IL-6 Trans-Signaling Drives Murine Crescentic GN

Gerald S. Braun,*† Yoshikuni Nagayama,*‡ Yuichi Maruta,*‡ Felix Heymann,§ Claudia R. van Roeyen,* Barbara M. Klinkhammer,* Peter Boor,*¶ Luigi Villa,* David J. Salant,** Ute Raffetseder,* Stefan Rose-John,†† Tammo Ostendorf,* and Jürgen Floege*

*Division of Nephrology and Immunology, Rheinisch-Westfälische Technische Hochschule (RWTH) Aachen University, Aachen, Germany; †Institute of Biochemistry and Molecular Biology, RWTH Aachen University, Aachen, Germany; §Division of Gastroenterology, Metabolic Diseases and Intensive Care, RWTH Aachen University, Aachen, Germany; ¶Institute of Pathology, RWTH Aachen University, Aachen, Germany; ‡Institute of Molecular Biomedicine, Comenius University, Bratislava, Slovakia; †Division of Nephrology, Showa University Fujigaoka Hospital, Yokohama, Japan; **Department of Medicine, Section of Nephrology, Boston University School of Medicine, Boston, MA; and ††Institute of Biochemistry, Christian-Albrechts-University, Kiel, Germany

ABSTRACT

The role of IL-6 signaling in renal diseases remains controversial, with data describing both anti-inflammatory and proinflammatory effects. IL-6 can act via classic signaling, engaging its two membrane receptors gp130 and IL-6 receptor (IL-6R). Alternatively, IL-6 trans-signaling requires soluble IL-6R (sIL-6R) to act on IL-6R-negative cells that express gp130. Here, we characterize the role of both pathways in crescentic nephritis. Patients with crescentic nephritis had significantly elevated levels of IL-6 in both serum and urine. Similarly, nephrotoxic serum-induced nephritis (NTN) in BALB/c mice was associated with elevated serum IL-6 levels. Levels of serum sIL-6R and renal downstream signals of IL-6 (phosphorylated signal transducer and activator of transcription 3, suppressor of cytokine signaling 3) increased over time in this model. Simultaneous inhibition of both IL-6 signaling pathways using anti–IL-6 antibody did not have a significant impact on NTN severity. In contrast, specific inhibition of trans-signaling using recombinant sgp130Fc resulted in milder disease. Vice versa, specific activation of trans-signaling using a recombinant IL-6–sIL-6R fusion molecule (Hyper-IL-6) significantly aggravated NTN and led to increased systolic BP in NTN mice. This correlated with increased renal mRNA synthesis of the Th17 cell cytokine IL-17A and decreased synthesis of resistin-like alpha (RELMalpha)-encoding mRNA, a surrogate marker of lesion-mitigating M2 macrophage subtypes. Collectively, our data suggest a central role for IL-6 trans-signaling in crescentic nephritis and offer options for more effective and specific therapeutic interventions in the IL-6 system.

Received November 26, 2014. Accepted March 20, 2015.
G.S.B. and Y.N. contributed equally to this work.
Published online ahead of print. Publication date available at www.jasn.org.
Correspondence: Dr. Gerald Braun, Medizinische Klinik II, Universitätsklinikum der RWTH, Pauwelstr. 30, D-52057 Aachen, Germany. Email: gbraun@ukaachen.de

IL-6 has long been recognized to contribute to inflammatory glomerular diseases.1–5 It is mostly produced by leukocytes but can also be secreted from glomerular endothelial and mesangial cells upon stimulation with, for example, angiotensin II and by podocytes following endotoxin administration in vivo.6–8 However, the role of IL-6 signaling in glomerular disease, in particular in rapidly progressive glomerulonephritis (RPGN), remains controversial. In rats, IL-6 infusion exerted a beneficial effect in the RPGN model of nephrotoxic nephritis (NTN),9 possibly by limiting macrophage activity. Likewise, IL-6 inhibition by either an IL-6- or an IL-6-receptor (IL-6R)-specific antibody aggravated NTN in C57Bi/6 mice leading to increased macrophage activity.10 However, research in models of
systemic lupus erythematosus (SLE) arrived at an opposite conclusion. In one study IL-6 infusion exerted an aggravating effect on GN.\textsuperscript{11} Although the opposite experiment of IL-6 antagonism did not turn out to be beneficial in this work,\textsuperscript{11} a second study described disease reduction in another SLE model by an IL-6R-specific antibody.\textsuperscript{12}

