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IL-1β promotes tubulointerstitial injury in MPO-ANCA-associated glomerulonephritis

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Key words
anti-neutrophil cytoplasmic antibody (ANCA) – tubulointerstitial injury (TII) – IL-1β – damage-associated molecular patterns (DAMPs) – NLRP3 inflammasome

Abstract. Background. It is widely accepted that tubulointerstitial injury (TII) is caused by glomerular injury (GI) in glomerular diseases. Glomerular endocapillary inflammation may result in crescent formation and exuded protein leakage, which may induce TII in antineutrophil cytoplasmic antibody-associated glomerulonephritis (ANCAGN). However, some reports have indicated a glomerulonephritis-independent mechanism of TII in ANCAGN. The aim of this study was to determine the principle cytokines correlated with TII severity and to elucidate a characteristic mechanism for TII in ANCAGN. Methods. 28 myeloperoxidase-ANCA-positive ANCAGN patients were enrolled, and their kidney biopsy specimens were histologically evaluated with regard to GI and TII. The mRNA expression of various cytokines was examined in 28 specimens. Results. Interleukin (IL)-1β was significantly correlated with the severity of TII. The mRNA expression of Toll-like receptor 4 (TLR4) and Nod-like receptor family pyrin domain-containing-3 (NLRP3) also correlated with TII severity. Immunohistochemical analysis demonstrated that TLR4 protein was positively stained in the tubulointerstitial infiltrating cells. NRLP3 protein was detected in macrophages in the severe infiltrating area but was absent or only very faintly expressed in the glomeruli. These results indicated that NLRP3 inflammasome-dependent processing in macrophages releases the mature active form of IL-1β, which may lead to the development and deterioration of TII. Conclusions. Sterile inflammation leads to the formation of ANCA-mediated neutrophil extracellular traps (NETs), which may stimulate macrophages and dendritic cells via TLR4 and induce NF-κB-dependent mRNA expression and translation of pro-IL-1β. Simultaneously, damage-associated molecular pattern signals resulting from NETs promote NLRP3 inflammasome-dependent processing and release mature active IL-1β. Sterile inflammation utilizing the NLRP3 inflammasome might be a characteristic reaction limited to the tubulointerstitium. Thus, neutralizing IL-1β may be a promising strategy to suspend the progress of TII and improve the prognosis of chronic kidney disease resulting from ANCAGN.

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Introduction

Glomerulonephritis causing progressive loss of renal function over a relatively short period of time is known as rapidly progressive glomerulonephritis (RPGN). In elderly people, myeloperoxidase (MPO) and proteinase-3 (PR3) antineutrophil cytoplasmic antibody (ANCA)-associated crescentic glomerulonephritis is a major cause of RPGN, and the principal histopathological features are glomerular extracapillary proliferation (crescents) and fibrinoid necrosis [1, 2]. In general, glomerulonephritis is associated with tubulointerstitial lesions to some extent, and it is universally agreed that glomerular damage is the cause of tubulointerstitial injury (TII). Nevertheless, the mechanism underlying the transfer of glomerular injury (GI) to tubulointerstitium remains controversial. The endocapillary aggressive process leads to breaks in the glomerular basement membrane (GBM) and exudation into Bowman's space and results in crescent formation [3, 4, 5]. The exuded protein leakage leads to excessive protein reabsorption in the proximal tubules resulting in TII [6, 7]. However, it was demonstrated that crescents in the early stage contain mainly epithelial cells [3, 8, 9], with the rest comprised of proliferating pari-

Table 1. Patient characteristics at the time of kidney biopsy.

n = 28	Average
Age	69.3 ± 10.1
Sex	male: 16; female: 12
WBC (/μL)	9,260 ± 4,770
Hb (g/dL)	9.65 ± 2.14
TP (g/dL)	6.51 ± 0.82
Alb (g/dL)	2.71 ± 0.74
BUN (mg/dL)	32.4 ± 15.7
Cr (mg/dL)	2.44 ± 1.42
eGFR (mL/min/1.73m^2)	29.22 ± 20.48
CRP (mg/dL)	6.82 ± 7.17
MPO-ANCA (U/mL)	227 ± 205
IgG (mg/dL)	1,568 ± 475
IgE (IU/mL)	280 ± 272
U-Pro (g/day)	1.38 ± 1.35
U-RBC (/HPF)	1-9:4, 10-49:10, 50-99:3, 100<:11
U-β2MG (μg/L)	15,238 ± 19,795
U-NAG (IU/L)	19.6 ± 13.6
Duration for Bx (day)*	39.5 ± 19.9

*The duration for kidney biopsy examination from disease development.

etal epithelial cells [10, 11]. Based on these findings, two mechanisms for the induction of TII by these crescents have been proposed. The first is that the crescents expand in the space between the tubular epithelium and the tubular basement membrane and then spread within this space along the entire proximal convolution [12, 13, 14]. The second proposed mechanism is that the growing crescents encroach upon the glomerulotubular junction directly [14, 15, 16]. Both mechanisms, i.e., abnormal filtrate spreading and cellular overgrowth, may lead to loss of the nephron.

