Haematopoietic stem cell migration to the ischemic damaged kidney is not altered by manipulating the SDF-1/CXCR4-axis

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Abstract

Background. Haematopoietic stem cells (HSC) have been shown to migrate to the ischemic kidney. The factors that regulate the trafficking of HSC to the ischemic damaged kidney are not fully understood. The stromal cell-derived factor-1 (SDF-1)/CXCR4-axis has been identified as the central signalling axis regulating trafficking of HSC to the bone marrow. Therefore, we hypothesized that SDF-1/CXCR4 interactions are implicated in the migration of HSC to the injured kidney.

Methods. HSC were isolated from mouse bone marrow and labelled with a cell tracker. Aceceptor mice were subjected to unilateral ischemia and received HSC intravenously directly after reperfusion. In addition, in separate groups of acceptor

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mice, endogenous SDF-1 or HSC-associated CXCR4 was blocked or kidneys were injected with SDF-1.

**Results.** Exogenous HSC could be detected in the tubules and interstitium of the kidney 24 h after ischemic injury. Importantly, the amount of HSC in the ischemic kidney was markedly higher compared to the contralateral kidney. Neutralizing endogenous SDF-1 or HSC-associated CXCR4 did not prevent the migration of HSC. No increase in the number of labelled HSC could be observed after local administration of SDF-1, as was also determined in bilateral kidney ischemia.

**Conclusion.** In conclusion, systemically administered HSC preferentially migrate to the ischemic injured kidney. This migration could not be prevented by blocking the SDF-1/CXCR4-axis or increased after local administration of SDF-1.

**Keywords:** HSC migration; ischemia/reperfusion; kidney; SDF-1/CXCR4-axis

**Introduction**

Acute renal failure (ARF) is a major clinical problem that affects up to 5% of hospitalized patients and has a mortality rate of 50–80% in these hospitalized patients [1]. Ischemia/reperfusion (I/R) injury is the major initiator of ARF and is caused by a sudden transient drop in blood flow to the kidney frequently occurring in shock, sepsis and during renal transplantation. I/R injury leads to a cascade of events affecting mainly tubular epithelial cells (TEC) in the corticomedullary region of the kidney, resulting in structural alterations including loss of brush border and cell polarity, and necrosis and apoptosis of damaged TEC [2]. Depending on the severity of injury, renal tubules have the capacity to regenerate and eventually restore the tubule with normal epithelium resulting in functional recovery of the kidney.

Haemato poetic stem cells (HSC) are suggested to be involved in this regenerative process. Several studies have found Y-chromosome-positive TEC in renal female grafts transplanted into male recipients, and in kidneys of female mice transplanted with male bone marrow [3–5]. However, this was a rare event, occurring only in a small percentage of tubules. Although initial reports showed a high number of bone marrow-derived cells (BMC) with an epithelial phenotype after renal I/R injury [6,7], carefully designed experiments by the same and other groups could only observe a few epithelial-like BMC in renal tubules after ischemic injury [8–10]. Since the levels of BMC that engraft the injured tubules and develop into functional TEC are very low, their contribution to renal repair is thought to be minor. Enhancing the migration of HSC to the injured kidney may result in a significant contribution of these HSC to renal repair, and hence has therapeutic potential.

The mechanisms that regulate the trafficking of HSC to the ischemic damaged kidney, however, remain unclear. A better understanding of the factors that regulate the migration of HSC into the injured kidney is essential for the development of strategies to improve HSC kidney engraftment and repair. The chemokine stromal cell-derived factor-1 (SDF-1, also known as CXCL12) and its receptor CXCR4 have been identified as the central signalling axis regulating the trafficking of HSC. In immune-deficient mice, the SDF-1/CXCR4-axis is essential for efficient homing of human stem cells as indicated by the impaired repopulation of the bone marrow after blocking stem cell-associated CXCR4 [11–13]. In addition, local administration of recombinant SDF-1 results in an increased homing of stem cells to the liver, spleen and bone marrow [11,12].

