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Genetic Susceptibility to Experimental Autoimmune Glomerulonephritis in the Wistar Kyoto Rat

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In experimental autoimmune glomerulonephritis (EAG), a model of Goodpasture's disease, Wistar Kyoto (WKY) rats immunized with collagenase-solubilized glomerular basement membrane (GBM) or the recombinant NC1 domain of the α3 chain of type IV collagen [α3(IV)NC1] develop anti-GBM antibodies and focal necrotizing glomerulonephritis with crescent formation. However, Lewis (LEW) rats, which share the same major histocompatibility complex (MHC) haplotype, are resistant to EAG development. A genome-wide linkage analysis of backcrossed animals with EAG revealed a major quantitative trait locus (QTL) on rat chromosome 13 (LOD = 3.9) linked to the percentage of glomerular crescents. To investigate the role of this QTL in EAG induction, reciprocal congenic rats were generated (LEW.W*Crgn1* congenic and WKY.L*Crgn1* congenic), immunized with recombinant rat α3(IV)NC1, and assessed for EAG development. WKY.L*Crgn1* rats showed a marked reduction in albuminuria, severity of crescentic nephritis, and number of glomerular macrophages compared with WKY controls. No reduction in antibody levels was observed. However, LEW.W*Crgn1* rats were resistant to EAG development, as were LEW controls. Macrophage activation *in vitro* was assessed in parental and congenic rat bone marrow–derived macrophages (BMDMs). WKY.L*Crgn1* BMDMs showed a significant reduction in Fc receptor–mediated oxidative burst, phagocytosis of opsonised polystyrene beads, and LPS-induced levels of MCP-1 secretion and iNOS mRNA expression compared with WKY rats. These results confirm the importance of *Crgn1* on chromosome 13 in EAG susceptibility, mediated partly through differences in Fc receptor-mediated macrophage activation. ***(Am J Pathol 2012, 180:1843–1851; DOI: 10.1016/j.ajpath.2012.01.029)***

Goodpasture's, or anti-glomerular basement membrane (GBM), disease is an autoimmune disorder characterized by rapidly progressive glomerulonephritis and lung hemorrhage.[1] The disease is caused by autoantibodies to basement membranes of glomeruli and alveoli,[2] and the pathogenicity of human antibodies has been demonstrated in passive transfer studies in primates.[3] The autoantigen has been identified as the the noncollagenous domain of the α3 chain of type IV collagen [α3(IV)NC1],[4,5] and the major epitope involved has been localized to the amino terminal of the α3(IV)NC1 molecule.[6–8] Goodpasture's disease is associated with certain major histocompatibility complex (MHC) class II alleles; in particular, a positive association has been shown with DR15 and DR4 and a negative association with DR7 and DR1.[9,10] T cells from patients with Goodpasture's disease proliferate in response to the Goodpasture antigen,[11] and it has been shown that the precursor frequency of autoreactive T cells specific for α3(IV)NC1 is higher in patients with active disease than in controls and declines following treatment.[12] The disease relapses extremely rarely, perhaps due to the influence of CD4+CD25+ regulatory T cells.[13]

Experimental autoimmune glomerulonephritis (EAG), an animal model of Goodpasture's disease, can be induced in Wistar Kyoto (WKY) rats by immunization with collagenase-solubilized glomerular basement membrane (GBM),[14–18] or the noncollagenous domain of the α3 chain of type IV collagen [α3(IV)NC1].[19–21] This model of EAG in the WKY rat is characterized by anti-GBM anti-

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body production directed toward α3(IV)NC1, accompanied by focal necrotizing glomerulonephritis with crescent formation. In contrast, when Lewis (LEW) rats, which share the same MHC background as WKY rats (Rt1-l), are immunized with GBM or α3(IV)NC1, they are resistant to the development of crescentic nephritis.[22] Interestingly, when LEW rats are immunized with whole GBM, they develop circulating anti-GBM antibodies, but these do not recognize α3(IV)NC1.[23]

In previous studies examining the genetic basis of susceptibility to EAG, we found that first-generation crosses (F1; WKY × LEW) were completely resistant to the development of EAG, whereas WKY backcross animals (BC1; WKY × F1) showed a range of responses, from severe crescentic glomerulonephritis to no histological evidence of disease.[22] These results indicate that EAG is inherited as a complex trait, with a role for WKY genes not linked to the MHC. In parallel studies, a full genome screen has been performed in a different model of glomerulonephritis, nephrotoxic nephritis (NTN), in WKY rats.[24] This study, using second-generation crosses (F2; F1 × F1), revealed two major quantitative trait loci (QTLs) on chromosomes 13 and 16 (designated crescentic glomerulonephritis 1 [*Crgn1*] and 2 [*Crgn2*]), both of which were linked to crescent formation and proteinuria. Infiltration of macrophages was also strongly linked to *Crgn1*. Several biological candidates were found in the *Crgn1* region of linkage, including genes encoding the activatory Fc receptor for IgG *Fcgr3* (also known as FcγRIII), the inhibitory Fc receptor *Fcgr2* (FcγRII), and the common γ-subunit *Fcer1g* (FcRγ). It was shown that copy number polymorphism of *Fcgr3* accounted for the predisposition to glomerulonephritis in the WKY strain at *Crgn1*.[24]

