Endogenous IL-22 is dispensable for experimental glomerulonephritis

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ABSTRACT

In recent years, the cytokine IL-22 attracted considerable attention due to its important immunoregulatory function in barrier tissues, such as the gut, lung and skin. While a regenerative role of IL-22 in renal tubular damage has been demonstrated, the role of IL-22 in the immunopathogenesis of glomerular injury is still unknown.

Here, we demonstrate that the IL-22 receptor is expressed in the glomerular compartment of the kidney and that IL-22 expression increases in the renal cortex after induction of glomerular injury in a mouse model for crescentic glomerulonephritis (cGN, nephrotoxic nephritis). We identified γδ T cells and T_H17 cells as major sources for IL-22 in the nephritic kidney. However, neither genetic or antibody-mediated deletion of IL-22, nor genetic deficiency in its endogenous inhibitor IL-22Rα2 (IL-22 binding protein) did result in substantial phenotypic differences in mice with cGN with respect to crescent formation, tubulointerstitial damage and kidney function impairment. Similarly, we did not observe significant differences between wildtype and IL-22-deficient mice in a mouse model of secondary focal and segmental glomerulosclerosis (Adriamycin-induced nephropathy). As shown previously, we detected concomitant upregulation of IL-17A and IFN-γ production by T cells during the course of cGN, providing alternative cytokine pathways that mediate glomerular injury in this model.

In conclusion, we show here that endogenous IL-22 expression is redundant in different forms of glomerular injury, indicating that the IL-22-directed therapies that are being tested in various human diseases might not affect the kidney in patients with glomerular disease.

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INTRODUCTION

Immune-mediated glomerular diseases are a heterogeneous group of disorders that are a major cause of end-stage renal diseases in the Western world (5). The common underlying mechanisms of glomerulonephritis encompass a disturbed immune reaction leading to pathogenic inflammation in the glomerulus of the kidney. Although important discoveries in this research field were made in the recent years, current treatment options for glomerulonephritis patients still rely on unspecific immunosuppressive agents that can cause severe side effects and are only partially effective. To overcome these therapeutic restrictions, the development of more effective and specific individual treatment strategies is crucial (9).

Cytokines are central mediators of tissue responses in inflammation and under homeostatic conditions. In the recent years, the cytokine Interleukin (IL)-22 received considerable attention due to its important function as an immunoregulatory cytokine at barrier surfaces (4, 18). Depending on the tissue location and type of the immune response, the cellular source of IL-22 can vary. In most settings, IL-22 production has been attributed to T_H17 cells, γδ T cells, and group 3 innate lymphoid cells (ILC3s) (4, 18), but non-lymphoid cells, such as macrophages and neutrophils, have also been described as a possible source of IL-22 (6, 8, 29).

In contrast to other interleukins, IL-22 acts exclusively on non-hematopoietic, epithelial and stromal cells via the heterodimeric IL-22 receptor (IL-22R) complex, consisting of the IL-22Rα1 and the ubiquitously expressed IL-10Rβ2 chain. The receptor complex is expressed on various barrier surfaces with highest expression levels in the skin, colonic mucosa and pancreas followed by liver, lung, and kidney (20, 26). Importantly, IL-22 function is also regulated by an endogenous inhibitor, the soluble IL-22 binding protein (IL-22bp or IL-22Rα2), adding another layer of complexity to IL-22 signalling biology (7).

Depending on the context, IL-22 can mediate pro- or anti-inflammatory functions and is therefore often described as a double-edged sword. In homeostasis and after acute injury (e.g.

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of the intestine), IL-22 expression promotes barrier integrity by induction of anti-apoptotic genes and anti-microbial peptides in epithelial cells, resulting in enhanced tissue regeneration and improved protection against invading pathogens (18). Conversely, IL-22 function has been linked to inflammatory tissue pathology in immune-mediated diseases, such as psoriasis. Here, the co-expression of IL-22 with other cytokines, such as IL-17A, induces pro-inflammatory molecules in parenchymal cells and thereby contributes to the recruitment of pathogenic effector cells promoting tissue inflammation (4).

The considerable expression of IL-22R mRNA in human and murine kidney tissue (20, 26) suggests a potential involvement of IL-22 in renal diseases. In line, two independent studies recently detected IL-22R expression on proximal tubular epithelial cells in the murine kidney and observed that IL-22 signaling promotes tubular regeneration after ischemia/reperfusion-induced acute kidney injury (8, 27).

Since studies addressing a potential involvement of endogenous IL-22 in glomerular diseases are still lacking (25), here, we focus on a potential role of IL-22 in glomerular injury.

MATERIAL & METHODS

Animals. All animals were raised under specific pathogen-free conditions. IL-22-deficient mice on the C57BL/6J background were kindly provided by J.-C. Renauld (Ludwig Institute for Cancer Research, Brussels, Belgium). To generate BALB/c IL-22-deficient mice, the same animal strain was backcrossed for at least 10 generations to the BALB/c background.

IL-22Rα2-deficient mice (7) on C57BL/6J background were provided by S. Huber with permission of Regeneron Pharmaceuticals, Inc. Sex- and age-matched C57BL/6J and BALB/c wildtype littermate controls were bred in the animal facility of the University Medical Centre Hamburg-Eppendorf. All animal experiments were performed according to national and institutional animal care and ethical guidelines and were approved by the local committees.

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Induction of experimental GN, experimental focal and segmental glomerulosclerosis and functional studies. Crescentic glomerulonephritis (cGN) was induced by intraperitoneal injection of nephrotoxic sheep serum (2.5 mg/g body weight) in 8 to 12 weeks old mice as previously described (2). For induction of experimental focal and segmental glomerulosclerosis (FSGS), BALB/c mice were injected intravenously with 12 µg Adriamycin per gram body weight (Cell Pharm GmbH, Germany). For urine sample collection, mice were housed in metabolic cages for 5 hours. Urinary albumin excretion was determined by standard ELISA (Mice-Albumin Kit; Bethyl Laboratories, Montgomery, TX). Urinary creatinine levels were measured with the Creatinine Jaffé Fluid (Hengler Analytik, Steinbach, Germany). Plasma cholesterol and blood urea nitrogen (BUN) were analysed by standard laboratory procedures.

Antibody-mediated neutralization of IL-22. The IL-22- blocking antibody (AM22.1) used in this study was previously described (23). IgG2a was used as an isotype control (BioXcell #BE0085; clone C1.18.4). IL-22 blocking antibody and its isotype control (both 100µg per mouse) were administered intraperitoneally one day before and at day 3 after induction of crescentic glomerulonephritis (cGN).

Histopathology and immunohistochemistry. Formalin-fixed, paraffin-embedded kidney sections were stained with Periodic acid-Schiff's (PAS) reagent according to standard laboratory procedures. Crescent formation in the cGN model was assessed in 30 glomeruli per mouse in a blinded fashion. Tubular injury was assessed by using photographs of non-overlapping cortical areas from PAS-stained kidney sections. In the GN model, percentage of interstitial area was determined by superimposition of the photographs with 40 apportioned dots and subsequently interstitially located dots were counted. In the progressive

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glomerulosclerosis model, percentage of tubular injury was assessed by counting of the area displaying dilated, atrophic or cast-filled tubules after superimposition of a grid.