The IL-6 system consists of IL-6 and two receptors that occur in membrane-bound and soluble forms.\textsuperscript{13} In classic signaling, IL-6 often has anti-inflammatory activity by binding to a membrane-bound heterotetramer of the IL-6R and glycoprotein 130 (gp130), the common signal transducing unit of the IL-6 family of cytokines.\textsuperscript{13} While gp130 is ubiquitous, IL-6R is largely restricted to immune cells and hepatocytes.\textsuperscript{13} In contrast with classic IL-6 signaling, virtually all cells of the body can be stimulated by trans-signaling, in which membrane-bound gp130 transduces a signal upon binding of a complex of IL-6 and the soluble isoform of IL-6R (sIL-6R).\textsuperscript{13} Trans-signaling markedly widens the spectrum of IL-6–susceptible cells and usually exerts proinflammatory actions, especially in the late course of diseases.\textsuperscript{14} A circulating soluble isoform of gp130 (sgp130) inhibits trans-signaling and is considered a buffer system limiting trans-signaling in health.\textsuperscript{14} Therapeutically, a fusion protein consisting of two sgp130 chains joined by an inactivated Fc receptor portion (sgp130Fc) exerted beneficial anti-inflammatory effects in several non-renal models.\textsuperscript{14}

Given that IL-6 can exert both anti-inflammatory (usually via classic signaling) and proinflammatory activity (trans-signaling), we hypothesized that this dual mode of action may explain the above controversial findings of previous attempts to target the IL-6 system in nephritis. We therefore first performed a comprehensive assessment of the IL-6 system in human and experimental glomerulonephritis and then either blocked both pathways or selectively inhibited or stimulated trans-signaling in the NTN model.

**RESULTS**

**Circulating IL-6 Levels are Increased in Patients with RPGN**

We first measured the levels of IL-6, sIL-6R, and sgp130 in sera and urine of 17 patients with a first presentation or a flare or a history of RPGN with a biopsy-proven necrotizing glomerulonephritis with extracapillary proliferations. This was associated with PR-3 ANCA (n=7), MPO ANCA (n=7), anti-GBM antibodies (n=2), or a negative autoimmune serology with pauci-immune necrotizing GN (n=1).

Samples were available from a period of an acute RPGN flare in 10 and from two different sampling time points in six patients (Supplemental Table 1). IL-6 but not sIL-6R was increased in serum and urine when compared with healthy controls (Figure 1). Urinary excretion of the soluble endogenous trans-signaling antagonist sgp130 was also increased in GN patients. Mean serum IL-6 levels were independent of disease etiology but there was an association of elevated serum IL-6 with high clinical activity (Supplemental Tables 2 and 3). Control patient characteristics are summarized in Supplemental Table 4. Collectively, the findings evidenced activation of the IL-6 system in various conditions of human RPGN.

**Activation of the Circulating and Renal IL-6 system during NTN in BALB/c Mice**

Next, we assessed the IL-6 system in experimental RPGN, i.e., NTN induced in BALB/c mice. The Th1-prone C57Bl/6 strains are usually more susceptible to this model.\textsuperscript{15} However, BALB/c mice offer the unique opportunity to study GN in a Th2-prone setting, which may have relevance to humans subject to a Western lifestyle.\textsuperscript{16} Severe crescentic GN developed within 7 days in mice given a single injection of nephrotoxic serum\textsuperscript{17} spiked with CpG oligonucleotides (Figure 2A). This crescentic response of the BALB/c strain was specific to the NTN serum used,\textsuperscript{17} since crescents were not inducible

![Figure 1](https://example.com/figure1.png)

**Figure 1.** The circulating IL-6 system during human biopsy-proven RPGN of various underlying etiologies. (A) Serum levels and (B) urine shedding of IL-6 and sgp130 are significantly increased in RPGN patients compared with healthy controls. IL-6, expressed as log10 for reasons of the large variance in the RPGN group and because the majority of controls ranging below the detection limit of the assay (<1.5 pg/mL) and thus arbitrarily assumed as 1 pg/mL yielding a log10 of 0. The corresponding absolute mean serum and urine IL-6 levels prior to logarithmic transformation in RPGN patients were 16.1 pg/mL and 165 pg/10 mg creatinine (urine IL-6 data point #9, Supplemental Table 1 excluded), respectively. *P≤0.05 (Mann–Whitney test). n.s., non-significant.
following injection with an alternative serum known to be effective in C57Bl/6 mice18 (data not shown).

To avoid artifacts from blood cells, organs were perfused prior to harvest. Immune responses in BALB/c mice consisted of an early transient increase in renal proinflammatory macrophages at Day 7, followed by the emergence of disease-mitigating macrophage subtypes, as evidenced by renal cortical mRNA expression of the respective M1 and M2 macrophage subtype markers iNOS and Relm-$\alpha$/Fizz-1 (Ref. 19) on the one hand and the Th1 and Th2 cell markers IFN-$\gamma$; T-bet20,21 and IL-4; GATA-3 (ref. 22) on the other hand (Supplemental Figure 1A–C; see Figure for abbreviations). This was accompanied by a continuous cortical increase of markers for dendritic and Th17 cells (Supplemental Figure 1, D and E), that have pathogenic roles in NTN.23,24 The validity of this analytic approach is supported by literature data indicating that the expression of subtype markers in inflamed kidneys is primarily derived from immune and not renal parenchymal cells.25,26 This was corroborated by stainings for iNOS, Relm-$\alpha$, and GATA-3 in our NTN kidneys (Supplemental Figures 2 and 3).

