

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 (Crgn) and this genetic susceptibility is mainly explained by two major quantitative trait loci (QTLs) (lod>8) on chromosomes 13 (*Crgn1*) and 16 (*Crgn2*) and five other loci (*Crgn3-7*, lod>3). Here, we have generated a double congenic strain by introgressing *Crgn1* and *Crgn2* from Crgn-resistant Lewis (LEW) rats onto the WKY genetic background (WKY.L*Crgn1,2*). WKY.L*Crgn1,2* rats showed a marked reduction in glomerular crescents, macrophage infiltration, and glomerular TNF- α and iNOS expression following induction of nephrotoxic nephritis (NTN). Bone marrow (BM) and kidney transplantation studies between parental and WKY.L*Crgn1,2* strains, together with *in vitro* mesangial cell experiments, demonstrated that *Crgn1* and *Crgn2* contribute exclusively to circulating cell-related glomerular injury by regulating macrophage infiltration and activation. Interestingly, the residual genetic susceptibility to Crgn in WKY.L*Crgn1,2* rats is associated with macrophage activity rather than infiltration, and in particular with enhanced macrophage metalloelastase (MMP-12) expression. These results clearly demonstrate that the genetically controlled activation status of macrophages determines glomerular damage independently of their number and offer a unique model for studying macrophage function in immune-mediated glomerulonephritis.

Key words : Crescentic glomerulonephritis, congenic strains, macrophages, MMP-12

Introduction

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. If untreated, Crgn rapidly progresses to irreversible renal scarring and end stage renal failure¹. In rodents there are marked strain differences in susceptibility to Crgn². In order 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 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 Crgn³.

In bone marrow and kidney transplant experiments, we have previously shown that susceptibility to NTN in the WKY rat is dependent on both circulating and intrinsic renal factors⁴. 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 show increased monocyte chemoattractant protein-1 (MCP-1) synthesis when compared to LEW ones⁴. In order 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 7 crescentic glomerulonephritis quantitative trait loci (QTL) (*Crgn1-7*)⁵. Two major QTLs, each with

highly significant LOD scores greater than 8, mapped on chromosomes 13 (*Crgn1*) and 16 (*Crgn2*)⁵. 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 iNOS synthesis in WKY bone marrow-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 have bred congenic rats. We have already reported the phenotypic effect of *Crgn2* on NTN-related phenotypes and demonstrated that congenic rats where LEW *Crgn2* was introgressed into the WKY genetic background (WKY.L*Crgn2*) showed reduced glomerular crescents, fibrinoid necrosis and macrophage infiltration⁶. We have now investigated the interaction between the two major Crgn loci (*Crgn1* and *Crgn2*) by generating a double congenic rat strain where both *Crgn1* and *Crgn2* from NTN-resistant LEW rats were introgressed into the genetic background of the WKY rat (WKY.L*Crgn1,2*). Our results show that *Crgn1* and *Crgn2* have an additive protective effect against glomerular crescent formation. We then used bone marrow 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 show that the

residual susceptibility to Crgn in WKY.*LCrgn1,2* is associated with differences in macrophage function compared with LEW and, in particular, that there is enhanced macrophage metalloelastase (MMP12) expression.

Results

We have previously generated a single congenic strain for the chromosome 16 congenic interval (WKY.L*Crgn2*) and assessed *Crgn* susceptibility in this strain compared to WKY rats⁶. Here, we have analysed 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.L*Crgn2*, WKY.L*Crgn1* and the double congenic rats (WKY.L*Crgn1,2*) (**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 2a, d**). Macrophage infiltration assessed by the measurement of ED-1 positive cells per glomerular cross section showed a significant reduction for WKY.L*Crgn1,2* rats when compared to parental WKY strain (**Figure 2b, d**). The interstitial macrophage numbers were not different between the WKY and all congenic strains (Supplementary **Figure 1**). Proteinuria was also measured in WKY, single and double congenic rats and we have 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.L*Crgn1* and WKY.L*Crgn1,2* rats (**Figure 2c**). Since macrophages infiltrating the glomeruli are the main source of inducible nitric oxide synthase (iNOS)^{9,10} and tumor

necrosis factor- α (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 congenics, 10d after NTN induction, confirming the combined protective effect of *Crgn1* and *Crgn2* on glomerular inflammation. In order 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* are 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 following LPS stimulation, when compared with WKY rats (**Figure 2h**). We have additionally assessed macrophage activation by measuring mRNA levels of interleukin-6 (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 to LEW strain⁶, and showed that both *Crgn1* and *Crgn2* control macrophage activation, as WKY.*LCrgn1*, WKY.*LCrgn2* and WKY.*LCrgn1,2* animals had similar BMDM iNOS (**Supplementary Figure 2a**) and IL-6 (**Supplementary Figure 2b**) expression as the NTN-resistant LEW rats. In order to assess the relative effects of these loci on circulating cells and on intrinsic renal cells, we carried out bone marrow and kidney transplant experiments. We have previously shown that in WKY rats that were given isologous WKY bone marrow,

