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Thrombopoietin-Dependent Myelo-Megakaryopoiesis Fuels Thromboinflammation and Worsens Antibody-Mediated Chronic Renal Microvascular Injury

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ABSTRACT

Background Chronic thromboinflammation provokes microvascular alterations and rarefaction, promoting organ dysfunction in individuals with various life-threatening diseases. Hematopoietic growth factors (HGFs) released by the affected organ may sustain emergency hematopoiesis and fuel the thromboinflammatory process.

Methods Using a murine model of antibody-mediated chronic kidney disease (AMCKD) and pharmacological interventions, we comprehensively monitored the response to injury in the circulating blood, urine, bone marrow, and kidney.

Results Experimental AMCKD was associated with chronic thromboinflammation and the production of HGFs, especially thrombopoietin (TPO), by the injured kidney, which stimulated and skewed hematopoiesis toward myelo-megakaryopoiesis. AMCKD was characterized by vascular and kidney dysfunction, TGFβ-dependent glomerulosclerosis, and microvascular rarefaction. In humans, extracapillary glomerulonephritis is associated with thromboinflammation, TGFβ-dependent glomerulosclerosis, and increased bioavailability of TPO. Analysis of albumin, HGF, and inflammatory cytokine levels in sera from patients with extracapillary glomerulonephritis allowed us to identify treatment responders. Strikingly, TPO neutralization in the experimental AMCKD model normalized hematopoiesis, reduced chronic thromboinflammation, and ameliorated renal disease.

Conclusion TPO-skewed hematopoiesis exacerbates chronic thromboinflammation in microvessels and worsens AMCKD. TPO is both a relevant biomarker and a promising therapeutic target in humans with CKD and other chronic thromboinflammatory diseases.

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INTRODUCTION

Thromboinflammation, a mixed process in which thrombosis is associated with inflammation, occurs in a broad range of human disorders and in various organs, including the kidneys.[1,2] This process disturbs vascular permeability and promotes a switch of vascular cells toward a prothrombotic, proinflammatory, and profibrotic

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phenotype,[1–4] contributing to capillary rarefaction, organ dysfunction, and fibrosis.[4] However, the mechanism by which chronic thromboinflammation is sustained and ultimately induces an irreversible loss of organ function is unknown.

Chronic thromboinflammation consumes both platelets and leukocytes and stimulates an emergency hematopoiesis that perpetuates thromboinflammation.[5] Hematopoiesis relies on the availability of hematopoietic growth factors (HGFs), such as stem cell factor (SCF) and thrombopoietin (TPO), to stimulate the production of multiple hematopoietic lineages and replenish the pool of consumed cells.[5–8] Of particular interest, TPO prompts the priming and expansion of hematopoietic stem cells (HSCs)[9] and megakaryocytes (MKs).[10] TPO can synergize with other HGFs and cytokines to promote megakaryopoiesis.[11] The production of TPO by hepatocytes is elicited by desialylated platelets under steady state and by IL6 under inflammatory conditions.[12–14] Increased TPO availability stimulates the production of platelets, which subsequently metabolize TPO when it binds to its receptor on their surface.[7] Kidneys also produce TPO,[14] but its function and the mechanism regulating its expression are unknown.

Platelets are critical in the context of fibrosis associated with chronic vascular injury because of their hemostatic properties and growth and profibrotic factors that platelets store in their α-granules, including TGFβ.[15,16] Its availability is regulated by the platelet mass,[16,17] and TPO regulates TGFβ production by MKs *in vitro*.[18] Furthermore, TGFβ receptor (TGFβR) signaling exacerbates vascular and organ dysfunction and promotes fibrosis.[19,20]

In the kidney, various insults can injure the microvascular network and trigger thromboinflammation.[1,2] In the context of autoimmunity or alloimmunity, antibodies trigger the injury.[1–4,21,22] In antibody-mediated chronic kidney disease (AMCKD), sustained microvascular injuries drive glomerulosclerosis, rarefaction of the downstream microvascular network, and renal insufficiency, which contribute to the development of end-stage kidney disease (ESKD).[23] The extent of fibrosis (glomerular or interstitial) in biopsies correlates with the decrease in the eGFR and predicts ESKD.[22] Few drugs halt or reverse organ fibrosis.[4,22] Despite the efficiency of immunosuppressive therapy in the acute phase of the disease, 50% of patients with antiglomerular basement membrane autoantibodies-induced extracapillary glomerulonephritis (ECGN) develop ESKD,[24] indicating the dire need for antifibrotic drugs in the post-acute phase.

We hypothesized that chronic thromboinflammation within the renal microvascular network triggers the production of HGFs, such as TPO, favoring myelo-megakaryopoiesis. In this scenario, chronic thromboinflammation is sustained by enhanced myeloid cell and platelet production, and platelet-derived TGFβ worsens fibrosis.

Significance Statement

Kidney-derived thrombopoietin (TPO) increases myeloid cell and platelet production during antibody-mediated chronic kidney disease (AMCKD) in a mouse model, exacerbating chronic thromobinflammation in microvessels. The effect is mirrored in patients with extracapillary glomerulonephritis associated with thromboinflammation, TGFβ-dependent glomerulosclerosis, and increased bioavailability of TPO. Neutralization of TPO in mice normalized hematopoiesis, reduced chronic thromboinflammation, and ameliorated renal disease. The findings suggest that TPO is a relevant biomarker and a promising therapeutic target for patients with CKD and other chronic thromboinflammatory diseases.

METHODS

Animal Model

Mice were purchased and bred in-house (Taconic, 129S6/SvEvTac). Sheep serum (Probetex, USA, PTX-000S [nonimmune] and PTX-001S-Ms [nephrotoxic serum (NTS)]) were injected i.v. on day 0 to induce AMCKD. Animal experiments conformed to Directive 2010/63/EU of the European Parliament, and the study was reviewed and approved by the Comité d'Ethique Paris Nord 121 (APAFIS n°17819).

RESULTS

Platelets Promote Experimental AMCKD

To test our hypothesis, we used a murine model of AMCKD induced by an injection of NTS. Antiglomerular basement membrane antibodies contained in the NTS specifically target glomerular capillaries and initiate thromboinflammation.[25] This microvascular injury drives glomerulosclerosis and induces a reduction in the eGFR.[26] First, we studied platelet biology in this model by immunodepleting them before AMCKD induction (Figure 1, A and B). Interestingly, during AMCKD, (isotype-treated) control mice developed thrombocytosis, starting on day 5 (Figure 1B). Control mice, but not platelet-depleted mice, displayed a significant increase in circulating soluble CD62P 24 hours postinjection (p.i.) of NTS and during the period of thrombocytosis (Figure 1C). Therefore, this result reflects acute and chronic platelet activation during AMCKD. In control mice, the plasma platelet factor 4 (PF4) concentration increased in parallel with thrombocytosis, whereas it remained extremely low in platelet-depleted mice (Figure 1D). Thus, the plasma PF4 concentration reflects the size of the platelet mass.

Platelet depletion prevented glomerular microvascular dysfunction (urinary albumin/creatinine ratio), and renal insufficiency (creatininemia and uremia) during both the acute and chronic phases of the disease (Figure 1, E–G). Platelet depletion significantly reduced the rise of plasma TGFβ concentration associated with thrombocytosis