Similar to humans with RPGN, pronounced increases in serum and urine IL-6 occurred in the early phase of NTN (Figure 2, B and C). In contrast with the human disease, however, increases in sIL-6R in serum paralleled the course of NTN. Meanwhile, serum sgp130 transiently decreased. Collectively, these findings are consistent with increased trans-signaling in the late disease phases. In urine, the IL-6 peak was accompanied by transient increases of both sIL-6R and sgp130 (Figure 2C). Thus, in the nephritic kidney, augmented

**Figure 2.** The circulating IL-6 system during NTN in BALB/c mice. (A) Histologic development of NTN over time. Glomerular crescents (arrow) as defined by the presence of at least two epithelial cell layers in Bowman’s space and fibrinoid necrosis (asterisk) are present from Day 7 onwards. Representative images, original magnifications, $\times 400$. (B) Serum levels for IL-6 increase 2000- to 5000-fold over baseline within hours of model induction, then subsequently fall rapidly but stay elevated 4-fold over control at Day 21. Levels of the soluble IL-6 receptor (sIL-6R) decrease initially but increase over time. The endogeneous antagonist, sgp130, is transiently significantly decreased. (C) In the urine, all three soluble components of the IL-6 system peak at Day 1–3. IL-6 and sIL-6R stay elevated when compared with control. $^*P$$\leq$$0.05$ versus control (analysis of variance [ANOVA]; Newman–Keuls post hoc test); $^P$$\leq$$0.05$ (subanalysis by two-sided t-test).
trans-signaling may be limited by the antagonist sgp130, similar to the human situation (Figure 1).

Activation of intrarenal IL-6 signaling in NTN was evidenced by increased levels of IL-6 (approximately 80-fold) and IL-6R (approximately 4-fold) mRNA (Figure 3A) and protein (Figure 3, B and C) at later stages. Transcriptional expression of the ubiquitous signal transducer, gp130, was unchanged. The continuous rise of IL-6 mRNA over time was paralleled by a marked transient peak in glomerular/cortical IL-6 protein on Day 3 (Figure 3, B and C). During NTN, pSTAT3 protein in kidney cortex increased as did mRNA of the endogenous pSTAT3-inducible suppressor of cytokine signaling, SOCS3, i.e., typical consequences of IL-6 downstream signaling activation (Figure 3, A and B). One source of increased IL-6 protein and signaling in nephritic glomeruli and cortical tissue were monocytes/macrophages (Figure 3D).

Total IL-6 Inhibition Does Not Alter the Course of NTN in BALB/c Mice
We first tested the effects of an antibody to IL-6, initiated after disease induction, which blocks both classic and trans-signaling. Using various outcome parameters, such as histologic changes (glomerular crescents, fibrinoid necrosis), renal function (blood urea nitrogen, BUN) and proteinuria, as well as cortical macropage and CD3 cell infiltration, we failed to observe a significant effect of total IL-6 inhibition in NTN (Figure 4). Bioactivity against IL-6 in vivo of this antibody and the batch used was evidenced previously by us and others.4,27,28

Selective Inhibition of Trans-Signaling Improves the Course of NTN in BALB/c Mice
Next we assessed the effects of selective blockade of IL-6 trans-signaling with sgp130Fc in NTN (Figure 5, left panels). sgp130Fc injections started after disease induction, i.e., in a therapeutic manner and in an attempt to avoid interference with disease induction.

At a dose of 3 mg/kg body wt, sgp130Fc reduced crescent formation (≈55%) (Figure 5, B and E) and glomerular fibrinoid necrosis (≈57%) (Figure 5, C and E), showing a trend for less fibrinogen deposition (Figure 5D). Other outcome parameters including BUN, proteinuria, and renal cortical leukocyte influx (F4/80 and CD3-positive cells) were numerically lower in nephritic mice treated with sgp130Fc, but the differences with PBS-treated mice failed to reach statistical significance (Figure 5, F,G,J and J). The mRNAs encoding SOCS3, IL-17A and the macrophage polarization-associated markers iNOS and Relm-α were not significantly affected by sgp130Fc (Figure 5, K–N). Blood pressure in NTN increased transiently and sgp130Fc mitigated this peak (Figure 5H).

