Early post-transplant urinary IP-10 expression after kidney transplantation is predictive of short- and long-term graft function

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The early identification of renal transplant recipients at enhanced risk of developing acute and subclinical rejection would allow individualized adjustment of immunosuppression before functional graft injury occurs and would exclude these patients from drug-weaning studies. Protein and reverse transcriptase-polymerase chain reaction-based analyses of candidate markers in urine open the opportunity to closely monitor kidney-transplanted patients non-invasively. The chemokine *interferon-inducible protein 10* (IP-10; CXCL10) might be an interesting candidate to uncover ongoing immune processes within the graft. Urine samples from kidney-transplanted recipients were retrospectively analyzed for IP-10 mRNA and protein expression. IP-10 levels were correlated with the incidence of acute rejection episodes proven by histology and long-term graft function assessed by the glomerular filtration rate 6 months post transplantation. IP-10 expression in urine identified patients with ongoing acute rejection episodes several days before a biopsy was indicated by rising serum creatinine levels. Most importantly, elevated levels of urinary IP-10 protein within the first four postoperative weeks were predictive of graft function at 6 months even in the absence of acute rejection. These data reveal a correlation between elevated IP-10 expression in urine at early time points post-transplantation and intragraft immune activation that leads to acute rejection and compromised long-term graft function.

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Acute rejection remains a serious and frequent complication after renal transplantation although immunosuppressive therapy and human leukocyte antigen matching have improved tremendously in recent years. Both early diagnosis and selective immunosuppressive therapy of acute rejection are essential for the long-term outcome. Measurement of serum creatinine (Cr) levels after renal transplantation is one of the approved methods for monitoring allograft function. However, the rise of serum Cr is a relatively late event of intragraft injury. Therefore subclinical rejection events may remain undiagnosed while untreated intragraft events increase the risk of development of chronic allograft nephropathy.[1] Acute rejection is diagnosed by examination of the immunological status of the graft by histological analysis of percutaneous biopsies at times when severe damage to the graft may have already taken place. The procedure is not only laborious, time-consuming, and expensive, but it can also lead to significant complications that necessitate its replacement by non-invasive diagnostic and prognostic assays.

Our group and others have shown by quantitative polymerase chain reaction that urinary mRNA expression of cytolytic molecules including Granulysin,[2] Perforin, and Granzyme B[3] is associated with acute rejection. Recent publications demonstrate that the expression of the CXC chemokine *interferon-inducible protein 10* (IP-10; CXCL10) and its receptor CXCR3 in urine is significantly elevated during kidney allograft rejection.[4–6] Chemokines are small peptides divided into C, CC, CXC, and CX_3C families and provide signals for the recruitment of different subsets of T cells through seven-transmembrane-spanning, G-protein-coupled receptors.[7] CXCR3, a receptor predominantly expressed by activated T- and natural killer cells, binds to the three CXC non-ELR (glutamate–leucine–arginine) chemokines *interferon-inducible T-cell-α chemoattractant* (CXCL11), IP-10 (CXCL10) and the *monokine-induced by γ-interferon* (CXCL9).[8] It has been shown that CXCR3 is a marker for T helper cells type-1 associated with inflammatory processes,[9] and that IP-10 and *monokine-induced*

by γ-interferon (CXCL9) attract activated, but not resting T cells.[10,11] IP-10 expression in serum has been the subject of several studies concerning autoimmune diseases including rheumatoid arthritis,[12] Hodgkin's lymphoma,[13] Graves' disease,[14] and multiple and systemic sclerosis.[15–17] This chemokine, which is secreted by a variety of cells including monocytes, fibroblasts, and endothelial cells, plays an important role during human solid-organ allograft rejection.[18–21] An upregulation of IP-10 mRNA in human renal allograft biopsies during acute rejection[22] and a correlation between elevated pre-transplant IP-10 serum levels and the risk of severe rejection has also been described.[23] In order to develop a non-invasive diagnostic strategy for acute rejection after renal transplantation, IP-10 mRNA[5] and IP-10 protein expression[4] were analyzed in urine at the time of biopsy-proven acute rejection. Elevated levels of IP-10 expression correlated with the histological and clinical findings of rejection in both studies.