SDF-1 is upregulated after injury in diverse experimental models including skin ischemia [14], toxic liver injury [11], myocardial infarct [15,16] and DNA damage [17]. We (unpublished data) and others [18] have shown that in the kidney, after I/R injury SDF-1 protein levels are upregulated. Tögel et al. also observed a selective homing of exogenous administered BMC to the injured kidney and could inhibit this by blocking bone marrow-associated CXCR4 [18]. Taken together, current literature suggests a role for the SDF-1/CXCR4-axis in HSC migration to injured organs.

In the present study, we thoroughly characterized the involvement of the SDF-1/CXCR4-axis in the migration of HSC to the injured kidney.

**Subjects and methods**

**Mice**

Eight-week-old male C57Bl/6 (B6) mice were purchased from Charles River (Maastricht, The Netherlands) and housed under specific pathogen-free conditions receiving food and water ad libitum. All experimental procedures were approved by the local Animal Care and Use Committee of the University of Amsterdam, The Netherlands.

**Isolation of HSC**

Total bone marrow was collected from male B6 mice by flushing femurs and tibiae. Erythrocytes were lysed in 160 mM NH₄Cl, 10 mM KHCO₃ and 0.1 mM EDTA (pH 7.4, 0.22 μm filtered). Cells were labelled with an APC-conjugated monoclonal antibody to c-Kit (BD Biosciences, Alphen a/d Rijn, The Netherlands) for 20 min, washed with FACS buffer (0.5% BSA, 0.35 mM EDTA in PBS), and the population of cells expressing high levels of c-Kit (c-Kithigh) was collected using a FACSAria cell sorter (Becton Dickinson, Breda, The Netherlands). The sorted c-Kithigh population was labelled with the fluorescent cell tracker CM-DiI (Invitrogen, Breda, The Netherlands) according to the manufacturer’s protocol. Unlike other membrane stains, this dye binds covalently to cellular thiols and therefore is well retained in cells throughout fixation steps. After washing, the cells were resuspended in sterile saline. For CXCR4 neutralization, 10⁶ c-Kithigh BMC were pre-incubated with 20 μg anti-murine CXCR4 neutralizing mAb (R&D, Abingdon, UK) for 30 min at 37°C [11].

Analysis of the expression of lineage markers, Sca-1 and CXCR4 on the sorted c-Kithigh BMC was performed by incubating the cells with a cocktail of rat-anti-mouse lineage markers (Ter119, CD45R, Ly6, CD8a, CD4, CD11b, all from BD Biosciences) for 30 min followed by a FITC-conjugated polyclonal antibody to rat-IgGs (Dako, Heverlee, Belgium) and a PE-conjugated monoclonal antibody to Sca-1 (BD Biosciences) for 30 min, or by incubating with an FITC-conjugated monoclonal antibody to CXCR4 (BD Biosciences) for 30 min. Analyses were performed on an FACS Calibur (Becton Dickinson) and showed that after cell sorting the c-Kithigh BMC expressed no lineage markers, as previously reported [19], and that at least 85% of the c-Kithigh population expressed Sca-1.

Therefore, the population of cells used in this study is highly enriched with c-Kit+, Sca-1− and Lin− cells, which are considered to represent HSC [20] and will hereafter be referred to as HSC. After cell sorting, 90% of our HSC population expressed CXCR4, the receptor for SDF-1.
In vitro migration assay

In vitro migration assays were performed in triplicate with 5 µm pore size transwells (Corning Life Sciences, Schiphol-Rijk, The Netherlands) coated with 0.5 µg/mL VCAM-1 (R&D) in PBS. To the lower compartment, medium (DMEM; Gibco, Invitrogen cell culture, Breda, The Netherlands) supplemented with 200 ng/mL recombinant murine SDF-1α (recSDF-1; PeproTech EC, London, UK) or medium alone was added. To the upper compartment, 0.1 × 10^6 CM-Dil-labelled HSC were applied and allowed to migrate for 2 h at 37°C. The neutralizing capacity of anti-SDF-1 was determined by adding 17 µg/mL neutralizing anti-human/mouse SDF-1α (anti-SDF-1, R&D) to the medium with recSDF-1 in the lower compartment. The neutralizing capacity of anti-CXCR4 was determined by adding HSC pre-incubated with CXCR4 (20 µg/10^6 HSC) to the upper compartment. The amount of migrated CM-Dil-labelled HSC was determined on an FACS Calibur (Becton Dickenson) and expressed as a percentage of the input.

