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Interleukin-9 release from human kidney grafts and its potential protective role in renal ischemia/reperfusion injury

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Abstract

Background: The pathophysiology of ischemia/reperfusion (I/R) injury is dominated by an inflammatory response. In the identification of new therapeutic agents, the role of individual cytokines may be essential. Interleukin (IL)-9 is a pleiotropic cytokine recently identified to be involved in various immune responses. In this study, the role of IL-9 in renal I/R injury was assessed.

Methods: We performed repeated direct measurements of arteriovenous IL-9 concentration differences over the reperfused graft in human kidney transplantation.

Results: Substantial renal IL-9 release was observed from deceased donor kidneys (P = 0.006). In contrast, living donor kidneys, which have a more favourable clinical outcome, did not release IL-9 during early reperfusion (P = 0.78). Tissue expression of IL-9 did not change upon reperfusion in both living and deceased human donor kidneys. To assess the role of IL-9 in I/R injury, an experimental study comprising IL-9 inhibition in mice undergoing renal I/R was performed. Although there was no difference in kidney function, structural damage was significantly aggravated in anti-IL-9 treated mice.

Conclusions: Deceased donor grafts show a substantial IL-9 release upon reperfusion in clinical kidney transplantation. However, inhibition of IL-9 aggravated kidney damage, suggesting a regulating or minor role of IL-9 in clinical I/R injury.

Introduction

Ischemia/reperfusion (I/R) injury is an inevitable consequence of organ transplantation and a major determinant of patient and graft survival. I/R injury induces delayed graft function, which complicates around 20%[1, 2] of deceased donor kidney transplantations and has a major influence in graft function and survival.[3] The pathophysiology of I/R injury is complex and incompletely understood, although both preclinical[4-6] and clinical studies[7-9] have shown that inflammation is an important mediator of I/R injury. The exact functions of the various cytokines involved in regulating the complex inflammatory events after I/R injury are not fully unraveled yet.[10] Insight in the role of individual cytokines may be essential in the identification of new therapeutic agents.

Interleukin (IL)-9 is a pleiotropic cytokine recently discovered to be involved in various immune responses. Studies on the etiology of asthma and allergies have demonstrated an evident pro-inflammatory role of IL-9.[11, 12] IL-9 is produced by T helper cells[13], regulatory T cells[14], and mast cells[15] and is able to modulate their production of various other cytokines.[12] In a preceding study, we described for the first time a renal release of IL-9 after kidney transplantation.[10]

The aim of the present study was to explore the potential pathophysiologal role of IL-9 in renal I/R injury. IL-9 release during reperfusion of both living and deceased human donor kidney grafts was assessed, and the effects of IL-9 inhibition on renal I/R injury were evaluated in an animal experiment.

Methods

Patient population

Twenty-four patients undergoing renal allograft transplantation were included; of these, eight patients received a kidney from a living donor and sixteen patients received a kidney from a deceased donor (nine brain-dead donors and seven cardiac-dead donors). Kidney transplantations were performed according to the local standardized protocol.[16] In living donors open nephrectomy was performed and Custodiol® histidine-tryptophan-ketoglutarate solution (Tramedico, Weesp, The Netherlands) was used for cold perfusion and storage of the kidney. Brain-dead and cardiac-dead donor kidneys were perfused and stored with either University of Wisconsin solution or Custodiol® histidine-tryptophan-ketoglutarate solution. The immunosuppressive regimen was based on induction therapy with basiliximab on day 0 and 4; and tacrolimus or cyclosporine A in addition with mycophenolate mofetil and

steroids. For technical reasons (renal vein sampling) only patients receiving a left kidney were included. Patient and graft characteristics and ischemia times are summarized in Table 1. The postoperative course was uneventful in all patients. One-year patient and graft survival was 100%. The study protocol was approved by the local ethics committee, and informed consent was obtained from each patient.

Table 1. Transplantation and outcome characteristics in living donor (LD) and deceased donor (DD) kidney transplantation (mean ± SD).