Most studies performed on urine or biopsy samples have relied on the measurement of IP-10 at the time of biopsy-proven rejection. Early detection or even prognosis of acute rejection prior to graft deterioration would be of special value and could be achieved by follow-up screening of patients post transplantation (Tx). To assess whether IP-10 exhibits predictive properties of acute rejection, we measured IP-10 mRNA expression in urine sediment and IP-10 protein expression in free urine after renal transplantation in a retrospective kinetic study. Firstly, we examined not only whether the expression of this molecule at the mRNA or protein level was increased at the time of acute rejection diagnosis but whether elevations could be observed before increased serum Cr indicated severe graft injury due to effector cell infiltration. Secondly, we addressed the question whether early intragraft immune activation is related to long-term function. As the glomerular filtration rate (GFR) is a good predictor of long-term allograft function,[24] we also ascertained whether persistently elevated IP-10 levels in urine post Tx would predict impaired kidney function as assessed by GFR (<45 ml/min per $1.73\,m^2$) at 6 months. Our data clearly demonstrate the prognostic value of IP-10 protein measurement after kidney transplantation for acute rejection diagnosis and that persistent elevation of IP-10 protein in urine is associated with limited graft function at 6 months even in the absence of rejection.

RESULTS

Elevated levels of IP-10 mRNA in urine sediment after kidney transplantation

To validate previous reports of heightened IP-10 expression in patients with acute rejection, we analyzed the mean IP-10 mRNA expression of urine specimens from kidney transplanted recipients diagnosed with a Banff I–III ($n=21$) or borderline (BL) rejection episode ($n=14$). In contrast to patients with urinary tract infection or cytomegalovirus (CMV) antigenemia, they showed significantly elevated levels of IP-10 mRNA compared to controls with stable graft function ($n=26$) ($P\leqslant0.001$) (Figure 1a).

Enhanced urinary IP-10 mRNA gene expression prior to acute rejection

Because patients developing either an acute rejection episode classified as Banff I–III or BL rejection demonstrated elevated mRNA IP-10 expression, we further analyzed the predictive value of urinary IP-10 mRNA monitoring in these patients. Significantly upregulated urinary IP-10 mRNA levels could be detected at all time points for patients diagnosed as acute rejection (Banff I–III) and BL rejection compared to the reference level of non-rejecting patients (Mann–Whitney *U*-tests, $P<0.05$). Nevertheless, the receiver-operating characteristic (ROC) -curve analysis of IP-10 mRNA expression demonstrated very low sensitivities for all time-points. The results for 6/7, 4/5, and 2/3 days prior to acute rejection diagnosis are displayed in Figure 1b–d and in Table 1.

Elevated levels of IP-10 protein in urine sediment after kidney transplantation

We further analyzed the mean IP-10 protein expression and found significantly upregulated levels of IP-10 in urines from patients diagnosed with Banff I–III ($n=26$) or BL rejection episode ($n=15$) compared to controls ($n=41$) ($P<0.001$) confirming our previous observations made for IP-10 mRNA measurement. Patients with urinary tract infection or CMV infections showed no significantly elevated levels of urinary IP-10 (Figure 2a).

Elevated IP-10 protein concentration in urine is predictive of acute rejection

Because of insufficient sensitivity of IP-10 mRNA measurement of urine sediment, we analyzed whether IP-10 protein expression might be of higher statistical power. The ROC-curve analysis for the three chosen time points prior to biopsy were based on urine samples derived from patients diagnosed with Banff I–III or BL rejection and revealed that protein IP-10 levels were predictive of cellular acute rejection already 6/7 days before biopsy-proven diagnosis. This result corresponds with our observation made for mRNA expression. However, applying a cutoff value of about 200 pg/ml for all time-points, the highest sensitivity of 71% and specificity of 95% for urinary IP-10 measurement could be observed 2/3 days prior to biopsy (Figure 2b–d, Table 1). In general, mean serum Cr of acute rejection patients was constantly elevated at the indicated time points in contrast to mean IP-10 expression, which showed a strong dynamic regulation over time (Figure 3).

As IP-10 protein expression in urine revealed a higher sensitivity and specificity compared to IP-10 mRNA expression, we monitored urine samples derived from 35 patients with biopsy-proven cell-mediated acute rejection from our patient cohort for urinary IP-10 protein expression over 6 months post Tx in an individual case study. Twenty-seven patients revealed significantly elevated levels of IP-10 during