In vivo migration assay

B6 mice received 0.6 × 10^6 CM-Dil-labelled HSC, or CM-Dil-labelled HSC pre-incubated with a CXCR4 blocking antibody (anti-CXCR4 group; 20 µg/10^6 HSC) [11] or CM-Dil-labelled HSC mixed with free anti-SDF-1 (anti-SDF-1 group; 2 mg/kg) [21] intravenously. Mice were killed 24 h after injection, and bone marrow aspirates were analysed by flow cytometry for the presence of injected HSC.

Renal I/R injury model

Unilateral renal I/R injury was induced by clamping the left renal artery for 55 min under general anaesthesia [0.1 mg/10 g mouse of fentanyl citrate fluanisone midazolam mixture, containing 1.25 mg/ml midazolam (Roche, Mijdrecht, The Netherlands), 0.08 mg/ml fentanyl-citrate, and 2.5 mg/ml fluanisone (Vetapharma, Leeds, UK)]. The contralateral (right) kidney was used as internal control. In the recSDF-1-treated group, mice received an intrarenal injection of 2 µg (25 µg/mL) recSDF-1 in a 0.2% peptidase hydrogel (PuraMatrix; BD Biosciences) in both the ischemic and contralateral kidney. Bilateral renal I/R injury was induced by clamping both renal arteries for 45 min under general anaesthesia, immediately followed by an intrarenal injection of 2 × 1 µg recSDF-1/0.2% hydrogel in the left kidney. The right kidney (control) was injected with the same volume of vehicle. After surgery, mice received intravenously 0.6 × 10^6 CM-Dil-labelled HSC, or CM-Dil-labelled HSC pre-incubated with a CXCR4 blocking antibody (anti-CXCR4 group, 20 µg/10^6 HSC) [11] or CM-Dil-labelled HSC mixed with free anti-SDF-1 antibody (anti-SDF-1 group; 2 mg/kg, R&D) [21] in a total volume of 200 µL. For analgesic purposes, mice received subcutaneously 50 µg/kg buprenorphin (Temgesic; Schering-Plough, Brussels, Belgium) after closing the abdomen and were killed 24 h after surgery. Kidneys were harvested, and blood samples were obtained via heart puncture and transferred to heparin tubes.

Detection of fluorescent-labelled HSC in the kidney

Formalin-fixed kidneys were embedded in paraffin. Four micrometre thick serial sections were cut, and every 10th section was used for examination. The sections were deparaffinized in fresh ethanol gradient series and subsequently embedded in the Vectashield HardSet mounting medium with DAPI (Vector Laboratories, Amsterdam, The Netherlands). The presence of injected HSC was examined by fluorescence microscopy using a Leica TCS5000 (Leica Microsystems, Rijswijk, The Netherlands) equipped with the Nuance multispectral imaging system (Cambridge Research, Woburn, MA, USA).

Kidney sections stored in a cold medium were used for flow cytometric analysis. The kidneys were minced through a 70 µm gauze, and a single cell suspension was made. Erythrocytes were lysed in 160 mM NH₄Cl, 10 mM KHCO₃ and 0.1 mM EDTA (pH 7.4). After washing, the kidney cells were analysed on a FACS Calibur (Becton Dickenson). For background correction, ischemic and contralateral kidneys of mice not injected with CM-Dil-labelled HSC were used.

Statistical analyses

Results are expressed as mean ± standard error of the mean (SEM), unless mentioned otherwise. Data were analysed using a non-parametric Mann–Whitney U-test. The values of P ≤ 0.05 were considered statistically significant.

Results

HSC preferentially migrate to ischemic damaged kidney

To study the migration of HSC towards the injured kidney, mice were subjected to unilateral I/R injury, after which they received 0.6 × 10^6 CM-Dil-labelled HSC into the tail vein. Twenty-four hours later, there was a significant (P = 0.03) increase in HSC migrating to the ischemic injured kidney compared to the contralateral kidney as assessed by flow cytometric analysis (Figure 1). The background fluorescence of ischemic and contralateral kidneys without HSC administration was subtracted to correct for autofluorescence of (dead) cells. Data are expressed as mean ± SEM, n = 4.

