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Brief Conclusive Report

P2X7 receptor-mediated Nlrp3-inflammasome activation is a genetic determinant of macrophage-dependent crescentic glomerulonephritis

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ABSTRACT

P2RX7, a mediator of IL-1β and IL-18 processing and release, is a ligand-gated cation channel that is expressed by macrophages. In experimental Crgn, P2RX7 deficiency attenuates renal injury, but the underlying mechanism is unknown. Here, we show that P2RX7 levels and the expression of several genes belonging to the Nlrp3-inflammasome pathway are up-regulated in the macrophages of the WKY rat, a strain uniquely susceptible to macrophage-dependent NTN. Importantly, following P2RX7 activation, WKY BMDMs produce markedly increased levels of active caspase-1, IL-1β, and IL-18 when compared with the NTN-resistant LEW rat BMDMs. P2RX7 and active IL-1β, IL-18, and caspase-1 protein levels were markedly increased in the WKY nephritic glomeruli 4 days following induction of NTN, and the use of a P2RX7 antagonist reduced the levels of secreted active IL-1β. Interestingly, the post-translational control of P2RX7-mediated inflammasome activation is under the genetic regulation of two previously identified Crgn quantitative trait loci in the BMDMs and nephritic glomeruli of the WKY rat. In conclusion, we propose a novel mechanism, whereby genetically determined P2RX7 levels in macrophages regulate Nlrp3-inflammasome activation and susceptibility to Crgn. *J. Leukoc. Biol.* 93: 127–134; 2013.

Abbreviations: ANTN=accelerated nephrotoxic nephritis, ASC=apoptotic speck protein containing a caspase recruitment domain (CARD), BMDM=bone marrow-derived macrophages, Crgn=crescentic glomerulonephritis, GBM=glomerular basement membrane, IL-1RN=IL-1R antagonist, IL-18BP=IL-18 binding protein, LEW=Lewis, Nlrp1, Nlrp3, Nlrc4=members of the nucleotide-binding oligomerization-like receptor family, NTN=nephrotoxic nephritis, P2RX7=P2X7 receptor, P2RX7−/−=P2X7 receptor-deficient, qRT-PCR=quantitative RT-PCR, QTL=quantitative trait locus, WKY=Wistar Kyoto, YVAD-cmk=Tyr-Val-Ala-Asp-chloromethylketone

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Introduction

Crgn is the most severe form of glomerular inflammation and presents clinically as rapidly progressive glomerulonephritis. Crgn is characterized by disruption of the GBM, leading to the infiltration and proliferation of inflammatory cells, such as macrophages in Bowman's space [1, 2]. Macrophages are important immune effector cells and are the predominant immune cell found within crescentic glomeruli playing pivotal roles. Numerous studies using different models of experimental Crgn have established a role for both macrophage activation and numbers in the progression of Crgn [3–5].

The rat model of NTN in the WKY strain is dependent on macrophage infiltration and activation in response to glomerular deposition of immunoglobulin. In this model, glomerular macrophage infiltration peaks at Day 4 following a single injection of rabbit anti-GBM antiserum [6]. The WKY NTN model is a highly reproducible model, which is very similar in its histological features to human Crgn. It is particularly useful for studying the role of cell-mediated innate immunity against the planted IgG in the GBM and specifically, for studying the role of macrophages in crescent formation. The WKY NTN model has been used to identify genetic loci that promote macrophage-dependent susceptibility to Crgn. Previous genome-wide linkage analysis carried out in the NTN-susceptible WKY and NTN-resistant LEW strains identified seven Crgn QTL [7]. The most significant are QTL controlling crescent formation and proteinuria mapped to chromosome 13 (Crgn1) and chromosome 16 (Crgn2) [7]. These loci showed highly significant LOD scores (LOD>8), and introgression of LEW Crgn1 or LEW Crgn2 into the WKY genetic background, generated by

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single congenic animals, resulted in a reduction of glomerular crescent formation of 18% and 8%, respectively [8]. Positional cloning studies led to the identification of genes controlling FcR-mediated macrophage activation [7, 9, 10]. Further genetic studies using congenic rat strains suggested that the polygenic regulation of NTN susceptibility in the WKY rat is under the control of macrophage activation rather than infiltration [8]. In addition to FcR-mediated macrophage activation, WKY and LEW BMDMs show differences in their superoxide anion production and LPS-stimulated NOS2 expression. Macrophage activation is also partly determined by the cell transcriptome, and we have reported that in addition to the observed differences in macrophage function, there is a major transcriptome difference (~800 differentially expressed transcripts; false discovery rate <5%) between the BMDMs of WKY and LEW rats [11]. In recent years, there have been numerous studies focusing on altering macrophage function in the pathophysiology of NTN. We have shown that blockade of proinflammatory (M1 phenotype) or promoting the anti-inflammatory (M2) phenotype of glomerular macrophages ameliorates disease in the WKY NTN model [12–15].