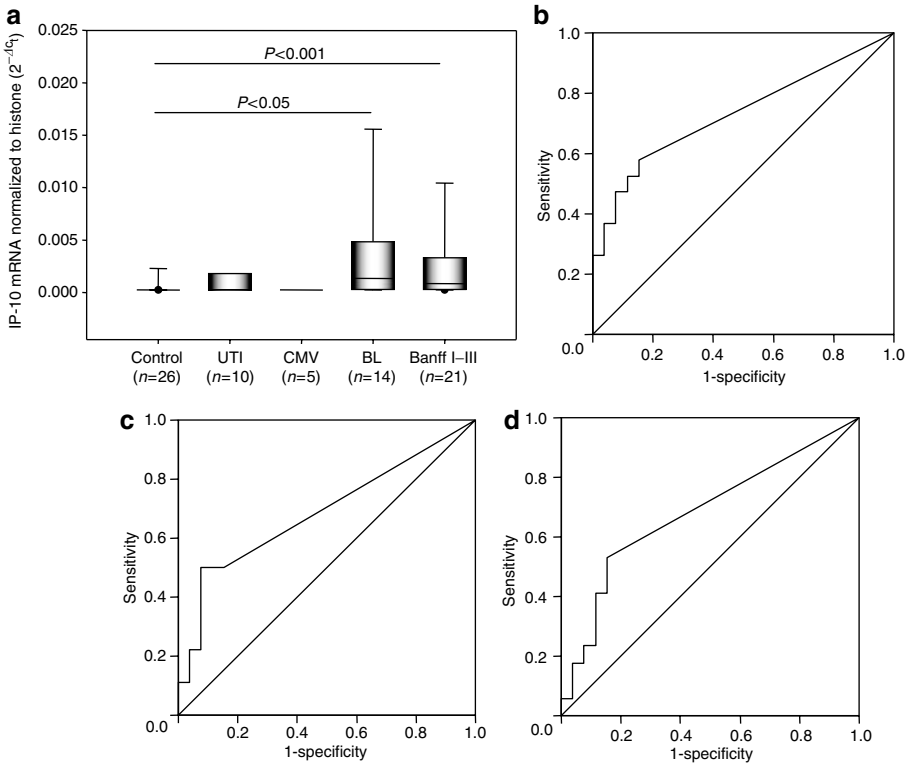

Figure 1 | IP-10 mRNA expression in urine sediment after kidney transplantation. (a) The mean level of IP-10 mRNA expression in urine sediment was significantly higher in patients with biopsy-proven rejection diagnosed as Banff I–III ($n = 21$; $P < 0.001$) or BL ($n = 14$; $P = 0.001$) compared to control patients with stable graft function ($n = 26$). There were no significant differences in IP-10 expression between control patients and patients with urinary tract infection (UTI) ($P > 0.05$) or CMV antigenemia ($P > 0.05$). (**b–d**) ROC curves of urinary mRNA IP-10 expression levels analyzed for patients diagnosed with Banff I–III/BL rejection demonstrating the sensitivity and specificity for various cutoff values for normalized IP-10 mRNA expression levels. Sensitivity defines true positive results and one-specificity true negative results for IP-10 mRNA in urine sediment as a predictive marker for acute rejection at the time points (**b**) 6/7 ($P < 0.05$), (**c**) 4/5 ($P < 0.05$), and (**d**) 2/3 ($P < 0.05$) days prior to biopsy-proven diagnosis of acute rejection. The mean control value was 0.0007 ($2^{-\Delta C_t}$).

Table 1 | Predictive properties of urinary IP-10 mRNA and protein for acute rejection

mRNA					Protein					
Time point (days prior to Rx)	*P*	Sens. (%)	Spec. (%)	AUC	Time point (days prior to Rx)	*P*	Sens. (%)	Spec. (%)	AUC	Cutoff (pg/m)
6/7	<0.05	58	85	0.731	6/7	<0.05	47	95	0.716	181.6
4/5	<0.05	50	85	0.686	4/5	<0.001	62	95	0.774	197
2/3	<0.05	53	85	0.681	2/3	<0.001	71	95	0.844	185

AUC, area under the curve; IP-10, interferon-inducible protein 10.

this observation period compared to the control group and 16 patients had significantly increased IP-10 protein concentrations in urine within a week before acute rejection was diagnosed by histology. The kinetics of IP-10 protein expression after Tx is shown for two representative patients in Figure 4. Patient No. 1 was diagnosed for acute rejection (Banff IIa) at day 17 post Tx (Figure 4a), and significantly increased levels of IP-10 protein were already observed at day 10, 13, and 15 post Tx. Antirejection therapy with methylprednisolone and treatment with OKT3 monoclonal antibody starting at day 17 and 21, respectively, lead to a marked reduction of serum Cr (<2 mg/dl) by day 29, but IP-10 protein levels were still detectable at day 22 (1924 pg/ml) and day 41 (949 pg/ml), suggesting persistent intragraft immune activation. The patient was again treated with methylprednisolone starting at day 44 post Tx, because of a persisting acute rejection episode revealed by biopsy analysis at day 48 (Banff IIa). At day 64 (1640 pg/ml), IP-10 protein expression remained elevated but at 115 and 178 days post Tx the patient displayed IP-10 levels within the range of the 95% confidence interval.