We have often encountered cases in which the severity of TII is much greater than that of glomerular damage. In fact, there have been several cases reported in which only tubulointerstitial nephritis is noted without any apparent glomerular lesions [17, 18] in ANCA-associated glomerulonephritis (ANCAGN). Histological evaluation of renal pathological changes using follow-up biopsies showed that renal function improved along with improvement in acute tubulointerstitial nephritis, while acute glomerular injuries developed into chronic glomerular injuries in other cases [19]. These reports suggest the existence of an independent mechanism for TII from that for glomerulonephritis in ANCAGN. It is well established that the extent of tubulointerstitial mononuclear infiltration correlates with kidney function and prognosis in many types of chronic kidney diseases (CKD) [20]. Indeed, the intertubular interstitium harbors dendritic cells (DCs), macrophages, lymphocytes, lymphatic endothelial cells, and various types of fibroblasts, the hallmark cell type of connective tissues [21]. In particular, DCs and macrophages survey against injury and infection and contribute to organ homeostasis and tissue repair but may also promote the progression of CKD. Therefore, it is reasonable that there is an alternative mechanism for TII besides that of the ripple effect of glomerulonephritis in ANCAGN.

Therefore, the aim of this study was to clarify this mechanism of TII independent of the ripple effects of glomerular damage. Toward this end, we conducted a retrospective pathological analysis of 28 patients with ANCAGN and evaluated the expression of various cytokine messages in biopsied kidney specimens to determine the relationship between cytokine message expression and the severity of TII. Specifically, we evaluated the correlations of the expression of the cytokines interleukin (IL)-17, IL-1β, interferon (IFN)-γ, and transforming growth factor (TGF)-β as well as Toll-like receptors (TLRs) with the severity of TII.

Materials and methods

This study was performed in accordance with the Declaration of Helsinki and was approved by the ethics committee of Fukuoka University. Written informed consent was obtained from all patients.

Patients

We enrolled 28 patients who were diagnosed with MPO-ANCA-positive ANCAGN in the Division of Nephrology and Rheumatology in Fukuoka University Hospital and Saiseikai Fukuoka General Hospital between November 2002 and October 2015. All patients received kidney biopsy examinations within 39.5 ± 19.9 days since subjective symptom appeared, and no steroid or immunosuppressant was prescribed at the time of kidney biopsy. The clinical background data of the patients are shown in Table 1.

Table 2. Patient characteristics of three TII severity categories.

TII	1+	2+	3+
Patients	5	12	11
Age	65.8 ± 6.1	69.6 ± 11.7	70.5 ± 9.9
Hb (g/dL)	11.2 ± 0.7	9.2 ± 1.4	9.4 ± 2.9
BUN (mg/dL)	23.7 ± 10.5	35.0 ± 18.3	33.5 ± 14.3
Cr (mg/dL)	1.39 ± 0.55	2.60 ± 1.42	2.74 ± 1.57
eGFR (mL/min/1.73m^2)	41.94 ± 18.69	24.26 ± 14.76	28.86 ± 25.34
CRP (mg/dL)	2.45 ± 5.23	8.12 ± 7.73	7.44 ± 7.07
U-Pro (g/day)	1.5 ± 1.0	1.3 ± 0.9	1.4 ± 1.9
U-β2MG (μg/L)	1,482 ± 2,418	16,382 ± 23,918	18,992 ± 17,176
U-NAG (IU/L)	12.6 ± 3.2	16.7 ± 9.3	25.9 ± 18.0
MPO-ANCA (U/mL)	252 ± 149	175 ± 212	228 ± 205
Cellular crescent (%)	8.1 ± 6.1	36.5 ± 26.9	29.0 ± 15.8
Fibrous crescent (%)	1.8 ± 2.6	12.4 ± 18.1	10.4 ± 12.8
Glomerulosclerosis (%)	9.2 ± 8.8	10.3 ± 17.3	11.8 ± 10.5
TIIf (0, 1+, 2+, 3+)*	2, 3, 0, 0	9, 1, 0, 2	8, 1, 1, 1

*The numbers of patients categorized in TIIf (0, 1+, 2+, 3+), respectively.