For detection of glomerular sheep IgG and mouse IgG deposition, paraffin-embedded kidney sections were stained with anti-mouse and anti-sheep antibodies (Jackson Immuno Research, UK) and developed with a polymer-based secondary antibody alkaline phosphatase kit (POLAP, Zytomed, Berlin, Germany). Glomerular deposition of mouse IgG was scored from 0 to 3 in 30 glomeruli per mouse. Scoring was performed with the Axioskop light microscope and photographs were taken with an Axiocam MRc camera (both Zeiss, Jena, Germany).

For staining of deposited C3 complement, paraffin-embedded kidney sections were stained with a FITC-conjugated anti-C3 antibody (Cappel Research Reagents, CA). Stained sections were evaluated by confocal microscopy using the Laser Scanning Microscope 800 and the appropriate software (all Zeiss, Jena, Germany).

Antigen-specific humoral immune response. Total mouse anti-sheep IgG titers were measured by ELISA using plasma of mice 7 and 21 days after induction of cGN. In brief, ELISA microtiter plates were coated with sheep IgG (100 µg/mL) (Sigma, St. Louis, USA) overnight at 4°C. After blocking with 1% Bovine serum albumin (BSA) Tris-buffered saline (Sigma), the plates were incubated with serial dilutions of mouse plasma (1:100 – 1:12,5000) for 1 hour, shaking at room temperature. Total bound mouse IgG was detected using peroxidase-conjugated goat anti-mouse IgG (R&D) in a 1:1000 dilution, TMB peroxidase substrate and absorbance readings (at 450 nm) on a spectrophotometer.

Cell isolation. For isolation of renal leukocytes, mouse kidneys were cut into small pieces and digested in complete medium (RPMI 1640, 10 % Fetal Bovine Serum, 1 % HEPES, 1% Penicillin/Streptomycin; all Gibco) with Collagenase D (0.4 mg/mL, Roche, Basel, Switzerland) and DNase I (100 µg/mL, Roche) for 45 min while rotating on a MACSmix®

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tube rotator (Miltenyi, Bergisch Gladbach, Germany) and then further dispersed by using gentleMACS® dissociation (Miltenyi). Further leukocyte purification was achieved by Percoll gradient centrifugation (37.5 %) (GE Healthcare, Chicago, IL). After subsequent erythrocyte lysis with ammonium chloride, cell suspensions were filtered through a 50µm strainer and used for further analyses.

Flow cytometry. Nonspecific staining was prevented by incubation with 10% normal mouse serum (Jackson ImmunoResearch Laboratories, West Grove, PA). To characterize leukocyte subsets, cell suspensions of mouse kidneys were stained with fluorochrome-coupled antibodies against CD45 (30-F11), Thy1.2 (CD90.2; 30-H12), CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), CD11b (M1/70), CD11c (HL-3), TCR-γδ (H57-597), TCR-αβ (GL3), Ly6G (1A8), SiglecF (E50-2440) and F4/80 (BM8) (all Biolegend; eBioscience; BD Biosciences). Intracellular staining was performed with IL-22 (Poly5164, Biolegend), IL-17A (TC11-18H10.1, BD Biosciences) and IFN-γ (XMG1.2, eBioscience). Isolated leukocytes were restimulated with phorbol 12-myristate 13-acetate (1 µg/mL, Sigma) and ionomycin (1 µg/mL, Calbiochem) in the presence of brefeldin A (10 µg/mL, Sigma) for 2.5 h. Subsequently, surface staining was performed as described above. Then, cells were fixed with formalin (3.7 %, Sigma), permeabilized with IGEPAL® CA-630 (0.1 %, Sigma) and stained with above mentioned intracellular fluorochrome-coupled antibodies. Dead cell staining was performed using LIVE/DEAD Fixable Read Dead Stain Kit (Invitrogen) or Zombie Dye (Biolegend). Absolute numbers of $CD45^+$ cells in kidney cell suspension were determined by staining with flourochrome-coupled anti-CD45 combined with cell count beads (Countbright®, Invitrogen). All samples were acquired on a LSRII flow cytometry (BD Biosciences) and analysed with the FlowJo Software (Treestar Inc.).

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Isolation of glomeruli and tubules. The isolation of glomeruli from the murine kidney was performed as described previously (21). In brief, mice were perfused with Dynabeads (Thermo Fischer) and after extraction of the kidney the inner and outer medulla were removed and discarded. Subsequently, after digestion of the kidney with Collagenase I for 20 min at 37°C, the glomeruli, containing the magnetic beads, were separated from the renal tubules by magnetic separation using the DynaMag-2 magnet (Invitrogen).

Real-time RT-PCR analysis. Total RNA of the renal cortex was prepared according to standard laboratory methods. After reverse transcription to cDNA, real-time PCR was performed for 45 cycles (initial denaturation at 95°C for 10 min, followed by cycles of denaturation at 95°C for 15 s, and primer annealing and elongation at 60°C for 1 min) with 1µl of cDNA samples in the presence of 0.5µl of specific murine primers. TaqMan® Gene Expression Assays and a StepOnePlus Real-Time PCR system (both Thermo Fisher Scientific) were used for quantification of the housekeeping gene (*Hprt1*) and the genes of interest. All samples were run in duplicates.

Statistics. The Student's *t*-Test was used for comparison between two groups. In case of three or more groups, One-way ANOVA was used followed by a post-hoc analysis with Newman-Keuls test for multiple comparisons. A *P*-value of <0.05 was considered to be statistically significant.

RESULTS

Expression of IL-22 and its receptor in experimental crescentic glomerulonephritis (cGN)

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To investigate the role of IL-22 in glomerular injury, we first wanted to address whether the IL-22R is expressed in the glomerular compartment of the kidney. In earlier studies, low constitutive expression of the IL22R was identified in the murine kidney (20) which was later located to proximal tubular epithelial cells (8, 27). Performing quantitative analysis of IL-22Rα1 and IL-10Rβ mRNA expression in the tubular and glomerular tissue isolated from the kidney from naïve C57BL/6 mice, we could confirm dominant IL-22Rα1 expression in the tubulointerstitial compartment. However, our data also revealed substantial expression of both IL-22R subunits in the glomerular compartment (Fig. 1A). Next, we addressed the question whether induction of glomerular inflammation leads to upregulation of IL-22 expression. mRNA expression analysis of the renal cortex during the course of cGN induced in C57BL/6 wildtype mice by injection of "nephrotoxic" sheep IgG demonstrated elevated IL-22 expression levels as early as 12 hours after induction of the model, followed by a rapid decline towards baseline levels (Fig. 1B). Induction of IL-22 expression was accompanied by upregulation of its natural inhibitor, the IL-22bp (IL-22rα2), and downregulation of the IL-22Rα1 receptor, indicating a tight regulation of IL-22 signaling in the kidney (Fig. 1B).