glomerular crescent formation in NTN was similar to that in unmanipulated WKY rats and that no crescents were seen in Lewis rats given isologous bone marrow⁴. When bone marrow (BM) was transferred from WKY.L*Crgn1,2* to WKY rats (WKY.L*Crgn1,2* =>WKY), followed by induction of NTN, the rats showed similar glomerular crescent formation to non-transplanted WKY.L*Crgn1,2* animals (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* contribute to mesangial cell MCP-1 production (**Figure 3c,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 *Crgn1* and *Crgn2*. We compared crescent formation in WKY rats transplanted with BM from LEW rats or from double congenic WKY.L*Crgn1,2* rats. We found that, although WKY.L*Crgn1,2* => WKY rats developed a significantly increased number of glomerular crescents when compared to LEW =>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. Macrophage

metalloelastase (MMP-12) is predominantly expressed in mature tissue macrophages¹³, and is a major factor for glomerular injury in anti-glomerular basement membrane (anti-GBM) nephritis^{14,15}. Based on these findings, we hypothesised that MMP-12 expression differences in parental and congenic primary macrophages could partly explain the observed macrophage infiltration-independent glomerular damage. MMP-12 expression was significantly increased in WKY and WKY.*LCr gn1,2* BMDMs when compared with LEW (**Figure 4b**). We then investigated whether MMP-12 expression correlated with crescent formation. We measured MMP-12 expression in glomeruli from three groups of BM-transplanted rats following NTN induction: LEW=>WKY, WKY.*LCr gn1,2* =>WKY and LEW=>WKY.*LCr gn1,2* (**Figure 5a**). The MMP-12 expression profile was found to mirror the severity of glomerular crescent formation in the three transplanted groups (**Figure 3a**) and this was confirmed by a strong positive correlation ($R=0.61$, $p<0.001$) between MMP-12 expression and % of glomerular crescents (**Figure 5b**). We also studied MMP-9 since this was previously reported for its capacity to degrade constituents of glomerular basement membrane (GBM) such as type IV collagen¹⁶ and to play a protective role in anti-GBM nephritis model in the mouse¹⁷. Although WKY and WKY.*LCr gn1,2* BMDMs express relatively increased MMP-9, when compared to LEW, we found that glomerular MMP-9 expression did not correlate with the glomerular crescent formation in the transplanted groups (**Supplementary Figure 3**).

It has been previously reported that CD8⁺ cells infiltrate the glomerulus at an early stage in the course of NTN and our previous studies have shown that they represent a subset of ED1⁺ macrophages³. We therefore asked whether there was a difference in the infiltration of CD8⁺ cells in WKY.*LCr gn1,2* =>WKY kidneys compared to

LEW=>WKY. We found similar numbers of CD8+ cells in the WKY.*LCrgn1,2* => WKY and LEW=>WKY glomeruli following NTN-induction (**Supplementary Figure 4**) and CD8 infiltration was markedly reduced compared to ED1+ cells 10 days following the NTS injection.

Discussion

Our previous work has identified 7 QTLs that control susceptibility to Crgn in the WKY rat⁵. 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 have introgressed these loci from the resistant LEW strain into the WKY genetic background and also generated a double congenic strain, WKY.L*Crgn1,2*. 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 (*Crgn3-7*). Our previous studies showed a modest protective effect of *Crgn2* on NTN-related phenotypes confirming the previously established linkage of *Crgn2* on chromosome 16^{5,6}. The current 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 to WKY rats.

We have previously shown that susceptibility to Crgn in the WKY rat was associated with differences in the phenotypes of macrophages and of glomerular mesangial cells⁴⁻⁶. Our results in the congenic rats suggest that *Crgn1* and *Crgn2* are exerting their effects predominantly on bone marrow-derived cells, and specifically on macrophages. Thus, when WKY rats were given bone marrow transplants from double congenic rats they developed similar numbers of crescents in NTN as 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 since both loci regulate glomerular macrophage infiltration and control IL-6 and iNOS expression in

