Kidney injury enhances renal G-CSF expression and modulates granulopoiesis and human neutrophil CD177 in vivo

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Summary

Kidney injury significantly increases overall mortality. Neutrophilic granulocytes (neutrophils) are the most abundant human blood leukocytes. They are characterized by a high turnover rate, chiefly controlled by granulocyte colony stimulating factor (G-CSF). The role of kidney injury and uremia in regulation of granulopoiesis has not been reported. Kidney transplantation, which inherently causes ischemia–reperfusion injury of the graft, elevated human neutrophil expression of the surface glycoprotein CD177. CD177 is among the most G-CSF-responsive neutrophil genes and reversibly increased on neutrophils of healthy donors who received recombinant G-CSF. In kidney graft recipients, a transient rise in neutrophil CD177 correlated with renal tubular epithelial G-CSF expression. In contrast, CD177 was unaltered in patients with chronic renal impairment and independent of renal replacement therapy. Under controlled conditions of experimental ischemia–reperfusion and unilateral ureteral obstruction injuries in mice, renal G-CSF mRNA and protein expression significantly increased and systemic neutrophilia developed. Human renal tubular epithelial cell G-CSF expression was promoted by hypoxia and proinflammatory cytokine interleukin 17A in vitro. Clinically, recipients of ABO blood group-incompatible kidney grafts developed a larger rise in neutrophil CD177. Their grafts are characterized by complement C4d deposition on the renal endothelium, even in the absence of rejection. Indeed, complement activation, but not hypoxia, induced primary human endothelial cell G-CSF expression. Our data demonstrate that kidney injury induces renal G-CSF expression and modulates granulopoiesis. They delineate differential G-CSF regulation in renal epithelium and endothelium. Altered granulopoiesis may contribute to the systemic impact of kidney injury.

Keywords: CD177, emergency granulopoiesis, ischemia–reperfusion injury, neutrophilic granulocyte

Introduction

Acute kidney injury increases long-term mortality, despite the fact that kidney functions in electrolyte and volume balance can be replaced by dialysis [1–4]. This correlates with the severity of injury and comorbid conditions, but is detectable also in patients with restored excretory kidney function. The underlying mechanisms are very incompletely understood. Recent studies have delineated long-term alterations in renal gene expression after experimental murine and clinical human kidney allograft injury [5,6], including numerous soluble mediators of inflammation.

Neutrophilic granulocytes (neutrophils) are the most abundant human blood leukocytes [7,8]. Despite their high turnover rate, individual baseline counts are remarkably stable. Neutrophils are positively controlled chiefly by granulocyte colony-stimulating factor (G-CSF) [8–10]. It maintains their baseline levels and enhances maturation and release in inflammation, a process termed ‘emergency granulopoiesis’ [9]. G-CSF can be produced by a number of cell types, including bone marrow, monocytes, and epithelial cells.
of cell types, including bone marrow stroma and various epithelia. However, there are comparatively few data on the relative contributions of different cell types to G-CSF production in pathophysiology [11]. In kidney tissue, both injury [12] and tumors [13] can induce G-CSF expression. Indeed, neutrophilia has been reported as a very rare paraneoplastic event in renal cancer [13]. A role for renal G-CSF in systemic granulopoiesis in humans with non-malignant renal conditions has not been demonstrated.

CD177 is among the most G-CSF-regulated neutrophil genes (alternate names: NB1, PRV-1) [14,15]. In human neutrophils, this GPI-linked glycoprotein has a unique expression pattern that distinguishes two populations and is stable in individuals [14,16–18]. Up-regulation of CD177 neutrophil surface levels and the proportion of positive neutrophils (CD177%) was found after G-CSF injection. However, longitudinal data that determine whether neutrophil CD177% reverts to the individual baseline are lacking.

