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Abstract

Crescentic glomerulonephritis is an important cause of human kidney failure for which the underlying molecular basis is largely unknown. In previous studies, we mapped several susceptibility loci, *Crgn1–Crgn7*, for crescentic glomerulonephritis in the Wistar Kyoto (WKY) rat1. Here we show by combined congenic, linkage and microarray studies that the activator protein-1 (AP-1) transcription factor JunD is a major determinant of macrophage activity and is associated with glomerulonephritis susceptibility. Introgression of *Crgn2* from the nonsusceptible Lewis strain onto the WKY background leads to significant reductions in crescent formation, macrophage infiltration, Fc receptor–mediated macrophage activation and cytokine production. Haplotype analysis restricted the *Crgn2* linkage interval to a 430-kb interval containing *Jund*, which is markedly overexpressed in WKY macrophages and glomeruli. *Jund* knockdown in rat and human primary macrophages led to significantly reduced macrophage activity and cytokine secretion, indicating conservation of JunD function in macrophage activation in rats and humans

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and suggesting in vivo inhibition of *Jund* as a possible new therapeutic strategy for diseases characterized by inflammation and macrophage activation.

Glomerulonephritis is a major cause of human kidney failure. In its most severe form, damage to glomerular capillaries leads to accumulation of inflammatory cells and proliferating epithelial cells in Bowman's space, giving rise to crescentic glomerulonephritis (CRGN), which, if untreated, usually progresses to irreversible renal scarring and end-stage renal failure. The Wistar-Kyoto (WKY) rat shows a marked susceptibility to CRGN, as demonstrated by susceptibility to experimentally induced nephrotoxic nephritis2 (NTN). Recent evidence indicates that both circulating cells and intrinsic renal factors play a role in genetic susceptibility to CRGN in the WKY rat3. Macrophages are central to the pathophysiology of CRGN. In the inflamed glomerulus, macrophages become activated, releasing proinflammatory cytokines, reactive oxygen species and proteases that disrupt the integrity of the glomerular basement membrane and lead to fibrin deposition4-6. In experimentally induced glomerulonephritis, selective depletion of macrophages ameliorates disease7,8 and macrophage activation is central to renal injury6,9,10. Furthermore, evidence supporting the importance of phenotypic properties of macrophages rather than their absolute number suggests that targeting macrophage activation could have considerable therapeutic importance in immune-mediated diseases, including glomerulonephritis6,10,11. We recently mapped quantitative trait loci (QTLs) for CRGN susceptibility in a genome-wide linkage analysis of F_2 offspring derived from NTN-susceptible WKY and NTN-resistant Lewis (LEW) rats1. Among seven significant QTLs, we identified two major loci (lod score > 8), *Crgn1* and *Crgn2*, on rat chromosomes 13 and 16, respectively, and deletion of the rat *Fcgr3-rs* gene was identified as the molecular basis for *Crgn1* (ref. 1). Here, we focus on the *Crgn2* locus and its effect on NTN-related phenotypes in the WKY rat.

We first evaluated the effect of *Crgn2* on NTN-related phenotypes in reciprocal congenic lines by introgression of LEW *Crgn2* onto a WKY genetic background (WKY.L*Crgn2*) and of 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 background strains, confirming that the *Crgn2* linkage region affects NTN susceptibility (Fig. 1a–d). Furthermore, *Crgn2* regulates macrophage activation and cytokine secretion, as bone marrow–derived macrophages (BMDMs) from WKY.L*Crgn2* rats showed less Fc receptor–mediated macrophage activation (Fig. 1e and **Supplementary Fig. 1a** online), diminished expression of the inducible nitric oxide synthase gene (*Nos2*) upon lipopolysaccharide (LPS) stimulation (**Supplementary Fig. 1b**), less basal and LPS-induced monocyte chemoattractant protein-1 (MCP-1) secretion and less LPS-induced interleukin (IL)-10 production (Fig. 1f) than WKY BMDMs.