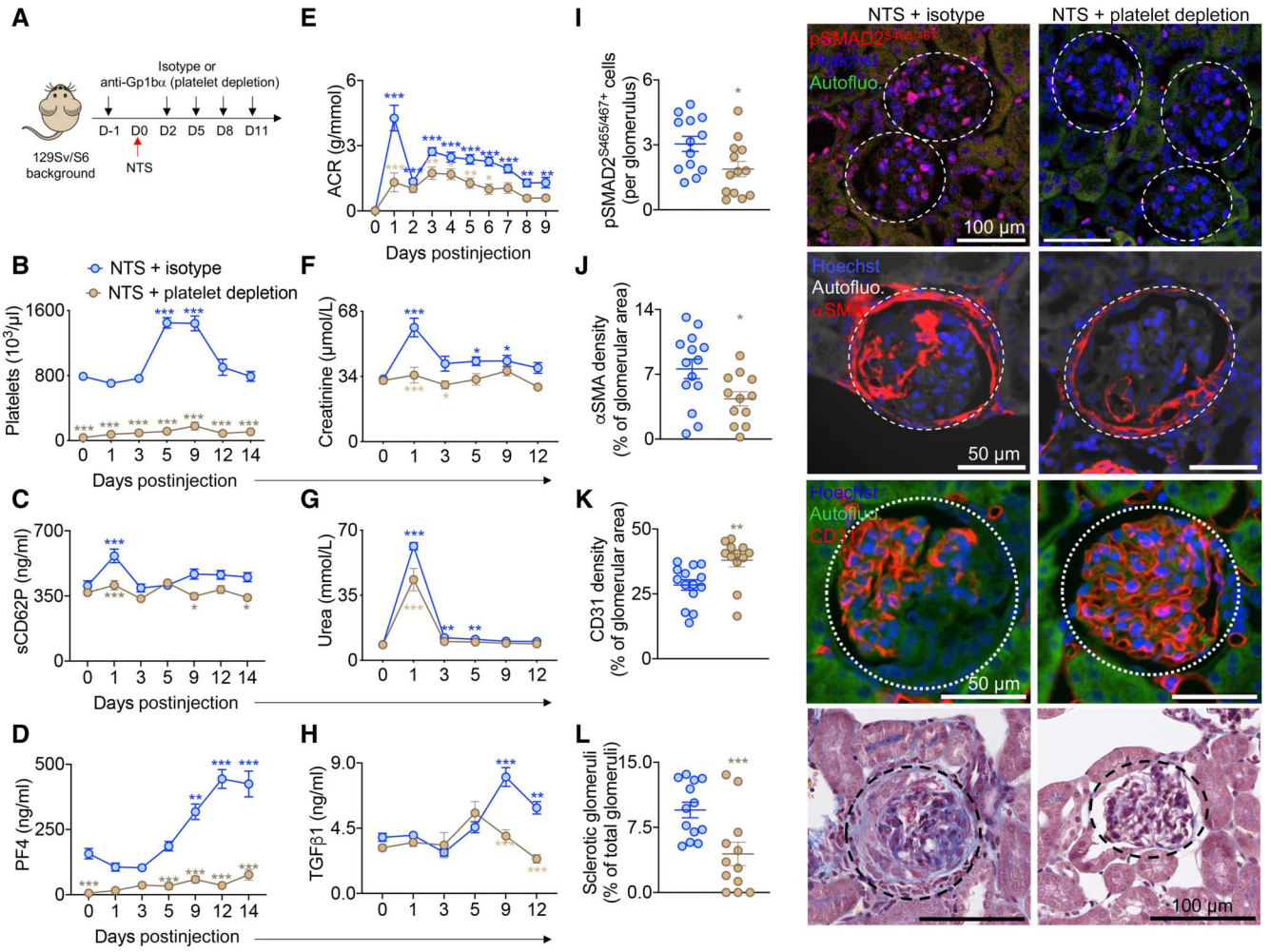

Figure 1. Platelet depletion prevents vascular and organ dysfunction, capillary rarefaction, and glomerulosclerosis in experimental AMCKD. (A) Experimental workflow: Platelet depletion was induced by i.v. injection of anti-Gp1bα antibodies (R300, 3 μg/g mouse). Control mice received the isotype. To maintain platelet depletion, mice received i.p. injections of anti-Gp1bα antibodies (R300, 3 μg/g mouse) on days 2, 5, 8, and 11. AMCKD was initiated on day 0 by NTS injection (i.v. 50 μl). (B–D) Kinetic analysis of circulating platelet count (B), sCD62P, (C) and platelet factor 4 (PF4) (D) in the plasma of mice during AMCKD, with or without platelet depletion. The data were pooled from two independent experiments (n=13–14 mice/group). (E–G) Kinetic analysis of the urinary ACR (E) and plasma creatinine (F) and urea (G) levels of mice during AMCKD, with or without platelet depletion. The data were pooled from two independent experiments (n=14–21 mice/group). (H) Kinetic analysis of the plasma TGFβ1 level of mice during AMCKD, with or without platelet depletion. The data were pooled from two independent experiments (n=13–14 mice/group). (B–H) Blue stars—*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$: NTS+ isotype on a specific day compared with NTS+ isotype at day 0; Kruskal–Wallis test, followed by the uncorrected Dunn test (NTS effect in non–platelet-depleted mice). Brown stars—*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$: NTS+platelet depletion versus NTS+isotype at matched time points; two-way ANOVA, followed by the uncorrected Fisher test (platelet depletion effect). (I–L) Quantification and representative images of immunofluorescence and histological analyses of kidney tissue sections on day 14 after induction of AMCKD and platelet depletion. The results of nuclear phosphorylated SMAD2$^{Ser465/467}$ (I), αSMA (J), CD31 (K), and Carstair (L) staining in glomeruli are shown. The data were pooled from two independent experiments (n=13–14 mice/group). Brown stars—*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$: NTS+platelet depletion versus NTS+isotype; Mann–Whitney test. αSMA, α smooth muscle actin; ACR, albumin/creatinine ratio; AMCKD, antibody-mediated chronic kidney disease; NTS, nephrotoxic serum; sCD62P, soluble CD62P. Figure 1 can be viewed in color online at www.jasn.org.

development during AMCKD (Figure 1H). Platelet depletion blunted the activation of the TGFβR signaling pathway and overexpression of α smooth muscle actin (αSMA) in glomeruli and reduced capillary rarefaction and glomerulosclerosis during AMCKD (Figure 1, I–L). Platelet depletion also reduced tubular injury and interstitial capillary rarefaction, without affecting interstitial inflammation (Supplemental Figure 1, A–C).

In the blood, platelet depletion significantly increased the AMCKD-associated anemia (Supplemental Figure 2A).

During the acute phase of AMCKD, platelet depletion reduced neutrophil mobilization without affecting the numbers of other circulating leukocytes (Supplemental Figure 2, B–H). During the chronic phase of AMCKD, platelet-depleted mice displayed a significant leukocytosis compared with control mice (Supplemental Figure 2, B–H), suggesting that platelet depletion stimulated hematopoiesis.

Thrombocytosis Accompanies the Development of AMCKD

Transient thrombocytopenic episode or systemic inflammation can induce reactive thrombocytosis. We first analyzed the induction of thrombocytopenia. The injection of a large bolus of NTS (200 μl), but not of nonimmune serum, induced a transient thrombocytopenia and platelet accumulation in glomeruli (Supplemental Figure 3, A and B). Lower doses of NTS ($\leq$100 μl) were nonthrombocytopenic but significantly activated platelets (Supplemental Figure 3, A, C, D). The magnitude of thrombocytosis depended on the dose of NTS injected (Supplemental Figure 3E). Hence, stimuli leading to platelet production must be generated during AMCKD.

Chronic thromboinflammation occurs during AMCKD, as shown by the accumulation of platelets, CD45$^+$ cells and fibrin(-ogen) in glomeruli (Figure 2, A–C) between day 5 and 14 p.i. of NTS. Degranulated platelets interacting with the injured endothelium were observed by transmitted electron microscopy in glomeruli during AMCKD (day 9, Figure 2D). This process paralleled capillary rarefaction (Figure 2E).

As platelet-derived TGFβ could contribute to glomerulosclerosis in AMCKD, we characterized the TGFβR signaling. The expression of TGFβRs remained unchanged in the injured kidney during AMCKD (Supplemental Figure 4, A and B). However, the activation of the TGFβR signaling pathway (phosphorylated SMADs) and the development of glomerulosclerosis paralleled the development of thrombocytosis (Supplemental Figure 4, C–E). Therefore, thrombocytosis might contribute to glomerulosclerosis development *via* platelet-derived TGFβ.

AMCKD Remotely Stimulates and Skews Hematopoiesis toward the Myelo-Megakaryocytic Lineage

We next aimed to identify the cues responsible for thrombocytosis and hypothesized that the injured kidney remotely stimulates hematopoiesis during AMCKD. In the bone marrow (BM), HSCs (Lineage$^-$Sca1$^+$cKit$^+$) with short-term repopulating abilities (CD34$^+$CD150$^-$) and with a myeloid/megakaryocyte (MK) bias (CD34$^-$CD150$^+$), as well as progenitor cell populations (granulocyte myeloid progenitors, common myeloid progenitors, MK-erythroid progenitors, and pre-MKs), expanded significantly during AMCKD (Figure 3, A–D). MKs significantly accumulated in

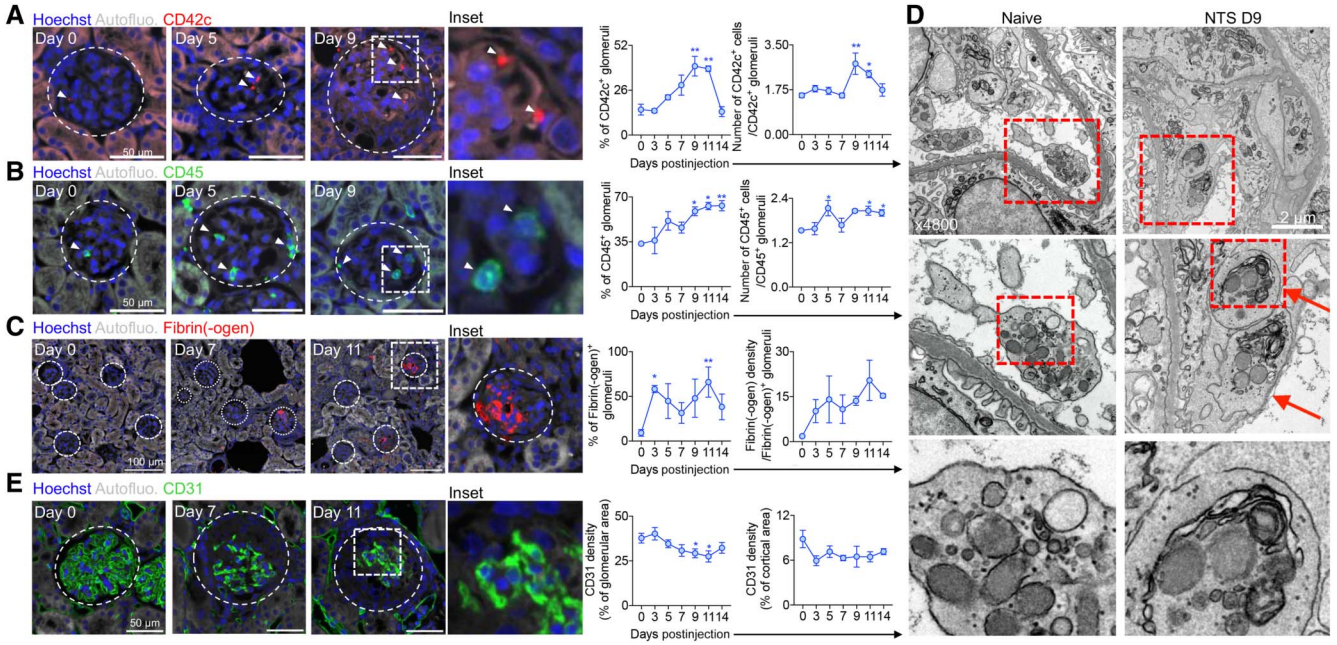

Figure 2. Kinetic analysis of chronic thromboinflammation and capillary rarefaction in AMCKD. (A–C) Kinetic analysis of platelet (A; CD42c$^+$), leukocyte (B; CD45$^+$ cells), and fibrin(-ogen). (C) Accumulation in glomeruli from mice injected with NTS. Representative images of immunofluorescent microscopy and quantification of the percentage of glomeruli positive for each staining, as well as the mean density of staining per positive glomerulus. The data are from one experiment (n=3 mice/time point). *$P < 0.05$ and **$P < 0.01$: NTS at a given time point versus day 0; Kruskal–Wallis test, followed by the uncorrected Dunn test. AMCKD, antibody-mediated chronic kidney disease; NTS, nephrotoxic serum. Figure 2 can be viewed in color online at www.jasn.org.