Cross-sectional studies report elevated neutrophil CD177% in cord blood, during pregnancy and also in sepsis and vasculitides, mainly in anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitis [14,19,20] and in Kawasaki disease [21]. In both sepsis and vasculitides, a high neutrophil CD177% is consistently associated with worse overall and renal outcomes. It is not known whether, in reverse, uremia or kidney injury impact upon this neutrophil phenotype.

In this study, we tested whether kidney injury affects renal G-CSF expression, systemic granulopoiesis and the human neutrophil CD177 phenotype in a longitudinal study in kidney transplant recipients, in uremic patients, mouse models of kidney injury and human cell culture.

Materials and methods

Patients and probands

The study was conducted in adult patients after informed consent according to the Declaration of Helsinki and local ethics board approval. Blood from stable hemodialysis out-patients without active infection or previous bone marrow transplantation was analyzed after a long interval. Healthy human granulocyte donor leukocytes were analyzed before surgery and on return to the out-patient department 3 and 12 months after transplantation. Patients receiving a first dose of rituximab for non-malignant nephrological conditions were recruited at MHH.

Mouse models of renal injury

Renal ischemia–reperfusion injury (IR) and unilateral ureteral obstruction (UUO) surgeries were performed according to the Directive 2010/63/EU of the European Parliament and approved by the state authority in male wild-type (C57BL/6J) mice kept in specific pathogen-free conditions. IR was induced by 27 min clamping; for UUO, proximal ureteral ligation was performed [22].

Leukocyte staining and flow cytometry

Murine full blood counts were obtained using an automated analyzer (VetABC; ScilVet, Viernheim, Germany). Mouse bone marrow cells were flushed from femurs. The following directly labeled antibodies were used: anti-CD11b (M1/70), anti-CD14 (HCD14), anti-CD16 (3G8), anti-CD115 (AFS98), anti-CD49d (9F10), anti-CD66b (G10F5), anti-CXCR4 (12G5), anti-Gr1 (RB6-8C5) and anti-CD177 (MEM166) (Biolegend, San Diego, CA, USA). Flow cytometry data obtained on a Becton-Dickinson fluorescence activated cell sorter (FACS)Canto were analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

Renal histology

Three months’ surveillance biopsies were scored using the current Banff criteria [23] in 3-μm formaldehyde-fixed paraffin-embedded serial sections stained with hematoxylin and eosin, periodic acid–Schiff, Jones’ methenamine silver and anti-C4d and anti-SV40 stainings by two nephropathologists unaware of all other parameters. For G-CSF staining, 3-μm sections were dewaxed and rehydrated. After endogenous peroxidase activity (3% H2O2) for 30 min at 98°C, murine kidneys were stained with polyclonal rabbit anti-G-CSF antibody (60 min, dilution 1 : 500; Sigma Aldrich, Darmstadt, Germany) and antigen retrieval in 0·01 M sodium citrate buffer (pH 6.0) for 30 min at 98°C, murine kidneys were stained with polyclonal rabbit anti-G-CSF antibody (60 min, dilution 1 : 500; Biostar, Woburn, MA, USA). Human samples were stained with polyclonal rabbit anti-G-CSF antibody (60 min, dilution 1 : 50; Sigma Aldrich, Darmstadt, Germany). Secondary antibody horseradish peroxidase (HRP)-conjugated polyclonal donkey anti-rabbit immunoglobulin (30 min, 1 : 100; Jackson ImmunoResearch Laboratories, Inc., West Baltimore Pike, PA, USA) was applied prior to 3,3′-diaminobenzidine detection (Zytomed Systems, Berlin, Germany). For negative controls, primary antibodies were omitted. Sections were evaluated using an Olympus U-DO3 microscope after mild hemalum counterstain. Representative photomicrographs were taken using a Zeiss Axioskop microscope with an MRT digital camera and Axiovision Software (Zeiss, Oberkochen, Germany). Renal tubuli were counted and assessed for G-CSF positivity in cortex and medulla separately. Values are expressed as percentage of positive tubuli.
Human renal tubular and endothelial cell culture