In further work focusing on the *Crgn2* locus on chromosome 16 and its effect on NTN-related phenotypes in the WKY rat, the AP-1 transcription factor Jund was shown to be a determinant of macrophage activation.[25] Reciprocal congenic rats were generated by introgressing LEW *Crgn2* onto a WKY genetic background (WKY.L*Crgn2*) and WKY *Crgn2* onto a LEW background (LEW.W*Crgn2*). WKY.L*Crgn2* rats showed significantly reduced glomerular crescent formation, fibrin deposition, and macrophage infiltration, whereas LEW.W*Crgn2* rats showed significantly more proteinuria and macrophage infiltration than the respective background strains, demonstrating that the *Crgn2* linkage region influences NTN susceptibility.[25] Furthermore, it was shown that *Crgn2* regulates macrophage activation; for example, bone marrow–derived macrophages (BMDMs) from WKY.L*Crgn2* rats showed reduced Fc receptor–mediated macrophage activation, and diminished expression of the inducible nitric oxide synthase gene (Nos2) on lipopolysaccharide (LPS) stimulation.[25]

In this study, we report for the first time a major quantitative trait locus (QTL) on chromosome 13 (LOD = 3.9) linked to glomerular crescent formation in WKY rats with EAG. Transferring the chromosome 13 QTL region identified in NTN from LEW rats to WKY rats (WKY.L*Crgn1* congenic) resulted in a marked reduction in susceptibility to EAG, and BMDMs from these congenic animals showed reduced Fc receptor–mediated macrophage activation, phagocytosis, and LPS-induced levels of MCP-1 secretion and iNOS mRNA expression. These results demonstrate the importance of the chromosome 13 QTL in susceptibility to EAG and should lead to insights into pathogenetic mechanisms, which may be applicable to human glomerulonephritis.

Materials and Methods

Experimental Animals

Male and female Wistar-Kyoto (WKY/NCr1BR) rats (*RT1-l*) were purchased from Charles River (Margate, UK), and male and female Lewis (LEW/SsNHsd) rats (*RT1-l*) were purchased from Harlan UK (Bicester, UK). All animals were housed in standard conditions and had free access to normal laboratory diet and water. All experimental procedures were conducted in accordance with the UK Animals (Scientific Procedures) Act.

Generation of Backcross Animals

First-generation intercross (F1) progeny were produced by the mating of WKY with LEW rats, and backcross progeny (BC1, $n = 196$) were produced by crossing the F1 population with the WKY parental strain.

Generation of Congenic Animals

Congenic rat lines were produced by introgression of the QTL region on chromosome 13, *Crgn1* (D13Rat86–D13Rat51) from the WKY donor onto the LEW recipient genome and vice versa. *Crgn1* congenics on both WKY (WKY.LEW-D13Arb15-D13Rat58, designated as WKY.L*Crgn1*), and LEW (LEW.WKY-D13Arb15-D13Rat58, designated as LEW.W*Crgn1*), genetic backgrounds were generated by backcrossing the (WKY × LEW) F1 rats to WKY and LEW parental strains for nine generations. Rats heterozygous for the chromosome-13 linkage region were brother–sister mated to fix the congenic interval and obtain the congenic lines.[26]

Production of Rat GBM

Collagenase-solubilized rat GBM (csGBM) was prepared from Sprague Dawley (SD) rat kidneys, as previously described.[17,18] Briefly, the kidneys were decapsulated, the medulla partly removed, and the cortex passed through a series of sieves to isolate the glomeruli. After examination by light microscopy, the glomeruli were disrupted ultrasonically, and the resulting material lyophilized and digested with purified type I collagenase (Sigma-Aldrich, Poole, UK) for 1 hour at 37°C.

Production of Recombinant Rat α3(IV)NC1

Recombinant rat α3(IV)NC1 (generated from cDNA encoding the full-length of α3(IV)NC1 from SD rats) was produced from a stably transfected HEK293 cell line, as previously described.[20,21] Purification of recombinant rat

α3(IV)NC1 from the supernatant was performed by affinity chromatography using an anti-FLAG M2 affinity column (Sigma-Aldrich, Poole, UK), Recombinant rat α3(IV)NC1 was then characterized by Western blotting, using serum from an animal with EAG and control serum, as previously described.[21]

Induction of EAG in BC1 Animals

Male and female WKY ($n = 5$), LEW ($n = 5$) and BC1 rats ($n = 196$), aged 6 to 8 weeks and weighing 120 to 150 g, were given a single intramuscular injection of rat GBM in an equal volume of Freund's complete adjuvant (FCA; Sigma-Aldrich) at a dose of 5 mg/kg body weight.[17,18] All animals were sacrificed at day 28 after immunization.

Induction of EAG in Congenic Animals

Female WKY ($n = 6$), LEW ($n = 6$), WKY/L*Crgn1*($n = 7$), and LEW/W*Crgn1* ($n = 7$) rats, aged 6 to 8 weeks and weighing 120 to 150g, were given a single intramuscular injection of recombinant rat α3(IV)NC1 in an equal volume of FCA (Sigma-Aldrich Company Ltd.) at a dose of 100 μg/rat.[20,21] All animals were sacrificed at day 28 after immunization.