Stimulation of Trans-Signaling Aggravates the Course of NTN in BALB/c Mice
Vice versa we tested, whether preferential stimulation of trans-signaling with Hyper-IL-6 (HIL-6) would worsen NTN. HIL-6 consists of joint IL-6 and sIL-6R protein sequences that directly activate trans-signaling with a 1000-fold higher efficiency compared with IL-6+sIL-6R alone.29

Daily administration of HIL-6 in NTN mice, again initiated after disease induction (Figure 5, right panels), aggravated glomerular fibrinoid necrosis and fibrinogen scores but not crescent formation (Figure 5, B–E). HIL-6 led to worse renal function and proteinuria as well as higher blood pressure on Day 13 after disease induction (Figure 5, F–H). As with sgp130Fc, renal cortical leukocyte infiltration (F4/80 and CD3) was not significantly affected (Figure 5, I and J). Renal cortical IL-17A mRNA increased significantly, whereas Relm-α mRNA decreased significantly, suggesting a shift toward potentially more deleterious T-cell types and a macrophage polarization into proinflammatory M1-like rather than immunosuppressive subtypes (Figure 5, K–N).

To exclude that the aggravation of NTN by HIL-6 was due to systemic effects of HIL-6 on blood pressure or other systemic parameters such as body weight, proteinuria or BUN, we administered the same dose of HIL-6 to healthy mice (Figure 6, A). There were no such effects (Figure 6, B–E).

DISCUSSION
In the present study we provide the first comprehensive description of the IL-6 system in experimental RPGN. Our data show an early, transient upregulation of circulating IL-6 combined with a downregulation of sIL-6R, which has also been noted in other models and should facilitate classic IL-6 signaling.30 In NTN, circulating IL-6 levels subsequently fell but still remained slightly elevated (4-fold) and, importantly, sIL-6R started to increase in parallel to glomerular crescent formation. This is consistent with the observation of 2–3-fold elevations of sIL-6R during later stages of inflammation in humans and should facilitate trans-signaling.13 Again, this sequence of early classic signaling followed by augmented IL-6 trans-signaling has been noted in non-renal models of inflammation as well.13,14 Our observations in active human RPGN best fit the transition phase between very early and late nephritis, given the isolated upregulation of circulating IL-6.

Within nephritic kidneys in NTN, regulation of the IL-6 system was more complex. We obtained strong evidence for increased intrarenal IL-6 signaling in late phases of the disease, as shown by the high amount of phosphorylated STAT3 in renal cortex. In addition, both IL-6 and, to a lesser extent, IL-6R mRNA and protein expression in renal cortex/glomeruli increased during NTN. Thus, both classic and trans-signaling may have increased at the tissue level. Except for the early stage (Day 3), which might reflect deposition of systemic IL-6, most of the glomerular/cortical IL-6 overexpression appeared to derive from intraglomerular cells other than podocytes and, may at least in part reflect monocyte/macrophage- or mesangial cell sources.5,31 While late restitution of circulating sgp130 in NTN may have antagonized IL-6 trans-signaling, intracellular regulators of IL-6R-gp130 signaling (e.g., SOCS3)
Figure 3. The local IL-6 system of the kidney during NTN in BALB/c mice. To exclude artifacts from intravascular components, animals were perfused with saline prior to organ harvest. (A) RT-qPCR showing marked increases of IL-6 and the IL-6–signaling-inducible suppressor of cytokine signaling 3 (SOCS3) mRNAs (30–80-fold and 5–15-fold, respectively). Slightly increased and unchanged mRNA expression of the IL-6R (3–5-fold) and gp130, respectively. All values normalized to transcript expression in healthy control animals (con). (B) Immunoblot analysis of the strongly upregulated mRNA events, i.e., IL-6 and IL-6 downstream signaling as evidenced by expression of STAT3 and its phosphorylation. Interestingly, IL-6 protein detection peaked as early as Day 3 while the corresponding mRNA expression at this time point was only moderately elevated (see A), suggesting the deposition of circulating IL-6 protein originating from extrarenal sources in the kidney. STAT3 and pSTAT3 indicate increasing downstream signaling over time. (C) Representative immunofluorescence images of glomeruli showing a transient increase of IL-6 at Day 3, thus supporting the view that IL-6 detected on immunoblot at Day 3 originates from transient extravascular deposition. Unchanged very low detection of the IL-6R throughout the course of NTN. Arrows exemplify sites of positive signal: distribution of both IL-6 and IL-6R was mesangial/endothelial and not significantly podocytic. Nestin as a podocyte marker. Original magnifications, ×400. (D) Flow cytometric identification of macrophages (Mφ) from kidney homogenates by CD45+CD11b+Ly6G−CD11c− and assessment of concomitant intracellular staining for IL-6 and pSTAT3, revealing increases in NTN in comparison with control. MFI, mean fluorescence intensity in arbitrary units. Correction for background intensity from the fluorescence minus one control. The corresponding relative amounts of IL-6- and pSTAT3-positive Mφ (con versus Day14): 0.8 versus 9.4% and 5.9 versus 14.9%, respectively. *P<0.05 versus control (ANOVA, Newman–Keuls post hoc test); *P≤0.05 (two-sided t-test). n.s., non-significant; con, healthy controls.
were also upregulated in nephritic kidneys and likely counteracted prolonged activation by IL-6.