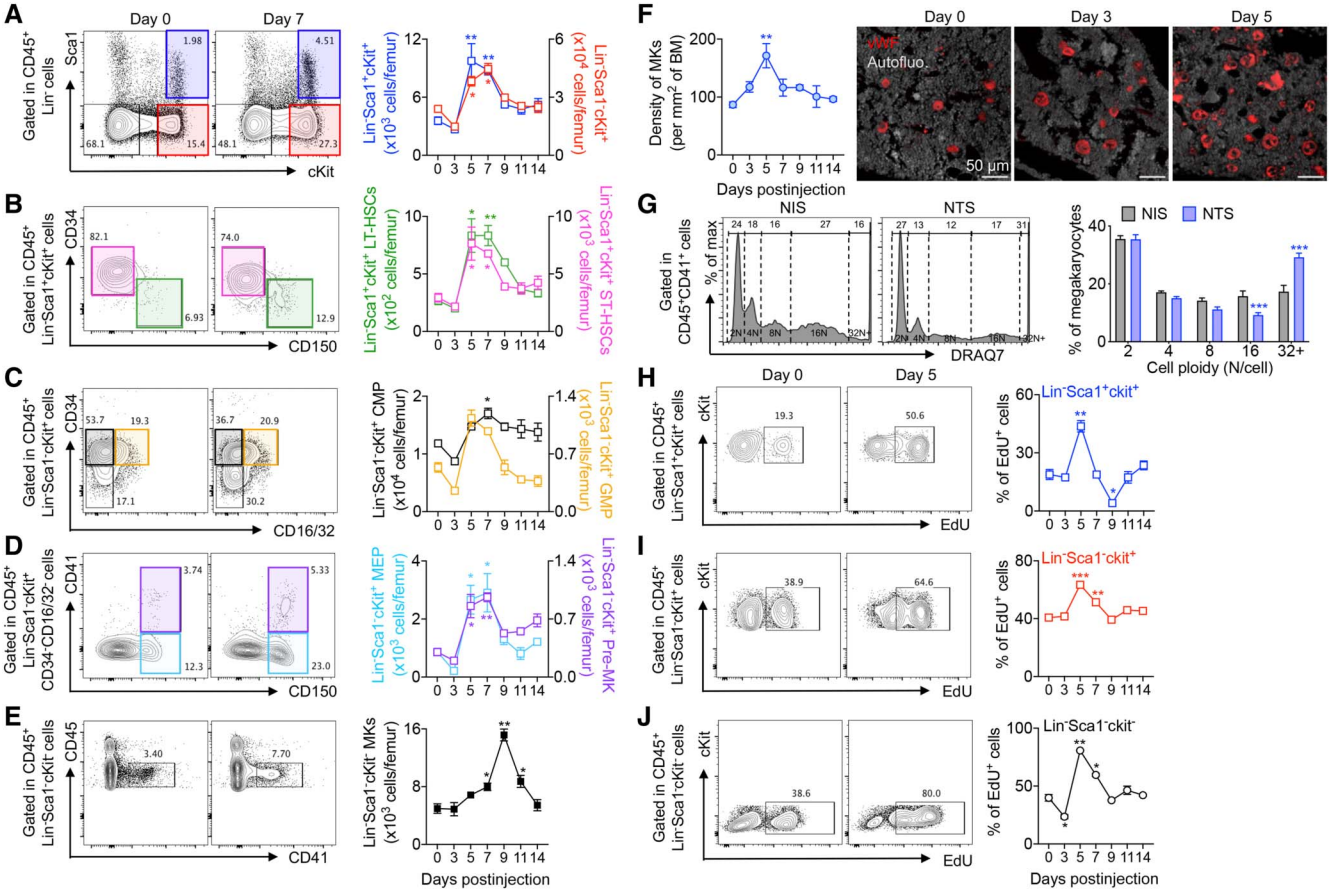

Figure 3. Experimental AMCKD is associated with remote induction of myelo-megakaryopoiesis in the BM. (A–E) Representative flow cytometry plots of hematopoietic progenitor cell populations in the BM of naïve mice (D0) and 7 days p.i. of NTS and kinetic quantification of the number of cells per femur. The following cell populations were identified in the $CD45^+$ lineage-negative ($CD3^-B220^-CD11b^-Ly6G^-$) gate: HSC-enriched ($Sca1^+cKit^+$) and multipotent progenitor-containing (Sca^-ckit^+) cell populations (A), HSCs with short-term ($Sca1^+cKit^+CD34^+CD150^-$) and long-term ($Sca1^+cKit^+CD34^-CD150^+$) repopulating capacities (B), GMPs ($Sca1^-cKit^+CD34^+CD16/32^+$), CMPs ($Sca1^-cKit^+CD34^+CD16/32^-$) (C), MEPs ($Sca1^-cKit^+CD34^-CD16/32^-CD150^+CD41^-$), pre-megakaryocyte progenitors (pre-MK prog., $Sca1^-cKit^+CD34^-CD16/32^-CD150^+CD41^+$) (D), and MKs ($Sca1^-cKit^-CD45^{low}CD41^+$) (E). The data are from one experiment ($n=3$ mice/group). $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$: number of progenitor cells per femur at a time point versus the number of cells in the same population from naïve mice. The colored stars match the color of the respective cell populations; Kruskal–Wallis test, followed by the uncorrected Dunn test. (F) Kinetic analysis of the density of MKs (vWF^+ cells) and representative images of BM of mice during experimental AMCKD. The data are from one experiment ($n=3$ mice/group). $^{**}P < 0.01$: density of MKs per femur versus naïve mice (D0); Kruskal–Wallis test, followed by the uncorrected Dunn test. (G) Representative flow cytometry histogram depicting DNA content in MKs from the BM of NIS-injected or NTS-injected mice on day 5 and quantification of the polyploidy of MKs. The data are from one experiment ($n=10$ mice/group). $^{***}P < 0.001$: NTS-injected versus NIS-injected; two-way ANOVA test, followed by the uncorrected Fisher test. (H–J) Analysis of DNA synthesis by the incorporation of EdU *in vivo* for 16 hours at different time points after NTS injection in $Lin^-Sca1^+cKit^+$ (H), $Lin^-Sca1^-cKit^+$ (I), and $Lin^-Sca1^-cKit^-$(J) cell populations. The data are from one experiment ($n=3$ mice/group). $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$: NTS-injected versus naïve mice; Kruskal–Wallis test, followed by the uncorrected Dunn test. AMCKD, antibody-mediated chronic kidney disease; BM, bone marrow; CMP, common myeloid progenitor; GMP, granulocyte myeloid progenitor; HSC, hematopoietic stem cell; MEP, megakaryocyte-erythroid progenitor; MK, megakaryocyte; NIS, nonimmune serum; NTS, nephrotoxic serum. Figure 3 can be viewed in color online at www.jasn.org.

the BM and increased their polyploidy (Figure 3, E–G) during AMCKD. DNA synthesis analysis *in vivo* revealed that all $CD45^+Lin^-$ cells proliferated with similar kinetics (Figure 3, H–J). Consistent with their numbers in the blood, myeloid cells accumulated in the BM, whereas lymphoid cells and red blood cell progenitors were depleted (Supplemental Figure 5, A–E). The spleen can support megakaryopoiesis, but splenectomized mice developed thrombocytosis during AMCKD, suggesting that the spleen is not mandatory in this setting (Supplemental Figure 6).