Assessment of EAG in BC1 and Congenic Animals

Albumin Excretion

Urinary albumin concentrations were measured in 24-hour collections from experimental animals at day 28 by rocket immunoelectrophoresis (Amersham Bioscience UK Ltd.), as previously described.[17,18] Briefly, urine samples from experimental animals were subjected to immunoelectrophoresis at 60 v in an electrophoresis tank containing Barbitone buffer (BDH Laboratory Supplies, Poole, Dorset, UK), pH 9.5, for 6 h, using a 1% agarose gel (BDH Laboratory Supplies) containing rabbit antisera to rat albumin raised in our laboratory. Results were calculated using rat serum albumin standards (which were run at the same time) and expressed in milligrams per 24 hours.

Light Microscopy

Kidney tissue was fixed in 10% neutral buffered formalin, processed, and embedded in paraffin wax for light microscopy. Briefly, 3-μm sections were stained with hemotoxylin and eosin, and periodic acid-Schiff. Fifty glomeruli per section were assessed by a blinded observer (HTC) and the severity of the crescentic nephritis graded as: crescents affecting more than 50% of circumference of the glomerulus, crescents affecting less than 50% of circumference of the glomerulus, or normal, and expressed as a percentage of glomeruli examined.[17,18]

Immunohistochemistry

Macrophage numbers were assessed in formalin-fixed, paraffin-embedded kidney sections stained with mouse monoclonal antibody ED1 (Serotec Ltd., Kidlington, UK), followed by biotinylated goat anti-mouse IgG secondary antibody (Dako Ltd., Cambridge, UK), and an avidin-biotin complex (Dako Ltd.). The cellular infiltrate was quantified by counting the number of positively stained cells per 50 consecutive glomeruli in cross section.[18,21]

ELISA

Circulating antibody concentrations to α3(IV)NC1 were measured in sera of experimental animals at day 28 after immunization by a direct solid-phase enzyme-linked immunosorbent assay (ELISA), as previously described.[20,21] Briefly, recombinant rat α3(IV)NC1 was coated on to microtiter ELISA plates (Life Technologies, Paisley, UK) at a concentration of 5 μg/mL by overnight incubation at 4°C. Sera from experimental animals were applied at a predetermined optimum dilution of 1/100 for 1 hour at 37°C. Bound anti-α3(IV)NC1 antibody was detected by alkaline phosphatase–conjugated sheep anti-rat IgG (Sigma-Aldrich.), and developed using the substrate p-nitrophenyl phosphate (NPP, Sigma-Aldrich). The absorbencies for each well were read at 405 nm using an Anthos Multiskan ELISA plate reader (Lab Tech International, Uckfield, UK), and results were calculated as mean optical density for each triplicate sample.

Direct Immunofluorescence

Deposits of IgG within the glomeruli were detected by direct immunofluorescence, as previously described.[17,28] Kidney tissue was embedded in OCT II embedding medium (Miles Inc., Elkhart, IN) on cork disks, snap frozen in isopentane (BDH Laboratory Supplies) precooled in liquid nitrogen, and stored at −80°C. Cryostat sections were cut at 5 μm and were incubated with fluorescein isothiocyanate (FITC)–labeled rabbit anti-rat IgG (Serotec Ltd.). The degree of IgG deposition was assessed by a blinded observer (J.R.), by grading the intensity of immunostaining from 0 to 3+ per 50 consecutive glomeruli in cross-section.

Genetic Mapping in the Backcross Population

Genomic DNA was extracted from the spleens of WKY, LEW, F1, and BC1 animals using the Genomix DNA extraction kit (VH BIO Ltd, Gosforth, UK), as previously described.[22] Microsatellite primers spanning the full rat genome, and within 20 cM of each other, were obtained from Genosys Biotechnology (Cambridge, UK). PCR amplifications were performed using a TouchDown subambient thermal cycler (Hybaid). In the first instance, microsatellite genotyping was performed on parental strain and F1 DNA to confirm published polymorphisms and to derive a panel of polymorphic microsatellites for this strain combination (WKY v LEW). PCR products were

amplified with incorporation of fluorescent dUTP (Perkin Elmer, Cambridge, UK) and scored on an ABI 377 automated sequencer (Perkin Elmer), using the GeneScan program (Perkin Elmer). BC1 progeny were genotyped as homozygous (WKY/WKY) or heterozygous (WKY/LEW) at each individual microsatellite marker.[22]

Bone Marrow–Derived Macrophages

Bone marrow–derived macrophages (BMDM) from WKY, LEW, and WKY.L*Crgn1* congenic rats were isolated, as previously described.[25,26] Femurs were excised and washed in 70% ethanol, sterile PBS, and Hanks' balanced salt solution (HBSS) (Sigma-Aldrich). Bones were transferred to a fresh Petri dish, where both ends were snapped and the bone marrow flushed out with 5−10 mL of Hank medium. Cells were washed three times with Hank medium, then resuspended in 20 mL of DMEM supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 20% FCS, 25% L929 medium, and cultured at 37°C in a total volume of 25 mL in 140-mm tissue culture Petri dishes (Corning, Ithaca, NY). On day 3 of culture, nonadherent cells were carefully removed and fresh sterile culture medium was added. Macrophages were harvested on day 5 by washing with sterile PBS and incubation with 5 mL cell dissociation buffer (Sigma-Aldrich) at 37°C for 10 minutes.