Table 3. Primer sequences for RT-PCR using SYBR green chemistry.

β-Actin	5′ GCA AAG ACC TGT ACG CCA AC 3′
	5′ CTA GAA GCA TTT GCG GTG GA 3′
IFN-γ	5′ GAG ACC ATC AAG GAA GAC AT 3′
	5′ GTA TTG CTT TGC GTT GGA 3′
IL-12	5′ CCT GAC CCA CCC AAG AAC TT 3′
	5′ GTG GCT GAG GTC TTG TCC GT 3′
IL-4	5′ CTG CCT CCA AGA ACA CAA CT 3′
	5′ CAC AGG ACA GGA ATT CAA GC 3′
IL-5	5′ CCA ACT GTG CAC TGA AGA 3′
	5′ TGG CCG TCA ATG TAT TTC 3′
IL-6	5′ GGC ACT GGC AGA AAA CAA 3′
	5′ CTC CAA AAG ACC AGT GAT GA 3′
IL-17	5′ GCA GGA ATC ACA ATC CCA C 3′
	5′ TCT CTC AGG GYC CTC ATT GC 3′
IL-10	5′ ACC CTG CCT AAC ATG CTT 3′
	5′ TCT CTC AGG GTC CTC ATT GC 3′
TGF-β	5′ CCC CCT ACA TTT GGA GCC TG 3′
	5′ TTG CGG CCC ACG TAG TAC AC 3′
NLRP3	5′ GAA GAA AGA TTA CCG TAA GAA GTA CAG AAA 3′
	5′ CGT TTG TTG AGG CTC ACA CTC T 3′
Caspase1	5′ GGG CAT AGC TGG GTT GTC 3′
	5′ CAA GGG TGC TGA ACA AGG 3′

Histological evaluations

The diagnosis was determined according to the classification of the Japanese Renal Biopsy Registry based on the classification of glomerular diseases. Biopsy specimens were processed and observed using routine methods, including light microscopy, immunofluorescence techniques, and electron microscopy. ANCAGN was defined according to laboratory and histological findings of rapidly progressive glomerulonephritis along with positive tests for MPO-ANCA. The severity of cellular crescent formation of glomerulus was evaluated in a blind manner by histologic examination with periodic acid-Schiff staining and periodic acid-methenamine-silver staining. A crescent occupying more than half of the total area by cellular elements was defined as a cellular crescent, and the percentages of glomeruli showing cellular crescent for all glomeruli were expressed as an index of the cellular crescent formation rate (cCFR). Concerning TII, cellular injury (TIIc) and fibrous injuries (TIIf) were respectively evaluated, and both were classified into 4 groups according to the histological diagnosis of the tubulointerstitial inflammation area (0: no inflammation; 1+: < 25% inflammation area; 2+: 25 – 50% inflammation area; 3+: > 50% inflammation area). The clinical characteristics of patients included in each category are shown in Table 2.

Reverse transcription-polymerase chain reaction (PCR)

Total RNA was extracted from the kidney biopsy specimens collected from 28 patients before commencing steroid therapy, using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Quantitative cDNA amplification was performed according to the manufacturer's instructions. All samples were stored at –20 °C until use. cDNAs of the cytokines were analyzed by real-time PCR using Power SYBR Green PCR Master Mix (Applied Biosystems Japan, Tokyo, Japan) or TaqMan Gene Expression Master Mix (Applied Biosystems Japan). Sequence-specific amplification was detected with an increased fluorescence signal during the amplification cycles using an ABI Prism 7500 sequence detection system (Perkin Elmer Japan, Yokohama, Japan). To provide a meaningful comparison between different samples, we calculated the amount of PCR products relative to the amount of β-actin in each sample. Oligonucleotide primers and probes were designed using the Primer Express program (Applied Biosystems Japan) or purchased directly. Oligonucleotides used for SYBR green chemistry methods are shown in Table 3. Purchased Taqman primers were as follows:

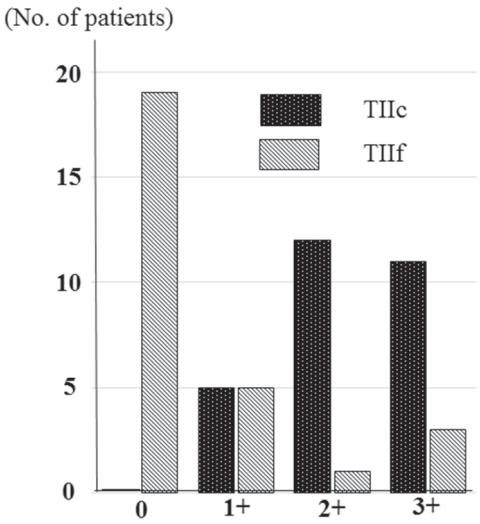

Figure 1. Distribution of patients in TII categories.