Fig. 1. HSC preferentially migrate to the ischemic injured kidney, and this migration is not altered by manipulating the SDF-1/CXCR4-axis. One day after unilateral ischemia for 55 min and subsequent intravenous administration of CM-Dil-labelled HSC, kidneys were harvested and analysed by flow cytometry for the presence of exogenous HSC (i.e. CM-Dil and c-Kit positive). Flow cytometry plots of (A) control, (B) ischemic kidney, (C) Exogenous HSC in contralateral kidney, and (D) ischemic kidney of mice with I/R injury and intravenously HSC (control) and of mice with additional neutralization of endogenous SDF-1 (anti-SDF-1), neutralization of HSC-associated CXCR4 (anti-CXCR4) and intrarenal recSDF-1 (recSDF-1), showed an increase in exogenous HSC to the ischemic kidney compared to the contralateral. However, this was only significant in the control and recSDF-1 mice (both *P = 0.03). Anti-SDF-1 or anti-CXCR4 treatment did not result in a decreased migration of HSC towards the ischemic kidney. Intrarenal recSDF-1 did not increase the migration of HSC towards the ischemic or contralateral kidney. Background fluorescence of ischemic and contralateral kidneys without HSC administration was subtracted to correct for autofluorescence of (dead) cells.

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Fig. 2. Exogenous HSC migrate to tubules and the interstitium. Fluorescent detection with TX2 filter of intravenously administered CM-DiI-labelled HSC in the injured kidney one day after I/R. (A and B) High magnification (40×) of the renal corticomedullary region. Two bright fluorescent cells are present in/close to injured tubules. (B) Spectral imaging was used to reduce the autofluorescent signal of the kidney, showing that the bright fluorescent cells are not endogenous kidney or inflammatory cells. (E) Overlay of the (C) TX2-filter image with (D) DAPI staining image corresponding to the boxed area in (A). Arrows point to the two bright exogenous cells; a clear overlay is seen with the DAPI staining.

and B). Of interest, exogenous HSC were mainly detected in the corticomedullary area, the part of the kidney most affected by I/R injury. More specifically, we found that these cells were localized in dilated tubules, in close proximity to dilated tubules and in the interstitium (Figure 2).

SDF-1 and CXCR4-neutralizing antibodies inhibit HSC migration in vitro and in vivo

To prove the efficacy of recSDF-1 as well as the neutralizing antibodies against SDF-1 and CXCR4, in vitro migration assays were performed. As depicted in Figure 3A, the medium supplemented with recSDF-1 resulted in an 8-fold increase of HSC migration compared to the medium alone. Adding anti-SDF-1 to the assay or pre-incubating the HSC with anti-CXCR4 resulted in reduced migration of HSC cells by 65 and 40%, respectively.

To validate whether anti-SDF-1 and anti-CXCR4 were also able to inhibit the migration of HSC in vivo, HSC were injected into the tail vein of naive mice with or without blocking either endogenous SDF-1 or HSC-associated CXCR4 with a monoclonal antibody as previously described [11,13,18,21]. Under physiological conditions, the bone marrow is the niche for HSC; the basal level of hypoxia in the bone marrow microenvironment may explain the constitutive and regional expression of SDF-1 in the bone marrow and the CXCR4-dependent stem and progenitor cell tropism to the bone marrow [22]. Twenty-four hours after administration, we could detect CM-DiI-labelled HSC in the bone marrow (Figure 3C). Subtraction of background signal from non-injected animals (Figure 3B) revealed that 0.21% ± 0.01% of total bone marrow consisted of CM-DiI-labelled HSC that had migrated towards the bone marrow. However, after neutralizing SDF-1 (Figure 3D) or CXCR4 (Figure 3E), no CM-DiI-labelled HSC could be detected in the bone marrow indicating that we have established a reliable in vivo system to disrupt the SDF-1/CXCR4-mediated migration of HSC.

Migration of HSC is not prevented by blocking the SDF-1/CXCR4-axis

In order to investigate the involvement of the SDF-1/CXCR4-axis in the migration of HSC to the injured kidney, we utilized two independent approaches to inhibit SDF-1/CXCR4 function. Unexpectedly, neutralization of endogenous SDF-1 or HSC-associated CXCR4 did not result in a significantly decreased migration of exogenous HSC to the ischemic injured or contralateral kidney (Figure 1C). In contrast, in both groups, there was an increase in exogenous HSC in the ischemic kidney compared to the contralateral kidney, although this was not significant.