Fc-Mediated Oxygen Burst Activity

BMDM from WKY, LEW, and WKY.L*Crgn1* congenic rats were assessed by Fc OxyBURST to investigate Fc-mediated function.[25,26] Briefly, cells (1×10^6 macrophages) were harvested and resuspended in Krebs-Ringer PBS (KRP buffer) with 1.0 mmol/L Ca^{2+}, 1.5 mmol/L Mg^{2+}, and 5.5 mmol/L glucose, warmed to 37°C for 10 minutes, then stimulated with the Fc OxyBURST immune complex (120 μg/mL; Invitrogen). Fc OxyBURST–induced oxidative burst was assessed at different time points using flow cytometry to measure the percentage of fluorescent cells on a FACScalibur (BD Biosciences), analyzed using Cellquest software (BD Biosciences).

Phagocytosis Assay

BMDM from WKY, LEW, and WKY.L*Crgn1* congenic rats were assessed for phagocytosis.[24,25] Latex polystyrene 6.0-μm microspheres (20 or 50 beads/macrophage; Polysciences Inc.) were incubated in 10 mg/mL BSA (Sigma-Aldrich) in PBS overnight at 4°C. The beads were then washed 3 times in PBS and resuspended in 100 μl of PBS. Rabbit anti-bovine albumin IgG fraction (Sigma-Aldrich), was added to a final dilution of 1:2 and incubated for 1 hour at room temperature. The beads were then washed three times in 1 mL of PBS and used immediately. Macrophages were cultured as described above and plated in eight-well glass chamber slides at 10^5 cells/well. Medium was changed to serum-free medium for 2 hours before adding the beads. Opsonized beads (20 or 50 beads/macrophage) were added to macrophages and incubated at 37°C in 5% CO_2 for 30 minutes. The medium was then aspirated, and slides were stained with Diff-Quick fix (Dade Behring). One hundred macrophages were counted to determine the number of beads ingested per cell.

Monocyte Chemotactic Protein–1 Determination by ELISA

The level of monocyte chemotactic protein–1 (MCP-1) cytokine production by LPS-stimulated (100 ng/mL) and unstimulated BMDM from WKY, LEW and WLY/L*Crgn1* congenic rats was assessed by a sandwich ELISA (BD Biosciences, UK), according to the manufacturer's instructions.[23,24] Briefly, supernatants from BMDMs plated in six-well plates at a density of 10^6 cells per well were incubated in 2 mL of culture medium for 24 hours. The supernatants were then added to the ELISA plate and incubated at RT for 2 hours. Bound antibody was detected by horseradish peroxidase–conjugated IgG, and developed using the substrate 3,3′,5,5′-Tetramethylbenzidine. The absorbencies for each well were read at 450 nm using an ELISA plate reader, and the results were calculated as mean optical density for each triplicate sample.

Inducible Nitric Oxide Synthase mRNA Expression by Real-Time RT-PCR

The level of inducible nitric oxide synthase (iNOS) mRNA expression by LPS-stimulated and nonstimulated BMDM from WKY, LEW and WLY/L*Crgn1* congenic rats was assessed by real-time RT-PCR, as previously described[25,28] Briefly, total RNA was isolated from BMDM using the trizol method. Real-time RT-PCR was performed on an ABI 7500 Sequence Detection System (Applied Biosystems, Warrington, UK) using SYBR Green (Stratagene, Cambridge, UK). A 200-ng quantity of total RNA was used, 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 30 seconds and 60°C for 45 seconds. Results were than exported to 7500 Fast system SDS software (ABS), and Ct values were determined for iNOS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The relative expression levels were then determined by using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

Differences between phenotypes in WKY, LEW, F1, BC1, and congenic animals, and BMDM activation and function studies, were determined by the Mann-Whitney *U* test. Analysis of variance was used to confirm differences between multiple groups of animals. The correlation between quantitative phenotypic traits (percentage crescents, number of glomerular macrophages, and albuminuria) in congenic animals was assessed by linear regression. The associations between extreme phenotypic traits (BC1 animals with >20% crescent formation versus those with no affected glomeruli) and polymorphic