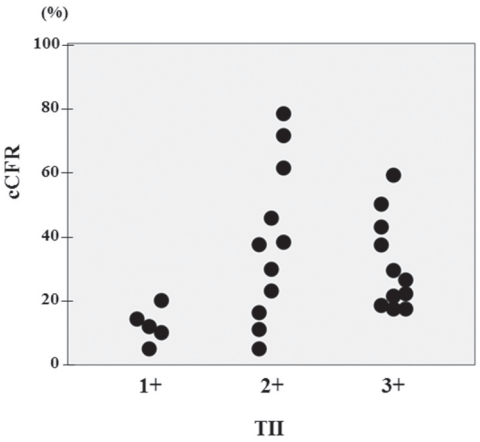

Figure 2. Severity of glomerular injury (GI) and tubulointerstitial injury (TII) in 28 ANCAGN patients. The cellular crescent formation rate (cCFR) is expressed as the percentage to represent the severity of GI along the Y-axis, and the severity grade for TII is shown on the X-axis according to 1+, 2+, 3+, as defined in the materials and methods section.

Hs01872448-s1 for TLR2, Hs01551078-m1 for TLR3, Hs01060665-g1 for TLR4, Hs01933259-m1 for TLR7, Hs00370913-s1 for TLR9, Hs01555410-m1 for IL-1β, Hs01038788-m1 for IL-18, Hs00356648-s1 for IFN-α, Hs99999043-m1 for TNF-α.

Immunohistological analysis

After deparaffinization in xylene and ethanol, and washing in phosphate-buffered saline (PBS), paraffin-embedded sections were incubated with mouse antihuman TLR-4 antibody (Ab), antihuman nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP3) Ab, and rabbit antihuman caspase1 (anti-CASP1) Ab (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at a concentration of 1 µg IgG/mL PBS, including 1% bovine serum albumin or 5% normal goat serum. Monoclonal and polyclonal staining was performed using antimouse or antirabbit IgG horseradish peroxidase-labeled polymer (Dako Cytomation, Inc., Carpinteria, CA, USA).

Statistical analysis

Statistical significance of the differences between groups was determined by Pearson's rank correlation coefficient. All statistical analyses in this study were performed using SPSS statistics software version 22 (IBM Corp, Armonk, NY, USA). p-values < 0.05 were considered to be statistically significant.

Results

Histological evaluation

Probably because kidney biopsies were performed with rapidity after disease development, the severities of TIIf were not conspicuous compared with those of TIIc. With more than 85% of patients being categorized in TIIf 0 or 1+, we decided to focus on TIIc in this study and designate TII for TIIc (Figure 1).

Correlation of the severity between GI and TII

We evaluated the relationship between GI and TII. Pearson's correlation coefficient was 0.253, and the severity of TII was not correlated with that of cCFR (Figure 2). This result supported the notion that there is likely another mechanism for TII other than a ripple effect from glomerular damage in ANCAGN. The mRNA expression levels of *IL-17*, *IFN-γ*, and *TGF-β* were correlated with cCFR (p-value 0.009, 0.005, and 0.002, respectively) (Figure 3), whereas only the expression of *IL-1β* correlated with the severity of TII (p-value 0.002) (Figure 4).