Injured Kidneys Produce HGFs during AMCKD

Because AMCKD stimulates hematopoiesis, we analyzed the expression of mRNAs encoding HGFs that might elicit this

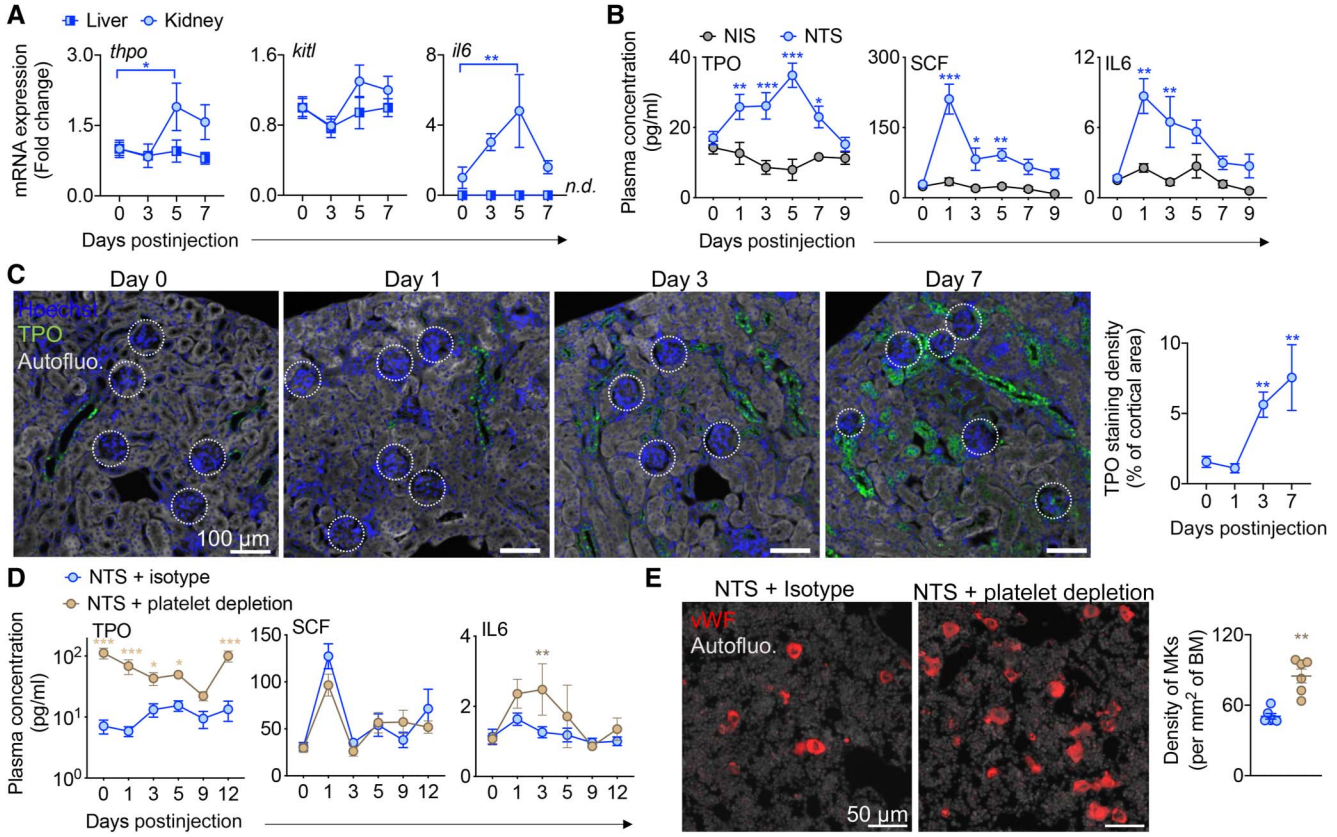

Figure 4. AMCKD-induced chronic thromboinflammatory microvascular injury is associated with kidney-specific expression of HGFs. (A) Kinetic analysis of the mRNA expression of *thpo*, *kitl*, and *il6* in the liver and kidney of mice injected with NTS using Quantigene technology. The data were pooled from two independent experiments (n=7–10 mice/time point). *$P < 0.05$ and **$P < 0.01$: versus day 0 (naïve mice); two-way ANOVA, followed by the uncorrected Fisher test. n.d.: not detected on day 0. (B) Kinetic analysis of the plasma concentrations of TPO, SCF, and IL6 in mice following NIS or NTS injection. The data were pooled from five independent experiments (n=7–31 mice/time point). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$: NTS versus NIS; two-way ANOVA, followed by the uncorrected Fisher test. (C) Representative images of immunofluorescence microscopy to detect TPO expression and quantification of TPO expression in the cortical area of the kidney at different time points after NTS injection. The data were pooled from three independent experiments (n=4–14 mice/time point). **$P < 0.01$: versus day 0; Kruskal–Wallis test, followed by the uncorrected Dunn test. (D) Kinetic analysis of the plasma concentrations of TPO, SCF, and IL6 in mice after NTS injection, with or without platelet depletion. The data were pooled from two independent experiments (n=13–14 mice/group). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$: NTS+platelet depletion versus NTS+ isotype; two-way ANOVA, followed by the uncorrected Fisher test. (E) Representative images and quantification of MKs (vWF$^+$ cells) in BM sections 14 days p.i. of NTS, with or without platelet depletion (R300). The data are from one experiment (n=6 mice/group). **$P < 0.01$, NTS+platelet depletion versus NTS+isotype; Mann–Whitney test. AMCKD, antibody-mediated chronic kidney disease; BM, bone marrow; HGF, hematopoietic growth factor; NIS, nonimmune serum; NTS, nephrotoxic serum; SCF, stem cell factor; TPO, thrombopoietin. Figure 4 can be viewed in color online at www.jasn.org.

effect, that is, *Tpo*, *Il6*, and *Scf*, (kidney [target organ] versus liver [control organ]). The expression of the *Tpo* and *il6* mRNAs significantly increased in the kidney, not in the liver, during AMCKD; *Kitl* expression remained unchanged (Figure 4A). Plasma concentrations of these HGFs significantly increased during AMCKD (Figure 4B). In the kidney, TPO production by tubular cells substantially increased during AMCKD (Figure 4C). Consistent with platelet metabolizing capacities, the plasma TPO concentration and megakaryopoiesis further increased in platelet-depleted mice during AMCKD, whereas SCF and IL6 were modestly affected (Figure 4, D and E). Our data suggest that kidney-derived TPO stimulates hematopoiesis, which might exacerbate AMCKD.

Inflammation Contributes to the Production of HGFs during AMCKD

Mice were pretreated with anti-inflammatory treatments to assess whether inflammation regulates HGF production and hematopoietic activity during AMCKD.

As kidney-resident and hematopoietic cells express IL-1R1[27] and IL1α stimulates emergency thrombopoiesis,[28] IL1R1 was neutralized. This strategy did not prevent AMCKD-induced thrombocytosis (Supplemental Figure 7, A and B), but it

significantly reduced neutrophilia and monocytosis (Supplemental Figure 7, C–E).

The anti-inflammatory dexamethasone treatment (DEX) blunted the increase of plasma IL6 and TPO and of their renal expression (mRNA), during AMCKD, without affecting SCF (Supplemental Figure 8, A–F). DEX prevented thrombocytosis and the rise in plasma PF4, without affecting platelet activation (Supplemental Figure 8, G–I). DEX did not affect circulating neutrophils but significantly reduced circulating monocytes and lymphocytes and renal inflammation (Supplemental Figure 8, J–M). Despite its effect on HGFs expression in the injured kidney, DEX did not reduce the expression of renal *Tgfb1* mRNA (Supplemental Figure 9A). DEX reduced the plasma level of TGFβ and the activation of the TGFβR signaling in glomeruli and glomerulosclerosis during AMCKD (Supplemental Figure 9, B–E), suggesting that DEX blunted glomerulosclerosis by preventing platelet-derived TGFβ to reach the kidney. Unfortunately, DEX did not reduce uremia and urinary albumin/creatinine ratio and even increased creatininemia, hypercholesterolemia, and weight loss during AMCKD (Supplemental Figure 9, F–J).

Because IL6 can regulate the production of TPO,[12] we neutralized it during AMCKD. This strategy failed to sustain long-term inhibition of TPO, did not affect the plasma level of SCF, and resulted in an increase in circulating platelets, plasma PF4, and soluble CD62P (Supplemental Figure 8, A–C, G–I). Despite an efficient reduction of the acute neutrophilia and of the renal inflammation (Supplemental Figure 8, J–M), IL6 neutralization failed to reduce plasma TGFβ concentration, glomerulosclerosis, and microvascular and renal dysfunction during AMCKD (Supplemental Figure 9, B, E–H). Hence, during AMCKD, IL6 modestly regulates TPO and thrombocytosis.

Therefore, inflammation regulates AMCKD-induced thrombocytosis. DEX efficiently prevented thrombocytosis, the rise in TGFβ bioavailability, and glomerulosclerosis, despite serious side effects.

TGFβ Activity Contributes to Organ Dysfunction and Fibrosis during AMCKD

To decipher if the increased TGFβ activity observed during thrombocytosis contributed to the pathophysiology of AMCKD, it was neutralized starting on day 3 p.i. of NTS (Figure 5, A and B).

Neutralization of TGFβ activity did not affect thrombocytosis or microvascular dysfunction but reduced uremia (Figure 5, C–E). Neutralization of TGFβ activity significantly reduced TGFβR signaling in glomeruli and the glomerular and interstitial expression of αSMA (Figure 5, F–I). Neutralization of TGFβ activity decreased interstitial, but not glomerular, inflammation and glomerular, but not interstitial, capillary rarefaction, and significantly reduced glomerulosclerosis during AMCKD (Figure 5, J–N). Unfortunately, it aggravated weight loss (Figure 5O).

Therefore, TGFβ activity contributes to the pathophysiology of AMCKD during the thrombocytosis period; however, the side effects associated with its global neutralization make this strategy inappropriate for therapy.