bone marrow-derived macrophages. In contrast neither *Crgn1* nor *Crgn2* control 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 have previously shown, *Crgn1* includes the gene coding for the alpha subunit of the activatory Fcγ receptor, *Fcgr3*. We showed that most laboratory rat strains express two forms of *Fcgr3*, but that in the WKY rat, a newly identified paralogue, *Fcgr3*-related sequence (*Fcgr3-rs*) was absent⁵. We then established an inhibitory role for *Fcgr3-rs* in macrophage activation as COS7 cells co-transfected with *Fcgr3* and *Fcgr3-rs* showed 70% inhibition of Fcgr3-mediated phagocytosis. This suggests that the deletion of *Fcgr3-rs* from the WKY genome explains partly the unchecked activation in the macrophages of this strain⁵. The locus on chromosome 16 (*Crgn2*) contains the AP-1 transcription factor *Jund* which is highly overexpressed in the macrophages (but not in mesangial cells) of the NTN-susceptible WKY rat⁶. We also established an important role for *Jund* in macrophage activation as knock down of *Jund* expression levels by siRNA in WKY bone marrow derived macrophages reduces Fc-receptor mediated macrophage activation⁶. Together these data indicate that *Fcgr3* in *Crgn1* and *Jund* in *Crgn2* are susceptibility genes for crescentic glomerulonephritis and act by regulating macrophage activation.

Whilst we have shown that *Crgn1* and *Crgn2* control susceptibility to Crgn through effects on macrophage infiltration and activation, the double congenic (WKY.L*Crgn1,2*) strain still showed significant glomerular crescent formation, indicating effects of other loci. In our linkage analysis we identified 5 other QTLs, *Crgn3-7*. Our current study provides insights into the role of these QTLs. In our bone marrow transplant experiments, we found that WKY rats given LEW bone marrow

developed 12% glomerular crescents in NTN whereas those transplanted with WKY.L*Crtn1,2* marrow 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 pro-inflammatory phenotype. We investigated this by focusing on macrophage activation molecules previously reported to be involved in glomerular basement membrane degradation. Matrix metalloproteinases (MMP), particularly the macrophage metalloelastase (MMP-12) was previously described to cause glomerular injury in the WKY anti-GBM nephritis model¹⁴. 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 to wild type controls¹⁷. We showed that MMP-9 and MMP-12 expression were significantly increased in WKY.L*Crtn1,2* and WKY BMDMs when compared to LEW demonstrating that they are under the control of genes outside *Crtn1* and *Crtn2*. However only MMP-12 expression correlated with the % of crescents in WKY glomeruli after transplantation with either LEW or WKY.L*Crtn1,2* BM and following NTN-induction. Since MMP-12 is mainly produced by macrophages infiltrating the glomerulus in anti-GBM nephritis¹⁴, our results suggest that macrophage activation status, partly explained by increased MMP-12, contributes to the susceptibility to glomerular injury encoded by *Crtn3-7*. We also investigated whether infiltration of CD8⁺ cells was the cause of relatively more glomerular crescents in WKY.L*Crtn1,2* =>WKY kidneys compared to LEW=>WKY but found that i) their infiltration was markedly reduced compared to ED1⁺ cells 10 days following the NTS injection and ii) there was no difference in CD8⁺ cell number between the WKY.L*Crtn1,2* => WKY and

LEW=>WKY glomeruli following NTN-induction. Given that our previous studies have shown that CD8+ cells represent a subset of ED1+ macrophages³, these results support the finding that macrophage phenotype is the more likely pathological source of glomerular injury.

Our studies in the WKY rat clearly demonstrate the importance of macrophage phenotype in susceptibility to glomerulonephritis. Previous work has also provided evidence on the heterogeneity of the macrophage activation suggesting that macrophage activation status is as important as their number in the outcome of inflammatory diseases¹⁸⁻²¹. Here, we have shown that there are multiple genetically determined differences between macrophages from WKY and LEW rats and that they are controlled at different genetic loci. In summary, WKY macrophages show enhanced ADCC and Fc-receptor mediated phagocytosis controlled by *Crtn1*, enhanced cytokine and iNOS expression and respiratory burst controlled by *Crtn2* and, as we demonstrate here, enhanced protease synthesis controlled by loci outside *Crtn1* or *Crtn2*. We have now shown that the loci controlling these different macrophage phenotypes have independent and additive effects on Crtn susceptibility. In future work we aim to identify the genes outside *Crtn1* and *Crtn2* that control the residual Crtn 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 Crtn *in vivo*.

In conclusion, our work emphasizes the importance of macrophage activation in the pathophysiology of crescentic glomerulonephritis. 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 crescentic glomerulonephritis.

Methods

Congenetic and control rat strains

Wistar-Kyoto (WKY/NCrl, designated here as WKY) rats were purchased from Charles River. Single congenic rats were generated as previously described⁶. 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 in order 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 Lewis *Crgn1* and Lewis *Crgn2* on a WKY background. All procedures were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act.

Nephrotoxic Nephritis (NTN)

Nephrotoxic serum (NTS) was prepared in rabbits by standard methods. NTN was 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 for 24 h with free access to food and water. Proteinuria was determined by the sulfosalicylic acid method²². On day 10 after induction of NTN, rats were killed under isoflurane anesthesia. Samples of kidney were fixed in 10% formal saline, processed, and embedded in paraffin wax. In some cases, glomeruli were isolated by sieving as described previously²³ and 2000 glomeruli were plated in six-well plates (Nunc, Roskilde, Denmark) in DMEM (Life Technologies, Paisley, UK). After 48h of incubation, glomeruli were collected for quantitative reverse transcription–PCR (qRT-PCR).