HSC migration is not influenced by exogenous SDF-1 in vivo

In contrast to other studies [11,13,18,21], we were unable to significantly influence the migration of HSC to the ischemic injured kidney by blocking the SDF-1/CXCR4-axis. Therefore, we hypothesized that endogenous SDF-1 levels in ischemic kidneys may be insufficient to properly attract HSC. To test this hypothesis, both contralateral...
Fig. 3. Neutralizing SDF-1 and CXCR4 antibodies inhibit migration of HSC towards recSDF-1 and the bone marrow. (A) In an in vitro migration assay, the amount of migrated CM-DiI-labelled HSC from the upper to the lower compartment was determined as a percentage of the input. After 2 h at 37°C, 1.4% passive (control) migration of HSC was observed. Addition of 200 ng/mL recSDF-1 to the medium in the lower compartment increased HSC migration 8-fold. Neutralizing SDF-1 in the lower compartment or neutralizing HSC-associated CXCR4 in the upper compartment resulted in a 65% and 40% reduction of recSDF-1-induced migration, respectively. All experiments were performed in triplicate, bars represent mean ± SD, n = 2. (B–E) In an in vivo migration assay, the percentage of exogenous HSC (i.e. c-kit and CM-DiI positive) in the bone marrow was determined by flow cytometry 24 h after intravenous injection. Flow cytometry plots of (B) a non-injected control, (C) a positive control, (D) anti-CXCR4-treated mice and (E) anti-SDF-1-treated mice reveals only exogenous HSC in the positive control; no exogenous HSC could be detected in the bone marrow of anti-CXCR4- or anti-SDF-1-treated mice.

Fig. 4. HSC migration is not influenced by locally administered SDF-1. One day after bilateral ischemia and local administration of saline or recSDF-1 followed by intravenous injection of CM-DiI-labelled HSC, no difference was observed in migration of the exogenous HSC. Flow cytometry plots of (A) a saline and (B) a recSDF-1-treated ischemic kidney. (C) Graphical representation of the mean (± SEM) percentage of exogenous HSC in the saline (control) and recSDF-1-treated ischemic kidneys shows no difference between the groups. Data are expressed as mean ± SEM, n = 4.

Subsequently, we hypothesized that a more severe and systemic type of danger might be necessary. Therefore, bilateral I/R injury was induced and directly after reperfusion local injections of recSDF-1 in the left and saline in the right kidney were performed. Immediately after surgery, mice received HSC into the tail vein and 24 h later the kidneys were analysed. Again, we could not observe any effect of the locally injected recSDF-1 on the migration of HSC since both kidneys were engrafted with the same amount of exogenous administered HSC (Figure 4).

Discussion

The aim of our study was to establish the importance of the SDF-1/CXCR4-axis in the migration of HSC to the ischemic injured kidney, in order to eventually manipulate this axis for therapeutic purposes. The contribution of HSC in post-ischemic renal tissue repair is controversial. First of all, the capacity of HSC to engraft tubules after ischemic injury is much lower than originally reported. Initial studies in chimeric mice stated that 21% [6] or even 80% [7] of the tubules contained BMC. However, some of these numbers are thought to be overestimated due to false-positive results as a consequence of β-gal leaking by the BMC [23]. Sex-mismatched and GFP-chimeric mice studies published in 2005 showed only a few BMC in the tubules after I/R injury [8,10], whereas Duffield and colleagues could not observe any BMC in post-ischemic tubules [24,25]. Secondly, the
biological significance of HSC in repair after I/R injury is challenged, since low numbers of BMC in the tubules do not make a significant contribution to renal functional recovery [8,10]. Therefore, mechanisms to increase the migration of HSC may result in a significant contribution of these cells to tubular repair and hence have therapeutic potential.