ATP is an important inflammatory mediator in macrophages [16]. P2RX7, a ligand-gated ion channel, was first cloned from the rat brain [17], and its stimulation in the presence of high ATP concentration leads to release of inflammatory mediators, such as IL-1β [18, 19] and IL-18 [19]. P2RX7 protein is expressed in normal kidney, and although glomerular expression of the molecule is scarce in normal kidney, it is up-regulated following injury, including acute and chronic inflammatory conditions [20, 21]. Notably, P2RX7 expression reaches its peak at Day 4 (which coincides with maximal glomerular macrophage infiltration) following injection of nephrotoxic serum in the glomeruli of WKY rats [22]. The same study also reported up-regulation of P2RX7 in human lupus-related glomerulonephritis [22]. The P2RX7-mediated release of IL-1β and IL-18 is a two-step phenomenon regulated at transcriptional and post-translational maturation levels. These cytokines are produced as proforms and require the activation of caspase-1, also known as the IL-1β-converting enzyme, for maturation and secretion. The inflammasomes are molecular platforms that can trigger the activation of inflammatory caspases, such as caspase-1 and processing of pro-IL-1β and pro-IL-18. Since their discovery 10 years ago [23], Nlrp1, Nlrp3, and Nlrc4 and the adapter protein ASC have been identified as critical components of the inflammasome by linking microbial and endogenous signals to caspase-1 activation and subsequent release of IL-1β and IL-18 [24]. The activation of the NLRP3 inflammasome induced by stimulation of the P2X7R requires two signals. The first signal is the stimulation with endogenous cytokines (i.e., TNF-α) or microbial ligands, such as LPS, and is necessary to induce the up-regulation of NLRP3, which is otherwise expressed at low levels in resting conditions [25, 26]. The second signal that leads to fully active NLRP3 inflammasome is characterized by the engagement of P2RX7 by high concentrations (5 mM) of ATP [27], which induce complete collapse of normal ionic gradients, including the release of intracellular potassium [19, 28]. This P2RX7-stimulated IL-β and IL-18 release is dependent on Nlrp3-inflammasome activation, as mice depleted of ASC or Nlrp3 are unable to process and secrete mature IL-1β in response to ATP [29, 30].

By using two distinct models of experimental Crgn, we have shown previously that P2RX7 has a proinflammatory role in the progression of renal injury. We reported that P2RX7−/− mice had less severe glomerular thrombosis, proteinuria, and macrophage infiltration when compared with WT controls following induction of ANTN [31]. In the WKY rat model of NTN, which unlike ANTN does not require preimmunization, early treatment of rats with a P2RX7 blocker (A-438079) resulted in significantly reduced proteinuria and macrophage infiltration when compared with vehicle-treated controls [31].

In the present study, we hypothesized that P2RX7 is a genetic determinant of macrophage activation and that its cellular levels regulate the processing of active to IL-1β and IL-18 in the inflamed glomerulus through the activation of Nlrp3 inflammasome. We studied P2RX7-mediated Nlrp3-inflammasome activation in the BMDMs and nephritic glomeruli of three strains of rat: the NTN-susceptible WKY rat, the NTN-resistant LEW rat, and the double-congenic WKY.LCrgn1,2 rat. In the latter strain, the two major QTL identified by genome-wide linkage analysis (Crgn1 and Crgn2) were introgressed from the resistant LEW rats into the genetic background of the WKY rat. We have shown previously that these double-congenic rats had 40% reduction in glomerular crescents in NTN when compared with parental WKY rats [8]. Based on our results, we propose a novel mechanism in the genetic control of macrophage function linked to Crgn, which acts through P2RX7-mediated Nlrp3-inflammasome activation.

MATERIALS AND METHODS

Animals

P2RX7−/−, which was originally generated by Pfizer (Groton, CT, USA), was purchased from The Jackson Laboratory (Bar Harbor, ME, USA). WKY (WKY/NCrl) and LEW (LEW/Crl) rats were purchased from Charles River Laboratories International (Wilmington, MA, USA). The generation of the double-congenic line WKY.LCrgn1,2 from single-congenic F1 animals was described previously [8]. All procedures were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act.

BMDM culture and stimulation

Murine and rat BMDMs were isolated from adult femurs and cultured for 5 days in the presence of L929 medium as described previously [8]. These cells were characterized as macrophages by immunohistochemistry for CD68, and >99% of the cells were CD68+. On Day 6 of differentiation, the medium was aspirated, and macrophages were primed for 5 h, unless specified otherwise, with 1 μg/mL LPS from *Escherichia coli* (Sigma, UK) in serum-free DMEM, supplemented with 100 IU penicillin and 100 μg/mL streptomycin. Following the LPS priming, the medium was aspirated, cells were washed with HBSS, and fresh, serum-free medium containing ATP at a concentration of 5 mM was added. In some experiments, 10 μg/ml Nigericin (Sigma) was added. After 30 min of ATP (or Nigericin) stimulation, culture medium and cell layers were collected by scraping. In some conditions, the P2RX7 inhibitor AZ10606120 dihydrochloride (Tocris Bioscience, Bristol, UK) was added before stimulating the cells with ATP.