Patient No. 2 was diagnosed with an acute rejection episode (Banff III) at day 31 and at day 68 (Banff Ib) post Tx (Figure 4b). Serum Cr declined after surgery, remained under 2 mg/dl until day 26 (1.73 mg/dl) and started to rise at day 27 (2.72 mg/dl). Interestingly, IP-10 protein expression was already upregulated significantly prior to acute rejection diagnosis by histology at day 19 post Tx (824 pg/ml) and

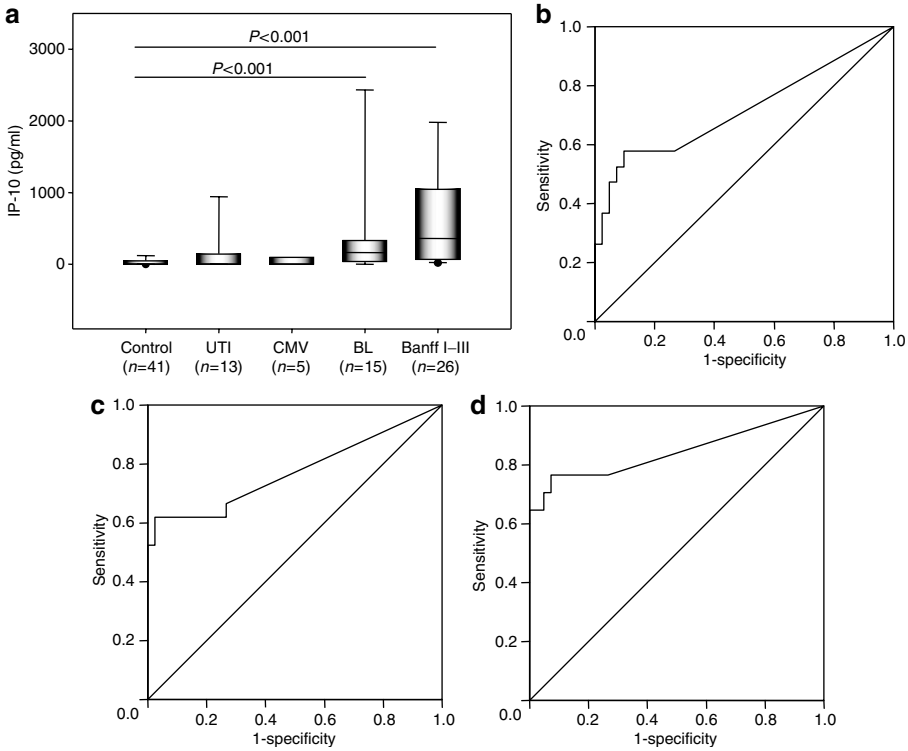

Figure 2 | IP-10 protein expression in urine after kidney transplantation. (a) The mean level of IP-10 protein expression in urine sediment of patients with biopsy-proven episodes of acute rejection were significantly higher in patients diagnosed with Banff I-III ($n = 26$; $P < 0.001$) or BL ($n = 15$; $P < 0.001$) compared to control patients with stable graft function ($n = 41$). There were no significant differences in IP-10 expression between control patients and patients with urinary tract infections ($P > 0.05$) or CMV antigenemia ($P > 0.05$). (**b–d**) ROC curves of urinary IP-10 protein expression levels analyzed for patients diagnosed with Banff I-III/BL rejection. Sensitivity defines true positive results and one-specificity true negative results for IP-10 protein in urine as a predictive marker for acute Rx at the time points (**b**) 6/7 ($P < 0.05$, cutoff 182 pg/ml, 47% sensitivity, 95% specificity), (**c**) 4/5 ($P < 0.001$, cutoff 197 pg/ml, 62% sensitivity, 95% specificity), (**d**) 2/3 ($P < 0.001$, cutoff 185 pg/ml, 71% sensitivity, 95% specificity) days prior to acute rejection diagnosis by pathohistological examination of the graft. Mean expression of controls was 31.8 pg/ml. UTI, urinary tract infection.