Flow cytometric analyses of leukocytes isolated from nephritic kidneys at the different time points verified induction of IL-22-producing cells in the kidney, which were mainly $CD3^+$ T cells (Fig. 1C). In line with the mRNA data, the abundance of IL-22-producing $CD3^+$ cells was elevated at 0.5 days, but also revealed a second peak of IL-22 production at day 7 after induction of cGN (Fig. 1C, D). As demonstrated by using *Il22*$^{-/-}$ mice as a staining control, the apparent positivity of $CD3^-$ cells for IL-22 that was observed at several time points was primarily due to unspecific binding of the IL-22 antibody by $CD3^-Ly6G^+$ neutrophils (Fig. 1E), arguing against a significant contribution of myeloid cells and ILC3s to IL-22 production in this model of cGN.

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Taken together, these data illustrate the presence of the IL-22 receptor in the glomerular compartment and an inflammation-dependent upregulation of IL-22 in the murine kidney in cGN.

Cellular sources of IL-22 in experimental cGN

To further characterize the cellular sources of IL-22 in experimental cGN, we performed more detailed flow cytometric analyses of the IL-22$^+$CD3$^+$ cells at days 0.5 and 7 after induction of the model (Fig. 2). These analyses revealed that ~70% of IL-22-producing cells at the early IL-22 expression peak were γδ T cells, whereas during the second expression peak at day 7, IL-22 was preferentially produced by CD4$^+$ T cells (Fig. 2A, B). Furthermore, co-expression analysis of IL-22 with the CD4$^+$ T helper cell lineage defining cytokines IL-17A and IFN-γ, showed that IL-22 was mainly expressed by T_H17 cells and IL-17A$^+$ γδ T cells, but not by IFN-γ$^+$ T_H1 cells (Fig. 2C, D). In summary, these data show a time-dependent cellular source of IL-22, switching from γδ T cells as the main IL-22$^+$ subset in the early phase to T_H17 cells in later stages of the model. As shown previously (12, 22), IL-17A- and IFN-γ-producing T cells subsets were significantly upregulated during the course of cGN (Figure 2E, F), providing alternative cytokine pathways that can mediate glomerular injury in this model.

Humoral and cellular immune responses in *Il22*$^{-/-}$ mice with cGN

In order to investigate the potential role of IL-22 in experimental cGN, we induced the nephrotoxic nephritis model in C57BL6/J *Il22*$^{-/-}$ mice and their wildtype littermates. In this cGN model, administration of "nephrotoxic" sheep IgG directed against components of the glomerular basement membrane (GBM) induces complement activation in the glomeruli leading to early neutrophil recruitment and glomerular damage in the heterologous phase (9). To exclude differences between *Il22*$^{-/-}$ and wildtype mice with respect to sheep IgG and complement C3 binding at the GBM, we analyzed kidney sections of the groups of nephritic

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mice by immunohistochemistry and immunofluorescence staining. These analyses revealed similar amounts of glomerular sheep IgG and complement C3 deposition in *Il22*$^{-/-}$ and wildtype mice after cGN induction at day 7 (Fig.3 A, B). In the subsequent autologous phase of the cGN model, a humoral immune response against the deposited sheep IgG results in glomerular mouse IgG deposition, further recruitment of immune cells and immune-mediated glomerular damage. To examine if IL-22 deficiency influences the humoral immune response against sheep IgG as the nephritic antigen, we performed immunohistochemistry for mouse IgG deposition in the glomeruli and measured serum levels of sheep-IgG-specific total mouse IgG in wildtype and *Il22*$^{-/-}$ mice at different time points of cGN. However, we did not detect differences in these parameters between the two groups of mice (Fig. 3 C-E).

Infiltration of immune cells into the kidney is the crucial driver of tissue damage in human and murine cGN (3). As IL-22 has been shown to influence leukocyte recruitment by inducing production of chemoattractants in epithelial cells (17), we assessed if IL-22 deficiency alters immune cell infiltration during the course of experimental cGN. To this end, we characterized the cellular infiltrates in *Il22*$^{-/-}$ and wildtype mice after induction of the model by flow cytometry (Fig. 4A-D). These analyses showed a similar abundance of $CD4^+$ T cells, $CD8^+$ T cells and γδ T cells, as well as the pathogenic T_H17 and T_H1 cell subsets in the two groups of mice at early and late time points (Fig. 4A-C). Similarly, the infiltration of the major myeloid cell subsets, e.g. neutrophils and mononuclear phagocytes, seemed to be unaffected by IL-22 deficiency in experimental cGN (Figure 4A, D). In summary, these data indicate a largely unaltered immune response in *Il22*$^{-/-}$ mice in this model for cGN.

IL-22 deficiency does not impact renal tissue damage in experimental cGN

IL-22 has been described to promote tissue protection at barrier surfaces by directly inducing epithelial cell regeneration (18). Therefore, we hypothesized that, although the immune response in *Il22*$^{-/-}$ mice was found to be unchanged, IL-22 expression might confer renal

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tissue protection in cGN by direct activation of IL-22R signaling in glomerular or tubular cells. To address this hypothesis, we analyzed renal histopathology and renal function parameters at days 7 and 21 after induction of cGN in *Il22*$^{-/-}$ mice and their wildtype littermates.

These analyses showed a similar degree of glomerular and tubulointerstitial tissue damage in wildtype and *Il22*$^{-/-}$ mice, as assessed by quantification of crescent formation and interstitial widening in Periodic acid-Schiff (PAS)-stained kidney sections (Fig. 5A, B). Moreover, albuminuria, hyperlipidemia and renal function impairment observed in the cGN model was unaltered by IL-22 deficiency (Fig 5C). In addition, we examined the long term effects of IL-22 deficiency on repair mechanisms in the cGN model. These analyses six weeks after induction of cGN showed that absence of IL-22 did not substantially alter renal histopathology, albuminuria, cholesterol levels and BUN (Fig. 5D, E and data not shown). These analysis indicate that endogenous IL-22 is dispensable for experimental cGN.

Antibody-mediated depletion of IL-22 does not impact renal tissue damage in experimental cGN

To circumvent activation of alternate compensatory mechanisms potentially evoked by genetic deletion of *Il22*, we investigated the effect of antibody-mediated neutralization of IL-22 on the course of experimental cGN. Similar to the results obtained in *Il22*$^{-/-}$ mice, renal histopathology (Figure 6A) and renal function parameters (Figure 6B) in anti-IL-22-antibody-treated mice at day seven after induction of cGN were similar to those in mice treated with the isotype control.

Deficiency of IL-22Rα2 does not impact renal tissue damage in experimental cGN

Since expression of IL-22 binding protein (IL-22Rα2) was found to be upregulated in parallel with IL-22 in experimental cGN (see Fig. 1B, D), we hypothesized that the IL-22 effect in this

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model might be strictly limited by its endogenous inhibitor. Therefore, to elucidate the effect of increased IL-22 signaling in the diseased kidney, we assessed mice that are genetically deficient in the endogenous inhibitor IL-22Rα2 at day seven after induction of cGN. In these analyses, neither renal histopathology (Fig. 7A), nor clinical parameters (Fig. 7B), were substantially different between wildtype and *Il22ra2*$^{-/-}$ mice, indicating that unrestricted signaling of endogenous IL-22 does not influence induction or repair mechanisms of experimental cGN.