TPO Contributes to Chronic Thromboinflammation during AMCKD

TPO has a unique role in hematopoiesis and might contribute to the physiopathology of AMCKD. Therefore, we tested an anti-TPO monoclonal antibody (Figure 6A). Efficient neutralization of plasma TPO did not affect the plasma SCF and IL6 concentrations, but it prevented thrombocytosis, the increase in plasma PF4, and significantly reduced chronic platelet activation (Figure 6, B–G). TPO neutralization significantly reduced the accumulation of platelets and immune cells in glomeruli (Figure 6, H–J), as well as thrombosis (Supplemental Figure 10, A and B), therefore showing that TPO contributed to chronic thromboinflammation during AMCKD.

TPO neutralization had no effect on circulating neutrophils or lymphocytes during AMCKD, but it significantly reduced monocytosis and interstitial $CD45^+$ cell accumulation, including $Iba1^+$ macrophages, in injured kidneys (Supplemental Figure 11, A–E). Therefore, TPO contributes to myelopoiesis and interstitial inflammation during AMCKD.

TPO Chronically Stimulates Hematopoiesis during AMCKD

We next addressed whether TPO stimulates hematopoiesis during AMCKD. TPO neutralization significantly reduced the accumulation of HSCs and myeloid and MK progenitors in the BM on day 14 (Supplemental Figure 12, A and B). At earlier time points, TPO neutralization prevented the expansion of HSCs with a myeloid/MK-bias ($Lineage^-Sca1^+cKit^+$, $CD150^+CD34^-$) before any significant reduction in the number of pre-MKs (Supplemental Figure 12C). TPO neutralization prevented the accumulation of MKs in the BM and their synthesis of DNA (Supplemental Figure 12, D and E). Furthermore, it prevented the AMCKD-induced splenic extramedullary hematopoiesis without affecting adaptive immune cell expansion (Supplemental Figure 13, A–E).

TPO Promotes Organ Dysfunction, TGFβ Bioavailability, Glomerulosclerosis, and Microvascular Rarefaction during AMCKD

TPO neutralization significantly reduced microvascular and kidney dysfunction, TGFβ bioavailability, and TGFβR signaling and αSMA expression in injured glomeruli (Figure 7, A–F). TPO neutralization reduced glomerular cell proliferation, capillary rarefaction, and glomerulosclerosis during AMCKD (Figure 7, G–J), suggesting that this treatment broadly reduced the levels of platelet-derived growth factors.

Neutralization of TPO in AMCKD Is Safe

The therapeutic potential of platelet depletion, TGFβ activity neutralization, and TPO neutralization was

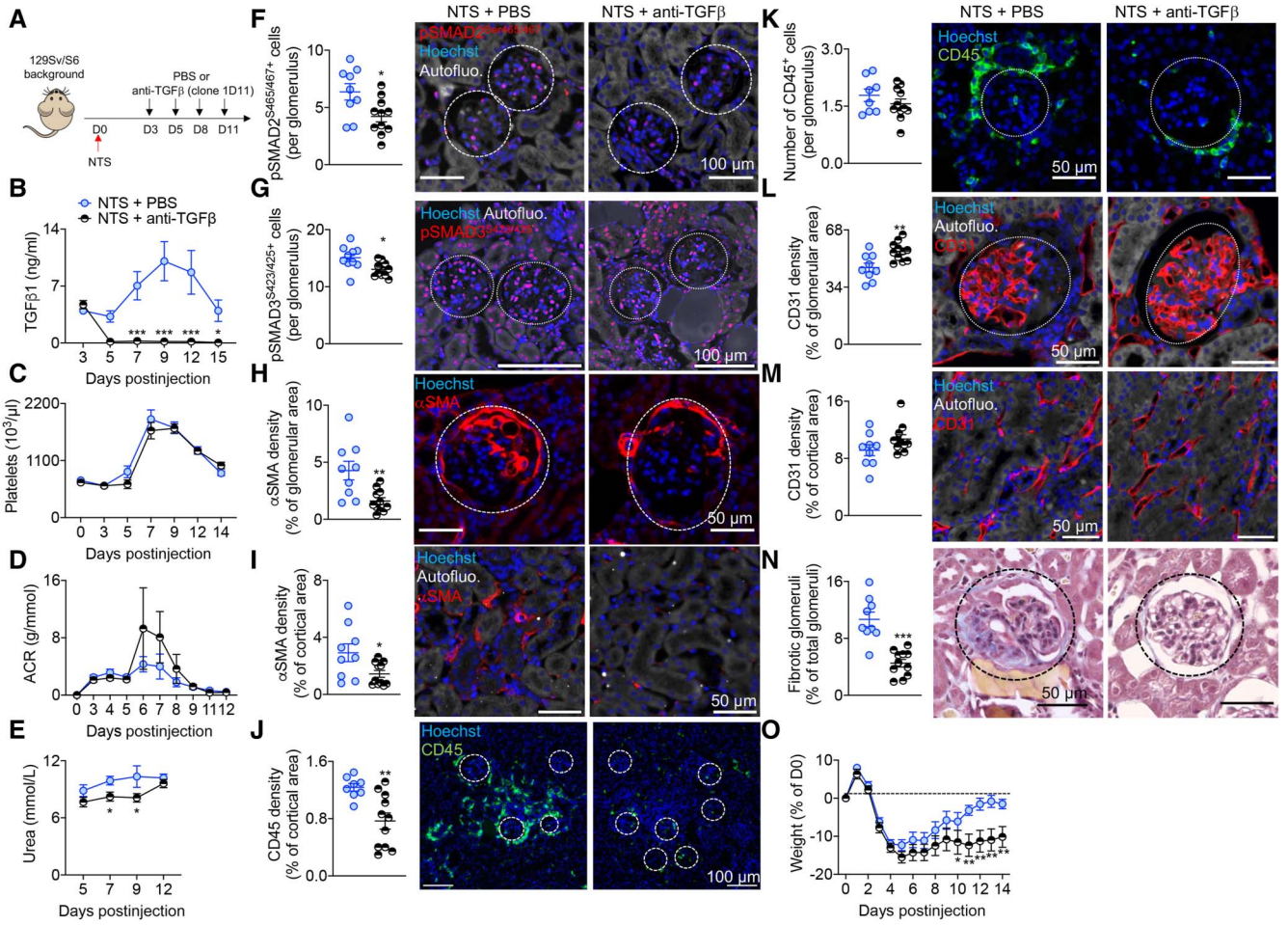

Figure 5. Neutralization of TGFβ activity reduces renal dysfunction, local inflammation, microvascular rarefaction, and glomerulosclerosis during the chronic phase of AMCKD, but increases weight loss. (A) Experimental workflow: Mice were injected with NTS to induce AMCKD on day 0. Neutralization of TGFβ activity was induced on day 3 p.i. of NTS (250 μg of antibody, clone 1D11, i.p.) and applied again on days 5, 8, and 11. Control mice received an equal volume of phosphate-buffered saline (PBS). (B) Kinetic analysis of plasma TGFβ in mice developing AMCKD and treated with PBS or anti-TGFβ. Plasma samples were taken from day 3 (before treatment) and onwards. The data are from one experiment (n=5 mice/group). *$P < 0.05$, ***$P < 0.001$: NTS+anti-TGFβ versus NTS+PBS; two-way ANOVA, followed by the uncorrected Fisher test. (C–E) Kinetic analysis of circulating platelet count (C), urinary ACR (D), and plasma urea (E) in mice developing AMCKD and treated with PBS or anti-TGFβ. The data were pooled from two independent experiments (n=10 mice/group). *$P < 0.05$: NTS+anti-TGFβ versus NTS+PBS; two-way ANOVA, followed by the uncorrected Fisher test. (F–N) Quantification and representative immunofluorescence staining of pSMAD2$^{Ser465/467}$ (F) and pSMAD3Ser423/425 (G), glomerular (H) and cortical (I) αSMA, cortical (J) and glomerular (K) inflammation, and glomerular (L) and cortical (M) capillary density. Glomerulosclerosis was analyzed using histological Carstair staining (N) of kidney sections 14 days p.i. of NTS in mice treated with anti-TGFβ or PBS. The data are pooled from two independent experiments (n=10 mice/group). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$: NTS+anti-TGFβ versus NTS+PBS mice; Mann–Whitney test. (O) Kinetic analysis of weight of mice developing AMCKD and treated with PBS or anti-TGFβ. The data were pooled from two independent experiments (n=10 mice/group). *$P < 0.05$ and **$P < 0.01$: NTS+anti-TGFβ versus NTS+PBS; two-way ANOVA, followed by the uncorrected Fisher test. αSMA, α smooth muscle actin; ACR, albumin/creatinine ratio; AMCKD, antibody-mediated chronic kidney disease; NTS, nephrotoxic serum. Figure 5 can be viewed in color online at www.jasn.org.

independent of immune complex accumulation and complement activation in glomeruli, unlike DEX and IL6 neutralization (Table 1). TPO neutralization did not affect weight change, creatininemia, hypercholesterolemia, nor anemia (Supplemental Figure 14, A–D). In summary, TPO significantly contributes to chronic thromboinflammation-induced microvascular dysfunction and pathological tissue remodeling during AMCKD.