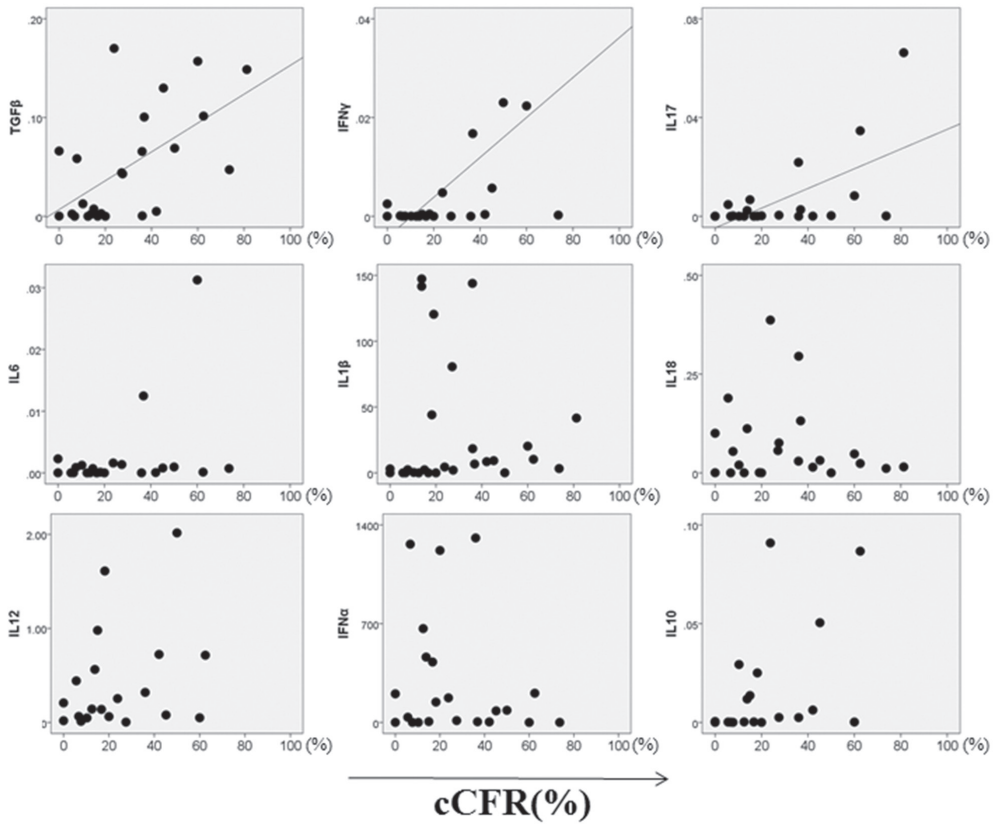

Figure 3. Correlation of the severity of GI and the mRNA expression of cytokines in ANCAGN. GI severity is expressed as cCFR (%) along the X-axis, and the expression level of each cytokine relative to *β-actin* expression is shown along the Y-axis. The mRNA expression levels of *TGF-β*, *IFN-γ*, and *IL-17* were significantly correlated with CFR.

Role of the inflammasome in TII

The results described above suggested that the severity of TII may be correlated with the mRNA expression level of IL-1β. IL-1β is an inflammasome-dependent cytokine, and the generation of mature IL-1β requires two separate processes: (i) the induction of NF-κB-dependent mRNA expression and translation of the pro-IL-1β, and (ii) inflammasome-dependent processing and release of the mature active IL-1β. First, we speculated that TLR signals might be related with NF-κB pathway activation, and we evaluated the mRNA expression levels of various TLRs. *TLR4* expression was most significantly correlated with the severity of TII (p-value 0.029) (Figure 5) and was also significantly correlated with *IL-1β* expression (p-value 0.039) (Figure 6A).

Next, we evaluated the role of the inflammasome in the development of TII.

NLRP3 expression was significantly correlated with the severity of TII (p-value 0.005) (Figure 6B) as well as with caspase1 mRNA expression (p-value 0.001) (Figure 6C), which is required for activation of IL-1β (Figure 6A).

Immunohistochemical analysis demonstrated that TLR4 protein was not detectable in the glomeruli, but some stained tubulointerstitial infiltrating cells were detected (Figure 7A). Although many NLRP3-positive cells were observed in the severe infiltrating area (Figure 7B), NRLP3 protein was absent or was only very faintly expressed in the glomeruli (data not shown). Similarly, caspase1-positive cells were scattered in the tubulointerstitium (Figure 7C), but the number of these cells was 1/5 that of NLRP3-positive cells. Almost all of these positive cells were also positive for CD68 expression (data not shown).