To determine whether the SDF-1/CXCR4-axis is the driving force for the recruitment of HSC to the injured kidney, we followed the migration of exogenous HSC in a unilateral renal I/R model. This model gives the opportunity to compare the migration of HSC in injured and normal tissue within one mouse, in contrast to experimental models such as cisplatin-induced renal injury and bilateral renal I/R injury. Here, we demonstrate that HSC migrate to the injured kidney and are even able to migrate into damaged tubules. In line with our data, Lin et al. [7] and Tögel et al. [18] have shown that the homing process of BMC is selective for the ischemic injured kidney. Our study, however, extends these findings as we have studied migration of purified HSC instead of whole bone marrow that is a heterogeneous population of cells consisting, amongst others, of inflammatory cells, mesenchymal stem cells and only a small percentage of HSC. Surprisingly, manipulating the SDF-1/CXCR4-axis in three different and independent manners did not result in a significantly altered migration of exogenous HSC.

Previous studies have shown the importance of the SDF-1/CXCR4-axis in the migration of BMC. This was done either by blocking endogenous SDF-1, blocking BMC-associated CXCR4 or increasing locally SDF-1 [11,12,18,21,26]. Our results are at variance with the current opinion that SDF-1 is the mediator of HSC trafficking to injured organs. The neutralizing antibodies were chosen based on their proven functionality in previous studies. Neutralization with the same function-blocking anti-SDF-1 monoclonal antibody and dose as used in the present study significantly attenuated HSC accumulation within the growing platelet-rich thrombus in an arterial thrombosis mouse model [21]. In order to neutralize HSC-associated CXCR4, the same protocol was used as reported previously to be effective in blocking the recruitment of BMC [18] or HSC [11,13,18]. In addition, our in vitro and in vivo HSC migration assays demonstrated a decreased migration towards high recSDF-1 levels and bone marrow, respectively, after neutralizing SDF-1 or CXCR4. Therefore, we believe that our experimental set-up is highly reliable to study the role of this axis. Despite this, we could not observe a significant effect of neutralizing SDF-1 or its receptor CXCR4 on the migration of HSC to the injured kidney. Since these results do not support the current reports on SDF-1-involvement in HSC-migration, we conducted a third independent experiment to determine the effect of local SDF-1 administration on the migration of exogenous HSC. Despite the previously reported preferred migration of HSC towards high SDF-1 gradient in liver, bone marrow and spleen [11,12], we could not observe this in both the unilateral and bilateral renal I/R injury model. However, in accordance with our results, it has been reported that BMC migration to the lung could not be influenced significantly by manipulating the SDF-1/CXCR4-axis in both a non-injured [12] and a bleomycine-injured [27] mouse model. This indicates that other mechanisms may be involved in HSC migration to the kidney and lung.

We also did not find an effect of the local administration of recSDF-1 in the non-injured (contralateral) kidney on renal engraftment of HSC compared to the non-treated contralateral kidney. These results suggest that injury is necessary for HSC migration and that SDF-1 alone is not sufficient for migration. This was also observed in the heart, where overexpression of SDF-1 without injury could not induce the homing of BMC [16]. In addition, the results from the bilateral I/R model, where both the SDF-1 and saline-injected kidney had the same percentage of migration, indicate that the exogenous administered HSC cells did not migrate preferentially towards high SDF-1 gradient.

Our results indicate that I/R injury induces the homing of HSC in the injured kidney and that this preferred migration is not exclusively dependent on the SDF-1/CXCR4-axis. Homing of HSC towards the ischemic damaged kidney could be much more complex. Several other factors are described to be involved in stem cell migration and in addition are known to be induced after I/R injury. These factors include the chemokine GROβ [28], the glycosaminoglycan hyaluronic acid [29], the nuclear factor HMGBl [30] and the growth factor HGF [11]. Interestingly, in a recently published report, a flexible hierarchy of homing molecules was proposed, providing an explanation for some of the contradictory findings in the literature [31].

Herein, the authors show that in a mouse model of HSC transplantation, SDF-1 was not required for stem cell homing due to compensatory action via the VLA-4/VCAM-1 interaction [31].

In conclusion, our initial hypothesis that SDF-1/CXCR4 interactions are implicated in the migration of HSC to the injured kidney is not supported by the results in this report. In sharp contrast to studies in other injured organs, our results indicate that migration of HSC to the ischemic damaged kidney is not solely dependent on the SDF-1/CXCR4 signalling axis.

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Conflict of interest statement. None declared.

(See related article by M. S. Goligorsky. This extraordinary extramendary haematopoiesis. Nephrol Dial Transplant 2009; 24: 2016–2017.)

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