The limits of the WKY.L*Crgn2* congenic interval corresponded to a genetic interval of 22.6 cM (**Supplementary Fig. 2** online), within which many candidate nucleotide variants may be responsible for the observed phenotypic variation. We previously combined global gene expression profiling with linkage analysis to identify positional candidate genes for insulin resistance in the spontaneously hypertensive rat12,13. To identify positional candidates for *Crgn2*, we carried out microarray analysis on normal glomeruli from WKY and LEW rats and on glomeruli from these two strains after induction of NTN. After correction for multiple testing, expression data of transcripts located in the *Crgn2* congenic interval showed three significantly upregulated and three significantly downregulated transcripts in non-nephritic glomeruli of the WKY rat compared to those of the LEW (**Supplementary Table 1** online). One of these genes, the activator protein-1 (AP-1) transcription factor gene *Jund*, showed a marked and highly significant increase in expression in WKY glomeruli

compared to LEW glomeruli, both at baseline and after induction of NTN (Fig. 2a). We confirmed *Jund* overexpression by quantitative RT-PCR (QRT-PCR) and also found it in WKY BMDMs compared to LEW BMDMs (Fig. 2b and **Supplementary Fig. 3a** online). Moreover, *Jund* expression segregated with the *Crgn2* congenic interval in the *Crgn2* reciprocal congenic strains. The introgressed congenic interval totally accounted for differences in gene expression between WKY and LEW in BMDMs (**Supplementary Fig. 3a**). We also saw increased expression of JunD at the protein level in WKY glomeruli at day 10 after induction of NTN (Fig. 2c) Notably, *Jund* was not differentially expressed between WKY and LEW basal (unstimulated) and tumor necrosis factor-α (TNF-α)-stimulated mesangial cells (**Supplementary Fig. 3b**), suggesting that the development of NTN is not secondary to changes in mesangial cell *Jund* expression. Sequence analysis of the WKY and LEW *Jund* promoters revealed a C/T polymorphism in the vicinity (+2 bp) of an octamer motif in the *Jund* promoter, 210 bp upstream of the transcription initiation site (Fig. 2d). To test whether the C/T polymorphism in the *Jund* promoter accounts for the interstrain differences in gene expression, we cloned *Jund* promoter sequences from LEW and WKY into a pGL3-Basic luciferase expression vector and assessed promoter activity in COS7 cells. The WKY promoter sequence containing the T allele showed greater relative luciferase activity than the LEW sequence containing the C allele (Fig. 2e). JunD binding to the AP-1 consensus sequence (5′-TGAGTCA-3′) was also significantly higher in WKY BMDMs than in WKY.L*Crgn2* (Fig. 2f). Because the C/T promoter polymorphism might have been be responsible, at least in part, for the increased *Jund* expression observed in WKY rats, we used this polymorphism to genotype 177 F_2 rats derived from WKY and LEW in a PCR-based amplification-refractory mutation system (ARMS) assay in order to define the location of *Jund* within the *Crgn2* interval. The *Jund* C/T promoter polymorphism showed complete linkage disequilibrium with the microsatellite marker *D16Rat78*, which previously mapped to the *Crgn2* peak of linkage1 and was significantly linked to glomerular crescents, proteinuria and macrophage infiltration (Fig. 3a and **Supplementary Fig. 4** online).

To refine the linkage to glomerular macrophage infiltration within *Crgn2*, we assessed macrophage infiltration after injection of nephrotoxic serum in four closely related Kyoto-derived strains of rat and found that IS/KYO had macrophage infiltration comparable to Lewis, whereas WKY, WTC, WKYO and DON had increased infiltration of macrophages (Fig. 3b). Haplotype analysis on chromosome 16 defined a maximal region of 430 kb that segregated with increased macrophage infiltration across the strains (Fig. 4a,b). Within this 430-kb region, *Jund* was the only transcript from the microarray analysis that showed overexpression in WKY compared to LEW glomeruli in either the basal state (Fig. 4a,b and **Supplementary Table 1**) or the NTN-induced state (data not shown). This excluded the other five transcripts that were observed to be differentially expressed, as they mapped outside the 430 kb interval (Fig. 4a). These results strongly implicate *Jund* as a positional candidate for increased macrophage activation and cytokine production and for glomerular macrophage infiltration encoded at the *Crgn2* locus.

JunD is an AP-1 transcription factor. It is ubiquitously expressed and reported to function as a negative regulator of cell proliferation and to protect against apoptosis and oxidative stress14-16. AP-1 is known to be activated in glomeruli in experimental glomerulonephritis in rats17-20. Although JunD regulates the transcriptional activity of T_H2 cytokines21, its role in macrophage activation and inflammatory disorders has not been investigated. To test whether JunD regulates rat macrophage activation, we reduced *Jund* expression by small interfering RNA (siRNA) in WKY BMDMs and measured subsequent macrophage activation. Knockdown of *Jund* in WKY BMDMs (Fig. 5a) resulted in significantly reduced Fc receptor–mediated macrophage activation (Fig. 5b) and LPS-induced *Nos2* expression (Fig. 5c), showing that *Jund* regulates macrophage activation and that its expression levels

play a key role in the pathophysiology of CRGN in the WKY rat by determining a specific macrophage phenotype.