Our second major finding was that pan-IL-6 inhibition, initiated after the early IL-6 peak, i.e., in a therapeutic fashion, had no detectable effect on the course of NTN. At first glance, these data are not consistent with other data describing renal benefits of IL-6 antagonism or deletion in murine lupus nephritis, hyperlipidemic ApoE⁻/⁻ mice and murine NTN nor with evidence that IL-6 induces or exacerbates renal disease. However, at the same time yet other reports noted a protective role of IL-6 in rat NTN or conversely worsening of NTN in C57Bl/6 mice by IL-6 antagonism. While it certainly is conceivable that differences in the experimental design and model of renal disease accounted for some of these discrepancies, it has also been shown that IL-6 can have a dichotomous role in renal disease. For example, in acute kidney injury, IL-6 contributes to early leukocyte-mediated damage but subsequently mediates tubular regeneration. Second, systemic effects of IL-6 on the immune system may exacerbate renal disease, whereas there are well-documented instances of intrarenal IL-6 production, for example by podocytes, mediating anti-inflammatory effects. Third, potential anti-inflammatory IL-6 effects exerted by classic signaling may be offset by proinflammatory trans-signaling of IL-6.

Our third major finding was a consistent set of data showing that specific blockade of trans-signaling improved whereas specific stimulation of trans-signaling aggravated murine NTN. This first demonstration of a particular role of trans-signaling in inflammatory renal disease extends to a growing list of studies describing the benefits of specific trans-signaling blockade in cerebral, lung, intestinal and other states of chronic inflammation (reviewed in) as well as in murine sepsis and lupus nephritis.

The first clinical trial on pan-IL-6 inhibition in human lupus nephritis turned out negative. So far, substantial benefit of IL-6 inhibition in human necrotizing GN has only been reported by two case vignettes on tocilizumab in rheumatoid arthritis-associated systemic vasculitis and without anti-MPO ANCA. This rare condition, however, substantially depends on the activity of the underlying rheumatic disease, making it difficult to judge if the benefits are GN-specific or just related to mitigation of rheumatic disease by the well

Figure 4. Total IL-6 antagonism during NTN in BALB/c mice. (A) Experimental design. Heterologous NTN was induced with a single injection of NTS for a duration of 14 days. Three times, either monoclonal anti-IL-6 antibody (a-IL-6) or the same amount of isotype control IgG were applied. (B–D) Histologic readouts, representative images, original magnifications, ×400. Arrows: glomerular crescents, asterisks: fibrinoid necroses. (E, F) Renal functional parameters, BUN, blood urea nitrogen. (G, H) Infiltration with macrophages and T cells, respectively. No difference between treatment groups was detected (two-sided t-test), n.s. = non-significant.

J Am Soc Nephrol 27: 132–142, 2016 IL-6 Trans-Signaling in Nephritis 137
established anti-rheumatic biologic tocilizumab. In this respect, trans-signaling inhibition in lupus nephritis and potentially in other GN entities holds promise for an alternative approach to pan-inhibition.

We observed opposite effects of trans-signaling blockade and activation on blood pressure during NTN, while we also confirmed that HIL-6 did not increase blood pressure in normal mice. Though it is conceivable that this simply reflects improvement or aggravation, respectively, of renal damage in NTN, there is also some evidence to directly invoke IL-6 in the control of blood pressure, and this may be one of the mechanisms by which trans-signaling blockade improved the course of NTN. IL-6 deficiency or trans-signaling blockade blunt the blood pressure increase following angiotensin II in mice. In murine preeclampsia, IL-6 was identified as a key mediator of the endothelin-1-induced blood pressure increase.

A second mechanism by which IL-6 affected the course of NTN may be via its actions on leukocytes. IL-6 suppresses myeloid cell proliferation. More importantly, classic but not trans-signaling drives macrophage polarization from a default proinflammatory (M1) toward an anti-inflammatory (M2) subtype in inflammatory models of C57Bl/6 mice. In line with this, deletion of the membrane-standing IL-6 receptor from myeloid cells required for classic signaling greatly facilitated the generation of proinflammatory M1-like macrophages and thus aggravated disease in NTN on a C57Bl/6 background. Using the same anti–IL-6 antibody, we failed to detect significant differences in outcome of NTN in our BALB/c mice. Conceivably, this difference relates to the choice of mouse strains, given that the BALB/c Th2-prone background leads to relatively mild macrophage and T-cell responses compared with mice on the C57Bl/6 background.