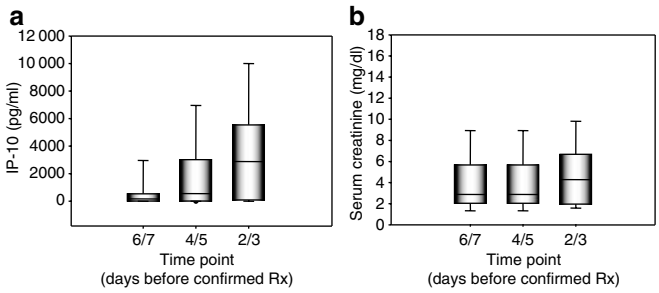

Figure 3 | Dynamics of serum Cr and urinary IP-10 protein concentrations prior to acute rejection. (**a**) IP-10 protein levels and (**b**) serum Cr levels in urine are shown at the time points 6/7, 4/5, and 2/3 days prior to acute rejection diagnosis by pathohistological examination of graft tissue ($n = 28$). Rx = rejection.

increased up to >10 000 pg/ml at day 28. After onset of antirejection treatment consisting of methylprednisolone and OKT3, serum Cr levels decreased. In contrast, IP-10 protein levels remained significantly elevated. A second biopsy on day 68 confirmed the persisting acute rejection episode, and repeated treatment with methylprednisolone and OKT3 lead to a rapid normalization of IP-10 protein expression and serum Cr (<2 mg/dl). Both patients No. 1 and No. 2 did not suffer from any urinary tract or CMV infections post Tx.

Elevated levels of IP-10 protein expression post Tx are associated with restricted graft outcome

To investigate whether elevated IP-10 protein expression in urine post Tx was predictive of graft function after 3 and 6 months, 67 patients were monitored for urinary IP-10 protein levels at six time points during the first month post Tx defined as 0–1, 2–7, 8–13, 14–19, 20–25, and 26–31 days post Tx and additionally once per month 2, 3, 4, 5, and 6 post Tx. The multivariate, nonparametric analysis of longitudinal data revealed a correlation between IP-10 concentrations in urine after Tx and the 6 month GFR, allowing further statistical investigations ($P < 0.05$). Especially, the mean IP-10 protein expression during the first month post Tx revealed significantly higher levels in urine of patients with a 6-month GFR <45 ml/min per 1.73 m^2 compared to patients with a 6 months GFR >45 ml/min per 1.73 m^2 ($P < 0.05$) (Figure 5a). The evaluation of the predictive value of mean urinary IP-10 expression during the first month post Tx for a 6 month GFR above or below 45 ml/min per 1.73 m^2 revealed a sensitivity of 58% and a specificity of 75% at a cutoff value of 196 pg/ml