The heterogeneity of activated macrophages is characterized by different functional activation states. These macrophage phenotypes reflect different genetic susceptibilities to experimentally induced autoimmunity in the rat[22]. To test whether *Jund* expression levels affect macrophage activation in humans, we measured cytokine secretion in basal and LPS-treated human primary macrophages after reduction of *Jund* expression by treatment with siRNA. We found that siRNA knockdown of *Jund* (Fig. 6a,b) in human primary macrophages significantly reduced LPS-induced secretion of IL-10, TNF-α and IL-6 (Fig. 6c), showing that *Jund* mRNA levels directly regulate the secretion of these cytokines in human primary macrophages.

Genome-wide linkage analysis from NTN-susceptible WKY and NTN-resistant LEW rats showed linkage to seven loci, including *Crgn2*, that influence glomerular crescents, proteinuria and macrophage infiltration (Fig. 3 and ref. 1). The present studies have confirmed, by introgression of the *Crgn2* locus onto the LEW or WKY genetic backgrounds in reciprocal congenic strains, that *Crgn2* influences development of glomerular crescents and proteinuria, fibrin deposition and macrophage infiltration and have also shown that *Crgn2* regulates activation and cytokine secretion of BMDMs. Gene expression studies in WKY and LEW rats and in the congenic LEW.W*Crgn2* strain showed marked differential expression of *Jund*, which was located at the *Crgn2* peak of linkage. Overexpression of *Jund* segregated with the WKY allele of *Jund*, indicating that *Jund* over-expression in the WKY strain is regulated in *cis* by polymorphism(s) within the *Crgn2* congenic segment. Haplotype mapping restricted the region of linkage to macrophage infiltration to a maximal interval of 430 kb, within which *Jund* was the only transcript from our microarray analysis showing different expression in WKY than in LEW glomeruli. Luciferase assay showed that *Jund* overexpression in WKY rats was mediated, at least in part, by a C/T polymorphism in the *Jund* promoter. In addition, increased *Jund* expression in WKY BMDMs was characterized by increased binding of JunD protein to the AP-1 consensus sequence. Taken together with our observations that *Jund* knockdown reduced macrophage activation in rats and cytokine production in humans, these data strongly implicate *Jund* as a primary determinant of macrophage activation and of CRGN in the WKY rat.

The reciprocal congenic lines showed significant, though small, alterations in NTN phenotypes (Fig. 1a–d), although not all these phenotypes were altered in both of the reciprocal congenic strains. This suggests that *Crgn2* is necessary but not sufficient to capture all the NTN-related phenotypic effects and that interaction between *Crgn2* and the other NTN-susceptibility loci may be required for full expression of or protection from the NTN phenotypes.

Although we have shown that intrinsic renal cells play a role in development of NTN in WKY rats[3], we found no evidence for dysregulated *Jund* expression in renal mesangial cells. JunD is known to regulate T cell activity[21], and the nephritic WKY kidney contains abundant CD8^{+} cells, but our previous studies indicated that these CD8^{+} cells are of the monocyte/macrophage lineage, with very few cells expressing other T-cell surface markers[2]. It is therefore most likely that *Jund* mediates NTN susceptibility through the effects that we have shown on macrophage activity and cytokine production.

In conclusion, our study suggests that overactivation of macrophages owing to genetically mediated overexpression of *Jund* is functionally linked to inflammation-mediated autoimmune disease susceptibility. Local or systemic knockdown of JunD should be

considered as a potential therapeutic approach to the treatment of inflammatory diseases characterized by macrophage infiltration and activation.

METHODS

Rats

Wistar-Kyoto (WKY/NCrl, designated here as WKY) and Lewis (LEW/Crl, designated LEW) rats were purchased from Charles River. WTC, WKYO, DON and IS/KYO rats were obtained from T. Serikawa (Institute of Laboratory Animals, Japan). Lewis/HanHsd rats for microarray experiments were purchased from Harlan. F_1 rats were generated by intercrossing the two strains. All procedures were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act.