	LD	DD	*P* value
n	8	16	
Recipient age (years) mean ± SD	41.1 ± 10.5	54.6 ± 12.2	0.02
Recipient gender (% male)	38	56	0.39
Donor age (years) mean ± SD	43.9 ± 10.6	53.5 ± 16.1	0.14
Donor gender (% male)	75	44	0.15
Preservation fluids	HTK* (n=8)	UW† (n=11) HTK* (n=5)	0.001
CIT‡ (min.) mean ± SD	179.1 ± 18.6	1117.7 ± 299.1	<0.001
WIT§ (min.) mean ± SD	34.0 ± 6.3	33.5 ± 6.1	0.85

* HTK: Histidine-tryptophan-ketoglutarate
† UW: University of Wisconsin
‡ CIT: cold ischemia time
§ WIT: warm ischemia time

IL-9 plasma measurements in arteriovenous samples

Arterial and renal venous blood samples were obtained during human kidney transplantation as described previously.[16] In short, blood aliquots were sampled at 30 seconds, 3, 10 and 30 minutes after reperfusion via a catheter placed in the renal vein. Paired arterial blood samples were obtained at 0, 3, 10 and 30 minutes after reperfusion. The same method was used to obtain a control arterial and venous blood sample during donor nephrectomy prior to the induction of renal ischemia. All samples were collected in tubes containing EDTA (BD Vacutainer, Plymouth, UK) and centrifuged twice (1550 *g*, 20 min, 4°C) to deplete it of leukocytes and thrombocytes. Plasma was aliquotted and stored at -70°C until analysis. IL-9 was measured in a custom-made multiplex assay in accordance with the manufacturer's instructions (X-plex, Biorad, Veenendaal, The Netherlands).

Immunohistochemical evaluation of IL-9 source in human kidney biopsies

A renal cortical biopsy was obtained before transplantation, after cold storage of a deceased donor graft. Kidney tissue was fixed in formalin for 24 hours and then embedded in paraffin for light microscopy. Longitudinal sections of 4 µm were prepared. After EDTA-retrieval, IL-9 deposition was assessed using a human IL-9 mAb in a 1:100 dilution (cat. no. 507602, Biolegend, Cambridge, UK). Staining was visualized using Nova RED (Vector Labs, Peterborough, UK). Sections were counterstained with hematoxylin (Merck, Darmstadt, Germany).

IL-9 expression in human kidney biopsies

A renal cortical biopsy was obtained before transplantation after cold storage, and a post-reperfusion biopsy was collected 45 minutes after reperfusion. Biopsies were immediately snap frozen in liquid nitrogen and stored at -70ºC. Total RNA was extracted from renal tissues using RNAzol (Campro Scientific, Veenendaal, The Netherlands) and glass beads.[17] The integrity of each RNA sample was examined by Agilent Lab-on-a-chip technology using the RNA 6000 Nano LabChip kit and a bioanalyzer 2100 (Agilent Technologies, Amstelveen, The Netherlands). RNA preparations were considered suitable for array hybridization only if samples showed intact 18S and 28S rRNA bands, and displayed no chromosomal peaks or RNA degradation products (RNA Integrity Number >8.0).[18] Subsequent microarray analysis was performed using Illumina whole-genome gene expression BeadChips (Illumina BeadArray®, San Diego, CA, USA) according to instructions of the manufacturer at Service XS (Leiden, The Netherlands). IL-9 expression data were obtained from this array.

Animals and surgical procedures

Male BALB/c Jico mice (Charles River, L'Arbresle, France) of 8 weeks old (20-30 g) were divided into the following four groups: (1) I/R pre-treated with anti-IL-9 antibody (Ab) (n=12); (2) I/R pre-treated with control Ab (n=12); (3) sham-operated, pre-treated with anti-IL-9 Ab (n=4), and (4) sham-operated pre-treated with control Ab (n=4). Monoclonal anti-IL-9 Ab (no. 504802, clone D9302C12, BioLegend, San Diego, CA, USA) or isotype control IgG (no. 400916, clone HTK888, BioLegend, San Diego, USA) was administered at 1 and 2 days before surgery. At 2 days before surgery, 2 ug/g body weight anti-IL-9 Ab or its equivalent of isotype control Ab was administered by i.p. injection, followed by 4 ug/g body weight the day before surgery. This dosage is based on previous research in which a significant effect of anti-IL-9 treatment was observed after administration of a similar dose of the antibody.[19] Mice were anesthetized with isoflurane. Bilateral kidney ischemia was induced by clamping the renal artery and vein for 35 minutes, followed by reperfusion. In the sham group, identical surgical procedures were used, except that clips were not applied.[16] Kidney function was measured by plasma urea concentration (Reflotron®, Roche

diagnostics, Almere, The Netherlands) the day before administration of antibodies (day -3), day of surgery (day 0), and two consecutive days after surgery (day 1 and 2). Two days after surgery, mice were killed and kidneys retrieved. Mice were maintained on standard diet and given water ad libitum throughout the whole experimental period. The study was approved by the veterinary authorities of our institute.