Figure 5. Respective inhibition and stimulation of trans-signaling during NTN in BALB/c mice. (A) Experimental designs. Heterologous NTN was induced for 14 days. In a first experiment, trans-signaling inhibitor sgp130Fc (or vehicle, i.e., PBS) was applied (left panel). In a second experiment, trans-signaling stimulator HIL-6 (or PBS) was applied (right panel). (B–E) Corresponding quantitative analysis of routine histology and representative PAS stains, original magnifications, ×400. Significant reduction of crescents and fibrinoid necrosis following sgp130Fc. Significant increase of fibrinoid necrosis and fibrinogen following HIL-6. Arrows, glomerular crescents; asterisks, fibrinoid necroses. (F, G) Functional parameters. Significant increase of blood urea nitrogen (BUN) and proteinuria by HIL-6. (H) Systolic blood pressure (SBP) rises during NTN. Sgp130Fc and HIL-6 leading to a significant decrease and increase on Days 4 and 13, respectively. (I, J) No statistical differences in renal macrophage and T-cell infiltration. (K–N) Further renal cortical RT-qPCR assessment of signaling and immune-cell-associated cytokines as likely (though not completely specific) surrogate parameters at Day 14 for IL-6 downstream signaling (SOCS3), Th17 population (IL-17A), M1 macrophages (iNOS) and M2 macrophages (Relm-α). Following HIL-6, significantly increasing IL-17A and decreasing Relm-α suggest Th17 activation and M2 macrophage reduction by trans-signaling stimulation.

*P<0.05. n.s., non-significant, all data one-sided t-test given the use of a one-sided hypothesis; H1, according to literature data improvement by sgp130Fc and worsening by HIL-6 were expected; $, opposite H1 and therefore considered non-significant. PAS, Periodic acid–Schiff.
M1-M2 and Th1-Th2 subtype markers we could confirm the known M2/Th2 skewness of BALB/c mice. Importantly, stimulation of IL-6 trans-signaling in NTN significantly suppressed renal cortical Relm-α mRNA, and thus a decrease in anti-inflammatory Relm-α–expressing macrophages may be one of the mechanisms underlying the aggravation of NTN by HIL-6. IL-17A mRNA also increased over time with NTN. During NTN, production of IL-17A is not exclusive to CD4+ cells which are ultimately defined as Th17 cells, but IL-17A has also been demonstrated in γδ T cells and CD3γδ cells that lack CD4, CD8, δT cell receptor, and NK1.1. However, these non-Th17-derived sources of IL-17A mostly occur during early NTN stages, suggesting that the increase in IL-17A mRNA in our NTN model in BALB/c animals originated from Th17 cells. Thus, stimulation of trans-signaling may additionally aggravate NTN by increasing the proinflammatory actions of IL-17A. A third potential source of IL-17A, remains to be defined in the kidney.

There are several limitations and strengths in the present study. One limitation is that we only studied one model of RPGN (NTN) induced on one genetic murine background. While our data clearly need confirmation in other RPGN models, e.g., the myeloperoxidase model, we believe the choice of Th2 BALB/c mice offers particular relevance to humans, given that Western lifestyle is associated with a more Th2-prone situation. Another limitation is that not all parameters studied in the specific trans-signaling interventions reached statistical significance. However, the consistency of the changes observed in antagonism and boosting of trans-signaling argues in favor of the relevance of this pathway. Finally, one strength of our approaches is that all interventions were designed in a therapeutic fashion. Thereby, we not only avoided interference of the interventions with disease induction but also increased the relevance to human trials. Sgp130Fc is now entering phase II clinical trials.

In summary, we show that the NTN model of crescentic glomerulonephritis in BALB/c mice exhibits a profound activation of the IL-6 system and that trans-signaling, as opposed to total IL-6 signaling, drives worsening of histologic and renal functional parameters as well as altered blood pressure. Our study thus points to a new, specific option for treating RPGN by selectively inhibiting IL-6 trans-signaling.

**CONCISE METHODS**

**Human Samples**
Human serum and urine samples originated from the Division of Nephrology Biobank. Informed consent was obtained, and the study was approved by the local ethics committee.

**Animals and Experimental Designs**
Twelve- to 14-week-old male BALB/c mice (Charles River Laboratories, Sulzfeld, Germany) were used. All experiments were approved by the local authorities. Nephrotoxic nephritis (NTN) was induced by a single injection of nephrotoxic serum spiked with 40 μg CpG oligonucleotides. Dosages were chosen following activity testing in a pilot set of mice. Monoclonal rat anti-mouse IL-6 inhibitory antibody MP5-20F3 and isotype control (rat monoclonal anti-horseradish peroxidase/HRPN) were purchased from Bio-X-Cell, West Lebanon, NH. Sgp130Fc and HIL-6 were synthesized as
described.58,59 See figures for used dosages of compounds. PBS was used as a vehicle for all compounds. Animals were perfused with saline prior to organ harvesting.