NTN, isolation of rat nephritic glomeruli, and immunostaining

NTN was induced in male WKY, LEW, and WKY.LCrgn1,2 rats by i.v. injection of 0.1 ml nephrotoxic serum, as described previously [9]. On Day 4

after induction of NTN, rats were killed under isoflurane anesthesia and kidneys placed immediately in cold PBS. Glomeruli were isolated by sequential sieving using stainless-steel meshes of 250, 150, and 75 μm in cold PBS, followed by centrifugation at 1500 rpm for 5 min. The glomerular pellet was then suspended in DMEM, supplemented with penicillin and streptomycin. A number of 1×10^4 glomeruli were seeded into each well of a six-well plate using 1 ml DMEM/well. After 48 h of incubation at 37°C, supernatants and glomerular layers were collected and stored at −20°C for further analysis. In some conditions, the nephritic glomeruli from the WKY rats were washed following 24 h of culture, and the remaining macrophages were cultured for an additional 24 h. For ED-1 (AbD Serotec, Oxford, UK) and Nlrp3 (Abcam, Cambridge, UK) immunostaining, formalin-fixed, paraffin-embedded rat tissues were microwaved in sodium citrate buffer for antigen retrieval. Following consecutive blocking steps (peroxide block for 15 min, 10% goat serum block for 15 min, followed by a 10% milk block for 15 min), sections were incubated with the primary antibody ED-1 (1:500) or NALP-3 (1:100) and the slides left for 1 h at room temperature. The slides were then washed and incubated in the polymer-HRP (Dako mouse EnVision kit, Dako, Denmark) for 30 min at room temperature. Staining was visualized using DAB (Dako EnVision kit) and sections counterstained with hematoxylin, dehydrated, and mounted with cover slips.

Western blotting and ELISA

The cell monolayer or glomerular preparation was washed once with cold 1× PBS and suspended immediately in lysis buffer. Lysates were clarified by centrifugation (13,500 g, 20 min), and the protein concentration in the supernatants was measured using the BCA protein assay kit (Pierce, UK), following the manufacturer's instructions. To analyze secreted proteins, cells were scraped, and cell layers and supernatant s were collected and filtered using Amicon ultra centrifugal filters (10 kDa cutoff; Millipore, UK) for protein purification and concentration. The concentrated samples were diluted with 5× sample buffer, boiled for 10 min, resolved by SDS-PAGE, and transferred to an Immobilon-P transfer membrane (Millipore). The primary antibodies used were as follows: rabbit polyclonal anti IL-1β (New England BioLabs, UK), rabbit polyclonal anti IL-18 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti caspase-1 (Santa Cruz Biotechnology), and rabbit polyclonal anti-P2X7 (Alomone Labs, Israel). IL-1β levels were quantified by sandwich ELISA (R&D Systems, Abingdon, UK), according to the manufacturer's instructions.

RNA extraction and qRT-PCR

Total RNA was extracted from BMDMs using the Trizol method. Total RNA concentration was determined using a NanoDrop spectrophotometer (Labtech International, Ringmer, UK). Real-time RT-PCR was performed on an ABI 7500 sequence detection system (Applied Biosystems, Warrington, UK) using SYBR Green (Stratagene, Cambridge, UK). A total of 100 ng total RNA was used for qRT-PCR, and all samples were amplified in triplicate using transcript-specific primers. The primers used in this study were as follows: P2RX7, forward 5′TCGGTTTGGCCACCGTGTGT-3′ and reverse 5′ CATTCACTGCACAGGGCTCGCA-3′; Il1RN, forward 5′CTTTTCTGTGTGATGCCCCT-3′ and reverse 5′GTGAAGATGGTGTTTGGGCT-3′; Il18, forward 5′ACCGCAGTAATACGGAGCAT-3′and reverse, 5′TAGGGTCACAGCCAGTCCTC-3′; IL18bp forward 5′ATGAGACACTGTGGCTGTGC -3′ and reverse 5′ACTGCTGGAGACCAGGAAGA-3′; Nlrp3, forward 5′GCTGCTCAGCTCTGACCTCT-3′ and reverse 5′AGGTGAGGCTGCAGTTGTCT-3′; ASC, forward 5′GCAATGTGCTGACTGAAGGA-3′ and reverse 5′TGTTCCAGGTCTGTCACCAA-3′; Casp1, forward 5′GGAGGGAATATGTGGGATCA-3′ and reverse Casp1 5′ CCCTCTTCGGAGTTCCCTAC-3′. Results were exported to 7500 Fast System SDS software and analyzed as described previously [8].

Statistical analysis

Data were expressed as mean ± SEM. Statistical difference was assessed by a single-factor variance (ANOVA), followed by Bonferroni multiple comparisons post-test; $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

P2RX7-Nlrp3-inflammasome gene expression in WKY, LEW, and WKY.LCrgn1,2 BMDMs

In our previous microarray analysis of WKY and LEW BMDMs, we identified P2RX7, IL-1RN, and IL-18 as markedly overexpressed transcripts in the WKY macrophages [11]. Based on these results, we hypothesized that the P2RX7-Nlrp3-inflammasome pathway genes may also be expressed differentially between the macrophages of the two strains of rat. In addition to P2RX7, IL-1RN and IL-18, the basal expression levels of caspase-1, IL-18BP, and LPS-stimulated Pycard (ASC) expression was found to be up-regulated significantly in WKY BMDMs (**Fig. 1A** and **B**). The transcriptional regulation of all of these transcripts was not under the control of Crgn1 and Crgn2, as the congenic WKY.LCrgn1,2 BMDMs showed similar expression levels to the parental WKY BMDMs (Fig. 1A). The P2RX7 protein levels were also increased in basal and LPS-stimulated WKY BMDMs when compared with LEW BMDMs (Fig. 1C). The WKY and LEW strains also show sequence variants within the P2RX7 gene (Supplemental Fig. 1). However P2RX7 protein levels are under partial genetic control of Crgn1 and Crgn2, as the LPS-stimulated WKY.LCrgn1,2 BMDMs showed reduced levels of P2RX7 protein levels (Fig. 1C). Furthermore, we confirmed that P2RX7 is essential for ATP-induced Nlrp3-inflammasome activation, and its blockade leads to reduced active IL-1β in WKY BMDMs and nephritic glomeruli (Supplemental Fig. 2).