IL-22 deficiency does not impact renal tissue damage in experimental focal and segmental glomerulosclerosis

Since kidney damage in the mouse model for cGN is primarily immune-mediated, we asked the question whether IL-22 might play a role in a mouse model for glomerular injury that is based on direct injury of glomerular epithelial cells. Therefore, we induced Adriamycin-induced nephropathy (AN), a mouse model for human secondary focal and segmental glomerulosclerosis (FSGS), in *Il22*$^{-/-}$ mice that were backcrossed to the BALB/c background for at least ten generations and in their BALB/c wildtype littermates. At day 14 after induction of AN, we could detect an upregulation of IL-22 expression by $CD4^+$ T cells in the kidney of wildtype animals (Fig. 8A, B). However, as in the cGN model, histopathological examination of PAS-stained kidney sections showed no difference between wildtype and *Il22*$^{-/-}$ mice in terms of glomerulosclerosis and tubular damage (Fig. 8C, D). Furthermore, the analyses of albuminuria, cholesterol and blood urea nitrogen levels did not reveal an impact of IL-22 deficiency on the renal outcome of the FSGS model (Fig 8E).

Taken together, we can conclude that endogenous IL-22 does not show a regenerative or proinflammatory function in the mouse models for glomerular injury investigated in this study.

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DISCUSSION

Depending on the tissue microenvironment and inflammatory context, the cytokine IL-22 can perpetuate inflammation or facilitate resolution of injury and promote restoration of tissue integrity. Especially the beneficial effects of IL-22 with respect to epithelial regeneration after injury identifies it as a promising therapeutic target also in kidney diseases. In fact, the kidney is one of the organs that shows substantial baseline expression of the IL-22 receptor (20, 26), indicating that there might be a role for IL-22 in renal homeostasis and/or inflammation. In line with this concept, recent studies have linked IL-22 signaling to tubular regeneration after renal ischemia reperfusion injury in mice (8, 27). In these studies, the IL-22 receptor was found to be expressed predominantly in tubular epithelial cells and to promote their survival by increasing expression of anti-apoptotic genes and reducing expression of inflammatory mediators. However, studies addressing a potential role of IL-22 in immune-mediated glomerular diseases have not been reported so far.

We show here that the IL-22 receptor is expressed in the glomerular compartment of the kidney and that γδ T cells and T_H17 cells time-dependently upregulate IL-22 expression in a mouse model for cGN. However, *Il22*$^{-/-}$ mice were able to mount an unaltered systemic and intrarenal immune response and did not show differences with respect to glomerular damage and kidney function impairment in two experimental mouse models of glomerular disease. Furthermore, neither antibody-mediated neutralization of IL-22, nor genetic deficiency in its endogenous inhibitor IL-22 binding protein, resulted in a substantial alteration of the course of experimental cGN.

In the cGN model, we observed an early upregulation of IL-22 mRNA expression already 12h after induction of injury, followed by a rapid decrease towards baseline levels. This expression pattern was similar to the IL-22 expression kinetic observed in acute tubular injury models (25), indicating that IL-22 might act as part of an immediate inflammatory response to different types of kidney damage. In line with the previous notion that γδ T cells are important

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366 early responders in experimental cGN (22), we found rapid induction of IL-22 expression in
367 γδ T cells, while T_H17 cells were the main producers of IL-22 at later stages. Similar changes
368 of the predominant source of IL-22 during the course of an inflammatory reaction have been
369 described in the intestine and skin. For example, in the mouse intestine, ILC3s are known to
370 be the main producers of IL-22 under homeostatic conditions and at the early stages of *C.*
371 *rodentium* infection, whereas IL-22^+CD4^+ T cells substantially expand at later stages (1). In a
372 mouse model for psoriasis, IL-22^+CD4^+ T cells expand together with γδ T cells when
373 inflammation progresses, whereas in the steady state IL-22 is mainly provided by γδ T cells
374 alone (1). In most of the studies, IL-22 production in $CD4^+$ T cells was found to be associated
375 with IL-17A expression and has therefore been attributed to the T_H17 cell subset, while the
376 evidence for a specialized `$T_H22$` (at least in the mouse) is still scarce (10). Production of IL-
377 22 by non-lymphoid cells has also been described (6, 29), but in contrast to previous reports
378 from ischemia-reperfusion-induced kidney injury (8), we were unable to detect a specific IL-
379 22 protein staining in the myeloid cell compartment of renal leukocytes in our study.
380 Furthermore, we did not detect IL-22-production by CD3-negative lymphoid cells, excluding
381 a major role of renal ILC3s as a potential source of IL-22, corroborating the previous
382 observation that the ILC3 subsets is very rare in the mouse kidney (15).
383 The evident expression of IL-22 in the inflamed kidney after induction of cGN raised the
384 question whether it might serve as an important regulator of immune-mediated glomerular
385 injury and so far there were no detailed studies addressing this issue (25). Our comprehensive
386 characterization of IL-22-deficient mice and their littermate controls in two well-established
387 mouse models provides substantial evidence that endogenous IL-22 is redundant for initiation
388 and control of glomerular injury. Potential compensatory mechanisms in mice with a genetic
389 deletion of IL-22 were excluded by using anti-IL-22 antibody treatment, which did not
390 influence immune-mediated renal injury in the cGN model, thereby supporting the data
391 obtained with *Il22*$^{-/-}$ mice. Although the results presented here are mainly negative findings,

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392 they clearly underscore the central importance of alternative cytokine pathways, such as the
393 IL-23/Th17 and Th1 axes that were both significantly upregulated in the cGN model, for
394 initiation and progression of crescentic GN. However, one previous study that investigated the
395 immunomodulatory function of a parasitic worm product in the MRL/MPJ-*Fas*^{lpr} model of
396 lupus nephritis showed that IL-22 might be able to promote autoimmune kidney injury in this
397 context (16). In a mouse model of diabetic nephropathy, in contrast, IL-22 overexpression by
398 therapeutic gene transfer resulted in alleviation of glomerular injury (24), an effect that was in
399 part mediated by IL-22-dependent suppression of the NLRP3 inflammasome. These
400 apparently divergent roles of IL-22 in glomerular injury may be explained by the different
401 context of IL-22 expression, since synergistic proinflammatory effects of IL-22 can depend on
402 co-production of other pro-inflammatory cytokines, presumably being more pronounced in
403 the lupus nephritis model than in experimental diabetic nephropathy. Moreover, excessive
404 overexpression of IL-22 by gene transfer has systemic effects, e.g. on metabolic control, that
405 might contribute to its beneficial effects in the diabetes model (24).
406 With respect to a potential involvement of IL-22 in human glomerulonephritis, the data is so
407 far very limited and partially controversial. One study found increased frequencies of IL-
408 22^+CD4^+ T cells in patients with renal involvement of systemic lupus erythematosus (SLE)
409 and showed that they correlate with disease activity (14). Conversely, other studies described
410 a decrease of IL-22^+CD4^+ T cells (28) or urinary IL-22 mRNA levels (11) in patients with
411 active lupus nephritis. Furthermore, a single nucleotide polymorphism in the IL-22R locus
412 was found to be associated with the development of IgA nephropathy in children (19) and
413 increased IL-22^+CD4^+ T cells and IL22 plasma levels in IgA nephropathy patients have also
414 been reported (13). However, the functional significance of these findings remains to be
415 elucidated.
416 Another noteworthy observation in the present study is the substantial mRNA expression of
417 the IL-22 binding protein (IL-22bp or IL-22Rα2), the endogenous inhibitor of IL-22, in the