Histological evaluation of murine kidney tissue

The mouse kidneys were fixed in formalin for 24 hours and then embedded in paraffin for light microscopy. Longitudinal sections of 4 µm were prepared and stained with periodic acid-Schiff (PAS). The degree of tissue injury was scored by two blinded observers. Kidney injury was scored semi-quantitatively using the amount of proximal tubule necrosis (score 0–3), band necrosis of the cortex and the medulla (score 0–3) and protein casts in the tubules (score 0–3).

Data collection and statistical analysis

Clinical donor data were retrieved from the Eurotransplant database. Delayed graft function was defined as the need for dialysis within the first week after transplantation. Statistical analysis was performed using SPSS 16.0 statistical analysis software (SPSS Inc, Chicago, IL, USA). Patient characteristics were compared by paired *t*-test and are expressed as mean ± standard deviation (SD). Area under the curve (AUC) was calculated for the arterial and venous curve of the human plasma measurements for the total of 30 minutes. The delta AUC was calculated (venous minus arterial) and the null-hypothesis (delta AUC is 0) was tested by a Wilcoxon signed rank test. Urea concentrations were compared using a Mann Whitney *U*-test, Wilcoxon signed rank test or Kruskal-Wallis test where appropriate. Histological score was tested by the Mann Whitney *U*-test. *P* value < 0.05 was considered significant.

Results

IL-9 is released from deceased donor kidneys during reperfusion

Living donor kidneys released no IL-9 during the first thirty minutes after reperfusion ($P = 0.78$, n = 8, Figure 1A). In contrast, deceased donor kidneys, which generally show more dysfunction after transplantation, released a vast amount of IL-9 in the first thirty minutes after reperfusion ($P = 0.006$, n = 16, Figure 1B). More specifically, only brain-dead donor kidneys showed a significant release of IL-9 ($P = 0.04$, n = 9). However, cardiac-dead donors showed the same trend ($P = 0.07$, n = 7), and therefore deceased donor data are clustered. IL-9 release was specific for I/R, as control measurements over the non ischemic kidney in the donor did not show a transrenal difference in IL-9 levels ($P = 0.89$, data not shown). This

indicates that IL-9 release is not a consequence of manipulation of the kidney during surgery or a reaction to anesthetics.