Discussion

It is widely accepted that glomerular damage causes TII. However, in the current study, no significant correlation between the

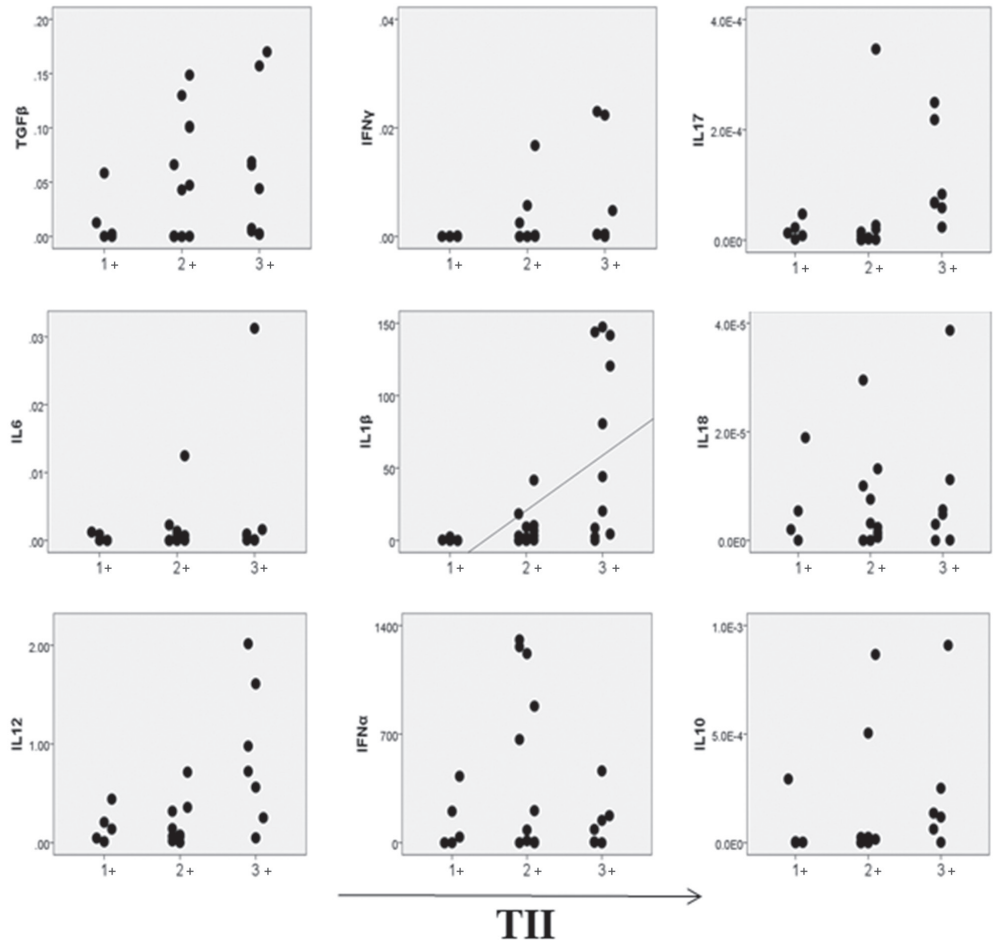

Figure 4. Correlation of the severity of TII and the mRNA expression of cytokines in ANCAGN. The severity grade of TII is shown along the X-axis, and the expression level of each cytokine relative to the β-actin expression level is shown along the Y-axis. Only *IL-1β* was significantly correlated with the severity of TII.

severity of GI and TII in patients with ANCAGN was observed. This indicated that mechanisms other than GI may provoke TII. Therefore, we assessed the mRNA expression levels of various cytokines in the kidneys of patients with ANCAGN to evaluate the immune response-related factors that are correlated with the severity of GI or TII, respectively. The expression of *IL-17*, *IFN-γ*, and *TGF-β* was correlated with cCFR increase, and *IL-1β* was correlated with TII severity. In fact, some studies have shown similar results for GI, in which Th1 and Th17 immune responses may be integral in human ANCAGN or in a mouse model of antigen-specific glomerulonephritis [22, 23, 24]. However, it was clarified in this study that *IL-1β* plays a central role for TII severity in ANCAGN. These findings mean that the principal cytokines responsible for the severity of GI and TII are different in ANCAGN.

IL-1β is first produced in an inactive proform (pro-IL-1β), which requires cleavage for its activation and the cysteine protease caspase-1 cleaves pro-IL-1β to form the mature IL-1β [25, 26, 27]. NLRP3 of the NLR family of innate immune cell sensors is a key component for caspase-1 production and activation of IL-1β [28], and its expression is limited to interstitial monocytic phagocytes/DCs, which express the components of the NLRP3 inflammasome inside the kidney [29, 30]. In this study, some tubulointerstitial infiltrating macrophages showed positive staining for NLRP3, and the *NLRP3* mRNA expression level correlated with the severity of TII. These results suggest that the NLRP3-inflammasome plays a role in TII. By contrast, we did not detect any NLRP3-positive staining in the glomeruli, indicating that glomerular injury develops independently of the NLRP3-inflamasome, as described in anti-GBM glomerulonephritis model mice [29].