P2RX7-Nlrp3-inflammasome-mediated IL-1β and IL-18 release in WKY, LEW, and WKY.LCrgn1,2 BMDMs

To activate the P2RX7-Nlrp3-inflammasome pathway, BMDMs from WKY, LEW, and WKY.LCrgn1,2 rats were first primed with LPS (1 μg/ml, 3 h). ATP (5 mM) was then added to the cells for 30 min. The active and pro forms of IL-1β, IL-18, and caspase-1 were assessed in the cell lysates and supernatants of BMDMs from the different rat strains by Western blotting (**Fig. 2**). The results show clearly that when WKY BMDMs are primed with LPS and stimulated with ATP, they produce markedly increased levels of active IL-1β (Fig. 2A), IL-18 (Fig. 2B), and mature caspase-1 (Fig. 2C) when compared with NTN-resistant LEW BMDMs. As with P2RX7 levels, the ATP-induced release of IL-1β and IL-18 is partially regulated by Crgn1 and Crgn2, as WKY.LCrgn1,2 BMDMs showed markedly reduced active caspase-1 and decreased IL-18 and IL-1β protein levels. The differential activation of the Nlrp3 inflammasome in WKY and LEW BMDMs is mainly a result of differences in P2RX7 levels, as the use of the potassium ionophore Nigericin, previously reported to induce the activation of Nlrp3 [32], did not result in increased active IL-1β (assessed by Western blot and sandwich ELISA) and caspase-1 secretion in LPS-primed WKY rat macrophages (Fig. 2D). Taken together, these results show that although Crgn1 and Crgn2 do not control the transcriptional regulation of the Nlrp3-inflammasome pathway (Fig. 1A), the maturation of IL-1β, IL-18, and caspase-1 is partially under the genetic regulation of both loci (Fig. 2).