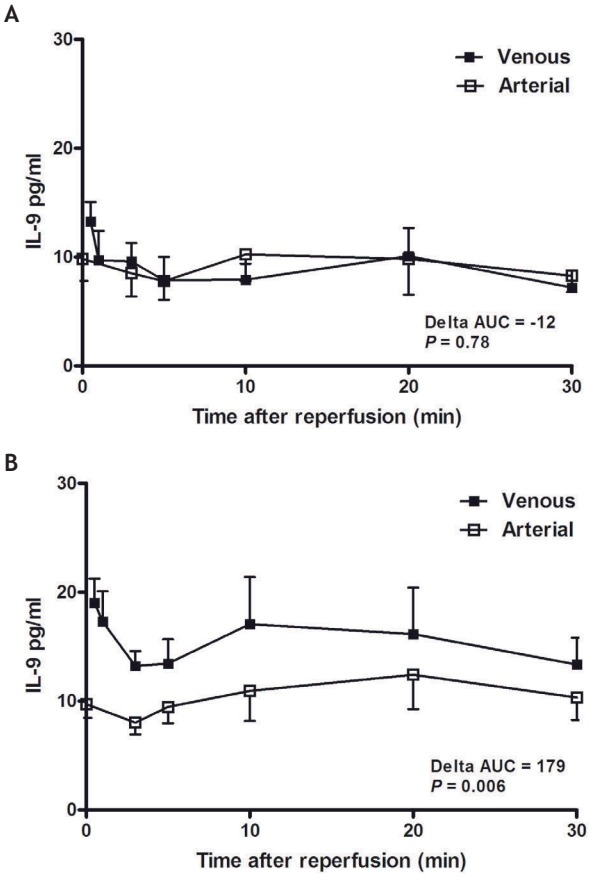


Figure 1. Arterial and renal venous plasma concentrations of IL-9 during the first 30 minutes of reperfusion.
(A) No significant release of IL-9 from living donor grafts was observed (P = 0.78, n = 8); (B) IL-9 was released significantly from deceased donor grafts (P = 0.006, n = 16).

Source of human IL-9 release is mainly tubular epithelial cells

The source of IL-9 released from deceased donor kidneys could be local resident cells or circulating cells. An immunohistochemical staining of IL-9 was performed on renal biopsy tissue from a deceased donor graft, which showed that local stores of IL-9 are present in the kidney. IL-9 signals were present predominantly in renal tubular epithelial cells and also in macrophages and dendritic cells (Figure 2).

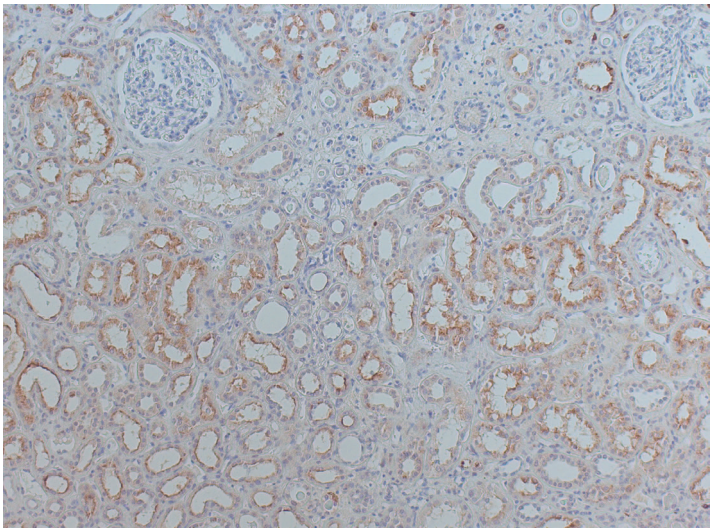


Figure 2. Typical example of IL-9 staining in deceased donor kidney biopsy collected before transplantation.
IL-9 signal was positive in renal tubular epithelial cells, macrophages, and dendritic cells. Original magnification x200.

No change in human renal IL-9 expression after reperfusion

No differences in IL-9 RNA expression were observed between pre- and post-reperfusion biopsies in living (P = 0.89, Figure 3A) as well as deceased donor kidneys (P = 0.59, Figure 3B). In addition, similar baseline expression of IL-9 in living and deceased donor kidneys was observed (P = 0.57).

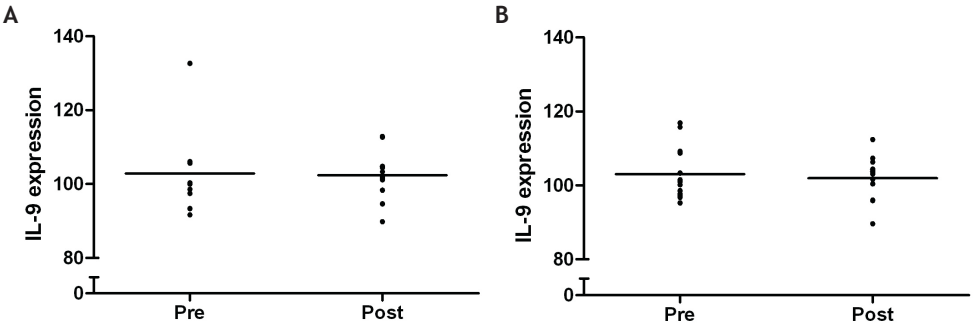


Figure 3. Interleukin-9 expression in human kidney biopsies.
IL-9 expression in human pre- and post-transplantation biopsies of (A) living donor kidneys; and (B) deceased donor kidneys. There was no difference in IL-9 RNA expression between pre- and post-reperfusion biopsies in living and deceased donor kidneys (P = 0.89 and P = 0.59, respectively) nor in baseline IL-9 expression between both donor types (P = 0.57).