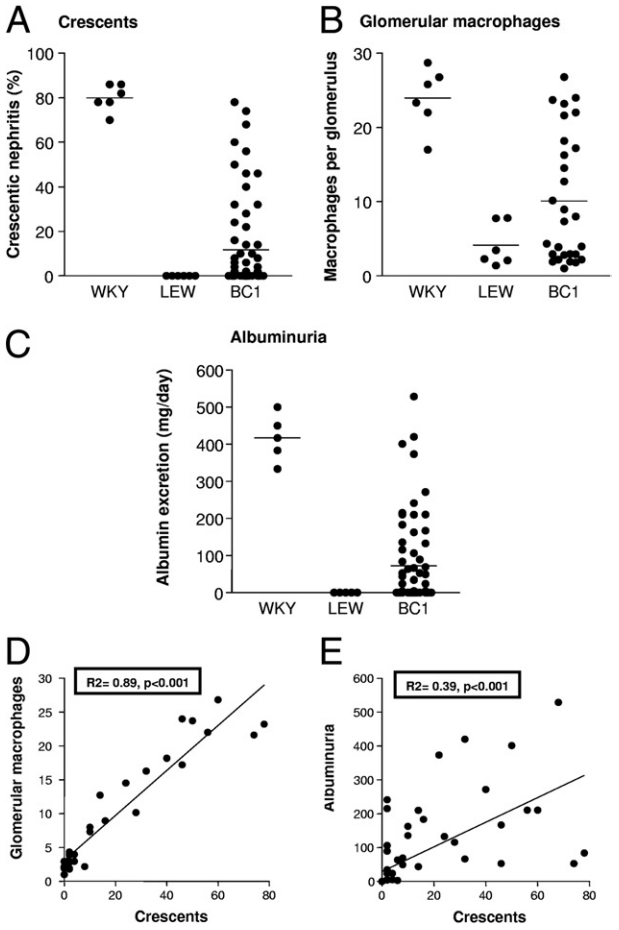

Figure 1. Phenotypic characterization of WKY, LEW, and BC1 animals after immunization with rat GBM, showing (**A**) crescents, (**B**) glomerular macrophages, and (**C**) albuminuria. Results shown represent the value for each animal at day 28 after immunization. Correlation analysis of phenotypic characterization of BC1 animals showing the correlation (**D**) between crescents and glomerular macrophages and (**E**) between crescents and albuminuria.

markers were assessed by a χ^2 test of genotype frequencies. Multipoint quantitative trait locus (QTL) analysis to test for linkage between genetic markers and severity of EAG was performed using the Mapmaker QTL program.[22]

Results

Assessment of EAG in BC1 Animals

WKY rats immunized with rat GBM in FCA developed high levels of albuminuria, a large glomerular infiltration of macrophages, and severe focal necrotizing glomerulonephritis with crescent formation, by day 28 after immunization. By contrast, LEW rats immunized with the same antigen showed no significant increase in the level of albuminuria or glomerular macrophages and no histological evidence of nephritis. BC1 animals developed a range in severity of EAG from no histological evidence of disease to severe crescentic glomerulonephritis. (Figure 1, A–C). The distribution of disease was the same in the male and female backcross animals.

There was a significant correlation between the percentage of glomerular crescents and the number of glomerular macrophages in individual BC1 animals (r^2 = 0.89, $P < 0.001$). There was also a significant correlation between the percentage of crescents and albuminuria (r^2 = 0.39, $P < 0.001$). (Figure 1, D and E).

Genome-Wide Linkage Analysis of EAG Phenotypes in BC1 Animals

Genome-wide linkage analysis for glomerular crescents following EAG induction revealed a significant QTL on chromosome 13 with a logarithm of the odds (LOD) score of 3.9. The peak of linkage was located at the marker D13Rat86 and the significant linkage was over an approximate length of 35 cM (Figure 2). This chromosomal region comprises many candidate genes including the Fc gamma receptor cluster. Importantly, this segment of chromosome 13 was previously found to be linked to glomerular crescents (LOD >8), proteinuria, and macrophage infiltration in the WKY NTN model (24). These results suggest that both EAG and NTN models in the WKY rat share common genetic architecture underlying glomerular inflammation.

Assessment of EAG in Congenic Animals

WKY rats immunized with recombinant rat α3(IV)NC1 in FCA developed circulating anti-α3(IV)NC1 antibodies, strong linear deposits of IgG on the GBM, high levels of albuminuria, severe focal necrotizing glomerulonephritis with crescent formation, and marked glomerular infiltration by macrophages, by day 28 after immunization. By contrast, LEW rats immunized with the same antigen were resistant to the development of EAG. WKY.L*Crgn1* congenic rats showed a significant reduction in severity of crescentic nephritis, number of glomerular macrophages, and albuminuria, when compared with WKY controls (Figures 3 and 4). No reduction in the levels of

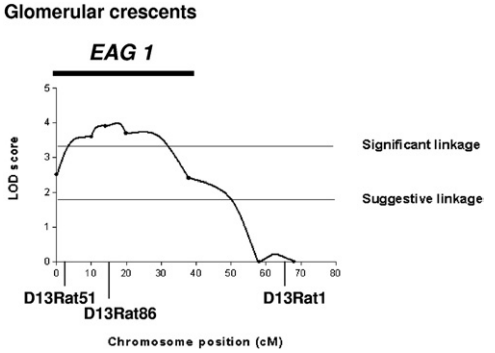

Figure 2. Logarithm of odds (LOD) score plot for glomerular crescents. Genetic map of chromosome 13, based on data from 196 BC1rats, was constructed using MAPMAKER/EXP version 3.0b. Positions of marker loci genotyped are indicated on the *x* axis. LOD scores at each position of the map were calculated using MAPMAKER/QTL version 1.9. The most likely position of the QTL (*EAG1*), determined by its 1-LOD support interval, is indicated by the solid black bar above the plot. Horizontal lines represent the threshold for significance of the LOD score. Map distances are given in centiMorgans (cM) determined with the Kosambi map function.