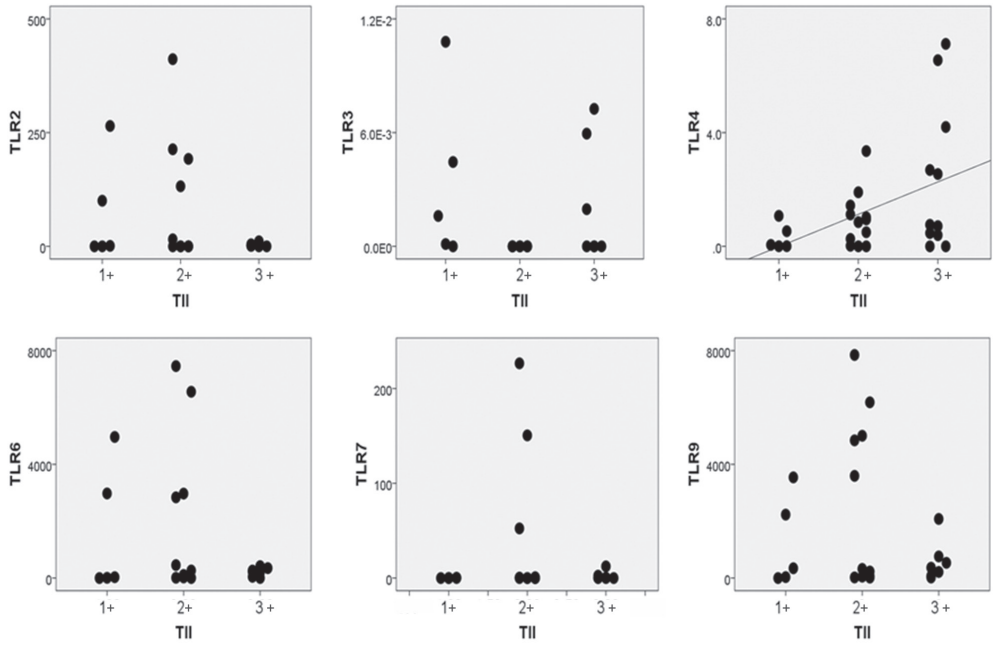

Figure 5. Correlation between the severity of TII and the mRNA expression of Toll-like receptor (TLR) in ANCAGN. The severity grade of TII is shown along the X-axis, and the expression level of each TLR relative to β-actin expression is shown along the Y-axis. Only *TLR4* was significantly correlated with the severity of TII.

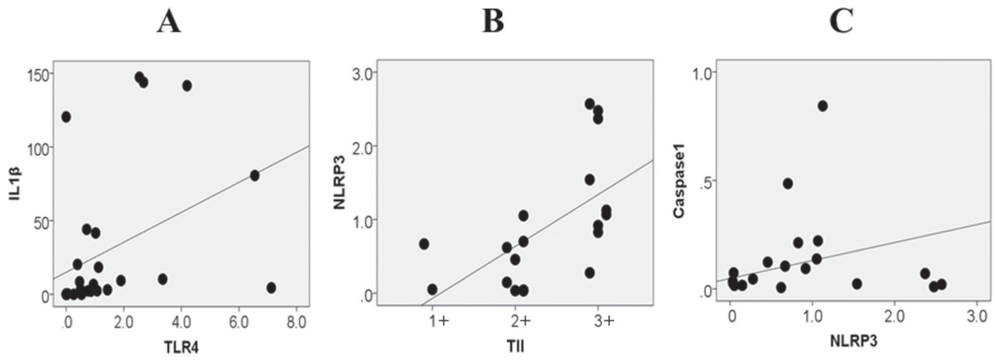

Figure 6. Relationship between the inflammasome and TII in ANCAGN. A: The expression levels of *TLR4* and *IL-1β* relative to *β-actin* expression are represented along the X- and Y-axis, respectively. B: The severity grade of TII is shown along the X-axis, and the expression level of NLRP3 relative to β-actin expression is shown along the Y-axis. C: Expression levels of *NLRP3* and caspase1 relative to *β-actin* expression are represented along the X- and Y-axis, respectively.