Anti-IL-9 treatment does not prevent functional and structural kidney I/R injury

Given the dominance of IL-9 release from deceased donor kidneys, we hypothesized that IL-9 is critical in the initiation of I/R injury. Therefore, mice were treated with anti-IL-9 Ab or isotype control Ab before undergoing renal I/R. All mice survived the end of the experiment. Baseline urea concentrations were identical in all animals (Figure 4). In both anti-IL-9 Ab and isotype control Ab treated mice that underwent renal I/R, urea concentration was significantly raised at 1 and 2 days after reperfusion as compared with baseline (all, P = 0.002). As expected, urea of sham-operated animals, both anti-IL-9 Ab and isotype control Ab pre-treated, did not change after surgery as compared with baseline (all, P = NS). Urea concentration was similar in anti-IL-9 treated and control Ab treated mice at day 1 (P = 0.91) and 2 (P = 0.53) after reperfusion (Figure 4).

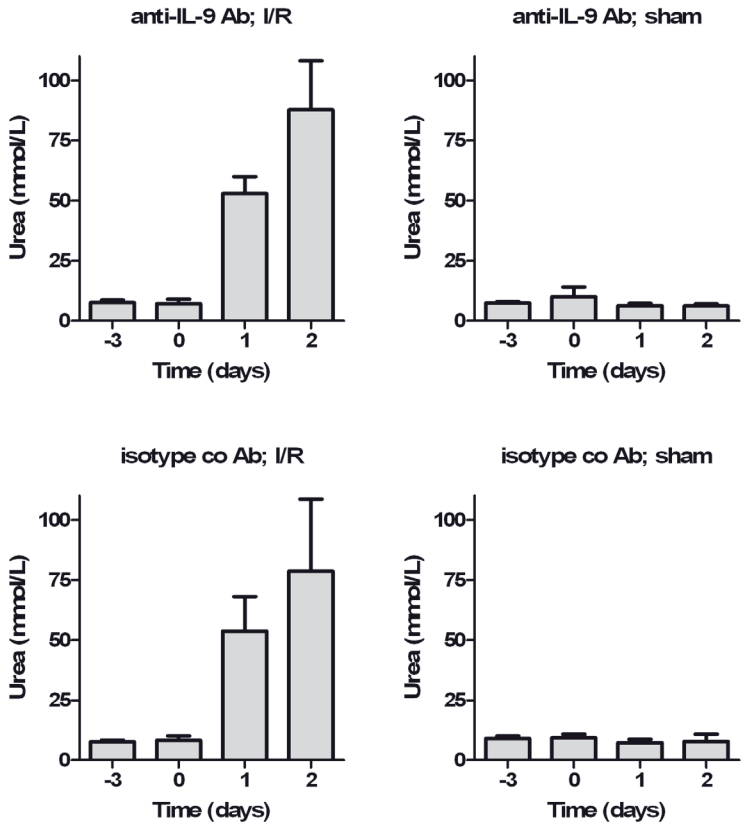
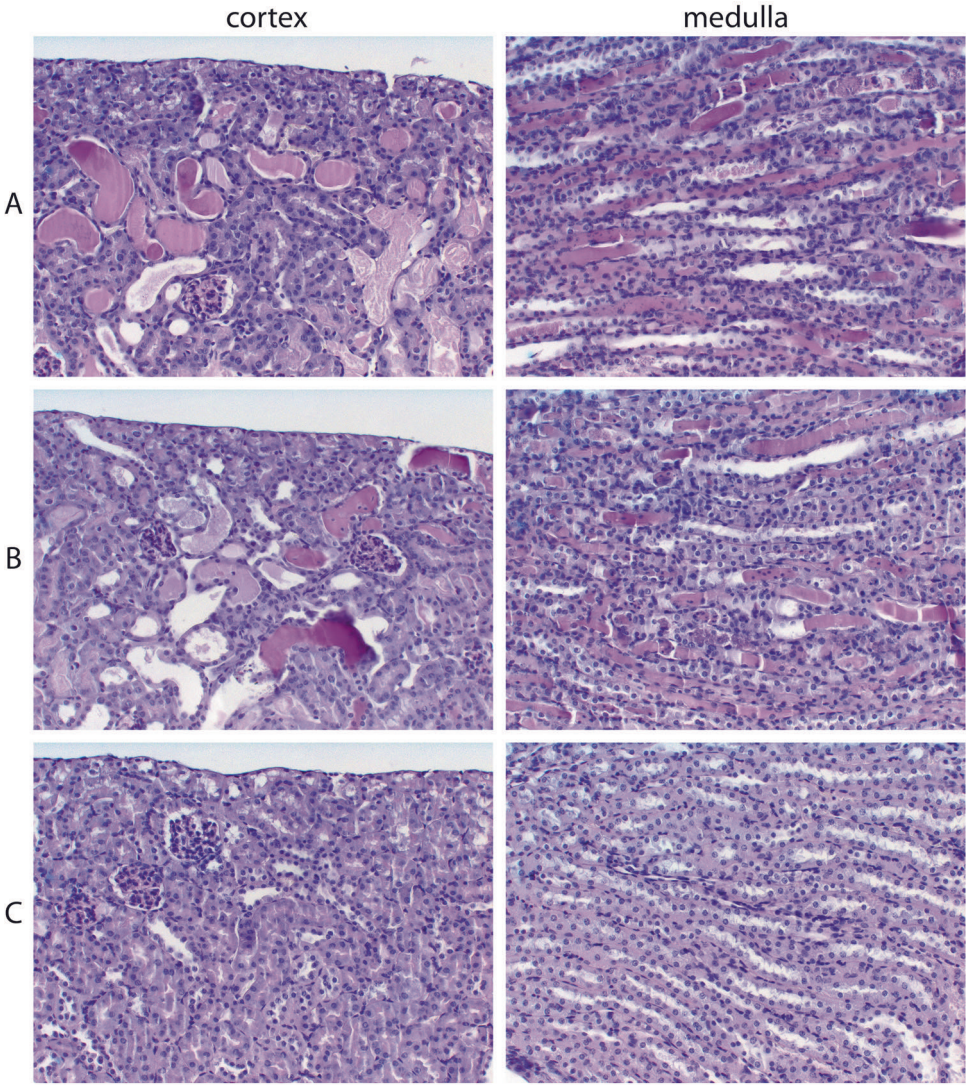