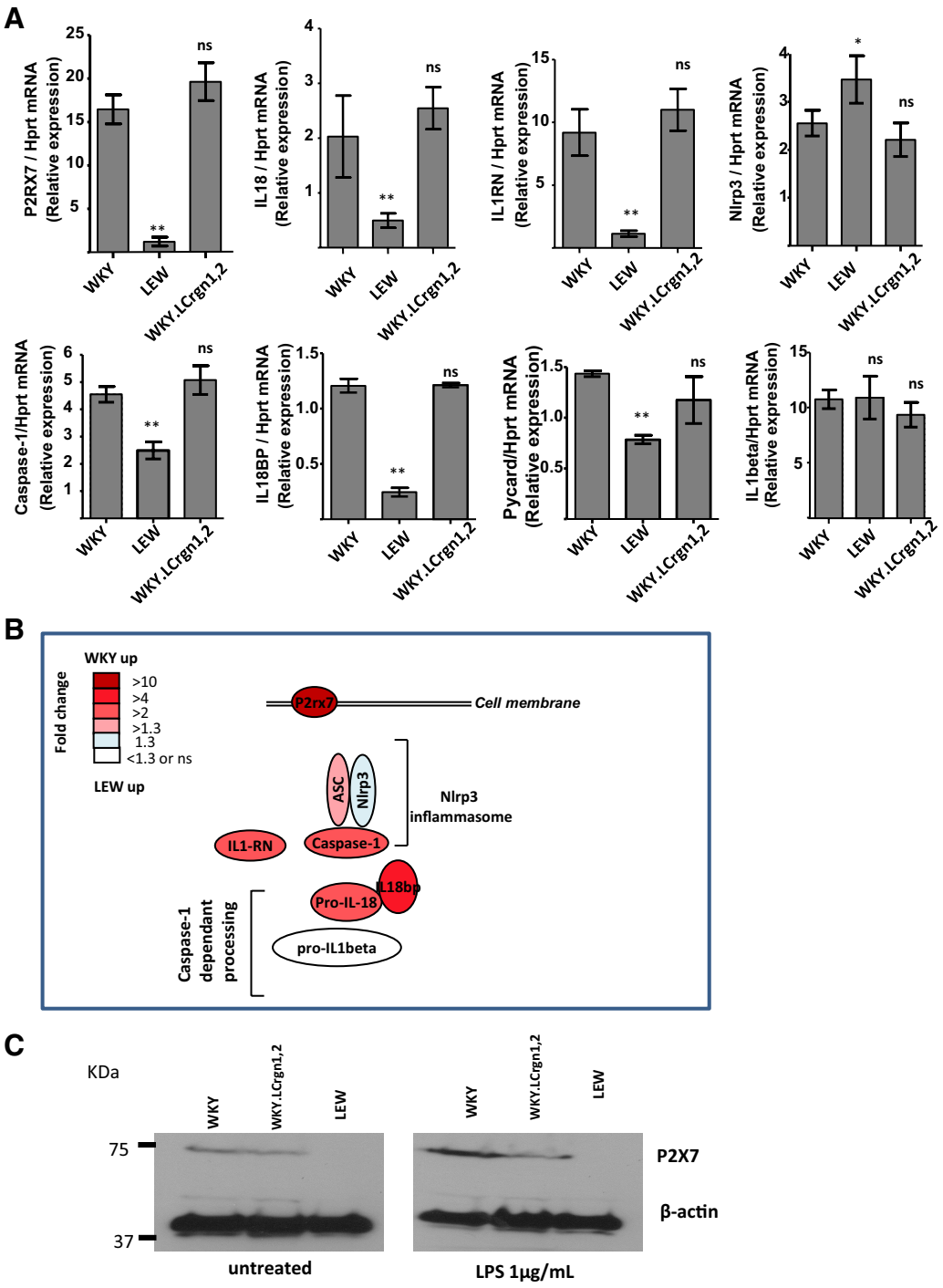

Figure 1. The expression of P2RX7-Nlrp3-inflammasome pathway genes is genetically controlled in the macrophages of rats showing different susceptibility to Crgn. (A) The expression of the major transcripts belonging to the P2RX7-Nlrp3-inflammasome pathway in the BMDMs of the WKY, WKY.LCrgn1,2, and LEW rats. All of the genes were assessed by qRT-PCR at basal levels, except for *Pycard*, for which expression levels were up-regulated significantly in LPS (100 ng/ml, 10 h)-stimulated cells. Hprt, Hypoxanthine guanine phosphoribosyl transferase. (B) Schematic and simplified representation of the P2RX7-Nlrp3-inflammasome pathway, highlighting the color-coded differential expression between the WKY and LEW BMDMs. (C) Western blot analysis of P2RX7 protein levels in basal (untreated) and LPS (1 μg/ml, 5 h)-treated BMDMs from WKY, LEW, and WKY.LCrgn1,2 rats; n = 6 rats were used for qRT-PCR. Western blot results are representative of three independent experiments. $^*P < 0.01$, $^{**}P < 0.001$ compared to WKY rats.

The increased P2RX7-mediated IL-1β and IL-18 secretion in WKY BMDMs is caspase-1-dependent and is not a result of an up-regulation of the pro forms of the cytokines

The differential activation of the P2RX7-Nlrp3-inflammasome pathway in WKY, LEW, and WKY.LCrgn1,2 BMDMs could be the result of the priming process, whereby LPS stimulation leads the enhanced TLR4-dependent NF-κB activation in the WKY rat. To dissect whether the priming (LPS) or the post-translational caspase-1-dependent maturation (ATP through P2RX7) accounts for the observed differences in the release of IL-1β and IL-18, we assessed pro-IL-1β and pro-IL-18 in the cell lysates (excluding the supernatants) of WKY, LEW, and WKY.LCrgn1,2 BMDMs (**Fig. 3A**). The results show that BMDMs from all of the rat strains produce similar amounts of pro-IL-1β and pro-IL-18 in response to LPS and that ATP stimulation does not modify the amount of either cytokine (Fig. 3A). In addition, the increased IL-1β and IL-18 levels are a result of increased caspase-1 activation in the WKY BMDMs, as the use of the specific caspase-1 inhibitor YVAD-cmk abolished