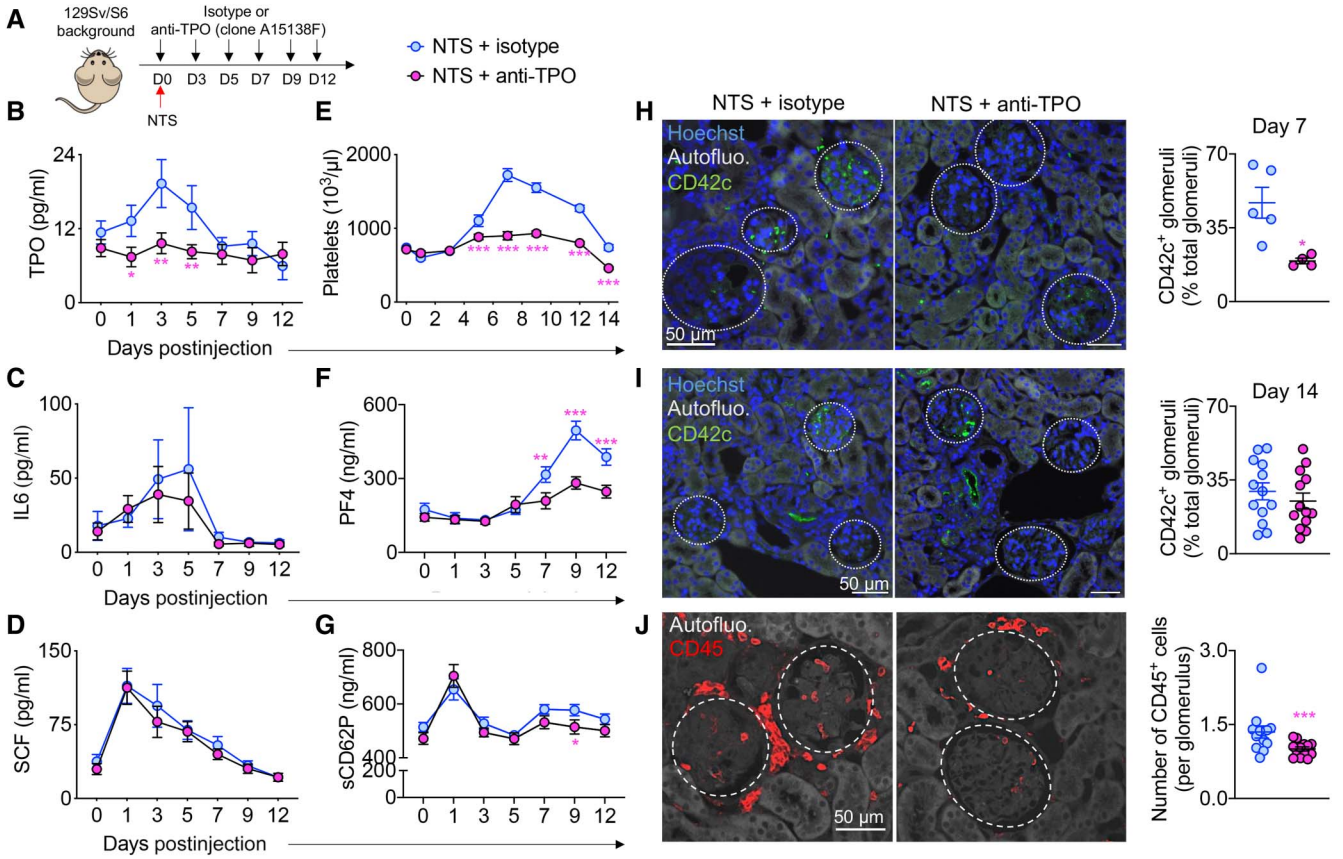

Figure 6. Neutralization of TPO in experimental AMCKD prevents chronic thromboinflammation. (A) Experimental workflow: neutralization of TPO started on day 0 before the injection of NTS (250 μg/mouse, clone A15138F, i.p.) and was repeated on days 3, 5, 7, 9, and 12. Control mice were injected with isotype at the same time points (250 μg/mouse, i.p.). (B–G) Kinetic analysis of the plasma concentrations of TPO (B), IL6 (C), SCF (D), of the levels of circulating platelets (E), and of the plasma concentration of PF4 (F), and sCD62P (G) in mice developing AMCKD, with or without neutralization of TPO. (H–J) Quantification and representative images of platelet accumulation, on day 7 (H) and day 14 (I) and CD45$^+$ cell accumulation, on day 14, in glomeruli (J). Immunofluorescent analysis of kidney sections from NTS-treated mice with anti-TPO or isotype treatment. The data were pooled from two independent experiments (n=13 mice/group). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$: NTS+anti-TPO versus NTS+isotype; Mann–Whitney test. AMCKD, antibody-mediated chronic kidney disease; NTS, nephrotoxic serum; sCD62P, soluble CD62P; SCF, stem cell factor; TPO, thrombopoietin. Figure 6 can be viewed in color online at www.jasn.org.

Accumulation of Platelets, Increased TGFβR Signaling in Glomeruli, and Increased HGF Levels Are Hallmarks of Human ECGN

To determine whether our experimental findings were relevant to the human disease, we studied tissue biopsies from patients with ECGN of different etiologies (Supplemental Table 1) characterized by sustained inflammation, fibrosis, and capillary rarefaction (Supplemental Figure 15, A–K). Platelet accumulation and TGFβR signaling significantly increased in glomeruli of patients with ECGN, compared with control glomeruli (Figure 8, A and B). The analysis of serum samples from a cohort of patients with granulomatosis, polyangiitis, and kidney involvement (granulomatosis with polyangiitis [GPA]-K$^+$, Supplemental Table 2)[29] showed that the active disease was characterized by increased serum concentrations of TPO, SCF, IL6, and IL1α and hypoalbuminemia. Follow-up analysis of a second serum sample obtained after treatment (remission) revealed that all parameters except SCF had normalized (Figure 8C).

To understand if the abovementioned parameters have a diagnostic and prognostic potential, we performed an unsupervised principal component analysis on data from healthy controls (n=16) and patients with GPA-K$^+$ (n=37), followed by hierarchical clustering analysis, thereby identifying 3 clusters (Supplemental Figure 16, A and B). The concentrations of IL1α and TPO were the two variables contributing the most to PCA1 (Supplemental Figure 16A). Cluster 1 (n=25) was a mixture of healthy controls (n=16) and patients with GPA-K$^+$ (n=9) with low-grade disease (unaltered serum albumin, creatinine, urea, proinflammatory cytokine, and HGF concentrations and leukocyte counts; Supplemental Figure 16, C–E). Clusters 2 (n=26) and 3 (n=2) were exclusively composed of patients with GPA-K$^+$ with active disease (mild-to-severe hypoalbuminemia, increased serum creatinine, urea,