Generation of congenic rat lines

To investigate the involvement of the chromosome 16 QTL in the NTN phenotype, we produced congenic rat lines by introgression of segments of interest on chromosome 16, *Crgn2* (*D16Rat88–D16Rat40*) from the WKY donor onto the Lewis recipient genome and vice versa. *Crgn2* congenics on both WKY (WKY.LEW-*D16Rat88/D16Rat40*, designated as WKY.L*Crgn2*) and Lewis (LEW.WKY-*D16Rat88/D16Rat40*, designated as LEW.W*Crgn2*) genetic backgrounds were generated by backcrossing the (WKY × LEW) F_1 rats to WKY and Lewis parental strains for nine generations. Rats heterozygous for the chromosome-16 linkage region were brother-sister mated to fix the congenic interval and obtain the congenic lines.

NTN induction

Nephrotoxic serum was prepared in rabbits as previously described[23]. We induced nephrotoxic nephritis in male rats by intravenous injection of 0.1 ml of nephrotoxic serum. 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 method[3]. On day 10 after induction of NTN, we killed rats under isoflurane anesthesia and collected blood from the abdominal aortas. Samples of kidney, skin, liver, colon and lung were fixed in formalin, buffered 10% (wt/vol in phosphate buffer), processed and embedded in paraffin wax.

Bone marrow–derived macrophage culture, Fc oxyBURST and bead phagocytosis assays

We isolated femurs from adult rats and flushed them with Hank's buffer (Gibco). Total bone marrow–derived cells were plated and cultured for 5 d in DMEM (Gibco) containing 25 mM HEPES buffer (Sigma), 25% L929 conditioned media, 25% decomplemented fetal bovine serum (F-539, M.B. Meldrum), penicillin (100 U/ml, Invitrogen), streptomycin (100 μg/ml, Invitrogen) and L-glutamine (2 mM, Invitrogen). Nonadherent cells were removed by changing the media and culturing the cells for another 2 d. Rat cells were characterized as macrophages by ED-1 staining. For Fc oxyBURST assay, 10^6 cells (in triplicate) were suspended in Krebs' Ringer's 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 (240 μg/ml, Invitrogen). Individual data points consisting of 10,000 fluorescence events were collected at 0, 15, 30, 45, 60, 90 and 120 s in a FACSCalibur after a baseline fluorescence reading was taken to determine the intrinsic fluorescence of the unstimulated cells. Percentage of fluorescent BMDMs corresponds to percentage of activated gated cells after Fc receptor–mediated phagocytosis.

We assessed macrophage bead phagocytosis as described[24]. Briefly, after differentiation, BMDMs were collected and cultured for 2 d in eight-well glass chamber slides (Nunc) at

10^5 macrophages per well. Two hours before adding the beads, we washed macrophages in warm Hank's (Gibco) and added serum-free DMEM. We then added 6-μm polystyrene beads opsonized and unopsonized with rabbit anti-BSA IgG (Sigma) (Polysciences) to wells at 20 beads per target cell. The chamber slides were then incubated at 37 °C, 5% CO_2 for ten minutes washed in PBS and fixed in cold methanol before a Diffquik stain (Medion Diagnostics) was performed. We counted 100 BMDMs in a blinded manner, noting the number of beads ingested. Each condition was carried out in duplicate, and the experiment was repeated with three separate rats.

Rat 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 25-cm^2 tissue culture flasks at 600 glomeruli per milliliter in RPMI 1640 medium (Invitrogen) that contained 20% decomplemented FBS (F-539), penicillin (100 U/ml; Invitrogen), streptomycin (100 μg/ml; Invitrogen), L-glutamine (2 mM, Invitrogen) and insulin-transferrin-selenite mixture (Sigma). The cultures were maintained at 37 ° with 5% CO_2 for 6 d, allowing glomerular mesangial cells to grow out. We characterized cells as mesangial cells as previously described3.

Primers

See **Supplementary Table 2** online.

Gene expression profiling and quantitative RT-PCR

Total RNA was extracted from WKY and LEW glomeruli and cRNA synthesized from 10 μg total RNA and purified according to Affymetrix's recommendations. We used the Affymetrix Rat Genome U34 Set on RNA extracted from WKY and LEW glomeruli ($n = 3$ rats per condition) with or without NTN induction. We extracted rat BMDM and glomerular RNA using Trizol (Invitrogen) and performed *Jund* QRT-PCR using gene-specific primers as previously described3.