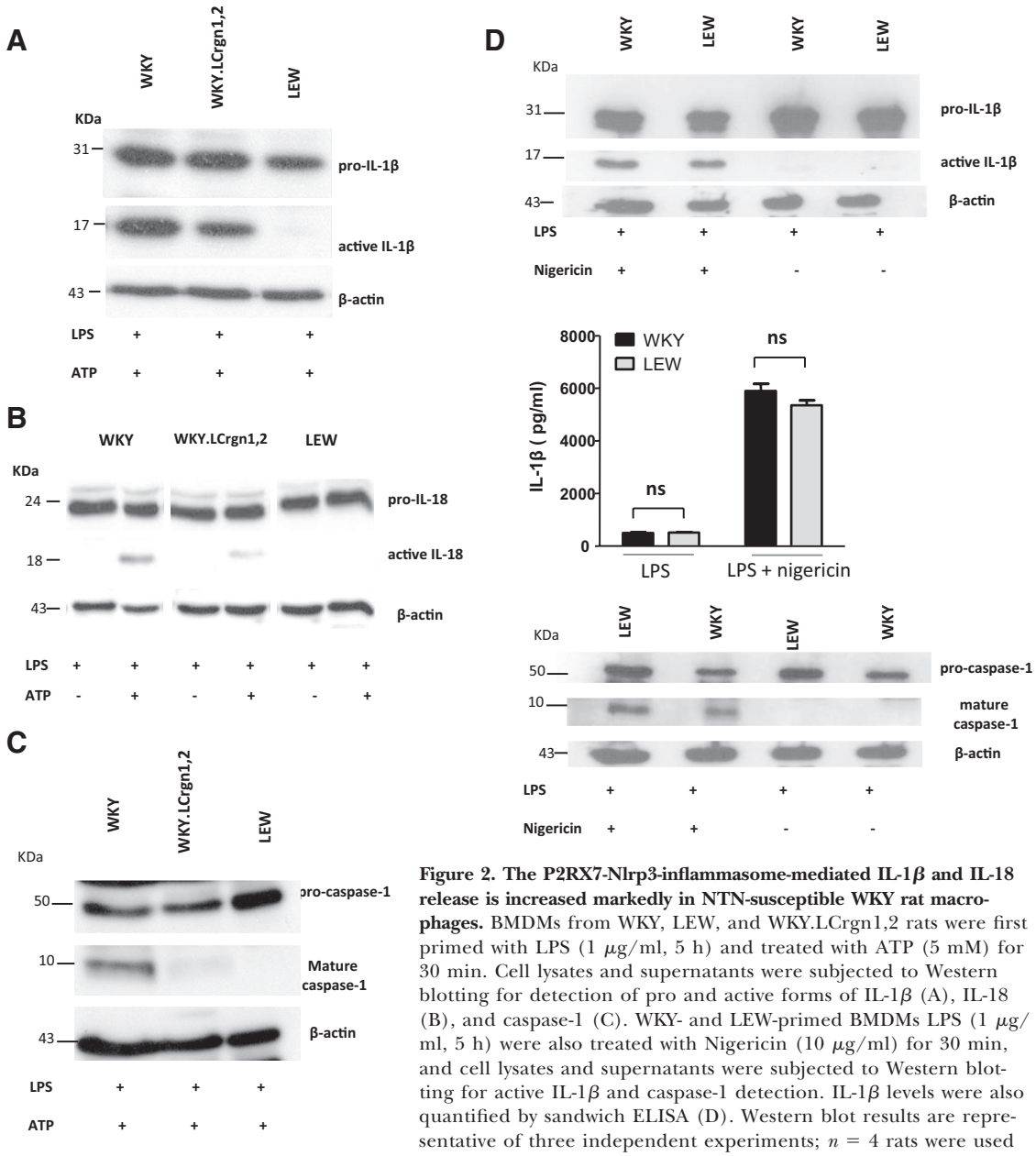

Figure 2. The P2RX7-Nlrp3-inflammasome-mediated IL-1β and IL-18 release is increased markedly in NTN-susceptible WKY rat macrophages. BMDMs from WKY, LEW, and WKY.LCrgn1,2 rats were first primed with LPS (1 μg/ml, 5 h) and treated with ATP (5 mM) for 30 min. Cell lysates and supernatants were subjected to Western blotting for detection of pro and active forms of IL-1β (A), IL-18 (B), and caspase-1 (C). WKY- and LEW-primed BMDMs LPS (1 μg/ml, 5 h) were also treated with Nigericin (10 μg/ml) for 30 min, and cell lysates and supernatants were subjected to Western blotting for active IL-1β and caspase-1 detection. IL-1β levels were also quantified by sandwich ELISA (D). Western blot results are representative of three independent experiments; n = 4 rats were used for IL-1β ELISA.

completely the production of the active forms of both cytokines (Fig. 3B).

P2RX7-Nlrp3-inflammasome activation and its genetic control in Crgn in the rat

We extended our in vitro observations obtained in BMDMs to the ex vivo analysis of Nlrp3-inflammasome activation in the nephritic glomeruli of WKY, LEW, and WKY.LCrgn1,2 rats. Glomeruli were isolated from the renal cortex 4 days after the NTN induction and were cultured for 48 h in serum-free DMEM media (to allow macrophage growth). We observed that P2RX7 and active IL-1β, IL-18, and caspase-1 were increased markedly in the nephritic glomeruli of WKY rats when compared with LEW (**Fig. 4**). In addition, we showed that the source of the increased P2RX7-mediated Nlrp3-inflammasome activation within the nephritic glomeruli is the infiltrating CD68+ macrophages (Supplemental Fig. 3). Interestingly, the WKY.LCrgn1,2 rats, which show reduced glomerular crescents and macrophage infiltration 10 days following NTN induction [8], also showed reduced P2RX7 (Fig. 4A) and active caspase-1 (Fig. 4B), IL-1β (Fig. 4C), and IL-18 (Fig. 4D), suggesting that Crgn1 and Crgn2 control P2RX7-mediated Nlrp3-inflammasome activation in the nephritic glomeruli. Immunohistochemical analysis of Nlrp3 and ED-1 on consecutive WKY kidney sections 10 days after NTN induction showed that glomerular Nlrp3 staining is primarily associated with ED-1+ cells (Fig. 4E).

Macrophages are important effector cells in the pathophysiology of Crgn. In the WKY NTN model, they infiltrate the