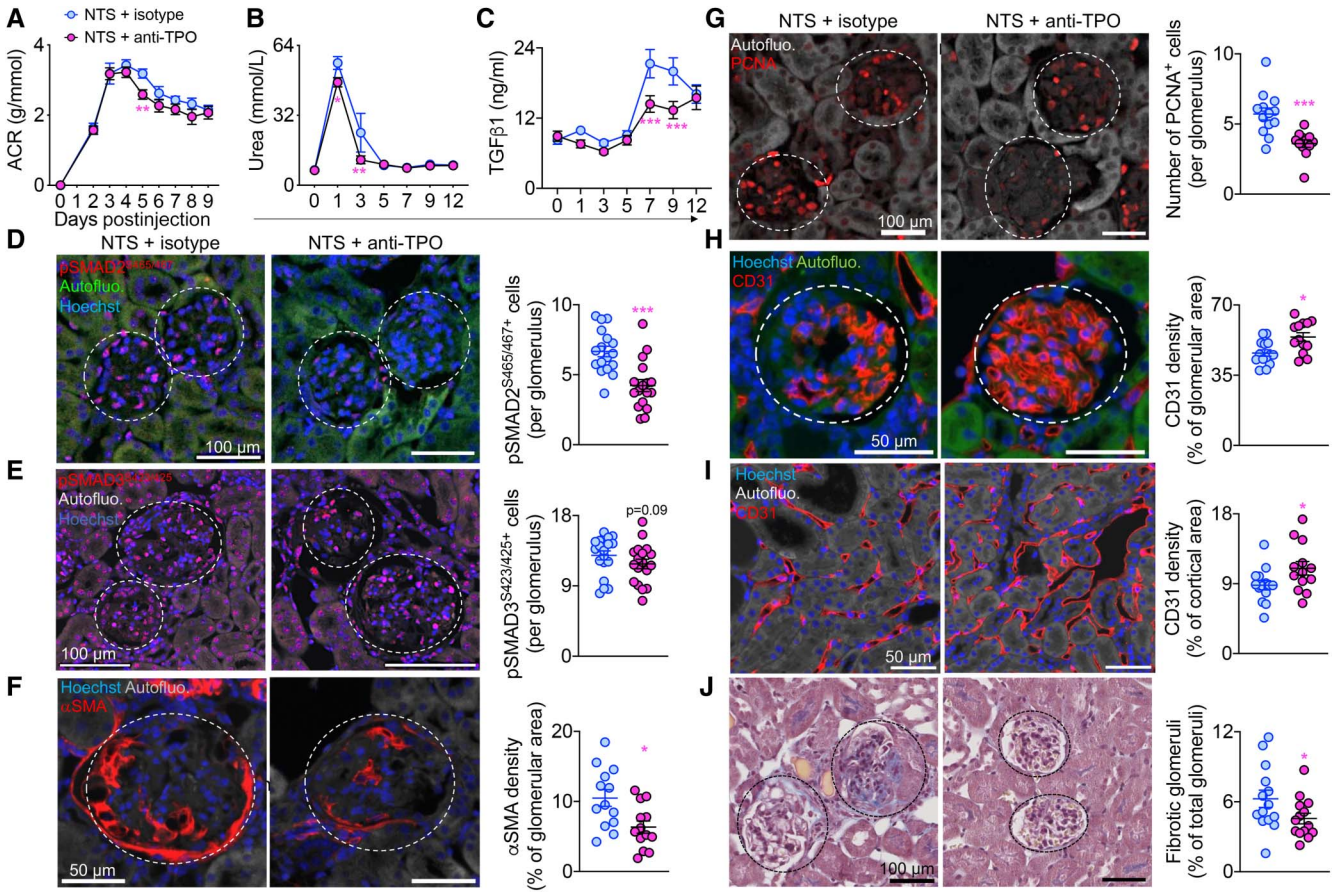

Figure 7. Neutralization of TPO in experimental AMCKD prevents vascular and kidney dysfunction, microvascular rarefaction, and fibrosis and reduces TGFβ bioactivity. (A–C) Kinetic analysis of urinary ACR (A), plasma urea (B), and TGFβ1 (C) concentrations in mice treated with anti-TPO or isotype during AMCKD development. The data were pooled from two independent experiments (n=13 mice/group). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$: NTS+anti-TPO versus NTS+isotype; two-way ANOVA, followed by the uncorrected Fisher test. (D and E) Quantification and representative immunofluorescence images of pSMAD2$^{Ser465/467}$ (D) and pSMAD3$^{Ser423/425}$ (E) in glomeruli of kidney sections of NTS-injected mice treated with anti-TPO or isotype. The data were pooled from three independent experiments (n=13 mice/group day 14 and n=5 mice/group from day 7). *$P < 0.05$ and ***$P < 0.001$: NTS+anti-TPO versus NTS+isotype; Mann–Whitney test. (F–J) Quantification and representative immunofluorescence image of αSMA (F), proliferating cell nuclear antigen (G), and CD31 staining in glomeruli (H), as well as of CD31 staining in the cortex (I) and glomerulosclerosis (J, histological Carstair staining) of kidney sections 14 days p.i. of NTS in mice treated with anti-TPO or isotype. The data were pooled from two independent experiments (n=13 mice/group). *$P < 0.05$ and ***$P < 0.001$: NTS+anti-TPO versus NTS+isotype; Mann–Whitney test. αSMA, α smooth muscle actin; ACR, albumin/creatinine ratio; AMCKD, antibody-mediated chronic kidney disease; NTS, nephrotoxic serum; TPO, thrombopoietin. Figure 7 can be viewed in color online at www.jasn.org.

proinflammatory cytokine and HGF concentrations, and high neutrophil counts; Supplemental Figure 16, C–E). Of note, most of these parameters were normalized by treatments in patients from cluster 2, but not in patients with GPA-K$^+$ from cluster 1. Hence, patients with GPA-K$^+$ from cluster 1 may have suffered the side effects of the immunosuppressive treatments without benefiting from them (Supplemental Figure 16, C–E). Finally, patients from cluster 2 had significantly higher serum TGFβ levels than patients with GPA-K$^+$ from cluster 1 (Supplemental Figure 16F).

Therefore, the serum TPO concentration is related to disease activity, can predict the treatment response in association with other parameters, and could be used to stratify patients with GPA-K$^+$ according to disease severity. Moreover, the increased serum TPO and TGFβ concentrations detected in patients from cluster 2 might be linked to an increased platelet production/turnover and glomerulosclerosis.

DISCUSSION

AMCKD is a situation wherein the renal microvascular network is targeted and damaged. As a consequence, glomerulosclerosis develops and renal functions are deteriorated, resulting in end-stage renal disease. Herein, we dissected the cellular and molecular chain of events linking the initial insult to the

Table 1. Sheep IgG, mouse IgG and C4d deposition in glomeruli

	NTS+platelet depletion			NTS+DEX			NTS+Anti-IL6			NTS+Anti-TGFβ			NTS+Anti-TPO		
	Isotype	Platelet Depletion	*P* Value	Control[a]	DEX	*P* Value	Control[a]	Anti-IL6	*P* Value	Isotype	Anti-TGFβ	*P* Value	Isotype	Anti-TPO	*P* Value
Sheep IgG (MFI per glomerulus)	88.55±5.20	82.32±6.92	0.2740	85.01±6.29	91.13±11.05	0.8392	85.01±6.29	75.98±8.99	0.5135	73.98±3.98	77.39±5.16	0.655	101.9±8.91	103.2±8.63	>0.999
Mouse IgG (MFI per glomerulus)	64.38±5.35	73.09±7.70	0.3217	125.7±11.80	108.1±11.24	0.5395	125.7±11.8	72.98±6.32	0.002[b]	64.66±7.32	66.99±6.63	0.717	87.87±4.70	94.72±4.68	0.287
C4d (MFI per glomerulus)	55.67±5.27	54±5	>0.999	85.97±5.26	37.02±5.66	0.002[b]	85.97±5.26	57.82±11.89	0.076	45.58±5.72	42.55±3.69	0.657	48.87±6.80	50.22±5.80	0.959

Data represent mean± SEM. IgG, Immunoglobulin G; NTS, nephrotoxic serum; DEX, dexamethasone treatment; TPO, thrombopoietin; MFI, mean fluorescence intensity.

[a]The control group is composed of Phosphate-buffered saline-treated and isotype-treated mice, pooled from two experiments.

[b]$P < 0.01$, NTS+treatment versus NTS control group, Mann–Whitney test.

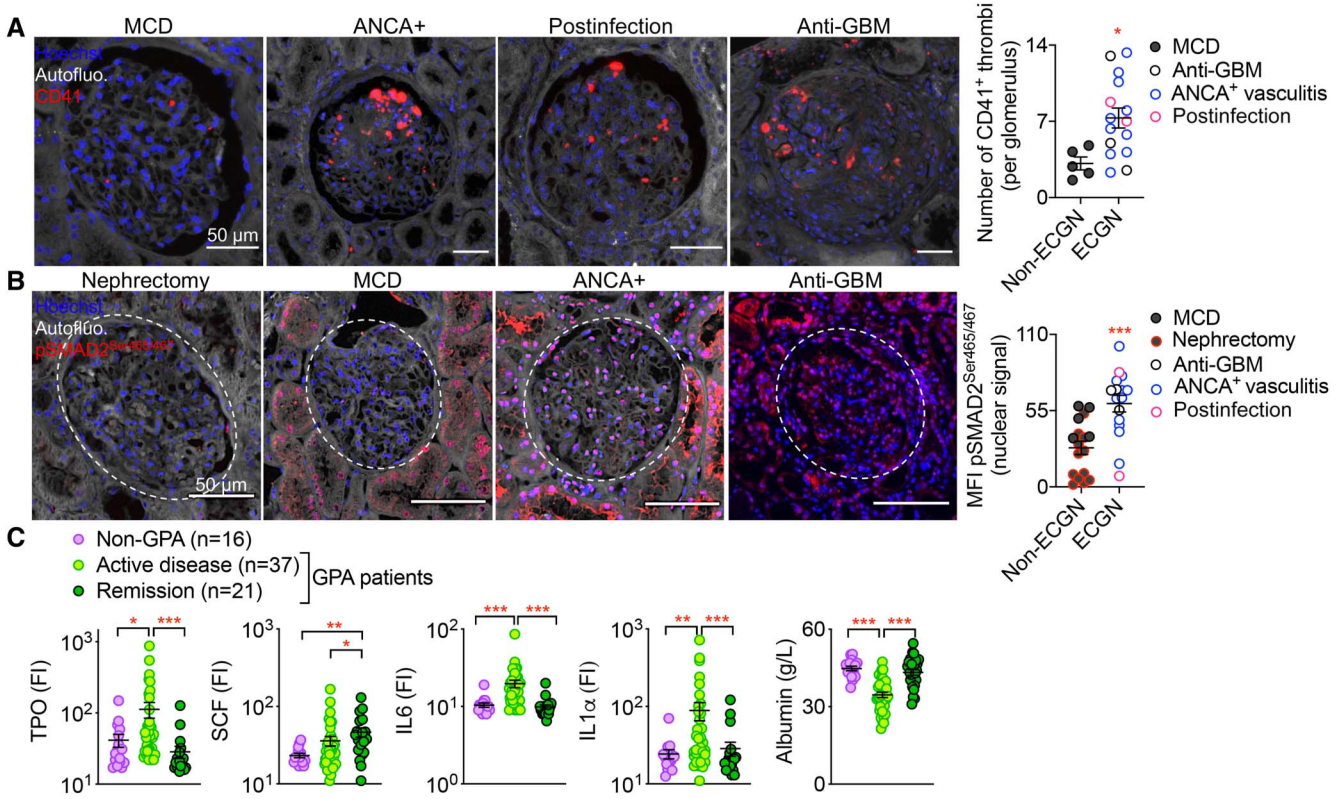

Figure 8. Platelet accumulation and activation of TGFβ receptor signaling in glomeruli, as well as circulating TPO, increase in humans with inflammatory CKD associated with ECGN. (A) Representative images of fluorescence microscopy and quantification of platelet accumulation in glomeruli in biopsies from control patients (proteinuria without ECGN; MCD, n=5) and patients with ECGN (anti-neutrophil cytoplasmic antibodies$^+$ vasculitis, n=10; postinfectious, n=2; anti-GBM, n=3). *$P < 0.05$, non-ECGN versus ECGN; Mann–Whitney test. (B) Representative images of fluorescence microscopy and quantification of the accumulation of pSMAD2$^{Ser465/467}$ in nuclei of cells from glomeruli from control patients (MCD, n=5; nephrectomy, n=12) and patients with ECGN (anti-neutrophil cytoplasmic antibodies$^+$ vasculitis ECGN, n=11; postinfectious ECGN, n=2; anti-GBM antibody ECGN, n=3). ***$P < 0.001$, non-ECGN versus ECGN; Mann–Whitney test. (C) Titration of the serum concentrations of TPO, SCF, IL6, IL1α, and albumin in non-granulomatosis with polyangiitis (GPA) patients (n=16), patients with active GPA disease and kidney involvement (n=37), and GPA patients 1 year later (considered in remission) (n=21). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$; Kruskal–Wallis test, followed by the uncorrected Dunn test. ECGN, extracapillary glomerulonephritis; GBM, glomerular basement membrane; MCD, minimal change disease; SCF, stem cell factor; TPO, thrombopoietin. Figure 8 can be viewed in color online at www.jasn.org.

development of a chronic injury and the resulting pathological tissue remodeling process.