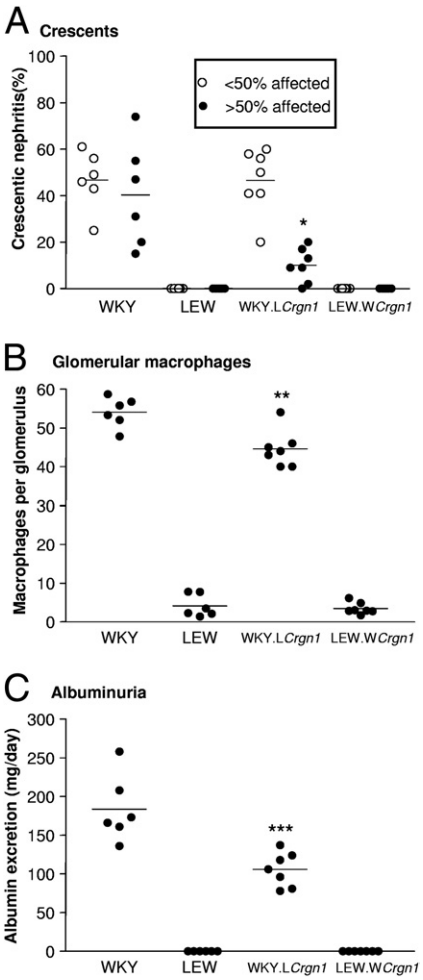

Figure 3. Phenotypic characterization of WKY, LEW, and reciprocal congenic animals for chromosome 13 QTL (*Crgn1*) after immunization with recombinant α3(IV)NC1, showing (**A**) crescents, (**B**) glomerular macrophages, and (**C**) albuminuria. Results shown represent the value for each animal at day 28 after immunization (*$P < 0.001$; **$P < 0.008$; ***$P < 0.002$, WKY versus WKY.L*Crgn1* animals).

circulating or deposited antibody was observed (Figure 5). LEW.W*Crgn1* congenic rats were resistant to the development of EAG, similar to LEW controls.

Assessment of Fc Receptor–Mediated Oxidative Burst, Phagocytosis, and LPS-Induced MCP-1 Synthesis and iNOS Expression in Macrophages of Congenic Rats

Bone marrow–derived macrophages (BMDM) from WKY rats showed a significant increase in Fc receptor–mediated oxidative burst, when compared with LEW rats. BMDM from WKY.L*Crgn1* congenic rats showed a significant reduction in Fc receptor mediated oxidation, when compared with WKY controls (Figure 6A).

BMDM from WKY rats showed a significant increase in phagocytosis of opsonized polystyrene beads, when compared with LEW rats. BMDM from WKY.L*Crgn1* congenic rats showed a significant reduction in phagocytosis of opsonised polystyrene beads, when compared with WKY controls (Figure 6B).

WKY BMDMs showed a significant increase in the LPS-induced MCP-1 secretion and iNOS mRNA expression, when compared with LEW rats, whereas WKY.L*Crgn1* BMDMs showed a significant reduction in the LPS-induced levels of MCP-1 and iNOS, when compared with WKY controls (Figure 6, C and D).

Discussion

The WKY rat shows a marked susceptibility to crescentic glomerulonephritis, both in the model of nephrotoxic nephritis (NTN), which is induced by administration of a heterologous antibody directed against the GBM,[27] and also in the model of experimental autoimmune glomerulonephritis (EAG), which depends on immunization with GBM or α3(IV)NC1.[18–20] The EAG model therefore differs from NTN in that it depends on the generation of an immune response against the administered autoantigen. It is important to appreciate that, although both of these

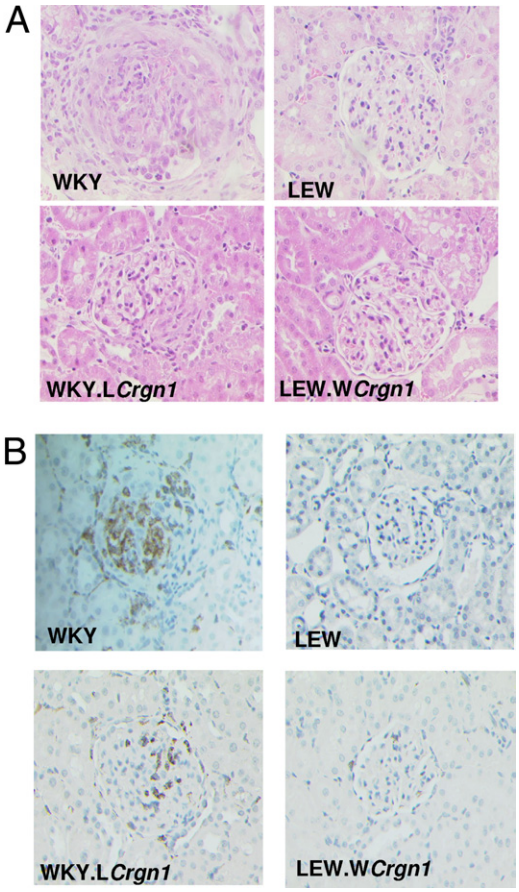

Figure 4. A: Hematoxylin and eosin stain of kidney sections at day 28 after immunization with α3(IV)NC1, showing severe crescentic glomerulonephritis in a WKY rat, normal glomerular architecture in a LEW rat, moderate glomerular damage in a WKY.L*Crgn1* congenic rat, and normal glomerular architecture in a LEW.*WCrgn1* congenic rat. Original magnification, ×200. **B:** Immunoperoxidase stain of kidney sections at day 28 after immunization with α3(IV)NC1, showing a large number of glomerular macrophages in a WKY rat, a few glomerular macrophages in a LEW rat, a moderate number of glomerular macrophages in a WKY.*LCrgn1* congenic rat, and a few glomerular macrophages in a LEW.*WCrgn1* congenic rat.