HK-2 human tubular cells (American Type Culture Collection, Manassas, VA, USA) and human umbilical vein endothelial cells (HUVEC), isolated after informed consent and ethics approval, were cultured under normal and hypoxic conditions as described previously [24]. Recombinant human interleukin (IL)-17A (Peprotech, London, UK) was applied at the indicated concentrations; no effects were seen for heat-degraded IL-17A used as control (data not shown). To test complement activation, HUVEC were exposed to normal human serum with and without anti-CD59 (BRIC229; International Blood Group Reference Laboratory, NHS Blood and Transplant, Bristol, UK) to block complement inactivation or immunoglobulin (Ig)G isotype control, as described previously [25].

RNA isolation and real time-polymerase chain reaction (RT–PCR)

RNA isolation, reverse transcription and quantitative PCR (qPCR) were performed as described previously [24]. Primers were as follows; mouse: Csf3: forward: CAGGCTCTATCGGGTATTT, reverse: GGAAGGCA-GAAGTGAAGG; Hprt: forward: CAGTCCCAGCTGTCGATT, reverse: AGCAAGTCCTTCAGTCCTGTC; human: Csf3: forward: GCTGCTTGAGCCAACTCCATA, reverse: GAACGCGGTACGACACCTC; and Hprt: forward: TGACACTGGCAAAACATGCA, reverse: GGTCCA-TTTCACGGCAAGCT. Products were confirmed by melting curve analysis and gel electrophoresis. Data were analyzed with hypoxanthine–guanine phosphoribosyltransferase (HPRT) as a reference gene using LinRegPCR software.

Renal ischemia–reperfusion gene expression data set

In agreement with the Creative Commons Attribution 4.0 International License, expression data were extracted from the supplemental gene expression data set of [6].

Statistical analysis

Two-tailed Student’s t-test or Mann–Whitney test for non-normally distributed samples was used to compare two conditions. If more than two conditions were compared, Bonferroni’s or Dunnett’s test was applied after analysis of variance (ANOVA), as indicated. P-values < 0.05 were considered significant. In the figures, data are expressed as mean ± standard error of the mean (s.e.m.). P-values are indicated as follows: *P < 0.05, **P < 0.01, ***P < 0.001.

Results

G-CSF transiently increases human neutrophil CD177 expression

Cd177 is a highly G-CSF regulated gene [14,15,26]. The presence of the CD177 protein distinguishes two human neutrophil subsets. Surface CD177+ cells also contain intracellular protein, while surface negative cells do not contain the protein in their intracellular granules, as illustrated by flow cytometry and confocal microscopy of cells from four individuals (Fig. 1a,b).

Other markers, including the beta-2 integrin subunit CD11b, which is considered a neutrophil activation marker [27], the Fc receptor CD16, which is higher in mature than immature neutrophils but again shed in activation or cell death [27,28], CD66b, which is a

Fig. 1. Granulocyte colony-stimulating factor (G-CSF) increases human neutrophil CD177 expression. (a,b) In human blood neutrophils, CD177 is expressed in intracellular granules and on the surface of a subset in most individuals. Four healthy donors with different percentages of CD177+ cells are shown as examples: [a: confocal microscopy of density gradient enriched neutrophils; green: CD177 and blue, 4′,6-diamidino-2-phenylindole (DAPI), 40× and 63× original magnification, bars indicate 100 μm; (b) flow cytometric CD177 assessment in blood of the same donors gated for CD11b+CD16high large granular cells]. (c,d) CD177+ neutrophils before (pre), 12 h and 40 ± 5 days after G-CSF administration to healthy volunteers were assessed as depicted in (b). The proportion (c) and mean fluorescence intensity (MFI, d) of CD177+ polymorphonuclear neutrophils (PMN) are shown [statistical analysis of n = 19, Bonferroni after analysis of variance (ANOVA)].
GPI-linked protein as CD177 [29], bone marrow homing receptor CXCR4 (CD184) [30] and CD49d, which is a reported marker of human myeloid derived suppressor cells [31], stained very similarly on the subsets (Supporting information, Fig. S1).