ARMS analysis

To determine genotype at the C/T polymorphism in the *Jund* promoter, we performed PCR using a WKY T allele–specific reverse primer or a LEW C allele–specific primer with a common forward primer, together with β-actin primers as a control of the PCR. Two PCR reactions were performed for a single DNA product (125 ng). After amplification, PCR products were analyzed on a 2.5% agarose gel and genotypes were determined according to the presence or absence of allele-specific bands.

Haplotype map

Genotypes and a subsequent haplotype map of the *Crgn2* QTL were generated from genomic DNA of WKY, LEW and Kyoto-derived strains for 12 markers (*D16Mit3, D16Rat43, D16Rat73, D16Rat76, D16Rat78, D16Rat82, D16Arb5, D16Rat32, D16Rat88, D16Got22, Jund1* and *oxsts5626*) spread across the 14-cM region of linkage. Genetic location of the markers was obtained from BN × SHRSP cross from Rat Genome Database (see URLs below). Physical marker locations on *Crgn2* were obtained from Ensembl (see URLs). The *Jund* promoter polymorphism was genotyped by direct sequence analysis of PCR products amplified from genomic DNA of WKY, LEW and Kyoto-derived strains. Fluorescently labeled microsatellite PCR products were separated on an ABI 3730xl (Applied Biosystems) and scored using GeneMapper Software v3.7.

Jund promoter polymorphism and luciferase assay

The *Jund* promoter sequence was determined by direct sequencing using 100 ng of WKY and LEW genomic DNA in an ABI 3730xl (Applied Biosystems).

At 48 h before transfection, we seeded COS7 cells onto 96-well plates at a density of 2.5×10^4 cells per well. In order to detect maximum and minimum promoter activities, we transfected the cells with either pGL3-Control (200 ng, Promega) or pGL3-Basic (200 ng, Promega), together with 200 ng of the internal control reporter *Renilla reniformis* luciferase driven under the SV40 promoter (pRL-SV40; Promega) for normalizing for the transfection efficiency. Transfections were performed using Lipofectamine 2000 reagent (Invitrogen). In order to compare WKY and Lewis *Jund* promoter activities, we amplified 300 bp of the *Jund* promoter containing the C/T polymorphism by PCR using KOD Hot Start DNA Polymerase (1 U/μl, Novagen) from WKY and LEW genomic DNA, introducing NheI and HindIII restriction sites in the 5′ end of forward and reverse primers. The PCR products obtained from WKY and Lewis genomic DNA were subsequently cloned into pGL3-Basic vectors and were transfected into COS7 cells, together with 200 ng of pRL-SV40. After washing them in PBS, we resuspended cells in 250 μl of lysis buffer. Luciferase assay was performed using the dual luciferase assay system kit according to the manufacturer's protocols (Promega). Values for the relative promoter activity were calculated from the ratio of firefly:Renilla luciferase activities using a luminometer (FluoStar).

siRNA inhibition of *Jund* expression

Human primary macrophages were derived from elutriated monocytes by culturing the cells with macrophage colony-stimulating factor at 100 ng/ml (Peprotech) in RPMI 1640 containing 10% heat-inactivated FCS for 3 d as previously described[25]. WKY BMDMs and human primary macrophages were plated in six-well plates (10^6 cells per well) in DMEM (Gibco) for 24 h and transfected for 48 h with the siGENOME SMARTpool (100 nM, Dharmacon), which consists of four unique siRNAs against rat *Jund* and double-stranded nontargeting siRNA, using Dharmafect 1 (1:50, Dharmacon) as a transfection reagent in OPTIMEM medium (Gibco).

Nuclear extraction

Nuclear extracts were prepared from control (unstimulated) or LPS-stimulated (100 ng/ml, Sigma) BMDMs. We washed cells once with PBS and lysed them in the presence of protease/phosphatase inhibitor cocktail using the Active Motif nuclear extract preparation kit. Extracts were stored at −80 °C until use.

JunD TransAM assay

Nuclear extracts, equivalent to 10 μg protein, obtained from control and LPS (100 ng/ml)-stimulated WKY, LEW and WKY.L*Crgn2* BMDMs were analyzed by TransAM assay (Active Motif). Nuclear proteins were incubated with consensus TPA-response element (TRE) oligonucleotides (5′-TGAGTCA-3′) of the AP-1 binding motif immobilized on 96-well assay plates and probed with antibodies specific to JunD. Plates were developed after addition of secondary antibody and colorimetric developing solution in accordance with manufacturer's instructions, and read on an Anthos ht III (Anthos Labtec) spectrophotometer at 450 nm.