Dying neutrophils have been shown to release chromatin fibers that trap and kill invading microbes extracellulary [31], and this phenomenon is generally referred to as neutrophil extracellular trap (NETs) formation. This glutinous DNA web can stick to the endothelium and cause tissue damage during sepsis [32]. In the context of sterile inflammation, ANCA-mediated NETs formation has been demonstrated [33], and the morphological changes of neutrophil nuclei clearly indicated that ANCA-induced NETs were of nuclear rather than of mitochondrial origin. TLR2 and TLR4 recognize self-compartments that are normally sequestered but are released in situations of stress, sterile inflammation, or cellular damage [34]. The results of the present study suggest that the stimulation of macrophages from TLR4 may induce NF-κB-dependent mRNA expression and translation of pro-IL-1β. Indeed, the mRNA expression level of *IL-1β* correlated with that of *TLR4*, which were both also correlated with the severity of TII. Simultaneously, DAMP signals, such as HMGB1, heat shock proteins, and fibronectin, also promote

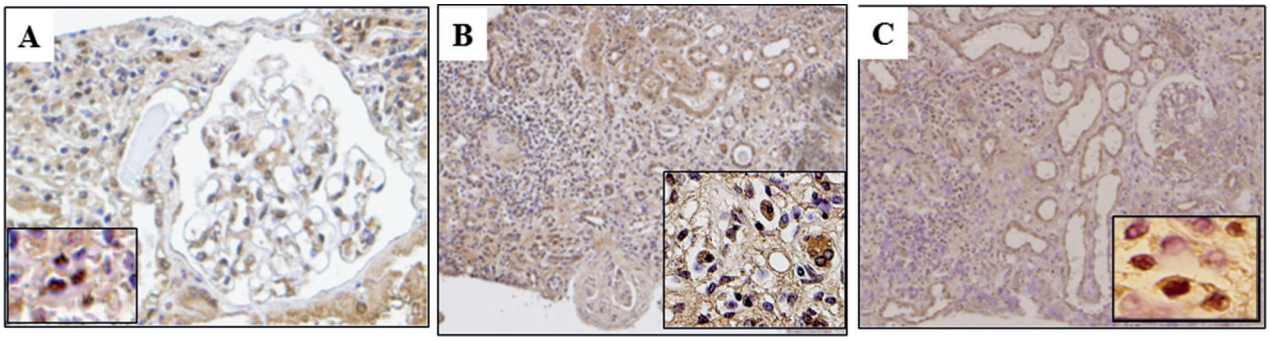

Figure 7. Representative immunohistochemistry results for the role of the inflammasome in ANCAGN. A: TLR4-positive cells were detected in the tubulointerstitium, and not in the glomerulus. Magnification, 400×, boxed area 1,000×. B: NLRP3: magnification, 100×, boxed area 800×. C: caspase1: magnification, 100×, boxed area, 1,000×.

NLRP3 inflammasome-dependent processing in macrophages or DCs, resulting in the release of the mature active IL-1β. Observation of the kidneys of mice showed that the tubulointerstitium of healthy kidneys contains numerous cells with predominant DC functionality, which are mostly absent from the glomeruli [35, 36]. In line with this finding, in the present study, there was no positive immunohistological staining for TLR4 or NLRP3 in the glomeruli, and there was also no correlation between cCFR and *IL-1β* mRNA expression. These observations suggest that sterile inflammation mediated by the NLRP3 inflammasome might be a characteristic reaction limited to the tubulointerstitium.

From a therapeutic standpoint, neutralizing IL-1β may represent a rational strategy for TII in ANCAGN. Some agents that target IL-1β are already in clinical use or in advanced stages of drug development. Anakinra, a nonglycosylated recombinant form of the naturally occurring IL-1 receptor antagonist that blocks inflammasome-dependent IL-1β signaling, has been successfully used in the treatment of type 2 diabetes, asbestosis, and other conditions in the United States [37]. Canakinumab is a monoclonal antibody that binds to and antagonizes IL-1β and is currently being studied in a number of clinical trials [38]. The prognosis of ANCAGN mainly depends on improvement of GI and TII, and thus targeting IL-1β appears to be a promising strategy to ameliorate TII and prevent interstitial fibrosis.

In summary, we evaluated the mRNA expression of cytokines in ANCAGN and found that *IL-1β* mRNA expression was correlated with the severity of TII. The proposed mechanism is that DAMPs from activated neutrophils may trigger sterile inflammation using the NLRP3 inflammasome in the DCs and macrophages from the tubulointerstitium, resulting in the release of mature IL-1β. Therefore, neutralizing IL-1β may be an excellent strategy to suspend the progress of TII and improve the prognosis of CKD resulting from ANCAGN.

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Conflict of interest

The authors have declared that no conflict of interest exists.

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