Cd177 mRNA, as many neutrophil genes, is functionally expressed in the progenitors in the bone marrow rather than in peripheral blood cells [14,15,26]. We therefore tested whether CD177 protein expression on blood neutrophils can serve as dynamic indicators of G-CSF action in humans. Indeed, a single dose of G-CSF significantly increased the percentage of CD177 neutrophils (CD177%) and the CD177 expression level on positive neutrophils in healthy human granulocyte donors (Fig. 1c,d), as described previously [14,32,33]. We further performed individual follow-up. This determined that CD177% reverted to baseline within 40 days after a single dose of G-CSF.

These data demonstrate that CD177 reversibly increased in response to G-CSF propose CD177% as a dynamic indicator of G-CSF action in vivo.

Kidney transplantation, but not uremia, enhances human neutrophil CD177 expression

To investigate the impact of kidney function and kidney damage, CD177 expression was studied in chronic renal impairment before and after kidney transplantation.

In a cohort of 51 patients receiving maintenance hemodialysis, CD177% and expression level did not differ from healthy blood donors (Supporting information, Fig. S2; clinical characteristics in Supporting information, Table S1). CD177 did not associate with age, gender, dialysis vintage, total leukocyte count or the use of erythropoietic agents in this cohort. Also, in a cohort of 48 patients scheduled for living donor kidney transplantation (clinical characteristics in Supporting information, Table S2), CD177% and expression level were very similar in patients already receiving dialysis for renal replacement or not (Supporting information, Fig. S3). The proportion of completely CD177-deficient individuals did not differ from healthy controls for either of the cohorts. CD177% did not correlate with any of the studied laboratory or clinical values that are reported in Supporting information, Tables S1 and S2. It was longitudinally stable in subgroup of 11 patients evaluated twice before transplantation (Supporting information, Fig. S4), as described in healthy donors [14]. Taken together, these results exclude a significant impact of uremia and type of renal replacement therapy on the neutrophil CD177 phenotype.

However, after kidney transplantation, CD177% and its surface expression level significantly increased (Fig. 2a). At this time-point, 3 months after transplantation, plasma G-CSF levels were elevated compared to reported normal values (n = 23, mean = 152 pg/ml, range = 6–724, literature normal range = 34–54 pg/ml) [34,35]. In the presence of intense immunosuppression, total white blood counts and absolute neutrophils were in the lower range [6 ± 0.4/ml (normal = 3.9–10.2) and 4.2 ± 0.3/ml (normal = 1.5–7.7)]. The increase in CD177% was unrelated

![Fig. 2. Kidney transplantation transiently increases human neutrophil CD177%. (a–c) The proportion of CD177+ blood neutrophils (PMN) was measured in a cohort of 48 living donor kidney recipients before (pre) and 3 months after kidney transplantation (a, paired t-tests). In all patients with a CD177+ neutrophil subpopulation, the change in CD177% compared to the baseline sample was assessed in additional samples before transplantation if available (pre) and 3 months and 1 year after transplantation (b, Bonferroni after analysis of variance (ANOVA)). The mean fluorescence intensity (MFI) on positive cells is shown before, 3 months and 1 year after transplantation (c, Bonferroni after ANOVA).](image-url)
to age, gender, excretory renal function or immuno-suppressive regimen (data not shown). Expressed as a change from baseline, the increase in CD177% reverted within 1 year (Fig. 2b). A similar trend was observed for its surface expression level (Fig. 2c).

These results introduce kidney transplantation as a novel inducer of a reversible up-regulation of human neutrophil CD177% in vivo.