Protein blot analysis of JunD

Human macrophage cell extracts were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore), which were blocked for 1 h with blocking buffer (5% (wt/vol) fat-free milk and 0.1% (vol/vol) Tween-20 in PBS) and then incubated

for 1 h with rabbit polyclonal JunD antibody (Santa Cruz) diluted 1:500 in blocking buffer. HRP-conjugated anti-rabbit IgG (Amersham Pharmacia Biotech) was used as a secondary antibody at a dilution of 1:2,000. Bound antibody was detected using the ECL kit (Amersham Pharmacia Biotech) and visualized using RX Medical X Ray film (Fujifilm).

Cytokine determination by ELISA

We carried out sandwich ELISAs for human IL-10, TNF-α, IL-6 (PharMingen International) and rat MCP-1, IL-10 (BD Biosciences), in accordance with the manufacturers' 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 24 h. For rat BMDMs, ELISA was performed with control (unstimulated) and LPS-stimulated (100 ng/ml) cells. For human primary macrophages, ELISA was performed with (10 ng/ml) or without LPS stimulation after siRNA transfection.

Statistical analysis

All data were analyzed by Student's *t*-test or analyses of variance followed by *post hoc* comparisons. Differences in relative mRNA levels and human cytokine quantities were tested for significance with the nonpara-metric Wilcoxon signed-rank test. Affymetrix U34 chip data was loaded into Rosetta Resolver and intensity profiles were normalized by the Affymetrix-Rosetta's Experiment Builder. Gene expression measurements at the probe level were analyzed using error weighted, one-way analysis of variance between WKY and LEW control glomeruli (WKY$_c$,LEW$_c$) and glomeruli after NTN induction (WKY$_{NTN}$ and LEW$_{NTN}$) followed by a Scheffé *post hoc* test. *P* values of the expression data reported in the text were adjusted for multiple testing using the Benjamini and Hochberg false discovery rate method.

URLs

Rat Genome Database, http://rgd.mcw.edu/. Ensembl, http://www.ensembl.org.

Accession codes

ArrayExpress: The microarray experiment on WKY, LEW control and NTN glomeruli has been submitted to ArrayExpress with accession code E-MEXP-1294.

Acknowledgments

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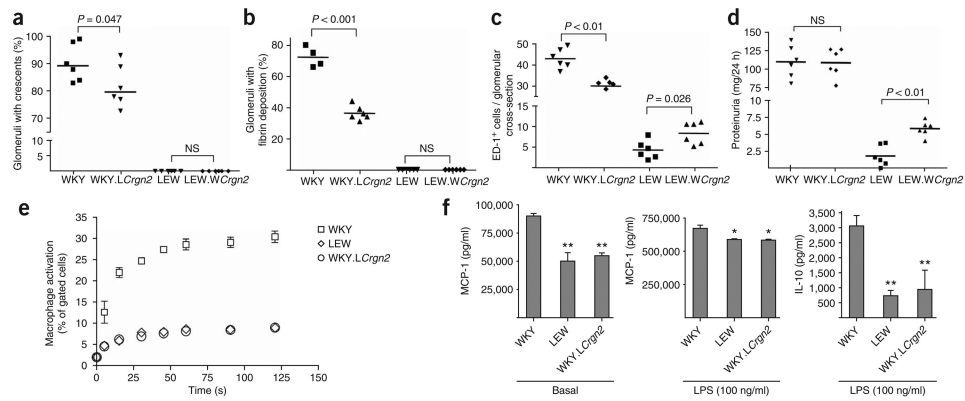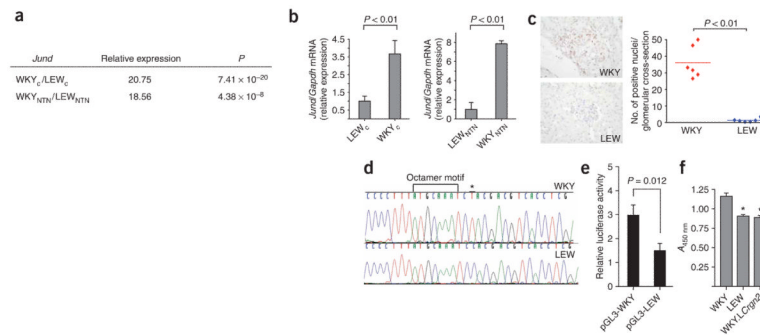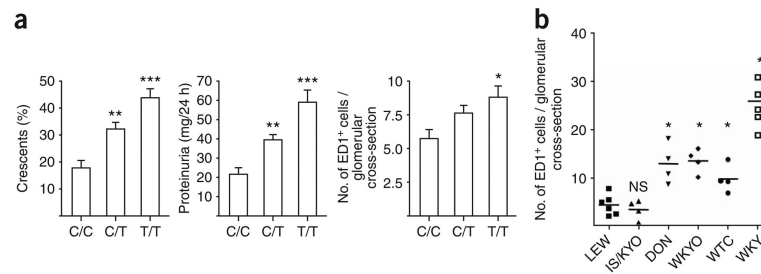
Figure 1.
Nephrotoxic nephritis (NTN) and macrophage phenotypes in parental (WKY, Lewis) and chromosome 16 congenic strains (WKY.L*Crgn2*, LEW.W*Crgn2*). For NTN phenotypes, rats were killed 10 d after injection of nephrotoxic serum. (**a–d**) Glomerular crescents (**a**), fibrin deposition (**b**), macrophage infiltration defined by ED-1–positive cells per glomerular cross-section (**c**) and proteinuria (**d**), measured using at least four rats per strain. (**e**) Macrophage activation, assessed by Fc receptor mediated phagocytosis and oxidization. WKY, LEW and WKY.L*Crgn2* BMDMs (three rats per strain) were stimulated with Fc oxyBURST. LEW and WKY.L*Crgn2* BMDMs showed significantly less activation than WKY at all time points ($P < 0.001$; error bars, s.e.m.). (**f**) Sandwich ELISA for secretion of MCP-1 in basal (unstimulated) and LPS (100 ng/ml)-stimulated BMDMs; secretion of IL-10 in LPS-stimulated (100 ng/ml) WKY, LEW and WKY.L*Crgn2* BMDMs. *$P < 0.05$ and **$P < 0.001$ compared to WKY; error bars, s.e.m.