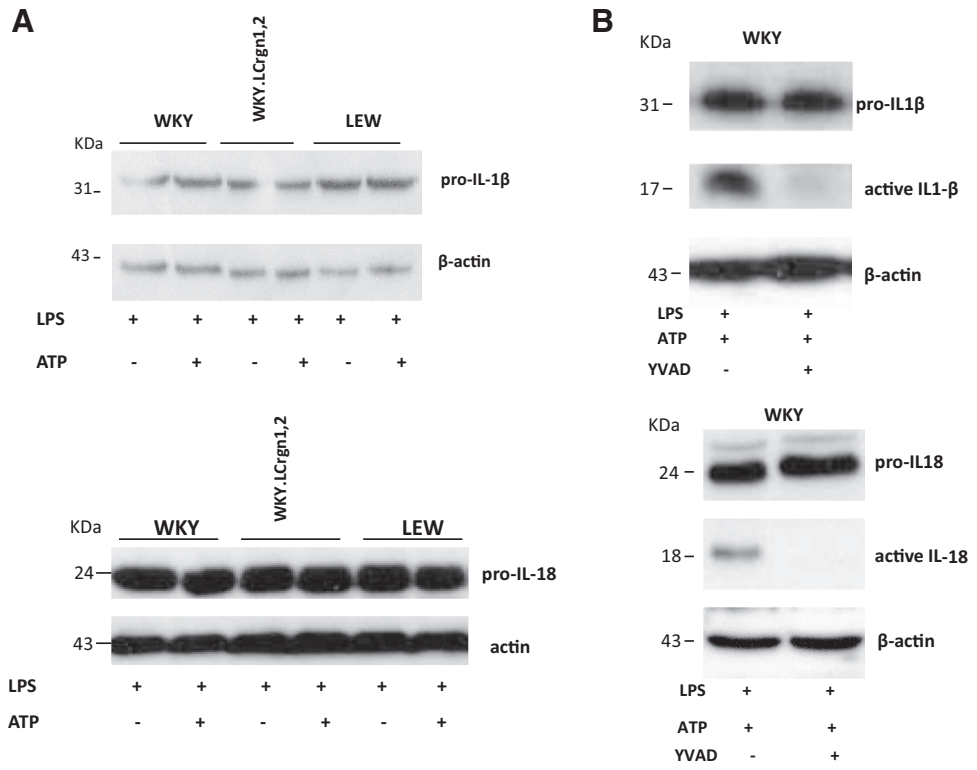

Figure 3. The increased P2RX7-mediated IL-1β and IL-18 secretion in WKY BMDMs is caspase-1-dependent and is not a result of an up-regulation of the pro forms of the cytokines. (A) BMDMs from WKY, LEW, and WKY.LCrgn1,2 rats were primed with LPS (1 μg/ml, 5 h) and treated 30 min with ATP (5 mM). To assess cellular levels of the pro forms of IL-1β and IL-18, cell lysates (not supernatants) were subjected to Western blotting. (B) The use of caspase-1 inhibitor (yVAD) and the assesement of IL-1β and IL-18 production in WKY BMDMs when the Nlrp3 inflammasome was activated with LPS (1 μg/ml, 5 h) followed by stimulation with ATP (5 mM, 30 min). These results are representative of three independent experiments.

glomeruli as early as 2.5 h following the injection of nephrotoxic serum, and their number increases and peaks at Day 4 [6]. We have previously shown by bone marrow transplantation experiments that genetically controlled macrophage infiltration and activation underpin glomerular injury in this model [8]. In this study, we focused on one particular proinflammatory pathway that partly explains the increased overactivity of WKY macrophages that is linked to susceptibility to Crgn. We previously conducted a microarray experiment in basal and LPS-stimulated BMDMs and identified P2RX7 as a markedly overexpressed transcript in the WKY cells when compared with LEW ones [11]. This led us to hypothesize that the members of Nlrp3-inflammasome pathway could also be differentially expressed between the NTN-susceptible WKY and NTN-resistant LEW BMDMs.

In the present study, we found that (1) there is genetic control of transcription of multiple genes belonging to the Nlrp3-inflammasome pathway in the WKY and LEW BMDMs and that this is not under the control of the two previously identified NTN QTL (Crgn1 and Crgn2); (2) there is genetic control of P2RX7 protein levels in BMDMs and nephritic glomeruli of WKY and LEW rats and that this is partly regulated by Crgn1 and Crgn2; and (3) caspase-1 activation and the release of active IL-1β and IL-18 are also under genetic control in WKY and LEW BMDMs and nephritic glomeruli. Crgn1 and Crgn2 partly regulate the Nlrp3-inflammasome activation in vitro and ex vivo. In summary, the P2RX7-Nlrp3-inflammasome pathway is genetically regulated, and its activation results in caspase-1-dependent IL-1β and IL-18 release in primary macrophages and glomeruli following NTN induction. In BMDMs, Crgn1 and Crg2 do not control the transcription but only the post-translational maturation of these cytokines. We have previously, positionally cloned Crgn1 and Crgn2 genes, which are *Fcgr3* and *Jund*, respectively. Although we have shown a role for these genes in FcR-mediated macrophage activation, we cannot exclude a potential role for these genes in P2RX7-mediated inflammasome activation. However, it is noteworthy that Crgn1 and Crgn2 contain hundreds of expressed genetic variants and may include genes other than *Fcgr3* and *Jund* implicated in Nlrp3-inflammasome activation.

The release of ATP by dying cells is a critical danger signal in tissues during organ damage, and the ensuing activation of P2RX7 is a key response in the injured organs [33, 34]. There are several studies that have examined the role of P2RX7 in renal inflammation and fibrosis [35]. For instance, P2RX7−/− mice exhibit reduced tubular injury, inflammation, and fibrosis when compared with WT mice following unilateral ureteric obstruction [36]. The proinflammatory role of P2RX7 in experimental Crgn in mice and rats led to the finding that the increased expression of this receptor in the glomeruli coincides with the peak of macrophage infiltration and IL-1β expression, and its absence or inhibition results in reduced glomerular thrombosis and macrophage infiltration [22, 31]. In humans, The P2RX7 gene is highly polymorphic, and single nucleotide polymorphisms underlie the variation observed in receptor function [37] and more broadly, the association with whole-body phenotypes and/or disease [38]. We have sequenced the protein-coding region of P2RX7 in WKY and LEW rats and found one synonymous coding variant, as well as single nucleotide polymorphisms and deletion/insertions in the promoter region. This suggests that the observed expression differences between the WKY and LEW rats could