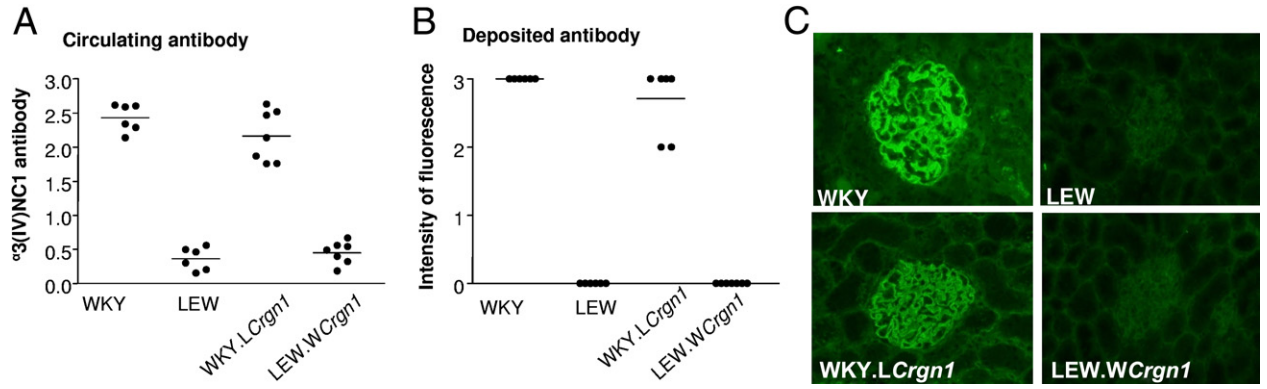

Figure 5. Phenotypic characterization of WKY, LEW, and reciprocal congenic animals after immunization with recombinant α3(IV)NC1, showing (**A**) circulating antibodies and (**B**) deposited antibodies on the GBM. (**C**) Direct immunofluorescence of kidney sections at day 28 after immunization with α3(IV)NC1, showing strong linear deposits of IgG on the GBM in a WKY rat, no deposits of IgG on the GBM in a LEW rat, strong linear deposits of IgG on the GBM in a WKY.*LCrgn1* congenic rat, and no deposits of IgG on the GBM in a LEW.*WCrgn1* congenic rat.

models result in severe crescentic glomerulonephritis, the genetics are distinct; in the NTN model F1 animals show intermediate susceptibility,[24] whereas in the EAG model F1 animals are completely resistant to disease.[22] In EAG, BC1 animals, generated by crossing WKY with F1 rats, showed a range of responses to immunization with GBM, from severe crescentic glomerulonephritis to no histological evidence of disease.[22] In that study, we also adopted a candidate gene approach to investigate whether the gene for the autoantigen α3(IV)NC1 (*Col4a3*), located on rat chromosome 9, was linked to the development of EAG, but no significant linkage was detected in the BC1 progeny.

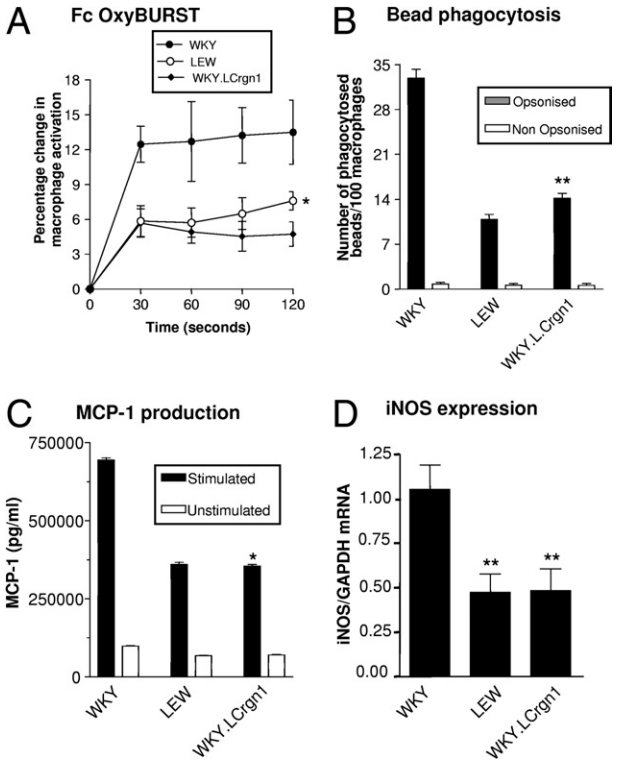

Figure 6. Analysis of Fc receptor–mediated macrophage activation and function in WKY, LEW, and WKY.*LCrgn1* congenic rats, showing (**A**) Fc oxyburst assay, (**B**) bead phagocytosis assay, (**C**) MCP-1 production after stimulation by LPS, and (**D**) iNOS mRNA expression after stimulation by LPS Results shown represent the mean ± SD for each group. (*$P < 0.02$; **$P < 0.001$, WKY versus WKY.*LCrgn1* congenic).