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renal cortex. Interestingly, the upregulation of IL-22bp closely paralleled the kinetic of IL-22 expression in the cGN model (see Figure 1B, D), suggesting a tight control mechanism that prevents excessive IL-22 signaling in renal inflammation. To assess whether this regulatory pathway prevents endogenous IL-22 from exhibiting pro- or anti-inflammatory effects in glomerular injury, we analyzed IL-22bp-deficient mice in the cGN model. However, even in this setting of unrestricted signaling, we did not find a substantial function for endogenous IL-22 production in immune-mediated glomerular damage.

In conclusion, our data demonstrate that the immunoregulatory cytokine IL-22 is dispensable for promoting tissue inflammation or protection in experimental glomerulonephritis, indicating that the IL-22-directed therapeutic approaches that are being tested in various human diseases (17) might not be beneficial in glomerulonephritis, but might also not show detrimental renal side effects.

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DISCLOSURES

The authors have declared that no conflict of interest exists.

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FIGURE CAPTIONS

Figure 1: Expression of IL-22 and its receptor in the kidney of naïve and nephritic mice. **(A)** Quantitative RT-PCR analysis of IL-22 receptor subunit transcripts in the tubular and glomerular compartment of the kidney in naïve wildtype (WT) C57BL/6 mice (*n*=7-8). **(B)** Quantitative RT-PCR analysis of IL-22, IL-22Ra1 and IL-22bp (IL-22Ra2) transcripts in the renal cortex of naïve WT mice and nephritic WT mice at different time points after induction of crescentic glomerulonephritis (cGN) (*n*=5–6 per group). **(C)** Representative flow cytometric analysis of kidney leukocytes after stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin at the different time points of cGN. Numbers indicate the percentage of cells in the respective gates. **(D)** Quantification of IL-22-producing T cells per kidney (*n*=5–6). **(E)** Specificity of IL-22 staining was examined by flow cytometric analysis of kidney leukocytes from WT mice and IL-22-deficient littermates at days 0.5 and 7 of cGN. Representative plots show a specific $CD3^+IL22^+$ population and unspecific IL-22 antibody binding by $Ly6G^+$ neutrophils. Numbers indicate the percentage of cells in the gates. Gating strategy is specified in brackets. All data are pooled from at least two independent experiments with similar results. Bars represent means ± SEM, open circles represent individual animals (**** $P<0.0001$, *** $P<0.001$, * $P<0.05$).

Figure 2: Time-dependent cellular source of IL-22 during cGN. **(A)** Representative flow cytometric analysis of IL-22-producing T cells in WT C57BL/6 mice at 0.5 and 7 days after induction of cGN. Numbers indicate the percentage of cells in the gates. **(B)** Frequency of γδ T cells and $CD4^+$ T cells within the $IL\text{-}22^+CD3^+$ gate at the respective time points (*n*=6). **(C)** Representative flow cytometric analysis of kidney leukocytes stimulated with PMA / ionomycin shows co-expression of IL-22 with IL-17A. Numbers indicate the percentage of cells in the quadrants. **(D)** Frequency of $IL\text{-}17A^+IL\text{-}22^+$

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and IL-17A$^-$IL-22$^+$ cells in the γδ T cell and CD4$^+$ T cell population of the murine kidney at days 0.5 and 7 of cGN (*n*=6). Gating strategies are specified in brackets. **(E, F)** Quantification of IL-17A- and IFN-γ-producing T cell subsets per kidney (n=5–6). All data are pooled from at least two independent experiments with similar results. Bars represent means ± SEM, open circles represent individual animals (*** P<0.001, * P<0.05).

Fig. 3: Humoral immune response in *Il22*$^{-/-}$ mice during the course of cGN.

(A-C) Representative photographs of kidney sections stained immunohistochemically with (A) anti-sheep IgG, (B) anti-complement C3 and (C) anti-mouse IgG (original magnification 400x) of WT and *Il22*$^{-/-}$ littermates 7 days after induction of cGN. **(D)** Quantification of glomerular mouse IgG deposition at day 7 and 21 after induction of cGN in WT mice and *Il22*$^{-/-}$ littermates (naïve WT controls *n*=7, WT d7 *n*=14, WT d21 *n*=5, *Il22*$^{-/-}$ d7 *n*=6, *Il22*$^{-/-}$ d21 *n*=7). **(E)** ELISA of sheep IgG-specific mouse IgG in the serum of WT mice and *Il22*$^{-/-}$ littermates at day 7 and 21 of cGN (naïve WT controls *n*=8, WT d7 *n*=7, WT d21 *n*=5, *Il22*$^{-/-}$ d7 *n*=15, *Il22*$^{-/-}$ d21 *n*=7). Symbols in D and E represent means ± SEM. Open and filled circles in D represent individual animals. Data are pooled from three individual experiments with similar results.

Fig. 4: Cellular immune response in the kidneys of WT and *Il22*$^{-/-}$ mice with cGN.

(A) Representative flow cytometry plots of renal leukocytes isolated from naïve and nephritic WT mice and their *Il22*$^{-/-}$ littermates at day 7 of cGN. Numbers indicate the percentage of cells in the gates or quadrants. **(B-D)** Quantification of the absolute numbers of T cell (B, C) and myeloid cell (D) subsets per kidney at the indicated time points (naïve WT controls *n*=8, WT d7 *n*=8, WT d21 *n*=6, *Il22*$^{-/-}$ d7 *n*=16, *Il22-/-* d21 *n*=7). Symbols in (B-D) represent mean ± SEM, open and filled circles represent individual animals. Data are pooled from three individual experiments with similar results.

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Fig. 5: Tissue damage and renal function parameters of WT and *Il22*$^{-/-}$ in cGN.

(A) Representative photographs of periodic acid-Schiff-stained kidney sections (original magnification 400x), **(B)** histopathological quantification of glomerular and tubulointerstitial damage and **(C)** analysis of renal function parameters in WT mice and *Il22*$^{-/-}$ littermates at 7 and 21 days after induction of cGN (naïve WT control *n*=5-8, WT d7 *n*=8, WT d21 *n*=5, *Il22*$^{-/-}$ d7 *n*=16, *Il22*$^{-/-}$ d21 *n*=4-6). Quantification of renal damage six weeks after induction of cGN assessed by histopathological analysis of crescent formation and interstitial widening **(D)** and renal function parameters **(E)** in IL-22-deficient mice and WT animals. Symbols in B - E represent mean ± SEM, open and filled circles represent individual animals. Data are pooled from at least three individual experiments with similar results.