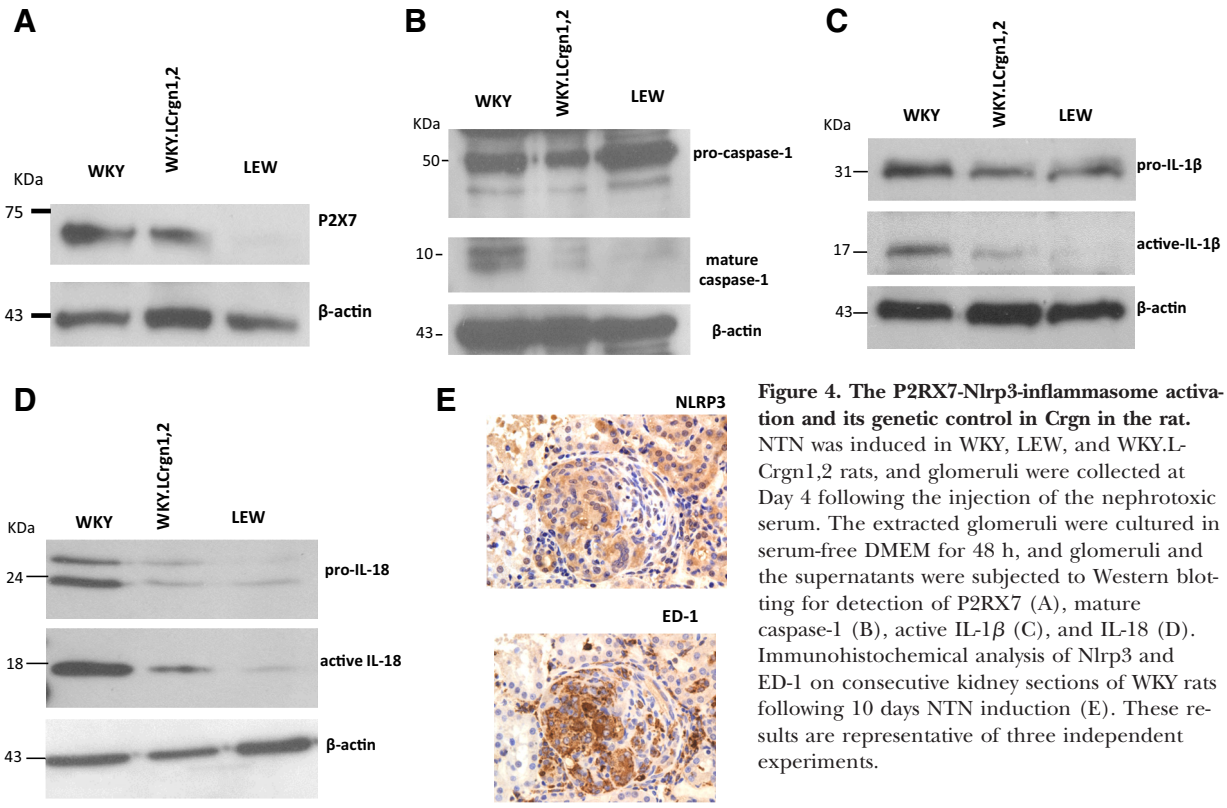

Figure 4. The P2RX7-Nlrp3-inflammasome activation and its genetic control in Crgn in the rat. NTN was induced in WKY, LEW, and WKY.LCrgn1,2 rats, and glomeruli were collected at Day 4 following the injection of the nephrotoxic serum. The extracted glomeruli were cultured in serum-free DMEM for 48 h, and glomeruli and the supernatants were subjected to Western blotting for detection of P2RX7 (A), mature caspase-1 (B), active IL-1β (C), and IL-18 (D). Immunohistochemical analysis of Nlrp3 and ED-1 on consecutive kidney sections of WKY rats following 10 days NTN induction (E). These results are representative of three independent experiments.

be a result of sequence variations within the vicinity of the gene (*cis* control) or elsewhere in the genome (*trans* control). The results described in the present study suggest that genetic loci control the expression of P2RX7 in macrophages and that fine-tuning of its expression levels is crucial for the activation of the Nlrp3-inflammasome and subsequent release of active IL-1β and IL-18 associated with susceptibility to Crgn.

Although there are reports implicating inflammasomes in chronic [39] kidney disease, there is no report showing the role of inflammasomes in macrophage-dependent Crgn. Our study is the first describing the P2RX7-Nlrp3-inflammasome activation in the NTN model in the WKY rat. Our current study highlights the importance of the Nlrp3-inflammasome pathway that is triggered by P2RX7 activation and results in IL-1β and IL-18 release in macrophages. Targeting the Nlrp3-inflammasome pathway could be considered as a therapeutic approach in Crgn.

AUTHORSHIP

S.D., H.T.C., F.W.K.T., and J.B. planned the study. S.D. optimized and performed the majority of Western blots for detection of the inflammasome activity in primary rat macrophages. R.R. performed the P2RX7 antagonist experiments in macrophages and nephritic glomeruli. S.S. performed experiments with nigericin stimulation and G.B. provided the immunostaining results for Nlrp3 and ED-1. L.F., C.D.P., H.T.C., F.W.K.T., S.D., R.R., R.J.U., and J.B. analyzed the results. J.B. prepared and wrote the manuscript.

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