS expression, and respiratory burst controlled by Crgn2, and, as we demonstrate here, enhanced protease synthesis controlled by loci outside Crgn1 or Crgn2. We have now shown that the loci controlling these various macrophage phenotypes have independent and additive effects on Crgn susceptibility. In future work, we aim to identify the genes outside of Crgn1 and Crgn2 that control the residual Crgn susceptibility seen in the double-congenic rats and to determine how they control macrophage accumulation and activation. We also need to identify which genes are responsible for the differences in mesangial cell phenotype between the two strains and how this contributes to susceptibility to Crgn in vivo.

In conclusion, our work emphasizes the importance of macrophage activation in the pathophysiology of Crgn. Understanding the mechanisms of macrophage infiltration and activation within the inflamed glomeruli may ultimately facilitate the design of more rational and targeted treatment of human Crgn.

**CONCISE METHODS**

**Congenic and Control Rat Strains**
WKY (WKY/NCr) rats were purchased from Charles River. Single-congenic rats were generated as described previously.6 We constructed a double-congenic line (i.e., a single strain in which both Crgn1 and Crgn2 were on the WKY genetic background) as follows: WKY/L.Crgn1 and WKY/L.Crgn2 strains were crossed to produce an F1 generation. The F1 rats were backcrossed with WKY/L.Crgn1. The F2 rats heterozygous for Crgn2 and homozygous for Crgn1 were crossed by brother–sister mating to obtain an F3 generation double congenic for LEW Crgn1 and LEW Crgn2 on a WKY background. All procedures were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act.

**Nephrotic Nephritis**
NTN was prepared in rabbits by standard methods. NTN induction of NTN, rats were killed under isoflurane anesthesia. Additionally, we induced in male rats by intravenous injection of 0.1 ml of NTS. Nine days later, urine was collected by placing rats into metabolic cages and cultured for 7 days in DMEM (Life Technologies) that contained Hanks buffer (Life Technologies). Total BM-derived cells were plated on a 48-well Costar plate at a density of 106 cells per well and incubated in 2 ml of culture medium containing 10% FBS (Biosera), penicillin (100 U/ml; Invitrogen), and L-glutamine (2 mM; Invitrogen). These cells were characterized as macrophages by ED-1 staining. BMDMs were made quiescent in serum-free medium for 24 hours and then stimulated with LPS (100 ng/ml). Control macrophages were unstimulated, and iNOS and IL-6 mRNA levels were measured by qRT-PCR. We carried out sandwich ELISA for rat TNF-α (BD Biosciences), in accordance with the manufacturer’s specifications, with supernatants from BMDMs plated in six-well plates at a density of 106 cells per well and incubated in 2 ml of culture medium for 24 hours with LPS (100 ng/ml). For Fc oxyBURST assay, 106 cells (in triplicate) were suspended in Krebs’ Ringer PBS with 1.0 mM Ca2+, 1.5 mM Mg2+, and 5.5 mM glucose; warmed to 37°C; and stimulated with Fc oxyBURST reagent (240 μg/ml; Invitrogen). Indi-

**Histology and Immunohistochemistry**
Crescent formation was assessed by counting the number of crescents in 100 consecutive glomeruli in periodic acid–Schiff–stained sections. Macrophages were identified by immunoperoxidase staining with mAb ED-1 (Serotec, Oxford, UK). Quantification of ED-1–stained macrophages in the renal interstitium was performed by photographing five randomly selected cortical interstitial areas using an Olympus BX40 microscope (Olympus Optical, London, UK) mounted with a Photonic Science Color Coolview digital camera (Photonic Science, East Sussex, UK). The percentage of each of the stained cross-sectional areas was calculated using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD) and was expressed as the mean percentage area stained.