Figure 2.

Jund expression and protein levels in the NTN-susceptible WKY rat. (**a,b**) Microarray (**a**) and QRT-PCR (**b**) analyses showed that *Jund* expression is higher in WKY than in LEW glomeruli at baseline (WKY$_c$, LEW$_c$) and in NTN-induced glomeruli (WKY$_{NTN}$, and LEW$_{NTN}$). At least three rats per strain and per condition (control or NTN) were used. Error bars, s.e.m. (**c**) Immunostaining for JunD protein in WKY and Lewis glomeruli after NTN induction (day 10), showing increased JunD in the WKY glomeruli. (**d**) Sequence analysis of the rat *Jund* promoter, showing a C/T polymorphism at −210 bp (asterisk) in the vicinity of an octamer binding motif (−219 to −212 bp). (**e**) Luciferase assay performed after transfecting COS7 cells with pGL3-Basic vector containing, 300 bp upstream of the transcription initiation site, either the WKY or LEW *Jund* promoters (pGL3-WKY and pGL3-LEW, respectively). Firefly luciferase activity, normalized to Renilla luciferase activity, is expressed relative to the activity of the empty pGL3-Basic vector. Error bars, s.e.m. for five different transfection experiments performed in replicates of six. (**f**) JunD binding to AP-1 site in BMDMs. Specific JunD binding to AP-1 consensus sequence nucleotides (5′-TGAGTCA-3′) was greater in WKY BMDM nuclear extracts than in LEW and WKY.L*Crgn2* by TransAM assay. A$_{450}$, absorbance at 450 nm. *$P < 0.05$; error bars, s.e.m.