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 primer, 5'TGACCCCCATTACTCTGACC-3'; TNF- α reverse primer, 5'-GGCCACTACTTCAGCGTCTC-3', iNOS forward primer, 5'GGACCACCTCTATCAGGAA-3'; iNOS reverse primer, 5'GGAGCACGCTGAACACCT-3'; MCP-1 forward primer, 5'ATGCAGTTAATGCCCCACTC-3'; MCP-1 reverse primer, 5'TTCCTTATTGGGGTCAGCAC-3'; IL-6 forward primer, 5'CCGGAGAGGAGACTTCACAG-3'; IL-6 reverse primer, 5'ACAGTGCATCATCGCTGTTC-3'; MMP-12 forward primer, 5'TGCAGCTGTCTTTGATCCAC-3', MMP-12 reverse primer, 5'GCATCAATTTTTGGCCTGAT-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 min at 50°C and 10 min at 95°C), the samples were cycled 40 times at 95°C for 15 s and 60°C for 1 min. Results were then exported to 7500 Fast system SDS software (ABS), and Ct values were determined for all the genes analysed. The relative expression levels normalised to GAPDH gene expression were then determined by using the $2^{-\Delta\Delta C_t}$ method.

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 5 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 area was calculated using Image Pro-Plus software (Media Cybernetics, Silver Spring, MD) and was expressed as the mean percentage area stained.

BM-Derived Macrophage Culture and Fc oxyBURST assay

Femurs from adult WKY and Lewis rats were isolated and flushed with Hanks buffer (Life Technologies). Total BM-derived cells were plated and cultured for 7 d in DMEM (Life Technologies) that contained 25 mM HEPES (Sigma), 25% L929 conditioned medium, 25% deplete FBS (Biosera, UK), 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 h 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 10^6 cells per well and incubated in 2 ml of culture medium for 24h with LPS (100 ng/ml). For Fc oxyBURST assay, 10^6 cells (in triplicate) were suspended in Krebs' Ringer PBS (KRP buffer) with 1.0 mM Ca^{2+} , 1.5 mM Mg^{2+}

and 5.5 mM glucose, warmed to 37 °C and stimulated with Fc oxyBURST reagent (240µg/ml, Invitrogen). Individual data points consisting of 10,000 fluorescence events were collected at 0, 15, 45, 75, 90, 105, 120 s in a FACSCalibur after a baseline fluorescence reading was taken to determine the intrinsic fluorescence of unstimulated cells. Percentage of fluorescent BMDMs corresponds to percentage of activated gated cells following Fc-receptor mediated phagocytosis.

Mesangial cell culture

Glomeruli from Lewis and WKY rats were isolated by sieving. Purified glomeruli were digested with collagenase type 1 (Sigma; 750 U/ml) for 20 min. Partially digested glomeruli were cultured in 25cm² 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 d, allowing glomerular mesangial cells to grow out. Medium was changed every 2 to 3 d 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 pancytokeratin, OX-1, ED-1, and OX-23.

In order to make the culture conditions comparable, passage 8 mesangial cells from different strains (n=4 rats/strain) of rats were plated into 6-well culture plates (10⁶ cells/well) at the same time. Confluent mesangial cells were stimulated with TNF-α (2 ng/ml, R&D, UK) in serum-free medium. After stimulation for 24 h, mesangial cell

supernatants were harvested and centrifuged to remove cellular contaminants. These supernatants were either examined immediately with sandwich ELISA or stored at -20°C. MCP-1 was measured in the supernatant by sandwich ELISA according to manufacturer's instructions (BD Biosciences, UK).

BM and kidney transplantation

Femurs were removed from donor rats (n=8 rats). Marrow 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 5×10^7 cells/ml, and kept on ice. Recipient rats were irradiated with 12 Gy at 0.74Gy/min, using gamma rays from a cesium source irradiator (IBL 637; CIS Bio International, Saclay, France). They were administered an intravenous injection of 0.2 ml of BM cell preparation within 2 h of irradiation. Rats were left 12 wk to recover and to reconstitute their BM. Assessment of BM chimerism was performed as previously described⁴ and the average percentage of donor DNA was >97% in all three groups of recipients. Orthotopic transplantation of the left kidney was performed as described previously⁴ with removal of the recipient's own left kidney at the time. After transplantation, rats were allowed to recover for 6 to 8 d before induction of NTN.

Statistics

Statistical differences in mean values between all the congenic strains and parental WKY rats were compared using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison post test where a P-value <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 analysed by linear regression.