Experimental kidney injury enhances renal G-CSF production and introduces neutrophilia

To determine renal G-CSF production as a major inducer of CD177 [14,15] under defined conditions in vivo, murine kidney injury models of ischemia–reperfusion injury and unilateral ureteral obstruction were employed. G-CSF was below detection level on both protein and mRNA level at rest (Fig. 3). However, both G-CSF protein and mRNA markedly increased in both types of injury (Fig. 3a–c), while G-CSF mRNA was below detection level in nearly all heart, lung, liver, spleen and bone marrow samples after injury (Supporting information, Fig. S5). Long-term follow-up data after ischemia–reperfusion injury demonstrated elevated renal G-CSF expression for at least 1 month after the event (Fig. 3d) [6].

Assessment of blood neutrophil counts revealed a significant increase in absolute leukocyte counts with relative and absolute neutrophil counts (Fig. 4a–d). This phenotype is consistent with emergency granulopoiesis [9].

These results show that kidney injury increases renal tubular G-CSF production with systemic effects on granulocytes in vivo.

Human kidney graft tubular G-CSF expression correlates with the increase in neutrophil CD177 expression

G-CSF was stained in human kidney grafts in surveillance biopsy samples obtained at the time of blood neutrophil assessment 3 months after transplantation. G-CSF protein was detected in renal tubuli in both cortex and medulla of the samples (Fig. 5a,b). The proportion of G–CSF expressing tubuli in both compartments closely correlated with the CD177% rise at this time-point (Fig. 5c,d).

This correlation is consistent with a mechanistic function of renal G-CSF for the neutrophil CD177 phenotype.

Hypoxia and IL-17A up-regulate G-CSF in renal tubular cells

To determine the underlying mechanisms of renal tubular G-CSF production we first performed hypoxia experiments, as ischemia was present in experimental and clinical kidney injury. We found that transient hypoxia indeed significantly increased human tubular cell G-CSF expression, while it had no detectable effect on endothelial cells (Fig 6a,b). IL-17A is a known strong inducer of G-CSF and has been detected in damaged human allografts [36]. It was also up-regulated from undetectable levels after isolated ischemia–reperfusion injury in the controlled murine system (Fig. 6c). In human tubular cells, it promoted G-CSF expression dose- and time-dependently (Fig. 6d,e). IL-17A also promoted human endothelial cell G-CSF expression, albeit only at a very high concentration (Fig. 6f).

These data propose tubular damage mechanisms for G-CSF production.

Increased rise of neutrophil CD177% in recipients of blood group incompatible grafts

We next investigated clinical factors that may further modulate the CD177% rise after human kidney transplantation.

CD177% did not correlate with human leucocyte antigen (HLA) mismatch number or donor-recipient relationship. There were no significant differences in the changes in CD177% or of tubular G-CSF in grafts with and without rejection (Supporting information, Table S3 and data not shown). The rise in CD177% was significantly higher in the ABO blood group-incompatible graft recipients (Fig. 7a). The therapeutic regimen of ABO blood group-incompatible graft recipients differed in B cell depletion by rituximab and plasma exchange and/or immunoadsorption before transplantation. We analyzed the effect of B cell depletion in patients with other kidney conditions, where it did not induce a significant change (Supporting information, Fig. S6). Also, in our cohort there was no differential effect of plasma-exchange or immunoadsorption (Supporting information, Fig. S7a). We also tested the effect of rejection therapies, including high-dose intravenous steroids and anti-thymocyte globulin as well as post-transplantation plasma exchange (Supporting information, Fig. S7b). None of these were significantly associated with the change in neutrophil CD177.

In summary, blood group-incompatible kidney grafts were associated with an increased neutrophil CD177%.