Fig. 6: Antibody-mediated neutralization of IL-22 in the cGN model.

(A) Histopathological quantification of glomerular and tubulointerstitial damage and **(B)** analysis of renal function parameters in anti-IL-22-antibody-treated and Isotype-treated C57BL/6 WT mice as well as naïve C57BL/6 WT animals 7 days after induction of cGN (naïve WT controls *n*=5, α-IL22 *n*=9, α-IgGa2 *n*=10). Symbols in A and B represent mean ± SEM, open and filled circles represent individual animals. Data are pooled from two individual experiments with similar results.

Fig. 7: Phenotype of *Il22ra2*$^{-/-}$ mice in the cGN model.

(A) Histopathological quantification of glomerular and tubulointerstitial damage and **(B)** analysis of renal function parameters in naïve C57BL/6 WT mice, as well as C57BL/6 WT and *Il22ra2*$^{-/-}$ mice at day 7 after induction of cGN (naïve WT controls *n*=5, cGN WT *n*=13, cGN *Il22ra2*$^{-/-}$ *n*=9). Symbols in A and B represent mean ± SEM, open and filled circles

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represent individual animals. Data are pooled from two individual experiments with similar results.

Fig. 8: Phenotype of *Il22*$^{-/-}$ mice in secondary FSGS.

(A) Representative flow cytometric analysis of IL-22 expression by renal CD4^{+} T cells at day 14 after induction of Adriamycin nephropathy (AN) in naïve BALB/c WT mice, AN WT mice and AN *Il22*$^{-/-}$ littermates. Numbers indicate the percentage of cells in the gates. **(B)** Absolute number of IL-17A^{+}IL-22^{+} CD4^{+} T cells per kidney (AN WT *n*=15, AN KO *n*=15). **(C)** Representative photographs of periodic acid-Schiff-stained kidney sections (original magnification 200x), **(D)** histopathological quantification of glomerular and tubulointerstitial damage and **(E)** analysis of renal function parameters in naïve BALB/c WT mice, as well as BALB/c WT and *Il22*$^{-/-}$ littermates at day 14 after induction of Adriamycin nephropathy (AN) (naïve WT controls *n*=4, AN WT *n*=14, AN *Il22*$^{-/-}$ *n*=16). Symbols in D and E represent mean ± SEM, open and filled circles represent individual animals. Data are pooled from two individual experiments with similar results.