Disclosure

None

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Figure legends

Figure 1. Genetic Map showing the transferred segment of chromosome 13 in WKY.*LCr gn1* and the transferred segment of chromosome 16 in the WKY.*LCr gn2* congenic lines. Map distances are based on the SHRSP X BN genetic map (<http://rgd.mcw.edu/>) and are in cM. The black bar represents the chromosomal region transferred from the donor LEW strain (designated as LL), the white bar from the recipient WKY strain (designated as WW) and the grey bar represents the recombination zone. Microsatellite markers *D13Rat86* and *D16Rat78* are underlined and represent the peak of linkage for *Cr gn1* and *Cr gn2* respectively.

Figure 2. NTN-related phenotypes and macrophage activation in single and double congenic lines (WKY, n=6 rats; all congenics, n=8 rats/strain)). **a.** Percentage of glomerular crescents in single (WKY.*LCr gn1*, WKY.*LCr gn2*) and double (WKY.*LCr gn1,2*) congenic rats in comparison with parental WKY rats **b.** Macrophage infiltration assessed by quantitative measurement of % of ED-1 positive area per glomerular cross section. **c.** Proteinuria levels measured in WKY, single and double congenic lines. **d.** Histology showing a crescentic glomerulus in a WKY rat and a mildly hypercellular glomerulus in a WKY.*LCr gn1,2* rat (Hematoxylin and Eosin. Magnification, x200). ED1 immunohistochemistry demonstrates extensive glomerular monocyte and macrophage infiltration in WKY whereas WKY.*LCr gn1,2* rats display reduced staining. Glomerular TNF- α (**e**) and iNOS (**f**) expression as assessed by qRT-PCR 10 days after injection of nephrotoxic serum. (n=6 rats/strain). **g.** Macrophage

activation was assessed in WKY, LEW and congenic BMDMs by Fc receptor-mediated phagocytosis and oxidization (n=5 rats/strain, without NTN induction). BMDMs were stimulated with Fc oxyBURST and the WKY rat showed significantly more activation than all the other strains at all time points ($P < 0.001$; error bars, s.e.m). **h.** Sandwich ELISA for secretion of TNF- α in LPS (100ng/ml) stimulated parental, single and double congenic BMDMs (n=6 rats/strain). ** $P < 0.01$ and * $P < 0.05$ compared to WKY.

Figure 3. *Crgn1* and *Crgn2* control exclusively the circulating cell-related glomerular inflammation in the WKY rat. **a.** % of glomerular crescents in parental and WKY.L*Crgn1,2* rats 10d following the injection of nephrotoxic serum with or without bone marrow transplantation. When bone marrow was transferred from WKY.L*Crgn1,2* to WKY rats (WKY.L*Crgn1,2* =>WKY, n=8 rats), the reduction in crescent formation was not significantly different from non-transplanted WKY.L*Crgn1,2* animals ($p = 0.28$, n=8 rats) 10d following NTN induction. All rats showed significant reduction ($P < 0.01$) in % of glomerular crescents compared to WKY; and WKY.L*Crgn1,2* =>WKY rats showed significantly increased % of glomerular crescents ($P < 0.01$) when compared to LEW =>WKY rats. (n=8 rats were used in all groups) **b.** Kidneys from WKY.L*Crgn1,2* rats transplanted into the WKY recipients (n=5 rats) did not show any reduction in the glomerular crescent formation. Mesangial cell MCP-1 production assessed in cell supernatants by sandwich ELISA (**c**) and cell layers by qRT-PCR (**d**) in parental (WKY, LEW), single (WKY.L*Crgn1*, WKY.L*Crgn2*) and double congenic lines (WKY.L*Crgn1,2*) 24h following TNF- α (2ng/ml) stimulation. Mesangial MCP-1 quantities are the result of five independent experiments and expressed as relative

quantities compared to WKY rats.*P<0.05; **P<0.01 compared to WKY; ns, non significant compared to WKY.

Figure 4. Macrophage activation (and not infiltration) controls genetic susceptibility to Crgn encoded by loci outside *Crqn1* and *Crqn2*. **a.** Number of ED1+ cells per glomerular cross section (gcs) in parental and WKY.L*Crqn1,2* rats 10 days following the injection of nephrotoxic serum with or without bone marrow transplantation. Although WKY.L*Crqn1,2*=>WKY and LEW=>WKY rats developed 52% and 12% glomerular crescents respectively (P<0.01) (**Figure 3**), ED1+ cells per gcs was similar (p=0.32) in the two groups. The ED1+ cells per gcs were significantly reduced (P<0.05) in all groups when compared to WKY rats. (n=8 rats were used in all groups). **b.** WKY and WKY.L*Crqn1,2* BMDMs express significantly higher levels of MMP12 when compared to Lewis BMDMs (n=6 rats/strain), indicating the genetically determined macrophage activation status in primary macrophages. WKY.L*Crqn1,2* BMDMs showed also increased MMP12 expression when compared to WKY ones. **P<0.01 compared to WKY; ns, non significant.