**Histology and Immunostaining**

Kidney sections were processed as described.4 See Table 1 for primary antibody specifications. Analyses were performed in a blinded fashion as described.4 In brief, 100 Periodic acid–Schiff-stained glomeruli and 20 anti-CD3-stained glomerular cross-sections were evaluated per mouse. F4/80 was quantified using software as percent of total area. Fibrinoid necrosis and fibrinogen scores were as follows: 0=absent; 1=less than 25%; 2=25–50%; 3=50–75%; 4=more than 75% of the glomerular tuft area.

**Western Blotting and RT-qPCR**

Western blot and quantitative real-time RT-qPCR were performed as described.4 The corresponding primary antibodies and primer sequences are listed in Tables 1 and 2, respectively.

**Flow Cytometry**

Blood leukocytes derived from 100 µl blood as well as isolated from one kidney were subjected flow cytometry as described.60 Antibodies for surface labeling of CD45, CD11b, Ly6G, and CD1 were purchased from Becton Dickinson, Heidelberg, Germany. Intracellular antibodies against IL-6 and pSTAT3-specific intracellular stainings, living cells were fixed with formalin 2% and permeabilized with saponin 0.1% and NP40 0.1%, respectively. In the case of IL-6, this procedure was preceded by a 4-h step of brefeldin A (eBioscience)-induced Golgi apparatus blockade in order to accumulate detectable amounts of this secreted cytokine. Analyses were performed on a Becton Dickinson Fortessa cytometer with APC-conjugated beads for relative quantification of overall events.60

**Other Parameters**

Creatinine, BUN, total protein, and albumin levels in mice4 or humans were measured by routine methods. Human IL-6 was measured using Table 1.

### Table 1. Primary antibodies for IF/IHC and immunoblotting

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specification, Supplier</th>
<th>Host species, type</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vinculin</td>
<td>Clone V284, SAB4200080; Sigma-Aldrich</td>
<td>Mouse, mc</td>
<td>1:2000</td>
</tr>
<tr>
<td>IL-6</td>
<td>sc-1265-R; Santa Cruz Biotechnology, Santa Cruz, CA</td>
<td>Rabbit, pc</td>
<td>1:50</td>
</tr>
<tr>
<td>IL-6</td>
<td>AF-406-NA; R&amp;D Systems, Minneapolis, MN</td>
<td>Goat, pc</td>
<td>1:500</td>
</tr>
<tr>
<td>IL-6R</td>
<td>sc-13947; Santa Cruz Biotechnology</td>
<td>Rabbit, pc</td>
<td>1:50</td>
</tr>
<tr>
<td>pSTAT3 (Tyr 705)</td>
<td>9145; Cell Signaling Technology, Danvers, MA</td>
<td>Rabbit, mc</td>
<td>1:1000</td>
</tr>
<tr>
<td>STAT3</td>
<td>9132; Cell Signaling Technology</td>
<td>Rabbit, pc</td>
<td>1:1000</td>
</tr>
<tr>
<td>F4/80</td>
<td>MCA497; AbD Serotech, Oxford, UK</td>
<td>Rat, mc</td>
<td>1:800</td>
</tr>
<tr>
<td>CD3</td>
<td>MCA1477; AbD Serotech</td>
<td>Rat, mc</td>
<td>1:100</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>F0111; DAKO, Hamburg, Germany</td>
<td>Rabbit, pc</td>
<td>1:1000</td>
</tr>
<tr>
<td>Nestin</td>
<td>NB100-1604; Novus Bioscience, Littleton, CO</td>
<td>Chicken, pc</td>
<td>1:100</td>
</tr>
<tr>
<td>iNOS-FITC</td>
<td>clone6/iNOS/NOS Type II; BD Biosciences, Heidelberg, Germany</td>
<td>Mouse, mc fc</td>
<td>1:100</td>
</tr>
<tr>
<td>Relm-α</td>
<td>ab39626; Abcam, Inc., Cambridge, UK</td>
<td>Rabbit, pc</td>
<td>1:25</td>
</tr>
<tr>
<td>GATA-3-Alexa649</td>
<td>Clone TWAJ; eBioscience</td>
<td>Mouse, mc fc</td>
<td>1:25</td>
</tr>
</tbody>
</table>

mc, monoclonal; pc, polyclonal; fc, fluorescent conjugate; IF, immunofluorescence; IHC, immunohistochemistry; WB, Western blot.