First, we report that experimental AMCKD and human ECGN are associated with chronic thromboinflammation. We observed that the development of a TPO-induced thrombocytosis contributed to the pathophysiology of AMCKD. We show that in this situation, platelets are a major source of profibrotic factors since their depletion, or the prevention of thrombocytosis by dexamethasone or TPO neutralization reduced TGFβ bioavailability and chronic thromboinflammation.

To trace back, in the BM, the origin of the pathological inflammatory thrombocytosis, we studied how hematopoietic progenitors behaved during AMCKD. We found that they were skewed toward myelo-megakaryopoiesis resulting in an enhanced platelet and myeloid cell production and the development of an inflammatory thrombocytosis. As TPO stimulates MK production[10,30] and is produced by the kidney,[14] we reasoned that the injured kidney during AMCKD might be a remote source of TPO. Strikingly, tubular cells from the injured kidneys produced large amounts of TPO, which correlated with its increase in the plasma, under inflammatory conditions. When we depleted platelets, TPO level was further increased and was associated with leukocytosis and accumulation of MKs in the BM, underscoring the importance of the TPO-metabolizing activity of platelets. The pathological involvement of platelets in glomerular diseases has long been recognized.[31,32] Patients with ECGN with high platelet turnover and/or thrombocytosis were also described.[33,34] Importantly, we found as other did[35] that the serum level of TPO increased in patients with active GPA-K$^+$ and contributed to predict response to treatment. TPO is hence a valuable biomarker in this context.

We next tried to identify the cues triggering the expression of TPO during AMCKD. We found that it can be blunted by

the injection of corticoids, such as dexamethasone. However, several inflammatory pathways may be involved, and our experiments performed with anti-inflammatory drugs could not identify the inducer. IL6 neutralization modestly reduced TPO and thrombocytosis during AMCKD, despite the regulatory role described for IL6 in the induction of TPO expression by the liver.[12] Although the IL1R signaling pathway was described to induce emergency platelet production,[28] IL1R1 neutralization failed to blunt thrombocytosis in our model. Neutralization of TGFβ activity did not affect thrombocytosis, suggesting that TGFβ is not involved in the regulation of TPO expression in AMCKD. Further studies will be required to identify the molecular and cellular pathways involved in the renal induction of TPO under inflammatory conditions. Interestingly, strategies to control platelet numbers (platelet depletion, DEX, or TPO neutralization) in AMCKD always resulted in a reduction of TGFβ bioavailability and fibrosis.

So far, two molecules seemed to be involved in the pathophysiology of AMCKD: TGFβ and TPO. Although TGFβ neutralization reduced organ dysfunction and fibrosis during AMCKD, we believe that the side effect associated with such a treatment (unexplained weight loss and systemic inflammation) disqualifies this strategy in a translational perspective. Instead, TPO neutralization is a promising approach. Indeed, *in vivo* TPO neutralization safely blunted the enhanced myelomegakaryopoiesis and chronic thromboinflammation. TPO neutralization also reduced microvascular dysfunction and pathological tissue remodeling. Although it could be surprising that prolonged TPO neutralization did not result in bleeding, it is known that spontaneous bleeding can be prevented by an extremely low number of platelets (for instance in cMPL and TPO knock out mice[10]).

We next questioned whether the released platelets in response to the TPO were displaying normal functions or were instead also skewed toward certain biological functions. Indeed, HGF-mediated hematopoiesis might favor the release of profibrotic and/or proinflammatory platelets, a point of major importance considering the continuous interactions between platelets and vascular cells in glomeruli through the touch and go mechanism (30 platelets/min),[36] which intensifies after NTS injection and contributes to inflammation and microvascular dysfunction.[36–38] We found that during AMCKD, platelets harbored an increased profibrotic potential because they carry TGFβ that they could release in the damaged glomeruli.

The small size of the human cohort that we have studied and the heterogeneity of the disease in patients constitute limitations of our findings and hence further studies will be required to ascertain that TPO and inflammatory thrombocytosis play a pathological role in ECGN and GPA, a true challenge given that these life-threatening conditions are rare. Another limitation of our study concerns the partial decryption of the signaling pathway of the TGFβ. We identified platelet-derived TGFβ as a potent molecular effector, downstream of inflammatory thrombocytosis and chronic thromboinflammation in AMCKD. TPO-induced thrombocytosis activated the TGFβR signaling pathway in glomeruli, during AMCKD, as shown by the phosphorylation and nuclear accumulation of SMAD proteins. This is an important information since SMAD3 was reported to significantly contribute to endothelial injury, inflammation, and fibrosis in AMCKD.[39,40] However, given that the canonical (SMAD2 and SMAD3) and noncanonical (extracellular signal-regulated kinase and mitogen-activated protein kinase) TGFβR signaling pathways contribute to renal fibrosis,[41] additional studies, using glomerular cell-specific deletion of molecular effectors involved in each pathway, will be required to identify which one contributes to platelet-induced glomerulosclerosis in AMCKD.

Overall, we showed that TPO-stimulated myelomegakaryopoiesis and the ensuing thrombocytosis contribute to the development of AMCKD by enhancing chronic thromboinflammation and TGFβ-induced pathological tissue remodeling. We show that TPO neutralization is a promising therapeutic strategy. Indeed, unlike classical immunosuppressive therapies (anti-TNF, anti-IL1β, and anti-CD20 antibodies; corticoids, cyclophosphamide, and Janus Kinase inhibitors), which are associated with many side effects (infections, cancer, anemia, and cardiovascular diseases), TPO neutralization attenuated hematopoietic activity during AMCKD and reduced inflammation without side effects. Our study hence paves the way for the development of drugs targeting TPO or other HGFs to prevent disturbed hematopoiesis in chronic thromboinflammatory diseases.

DISCLOSURES

A. Nicoletti reports Ownership Interest: Tridek-On Therapeutics; Research Funding: Tridek-On Therapeutics; and Patents or Royalties: Kapto-Medicals, Tridek-On Therapeutics. G. Caligiuri reports Patents or Royalties: Kapto Medical, Tridek One Therapeutics; and Advisory or Leadership Role: Kapto Medical, Tridek One Therapeutics. All remaining authors have nothing to disclose.

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DATA SHARING STATEMENT

All data used in this study are available in this article.

SUPPLEMENTAL MATERIAL

This article contains the following supplemental material online at http://links.lww.com/JSN/E391 and http://links.lww.com/JSN/E473.

Supplemental Methods

Supplemental Table 1. Patient's characteristics from the NeutroVasc cohort.

Supplemental Table 2. Patient's characteristics for the biopsy cohort.

Supplemental Table 3. Antibodies used for immunofluorescence microscopy.

Supplemental Figure 1. Histological analysis of kidney sections after platelet depletion in experimental AMCKD.

Supplemental Figure 2. Platelet depletion affects blood cell counts during experimental AMCKD.

Supplemental Figure 3. NTS dose-dependently induced platelet consumption, accumulation in glomeruli, and activation and thrombocytosis during AMCKD.

Supplemental Figure 4. Kinetic analysis of TGFbR signaling and fibrosis in AMCKD.

Supplemental Figure 5. Kinetic analysis of myeloid and lymphoid cells and RBC progenitors in the BM during experimental AMCKD.

Supplemental Figure 6. Increased platelet count in experimental AMCKD is still observed in splenectomized mice.

Supplemental Figure 7. IL1R signaling does not contribute to thrombopoiesis in AMCKD, despite its significant contribution to myelopoiesis.

Supplemental Figure 8. DEX treatment and IL6 neutralization affect platelet production and inflammation during AMCKD.

Supplemental Figure 9. DEX treatment, but not IL6 neutralization, reduced TGFb bioavailability and fibrosis in AMCKD, despite side effects.

Supplemental Figure 10. Neutralization of TPO in experimental AMCKD reduces chronic glomerular thrombosis.

Supplemental Figure 11. Neutralization of TPO in experimental AMCKD reduces circulating blood monocytes and renal inflammation.

Supplemental Figure 12. Neutralization of TPO in experimental AMCKD prevents the activation of myelo-megakaryopoiesis in the BM.

Supplemental Figure 13. Neutralization of TPO prevents splenic extramedullary hematopoiesis associated with AMCKD without affecting adaptive immunity.

Supplemental Figure 14. Neutralization of TPO in experimental AMCKD does not affect fixation of sheep and mouse Immunoglobulin G, complement activation in glomeruli, weight, plasma creatinine, or cholesterol.

Supplemental Figure 15. Characterization of inflammation, fibrosis, and capillary density in glomeruli and in interstitium in during ECGN in human.

Supplemental Figure 16. Circulating level of TPO, in association with the levels of IL6, IL1a, SCF, and albumin, identifies patients with GPA and kidney involvement responding to treatment.

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