Complement activation increases endothelial G-CSF production

In ABO-incompatible grafts, endothelial complement C4d deposition is regularly detected [37]. This phenotype was also present in our patient cohort (Fig. 7b,c). We therefore tested endothelial G-CSF production in response to complement (Fig. 7d). Indeed, blockade of complement decoy receptor CD59 significantly enhanced endothelial G-CSF mRNA expression in the presence of normal human serum.
In mice, expression of the protective CD59b isoform [38] decreased after ischemia–reperfusion injury (Supporting information, Fig. S8). As complement is activated and CD59 over-expression protects from damage in this murine model [39], endothelial activation by complement may also promote G-CSF in this setting.

These data propose complement-mediated endothelial injury as an additional source of renal G-CSF.
Discussion

This study demonstrates that kidney injury enhances renal G-CSF expression and regulates granulopoiesis. Our experiments delineate differential G-CSF regulation in renal tubuli and endothelium and show its impact on the human neutrophil CD177 phenotype in vivo.

CD177 that was discovered in alloimmune neutropenia [40], and again in myeloproliferative disease [41], is peculiar for its bimodal expression pattern on human neutrophils [42], and was recently found to follow a previously unknown unique monoallelic expression pattern [43]. For the present project, we made use of the fact that CD177

![Fig. 4. Increased neutrophil counts after experimental kidney injury.](image-url)

![Fig. 5. Tubular granulocyte colony-stimulating factor (G-CSF) protein expression correlates with the increase in neutrophil CD177% 3 months after kidney transplantation.](image-url)
is among the most G-CSF regulated genes in human neutrophils [15]. Our longitudinal data in healthy subjects treated with exogenous G-CSF add to this knowledge by demonstrating that the individual CD177% setpoint is maintained after excursion.

We subsequently used the CD177 protein as a sensor of G-CSF action, as absolute neutrophil counts in peripheral blood may not reflect the full G-CSF impact on the bone marrow in complex conditions that include the use of anti-proliferative substances, e.g. autoimmune...
Neutrophil regulation by the kidney

...interaction with proteinase 3 and integrins do not apply to its murine homologue have impaired functional studies of this specific subtype [56]. Very recent data on a differential role in migration depending on the underlying stimulus for murine Ly6G [57], a protein of similar structure as CD177, should be considered when planning prospective human studies with parallel assessment of systemic and local CD177 phenotypes in inflammation. Renal transplant recipients may be considered for such a study, as the kidney graft is frequently biopsied and different forms of inflammation are observed. Both tubulointerstitial inflammation in infection and glomerulitis in antibody-mediated rejection are sufficiently frequent and contain significant numbers of neutrophils, and their CD177 expression could be compared to peripheral blood at the same time-point.

Defective erythropoiesis due to lack of erythropoietin and altered monocyte numbers and composition are well-known systemic hematopoietic effects of uremia [1,58,59]. Also, neutrophil functional changes have been associated with the degree of uremia [60]. Our findings in neutrophils differ from this: uremia or dialysis did not have a detectable effect on neutrophil CD177%. In contrast, renal transplantation, which transfers a kidney with ischemia–reperfusion injury into the body significantly transiently enhanced CD177. Our data from experimental kidney injury show that, indeed, the damaged kidney produces G-CSF and kidney damage increases granulopoiesis. As local long-term up-regulation has been shown for other cytokines after renal injury [6], effects of a ‘toxic kidney’ should also be investigated for other immune cells and in disease conditions.

In summary, our data are consistent with a model of G-CSF as a systemic injury signal emitted by the kidney parenchyma.

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Disclosures

None.

Author contributions

J. V., J. S., J. H. B., R. S., H. H. and S. v. V. designed research; J. V., J. S., J. N., L. D., A. H., P. S. and J. H.
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38 Qian YM, Qin X, Miwa T, Sun X, Halperin JA, Song WC. Characterization of the dialysis patient cohort Table S1. Additional supporting information may be found in the online version of this article at the publisher’s web site: Table S1. Characterization of the dialysis patient cohort (n = 51)
Table S2. Characterization of kidney transplant recipients (n = 48)
Table S3. Pathologic diagnoses of three-month surveillance biopsies (n = 34)
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