It has previously been shown that differences in the characteristics of the anti-GBM antibodies between WKY and LEW rats account in part for the difference in susceptibility to EAG.[23] This study demonstrated that anti-GBM antibodies in WKY rats immunized with GBM were present in a higher concentration and showed greater specificity for α3(IV)NC1 when compared with antibodies in LEW rats. In addition, passive transfer of eluted anti-GBM antibodies from kidneys of WKY rats with EAG led to similar deposition of IgG on the GBM of both WKY and LEW rats, but resulted in the development of crescentic glomerulonephritis only in WKY rats. These findings illustrate the importance of both the autoimmune response, and also the inflammatory response to deposited antibody, in susceptibility to glomerulonephritis. In addition, it has been demonstrated, using bone marrow and kidney transplantation, that susceptibility to NTN in the WKY rat depends on both circulating and intrinsic renal cells, and that there are genetic differences between the strains in mesangial cell responses to inflammatory stimuli.[28]

In the present study of the genetics of EAG, we bred, immunized, and phenotyped a new cohort of BC1 animals ($n = 196$), which showed a similar range in the severity of nephritis to that previously reported.[22] We performed a genome-wide linkage analysis on this cohort of animals using polymorphic microsatellite markers spanning the whole of the rat genome. This revealed a major quantitative trait locus (QTL) on chromosome 13 (LOD = 3.9) linked to the severity of glomerulonephritis. Several biological candidates are present in the region of linkage, including genes encoding the Fcγ receptors (FcγR).

Because many forms of glomerulonephritis involve antibody or immune complex localization in the kidney, FcγR are logical candidates for susceptibility, as they are responsible for initiating a wide range of cellular responses when engaged by the Fc region of IgG. Several FcγR genes are located in the region of linkage on chromosome 13 in EAG, including genes encoding the acti-

vatory Fc receptor, *Fcgr3* (FcγRIII), the inhibitory Fc receptor *Fcgr2* (FcγRII), and the common γ-subunit *Fcer1g* (FcRγ). In parallel studies by our group, it was shown that copy number polymorphism in *Fcgr3* predisposes to the development of NTN in the WKY rat, a related rat model of glomerulonephritis.[24] It therefore seems likely that Fcgr3, or other FcγR genes, account for the susceptibility to EAG conferred by the chromosome 13 QTL.

To investigate the biological relevance of the QTL on chromosome 13, congenic rats were generated by transferring the chromosome 13 QTL region from WKY rats to LEW (LEW.W*Crgn1*) and the same region from LEW rats to WKY (WKY.L*Crgn1*).[26] After immunization with α3(IV)NC1, WKY.L*Crgn1* congenic rats showed a marked reduction in the severity of crescentic nephritis, especially in those crescents affecting more than 50% of the circumference of the glomerulus, and in the number of glomerular macrophages, when compared with WKY rats. This demonstrates that WKY.L*Crgn1* congenic rats develop a less severe form of crescentic nephritis than WKY controls. Interestingly, there was no reduction in anti-α3(IV)NC1 antibody levels, suggesting that protection from disease in congenic rats is due to events downstream of or independent of antibody deposition. In addition, the protective effect of *Crgn1* in EAG is more pronounced than that seen in NTN,[26] suggesting that different susceptibility genes may be involved in these two models of glomerulonephritis. By contrast, LEW.W*Crgn1* congenic rats were completely resistant to the development of EAG, as were LEW controls. Results from the WKY.LCrgn1 animals demonstrate the importance of the chromosome 13 QTL in the development of EAG, whereas those from the LEW.WCrgn1 animals show that other genes must be involved.

Because macrophage infiltration of the glomeruli is one of the key features in the pathogenesis of EAG, we assessed Fc receptor mediated macrophage activation and function in WKY.L*Crgn1* congenic rats. Bone marrow–derived macrophages (BMDM) from these animals showed a significant reduction in Fc receptor–mediated oxidation and phagocytosis of opsonized polystyrene beads, and in the LPS-induced levels of MCP-1 secretion and iNOS mRNA expression, when compared with BMDM from WKY controls. This suggests that genetically determined Fc receptor–mediated macrophage activation is particularly important in susceptibility to EAG. However, it is known that other genes are involved in macrophage activation. For example, we previously identified the AP-1 transcription factor Jund as a determinant of macrophage activity in the second highly significant QTL (Crgn2) in NTN. Jund transcription is markedly increased in WKY BMDMs as compared with LEW, and siRNA knockdown of JunD led to reduced Fc receptor–dependent oxidative burst.[25] We have recently generated double congenic rats for *Crgn1* and *Crgn2* from LEW on a WKY genetic background, and confirmed the additive effect of both loci on macrophage infiltration and activation in NTN.[26]

In conclusion, we have revealed a major QTL on rat chromosome 13 linked to the percentage of glomerular crescents in EAG. We have shown a reduction in the severity of EAG and in Fc receptor-mediated macrophage activation in congenic rats in which the LEW chromosome 13 congenic interval is introgressed onto the WKY background. These results suggest that *Crgn1*, as identified in NTN, is also involved in susceptibility to EAG. The finding that the same genetic interval is involved in two similar but genetically distinct models of glomerulonephritis adds weight to its importance. These insights into the susceptibility to experimental glomerulonephritis may prove to be relevant to the immunopathogenesis of human autoimmune glomerulonephritis.

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