Figure 5. MMP-12 expression correlates with the severity of crescent formation in the glomeruli of WKY rats transplanted either with Lewis or WKY.L*Crqn1,2* bone marrow (BM) and the glomeruli of WKY.L*Crqn1,2* rats transplanted with Lewis BM. **a,** MMP12 expression was assessed by qRT-PCR in cultured glomeruli 10 days following the injection of nephrotoxic serum and after bone marrow transplantation (n=8 rats in each group); and linear regression analysis (**b**) showed positive correlation ($R^2=0.61$, P<0.01)

with the % of glomerular crescents shown in **Figure 3a**. ** $P < 0.01$ compared to LEW=>WKY; ns, non significant compared to LEW=>WKY.

Figure 1

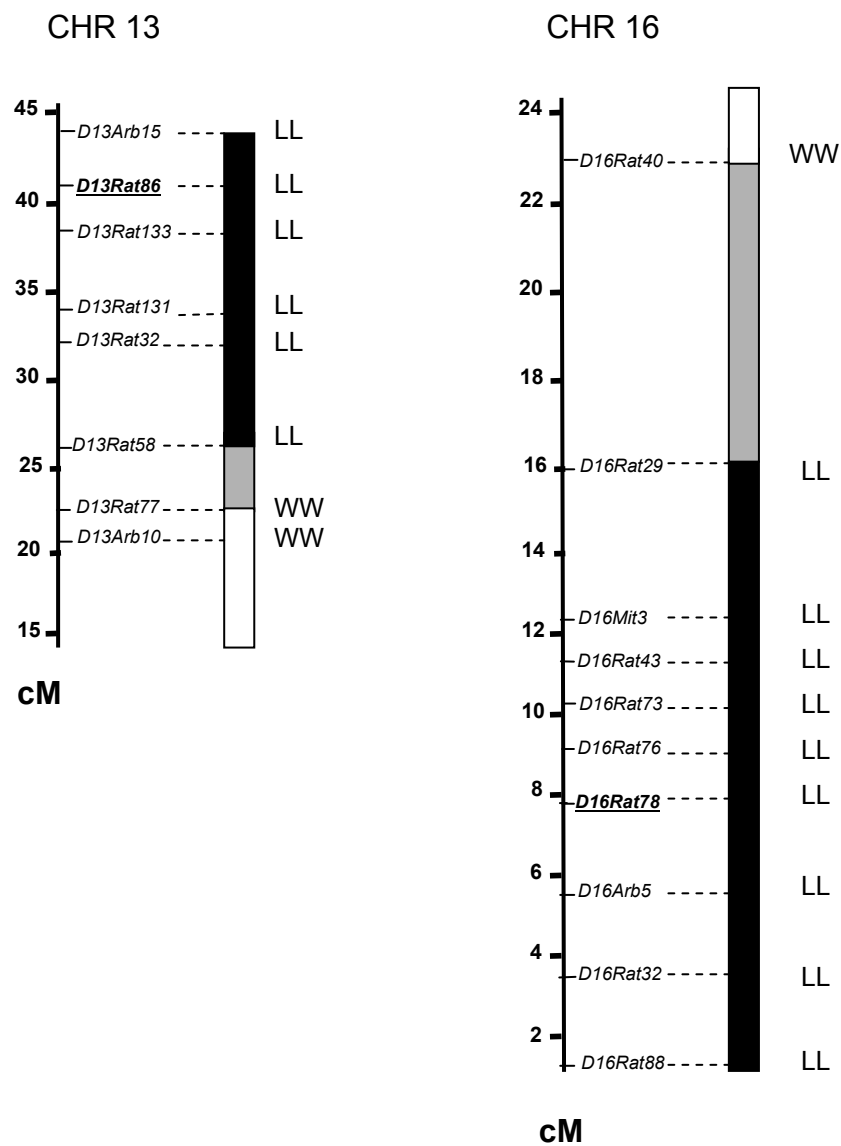


Figure 2

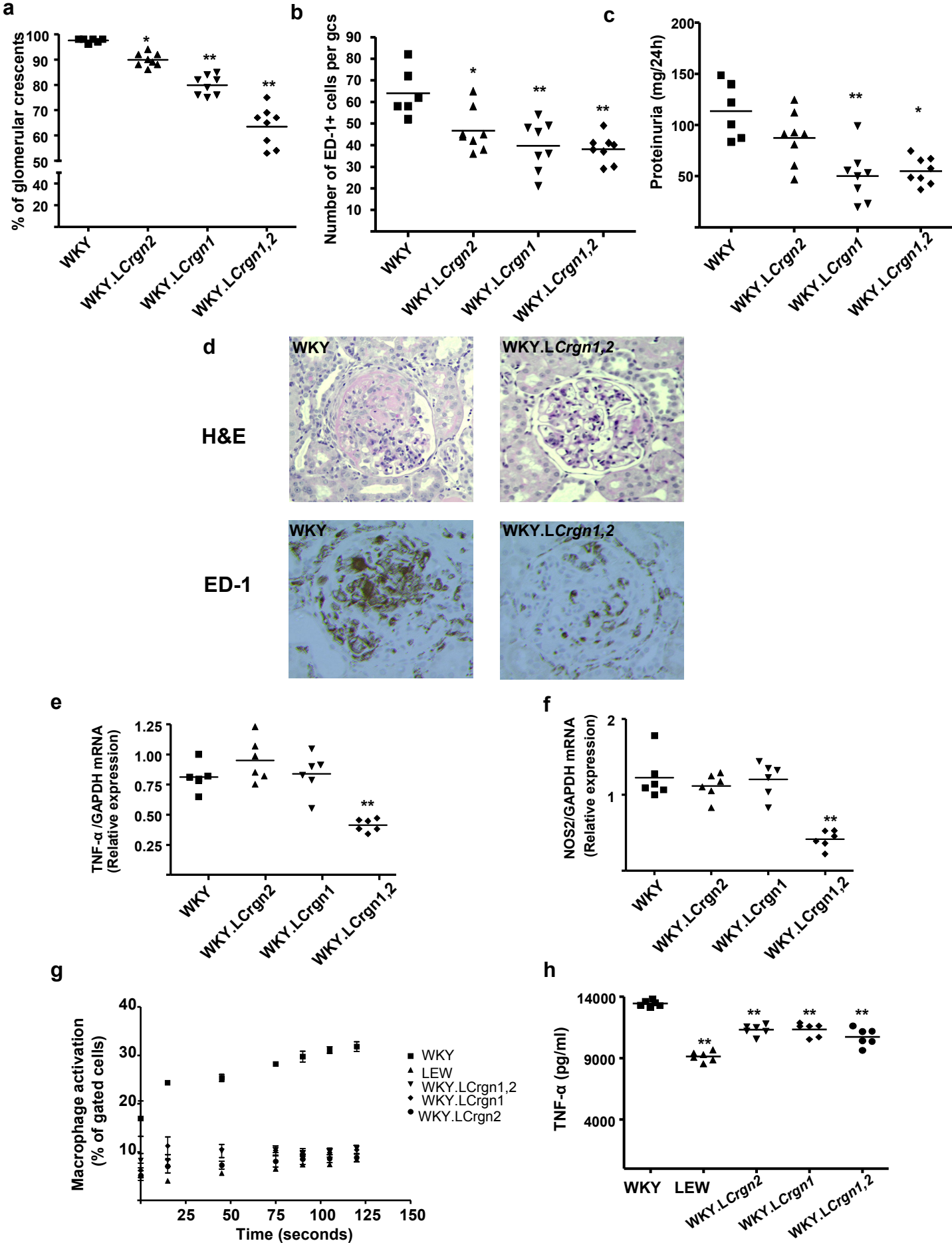


Figure 3

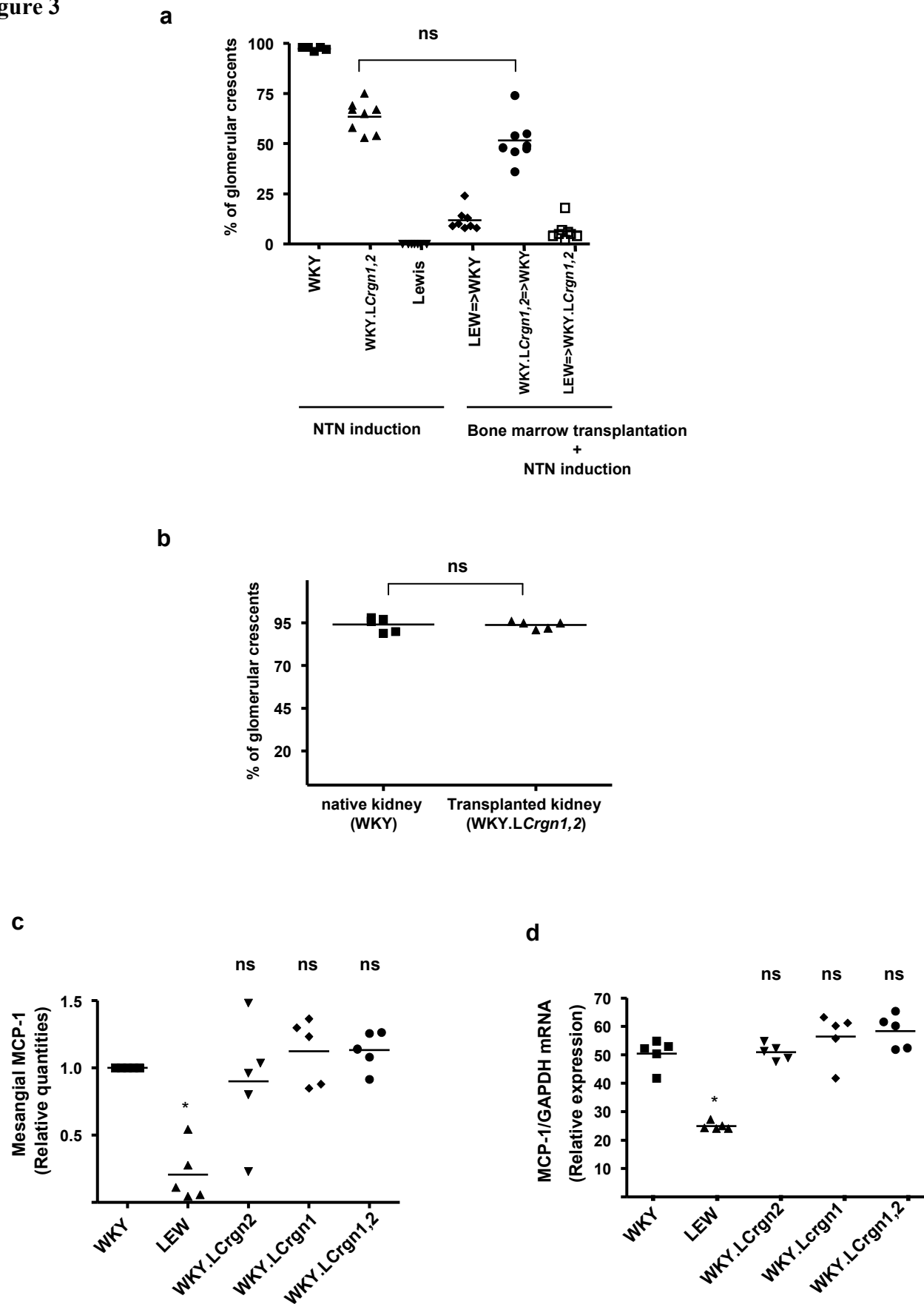
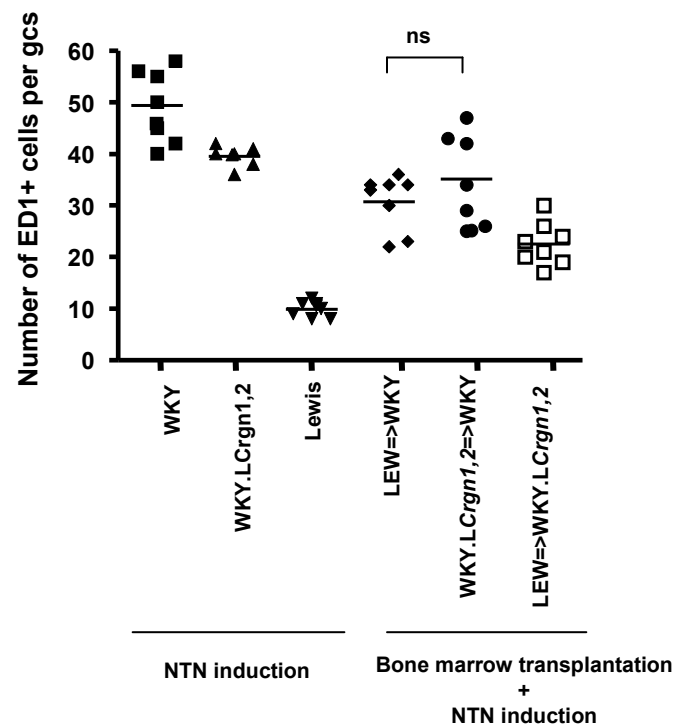


Figure 4

a



b

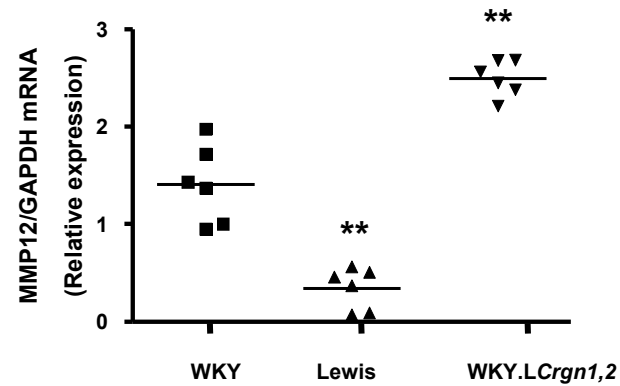


Figure 5