**BMDM Culture and Fc oxyBURST Assay**
Femurs from adult WKY and LEW rats were isolated and flushed with Hanks buffer (Life Technologies). Total BM-derived cells were plated on a 48-well Costar plate at a density of 106 cells per well and incubated in 2 ml of culture medium containing 10% FBS (Biosera), penicillin (100 U/ml; Invitrogen), streptomycin (100 μg/ml; Invitrogen), and l-glutamine (2 mM; Invitrogen). These cells were characterized as macrophages by ED-1 staining. BMDMs were made quiescent in serum-free medium for 24 hours and then stimulated with LPS (100 ng/ml). Control macrophages were unstimulated, and iNOS and IL-6 mRNA levels were measured by qRT-PCR. We carried out sandwich ELISA for rat TNF-α (BD Biosciences), in accordance with the manufacturer’s specifications, with supernatants from BMDMs plated in six-well plates at a density of 106 cells per well and incubated in 2 ml of culture medium for 24 hours with LPS (100 ng/ml). For Fc oxyBURST assay, 106 cells (in triplicate) were suspended in Krebs’ Ringer PBS with 1.0 mM Ca2+, 1.5 mM Mg2+, and 5.5 mM glucose; warmed to 37°C; and stimulated with Fc oxyBURST reagent (240 μg/ml; Invitrogen). Indi-

**RNA Extraction and qRT-PCR**
Total RNA was extracted from isolated glomeruli, mesangial cells, or macrophages using the Trizol method. Total RNA concentration was determined by using Nanodrop spectrophotometer (Labtech Int., Ringmer, UK). TNF-α, iNOS, MCP-1, and IL-6 primers were as follows: TNF-α forward 5′-TGACCCCATATTGAGACCC-3′ and reverse 5′-GCGCAGTGACTTACGGTCTTC-3′, iNOS forward 5′-GGACCACCTCTATGAGGA-3′ and reverse 5′-GGAGCAGCTGACCT-3′; MCP-1 forward 5′-ATGCAGTTAATGCCCCACTC-3′ and reverse 5′-TCTCTATGGGCTGACAC-3′; IL-6 forward 5′-CCGGAGAGGAGACTTCACAG-3′ and reverse 5′-ACATGTCGACTCGCTGTTC-3′; and MMP-12 forward 5′-TGCAGCTGTCTTTGATCCAC-3′ and reverse 5′-GATCAATTITTGCCGCTAT-3′. Real-time RT-PCR was performed on a ABI 7500 Sequence Detection System (Applied Biosystems, Warrington, UK) using SYBR Green (Stratagene, Cambridge, UK). A total of 100 ng of total RNA was used for qRT-PCR, and all of the samples were amplified in triplicate. After the initial reverse transcription (30 minutes at 50°C and 10 minutes at 95°C), the samples were cycled 40 times at 95°C for 15 seconds and 60°C for 1 minute. Results were then exported to 7500 Fast system SDS software (ABS), and Ct values were determined for all of the genes analyzed. The relative expression levels normalized to glyceraldehyde-3-phosphate dehydrogenase gene expression were then determined by using the 2−ΔΔCt method.
vial data points consisting of 10,000 fluorescence events were collected at 0, 15, 45, 75, 90, 105, and 120 seconds in a FACSCalibur after a baseline fluorescence reading was taken to determine the intrinsic fluorescence of unstimulated cells. Percentage of fluorescence BMDMs corresponds to percentage of activated gated cells after Fc receptor–mediated phagocytosis.

Mesangial Cell Culture
Glomeruli from LEW and WKY rats were isolated by sieving. Purified glomeruli were digested with collagenase type 1 (750 U/ml; Sigma) for 20 minutes. Partially digested glomeruli were cultured in 25-cm² tissue culture flasks at 600 glomeruli/ml in RPMI 1640 medium (Invitrogen) that contained 20% decomplemented FBS (F-539), penicillin (100 U/ml; Invitrogen), streptomycin (100 µg/ml; Invitrogen), and l-glutamine (2 mM; Invitrogen) and was supplemented with insulin-transferrin-selenite (Sigma). The cultures were maintained at 37°C with 5% CO₂ for 6 days, allowing glomerular mesangial cells to grow out. Medium was changed every 2 to 3 days thereafter. By days 21 to 28, when the cell outgrowth reached confluence, cells were subcultured. These cells were characterized by immunofluorescence staining using cells that were cultured on coverslips. They were positive for Thy-1.1 antigen, myosin, and desmin and negative for panckytokeratin, OX-1, ED-1, and OX-23.