Figure 3.
Combined gene expression and genetic mapping for NTN susceptibility in rat. Rat *Jund* promoter polymorphism between WKY and LEW was used to genotype 177 F_2 rats derived from WKY and LEW by PCR-based ARMS assay (see also **Supplementary Fig. 4**). (**a**) NTN phenotypes versus *Jund* genotype. $*P < 0.05$; $**P < 0.01$; $*** P < 0.001$, compared to C/C genotype. Error bars, s.e.m. (**b**) Macrophage infiltration determined by quantitative analysis of ED-1–positive cells in the glomeruli (including the Bowman's space) in WKY, LEW and four Kyoto-derived rat strains, 10 d after NTN induction. $*P < 0.001$ compared to LEW; NS, nonsignificant.
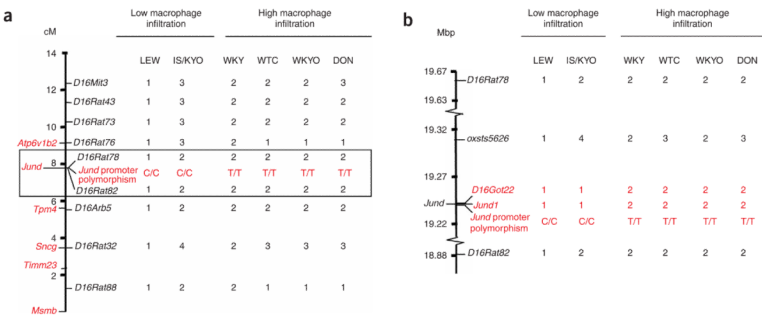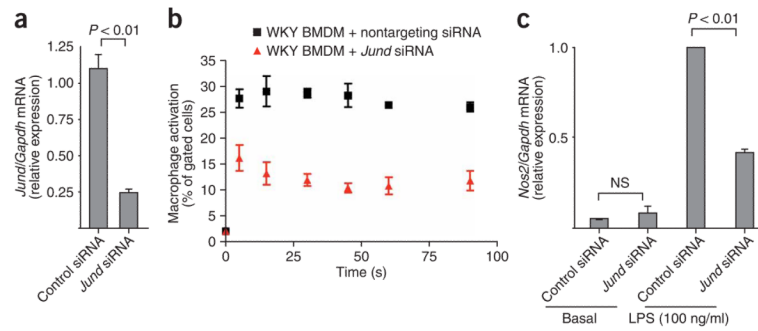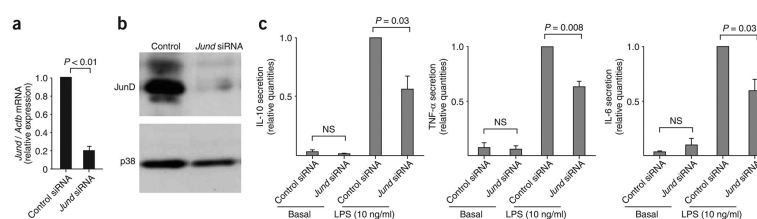
Figure 4.
Chromosome 16 haplotype analysis. (**a**) *Crgn2* genetic map. High and low macrophage infiltration denote, respectively, strains with macrophage infiltration that is or is not significantly greater than that in the LEW strain. Numbers denote classification of alleles: 1, LEW; 2, WKY; 3 and 4, other alleles. Genetic locations of other significantly differentially expressed genes in the overall *Crgn2* congenic interval are also indicated. The boxed area corresponds to microsatellite markers (*D16Rat78* and *D16Rat82*) mapping to the peak of linkage in *Crgn2*. Genetic distances are indicated in cM. (**b**) Fine haplotype mapping delineates a maximal 430-kb region of haplotypic identity segregating with macrophage infiltration. Physical location of the markers is indicated in Mbp. The *Jund* C/T promoter polymorphism showed the same haplotype as *D16Got22* and *Jund1*.

Figure 5.
Effect of modulating *Jund* expression on macrophage activity. (**a–c**) WKY BMDMs were cultured (*n* = 3 rats) and transfected either with *Jund* siRNA or control (nontargeting) siRNA. *Jund* mRNAs were measured 48 h after transfection by QRT-PCR (**a**) and macrophage activation was assessed by Fc oxyBURST assay (**b**) and by measuring *Nos2* expression in basal (unstimulated) and LPS-stimulated (100 ng/ml) cells (**c**). WKY BMDMs with *Jund* knockdown showed significantly less Fc receptor–mediated activation at all time points than BMDMs transfected with control siRNA (*P* < 0.001); NS, nonsignificant. Error bars, s.e.m.

Figure 6.
JunD expression and cellular activation in human primary macrophages. Human macrophages from five healthy donors were derived from elutriated monocytes. Five independent experiments were performed in which cells were incubated either with *Jund* siRNA (100 nM) or control (nontargeting) siRNA (100 nM) for 48 h and stimulated with LPS (10 ng/ml) for 24 h. (**a**) QRT-PCR for human *Jund*. (**b**) JunD and p38 protein blotting. (**c**) IL-10, TNF-α and IL-6 relative secretion were assessed by sandwich ELISA in basal (unstimulated) and 24 h LPS-stimulated (10 ng/ml) cell supernatants. Error bars, s.e.m.