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Figure 1

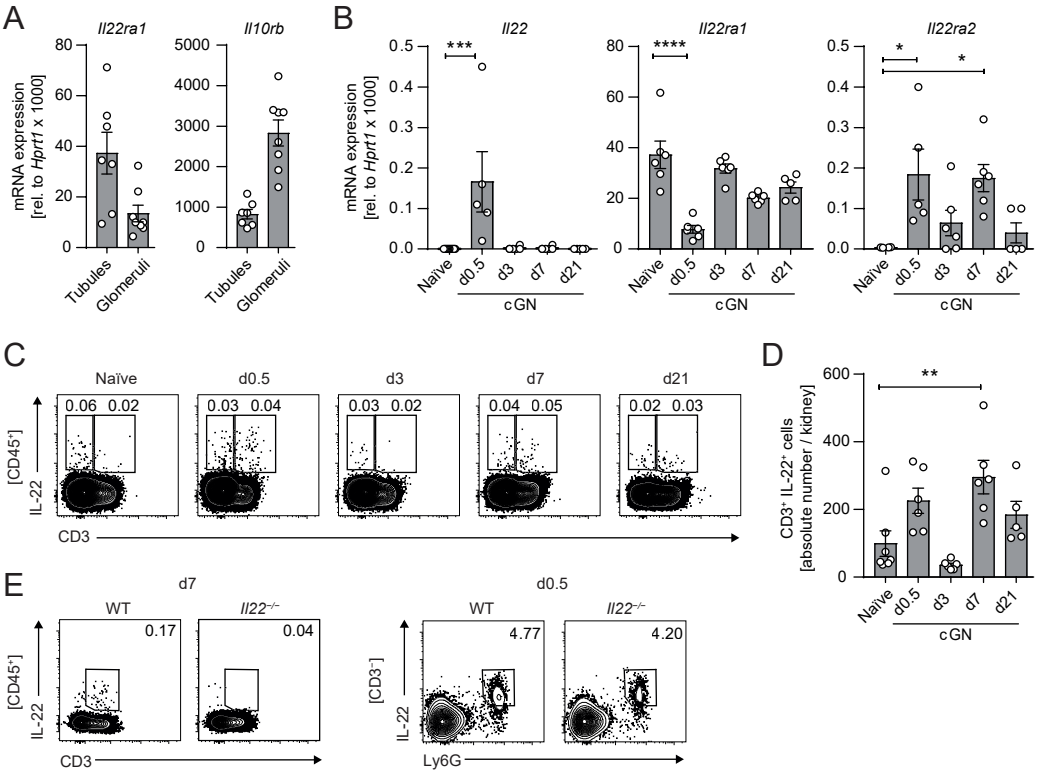

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Figure 2

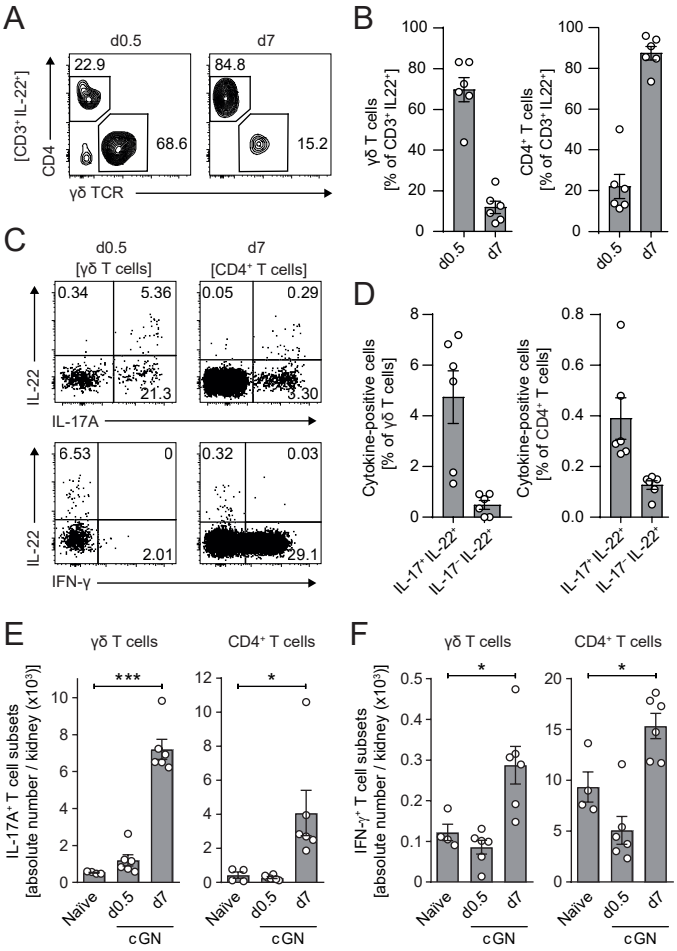

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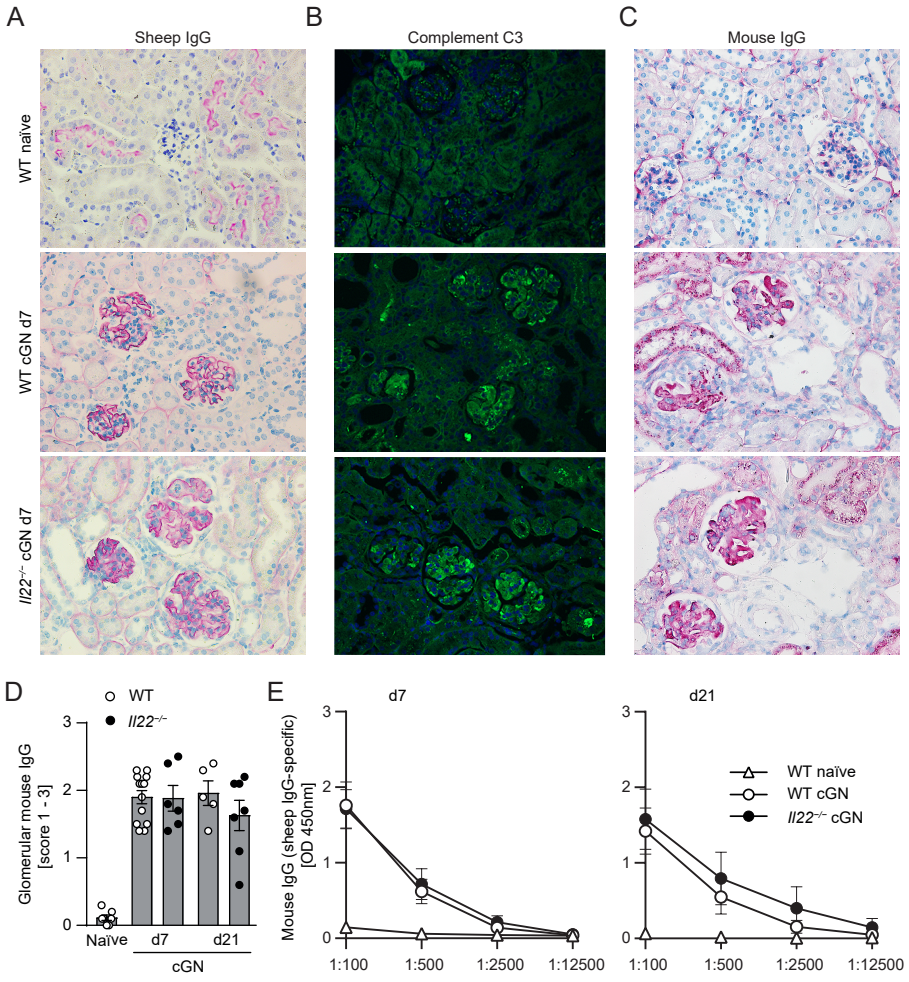
Figure 3



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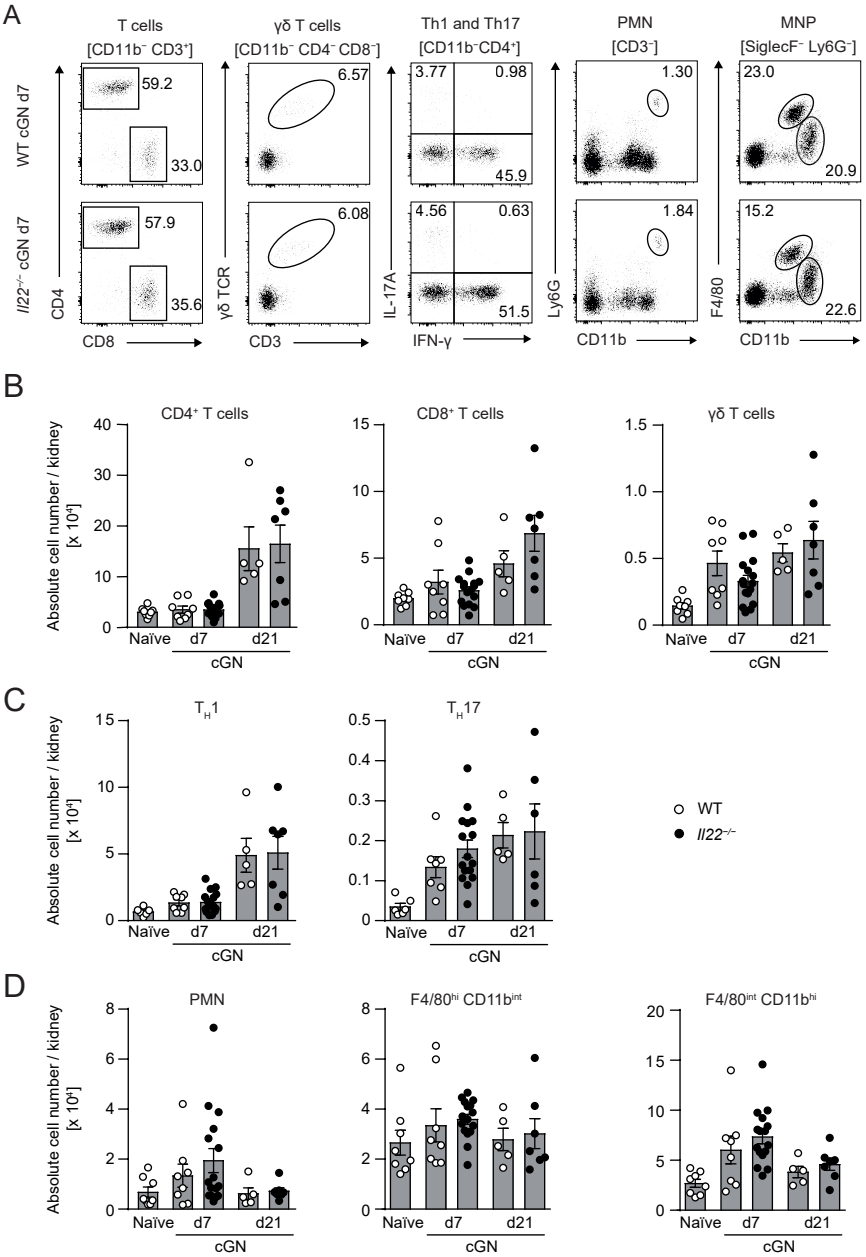