Figure 4. Plasma urea levels after murine renal ischemia/reperfusion.
Plasma urea levels measured at baseline (day -3), at the day of surgery (day 0), and two consecutive days after surgery (day 1 and 2). Plasma urea levels were similar in anti-IL-9 Ab treated mice compared with isotype control Ab treated mice after I/R at day -3 (P = 0.77), day 0 (P = 0.69), day 1 (P = 0.91), and day 2 (P = 0.53). The median and interquartile range are plotted.

Structural kidney damage was moderate to severe in all kidneys that underwent I/R, whereas kidneys of sham-operated mice showed normal histology. Kidney injury was scored on several characteristics and then quantified. There was significantly more structural damage in kidneys of anti-IL-9 Ab treated mice than in the control Ab treated group after I/R. In detail, anti-IL-9 Ab treated mice showed more proximal tubule necrosis (P = 0.02), band necrosis of the cortex and medulla (P = 0.01) and protein casts in the tubules (P = 0.005) (Figure 5).



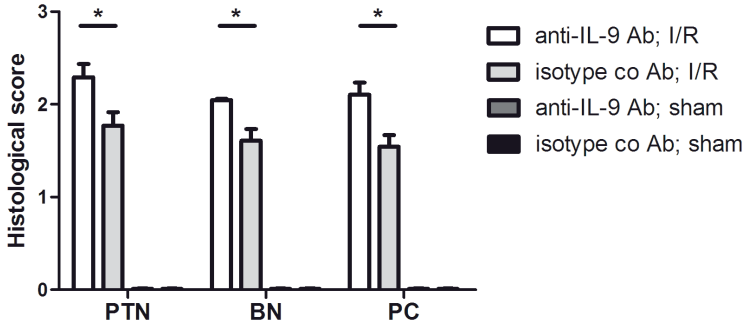


Figure 5. PAS-staining of murine kidney biopsies.
Representative PAS-stained kidney sections harvested 2 days after surgery of (A) mice that received anti-IL-9 Ab before renal I/R; (B) mice that received control Ab before kidney I/R; (C) sham-operated mice that received anti-IL-9 or control Ab treatment before operation (no difference). Original magnification x200. Mice receiving anti-IL-9 Ab before I/R had evident protein casts and tubule necrosis, more than isotype controle Ab treated mice. Sham-operated mice receiving either anti-IL-9 Ab or control Ab had no PAS-positive deposits and no structural kidney damage. (D) Semi-quantitative histological damage score for the severity of kidney damage is shown. Damage was scored (0-3) on presence of proximal tubule necrosis (PTN), band necrosis (BN) and protein casts (PC). Kidneys of all mice that underwent kidney I/R showed moderate to severe damage. There were significant differences between anti-IL-9 Ab-treated and isotype control Ab-treated groups (PTN, $P = 0.02$; BN, $P = 0.01$; PC, $P = 0.005$). Kidneys of sham-operated mice scored 0 for all measures. Median and interquartile ranges are plotted. Significant differences are indicated by an asterisk.

Discussion

In this study it is shown that IL-9 is released exclusively from human deceased donor grafts directly after reperfusion. Since deceased donor grafts are clinically more affected by I/R injury, we hypothesized that IL-9 is a mediator of renal I/R injury. In a subsequent mouse experiment, inhibition of IL-9 did not influence kidney function after I/R and even aggravated structural kidney injury.

IL-9 was released instantly after reperfusion from deceased donor grafts and its release persisted during the next thirty minutes. The substantial and immediate character of this IL-9 release suggests a pre-stored pool, since no change in IL-9 expression in kidney tissue upon reperfusion was observed. Pre-transplantation infiltrated cells are probably not involved, since baseline biopsies showed no difference between human living and deceased donor IL-9 expression, although there was a vast difference in cellular infiltrate before transplantation.[10] Immunohistochemical staining of IL-9 showed its presence in tubular epithelium and resident macrophages and dendritic cells. These cells are likely responsible for secretion of their pre-stored IL-9.

IL-9 is considered a pro-inflammatory cytokine, although as yet little is known about its exact functions. Preclinical studies have shown that IL-9 promotes mast cell growth and plays a crucial role in the nephroprotective effects of regulatory T cells.[12, 20] In addition, a potential role in the stimulation of erythropoiesis has been suggested[21, 22], as well as an antiapoptotic effect.[23] The observation in our study that IL-9 was released exclusively from deceased donor kidneys, in which tissue damage is most severe, argues for an active role of IL-9 in initiating I/R injury. Therefore, the effect of IL-9 inhibition on kidney I/R injury was assessed in a mouse experiment. Remarkably, results showed aggravated structural kidney damage after I/R in anti-IL-9 Ab treated animals. When extrapolating these findings, it can be suggested that IL-9 plays a regulating role in clinical renal I/R injury or can be an initial trigger for salvaging or limiting injury.

Although no previous data are available regarding the role of IL-9 in I/R injury, the few studies on allograft rejection after transplantation are conflicting. An association between IL-9 and acute, eosinophil-driven rejection has been suggested by Poulin et al. in mismatched heart allografts.[24] However, no involvement of IL-9 was observed in rejection on a longer term in both mouse islets and human kidney allografts.[25]

Our arteriovenous sampling time was limited to thirty minutes after reperfusion, the time needed to complete the operative procedure. Deceased donor kidney grafts showed an extensive release of IL-9 within the studied timeframe; therefore it is highly unlikely that living donor kidney grafts will start releasing IL-9 after the studied period. Finally, one might argue that a mouse model of renal I/R injury can only approximate the human situation. Nevertheless, the effects of IL-9 inhibition could be accurately assessed in our animal experiment and a negative effect of anti-IL-9 treatment was indeed observed.

In conclusion, this study shows a substantial and exclusive release of IL-9 from deceased donor grafts in human kidney transplantation, while living donor kidneys, which generally have a more favourable clinical outcome, did not release IL-9 during early reperfusion. The source of released IL-9 was its pre-stored pool in renal tubular cells. Experimental inhibition of IL-9 in mice aggravated kidney damage after I/R injury. Altogether, these results suggest a regulating or minor role of the released IL-9 on I/R injury in human kidney transplantation.

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