### Table 2. Primers for real-time PCR

<table>
<thead>
<tr>
<th>Gene or transcript</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gapdh</td>
<td>GGCAATTCAACGGCCACAGT</td>
<td>AGATGTTGATGGGCTTCCC</td>
</tr>
<tr>
<td>Il6 (IL-6)</td>
<td>TGTTCCATAACATAATGACCAT T</td>
<td>AGTCGGAGGTATTATACCATGT T</td>
</tr>
<tr>
<td>Il6ra (IL-6-R)</td>
<td>GGAGATCCCTGGAGGGTGACA</td>
<td>CGTTGTTGCTGAGGGCTT</td>
</tr>
<tr>
<td>Il6st (gp130)</td>
<td>GGAGCGAGTCACTGCACTACAC</td>
<td>GCTCGATCGGCCGAAAGGA</td>
</tr>
<tr>
<td>Socs3</td>
<td>GCCACCTGGACTCCTATGAAA</td>
<td>GAGCATCATCAGTGACCAAGCT</td>
</tr>
<tr>
<td>Il17a (IL-17A)</td>
<td>QuantiTect Primer Assay, Mm_Il17a_1_SG, Catalog No. QT00103278, Quiagen, Hilden, Germany</td>
<td>CCGCATAGCTTGCTTGGT</td>
</tr>
<tr>
<td>Nos2 (INOS)</td>
<td>TCTCCTGACATTTCCTCATGAG</td>
<td>CCGCATAGCTTGCTTGGT</td>
</tr>
<tr>
<td>Ifng (IFN-γ)</td>
<td>CTACACTGCTACTTGGGTCTT</td>
<td>TGACTGCGGTGGAAGTA</td>
</tr>
<tr>
<td>Tbx21 (T-bet)</td>
<td>TTGTGGTGTGCTGCTTGGT</td>
<td>CATGCTGCTCATGGTCCTT</td>
</tr>
<tr>
<td>Retnla (Relm-α)</td>
<td>GCACAGGAGTGGAAATGACTC T</td>
<td>GATGACTGCTACTTGGGTGGT</td>
</tr>
<tr>
<td>Il4 (IL-4)</td>
<td>GAGAAGGGAGGAGGCCCAGCA</td>
<td>CTCACCTGCTTGGTCTTGGT</td>
</tr>
<tr>
<td>Gata3 (GATA-3)</td>
<td>GACGTCCACCTGCACTTATGCTAC</td>
<td>GCCAGCACAGATAGAAGAG</td>
</tr>
<tr>
<td>Itgax (CD11c)</td>
<td>QuantiTect Primer Assay, Mm_Itgax_1_SG, Catalog No. QT00113715, Quiagen, Hilden, Germany</td>
<td>GCCAGCACAGATAGAAGAG</td>
</tr>
</tbody>
</table>

Gapdh, glyceraldehyde-3-phosphate dehydrogenase; gp130, glycoprotein 130; Socs3, suppressor of cytokine signaling 3; INOS, inducible nitric oxide synthase; T-bet, T-box transcription factor 21; Relm-α, resistin-like molecule alpha; GATA-3, GATA binding protein-3; Itgax/CD11c, cluster of differentiation 11c, integrin alpha X.
an electrochemiluminescent immunoassay (Cobas Elecsys e411; Roche Diagnostics, Mannheim, Germany). ELISAs for the remaining circulating molecules of the IL-6 system were obtained from R&D Systems. Blood pressure was measured by tail plethysmography as described.4

Statistical Analyses
Values are means ± SEM. Statistical significance was evaluated using Graph Pad Prism version 6.0b (Graph Pad Software, San Diego, CA). Statistical significance was defined as P <0.05.

ACKNOWLEDGMENTS
G.S.B. designed the study and research, performed experiments, analyzed data and prepared the manuscript. Y.N., Y.M., F.H., C.R.V.R., S.R.J., and T.O. contributed intellectual input and reagents. T.O. and J.F. conceived, supervised and co-wrote the study.

This study was supported by grants of the Deutsche Forschungsgemeinschaft (SFB TTR 57, projects P17 and P25, and FL 178/4–1) to J.F., P.B., and T.O. and by RO4036/1-1 to C.R.V.R. and BO 3755/2-1 to P.B. Further support came from a grant of the Interdisciplinary Center for Clinical Research (IZKF) at RWTH Aachen University to T.O. and J.F. (E7-2) and from an intramural program to G.S.B. (Rotationsprogram der Medizinischen Fakultät der RWTH). D.J.S. was supported by research grant DK90929 from the US National Institutes of Health. The work of S.R.J. was supported by the Deutsche Forschungsgemeinschaft (SFB 841, project C1 and the Cluster of Excellence Inflammation at Interfaces). The expert help of Christina Gianussis, Nicole Bataille, Gabriele Dietzel, Gertrud Minnartz, Dagmar Wieland, Esther Stüttgen and Lydia Zimmermanns in performing these studies is gratefully acknowledged.

DISCLOSURES
S.R.J. is a shareholder of the CONARIS Research Institute AG (Kiel, Germany), which is commercially developing sgp130Fc as a therapy for inflammatory diseases, and is an inventor on gp130 patents owned by CONARIS.

REFERENCES