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Figure 5

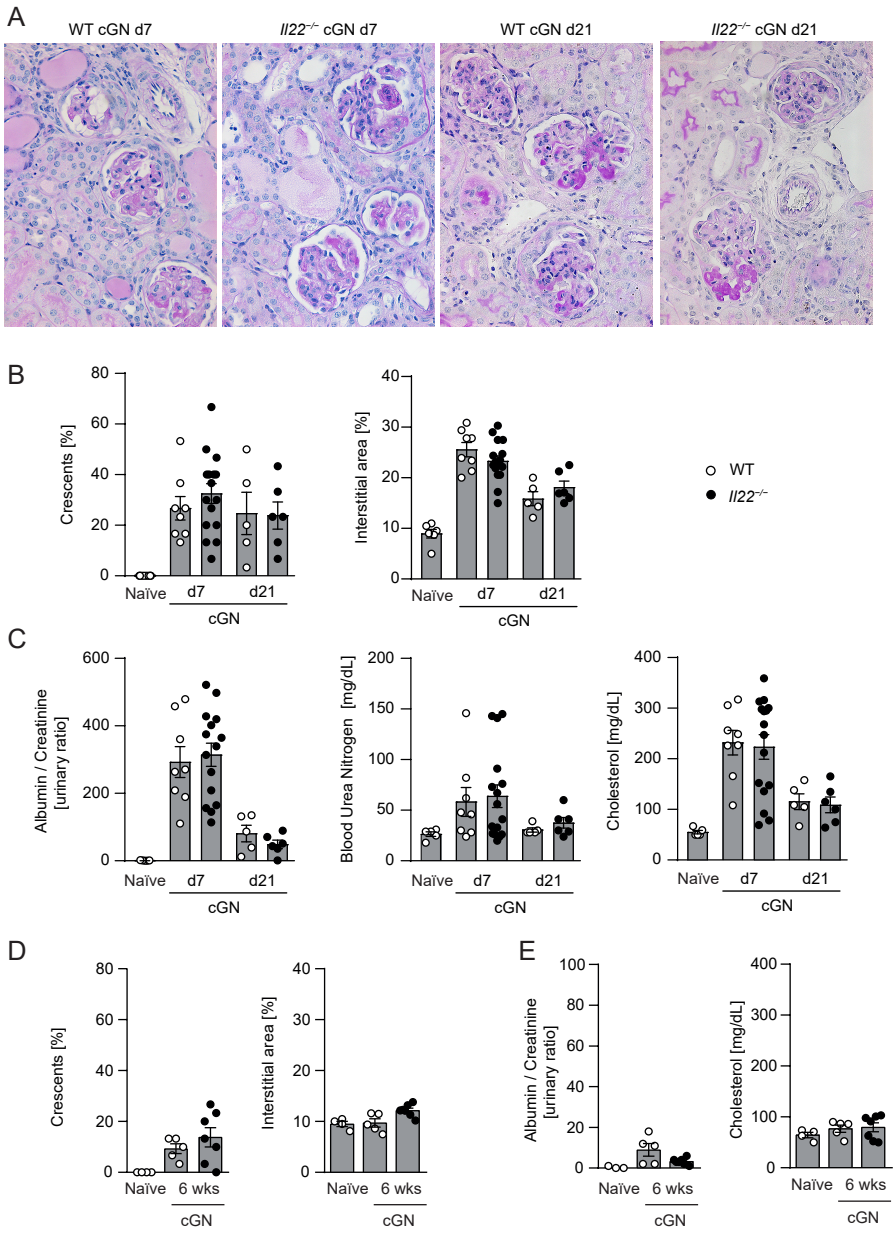

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Figure 6

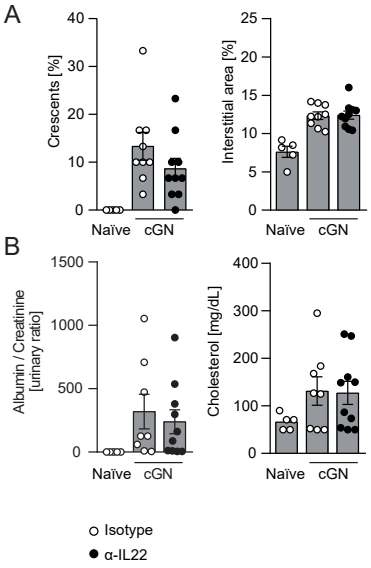

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Figure 7

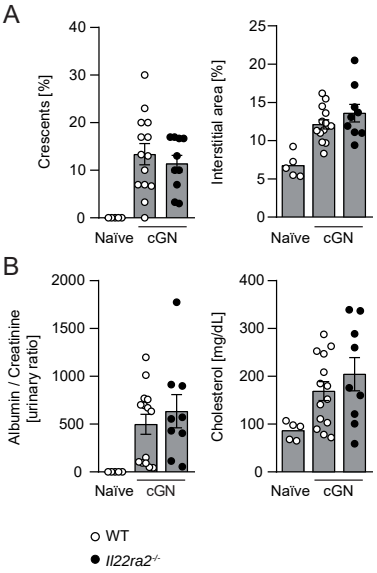

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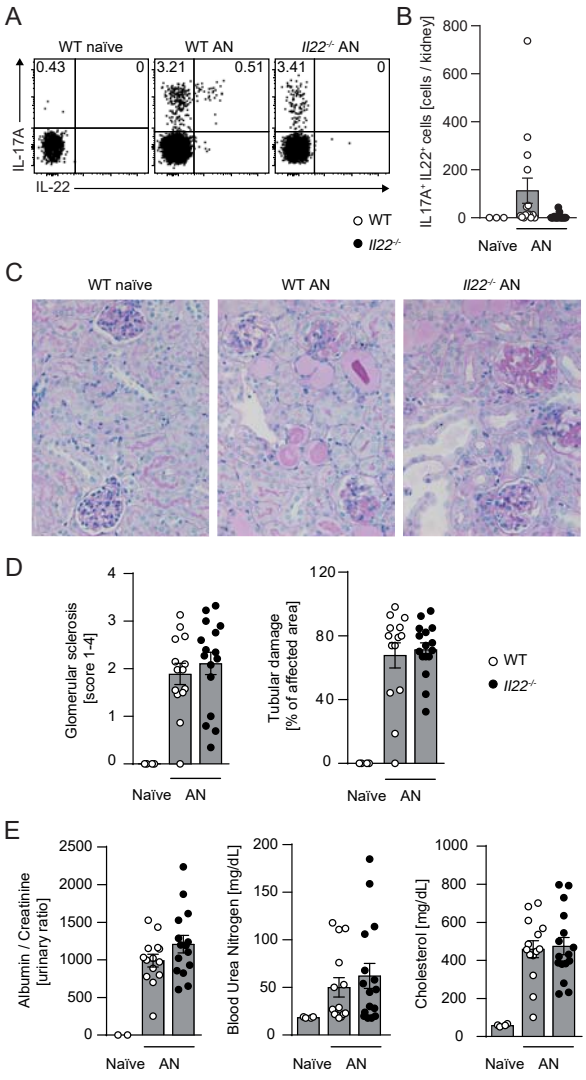
Figure 8



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