To make the culture conditions comparable, passage 8 mesangial cells from different strains (n = 4 rats per strain) of rats were plated into six-well culture plates (10⁶ cells/well) at the same time. Confluent allowed to recover for 6 to 8 days before induction of NTN.

BM and Kidney Transplantation
Femurs were removed from donor rats (n = 8 rats). BM was flushed out using RPMI with 10% FBS (Sigma-Aldrich, Poole, UK), 100 U/ml penicillin, and 100 g/ml streptomycin (Invitrogen, Paisley, UK). Cells were then washed, resuspended in fresh medium at 5 × 10⁷ cells/ml, and kept on ice. Recipient rats were irradiated with 12 Gy at 0.74 Gy/min, using gamma rays from a cesium source irradiator (IBL 637; and kept on ice. Recipient rats were irradiated with 12 Gy at 0.74 Gy/min, using gamma rays from a cesium source irradiator (IBL 637; and kept on ice. Recipient rats were irradiated with 12 Gy at 0.74 Gy/min, using gamma rays from a cesium source irradiator (IBL 637; and kept on ice. Recipient rats were irradiated with 12 Gy at 0.74 Gy/min, using gamma rays from a cesium source irradiator (IBL 637; and kept on ice. Recipient rats were irradiated with 12 Gy at 0.74 Gy/min, using gamma rays from a cesium source irradiator (IBL 637; and kept on ice. Recipient rats were irradiated with 12 Gy at 0.74 Gy/min, using gamma rays from a cesium source irradiator (IBL 637; and kept on ice. Recipient rats were irradiated with 12 Gy at 0.74 Gy/min, using gamma rays from a cesium source irradiator (IBL 637; and kept on ice. Recipient rats were irradiated with 12 Gy at 0.74 Gy/min, using gamma rays from a cesium source irradiator (IBL 637; and kept on ice. Recipient rats were irradiated with 12 Gy at 0.74 Gy/min, using gamma rays from a cesium source irradiator (IBL 637; and kept on ice. Recipient rats were irradiated with 12 Gy at 0.74 Gy/min, using gamma rays from a cesium source irradiator (IBL 637; and kept on ice. Recipient rats were irradiated with 12 Gy at 0.74 Gy/min, using gamma rays from a cesium source irradiator (IBL 637; and kept on ice. Recipient rats were irradiated with 12 Gy at 0.74 Gy/min, using gamma rays from a cesium source irradiator (IBL 637; and kept on ice. Recipient rats were irradiated with 12 Gy at 0.74 Gy/min, using gamma rays from a cesium source irradiator (IBL 637; and kept on ice. Recipient rats were irradiated with 12 Gy at 0.74 Gy/min, using gamma rays from a cesium source irradiator (IBL 637; and kept on ice. Recipient rats were irradiated with 12 Gy at 0.74 Gy/min, using gamma rays from a cesium source irradiator (IBL 637; and kept on ice. Recipient rats were irradiated with 12 Gy at 0.74 Gy/min, using gamma rays from a cesium source irradiator (IBL 637; and kept on ice. Recipient rats were irradiated with 12 Gy at 0.74 Gy/min, using gamma rays from a cesium source irradiator (IBL 637; and kept on ice. Recipient rats were irradiated with 12 Gy at 0.74 Gy/min, using gamma rays from a cesium source irradiator (IBL 637; and kept on ice. Recipient rats were irradiated with 12 Gy at 0.74 Gy/min, using gamma rays from a cesium source irradiator (IBL

Statistical Analysis
Statistical differences in mean values between all of the congenic strains and parental WKY rats were compared using one-way ANOVA followed by Bonferroni multiple comparison posttest; P < 0.05 was considered statistically significant. Differences in relative MCP-1 quantities were tested for significance with the nonparametric Wilcoxon signed-rank test. Comparisons between native and transplanted kidney groups were analyzed by Mann-Whitney U test. Correlation between MMP-12 expression and glomerular crescents was analyzed by linear regression.

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DISCLOSURES
None.

REFERENCES


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