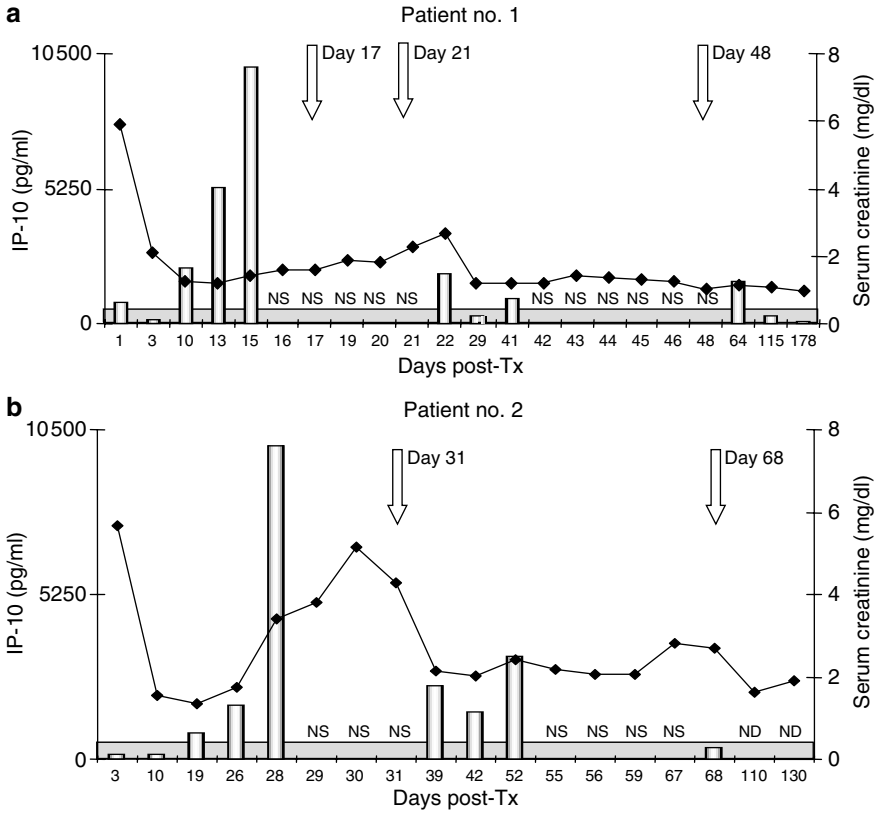

Figure 4 | Representative kinetic expression of IP-10 protein in urine. Arrows indicate time point of biopsy-proven acute rejection. A confidence interval was calculated based on the arithmetic mean of IP-10 expression in 292 urine samples from patients with an uncomplicated course. Columns represent IP-10 concentrations, the curve (◆) shows serum Cr levels. (**a**) Patient No. 1 was diagnosed for an acute rejection episode at day 17 post Tx (Banff IIa), which persisted and was proven again by biopsy histology post Tx at days 21 and 48. (**b**) Patient No. 2 experienced biopsy-proven acute rejection episodes at days 31, 68, and 168 (Banff III, Ib, and 1a, respectively); ns, no urinary sample available; nd, no detectable IP-10 levels.

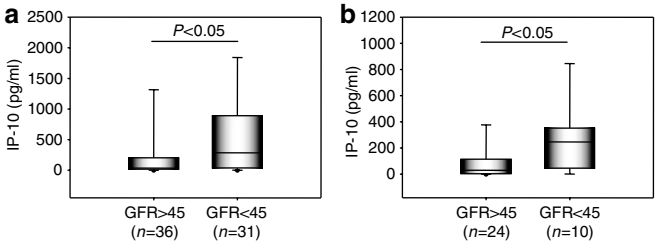

Figure 5 | Patients with an impaired 6 month graft function reflected by a GFR <45 ml/min per 1.73 m^2 demonstrate significantly higher mean values of urinary protein expression during the first month post Tx compared to patients displaying a GFR >45 ml/min per 1.73 m^2 independently of acute rejection. (**a**) Analysis of patients with rejection episodes (BL, Banff I–III) and stable graft function ($P<0.05$). (**b**) Separate analysis of patients without signs of rejection ($P<0.05$).

(ROC-curve not shown, area under the curve was 0.68). To investigate whether elevated levels of IP-10 during the first month post Tx correlated with poor graft function independently of acute rejection, IP-10 protein expression of the patient group without any signs of clinical rejection was analyzed separately. The analysis revealed a significant induction ($P<0.05$) of IP-10 levels in those patients with restricted kidney function after 6 months ($n=10$, GFR <45 ml/min per 1.73 m^2) compared to patients with a well-functioning graft ($n=24$, GFR >45 ml/min) (Figure 5b). In contrast, no significance could be estimated between elevated IP-10 levels during the first month and a 3 months GFR.

DISCUSSION

Although acute rejection episodes have decreased because of advanced clinical care and improved immunosuppressive therapies, the development of new non-invasive diagnostic strategies after renal Tx is still essential for a more individualized therapy to optimize cost/benefit/risk ratios. In particular, drug-weaning studies would benefit from early identification of patients at enhanced risk of clinical and subclinical rejection. Acute rejection episodes can lead to severe and irreversible graft injury and the gold standard for diagnosis is still the histological evaluation of graft tissue obtained through the invasive procedure of needle biopsy. Therefore, non-invasive monitoring of peripheral blood and urine could lead to further diagnostic information regarding the condition of the allograft at earlier time points and allow antirejection therapy to be adjusted at an early stage before

severe graft injury ensues. Chemokines participate in the inflammatory response within allografts and it has become clear that the interaction between the receptor CXCR3, which is expressed by Th1 cells, and its ligand IP-10, leads to the recruitment of activated T cells to sites of inflammation.[25–29] Previous experimental studies indicate that the CXCR3 ligand IP-10 plays an important role in acute cardiac allograft rejection[30] and several clinical studies indicate an upregulation of IP-10 gene[22,31] and protein[32] expression in renal biopsies with allograft dysfunction. Recently, two groups identified acute rejection processes after kidney transplantation non-invasively by measurement of IP-10 mRNA[5] and protein expression[4] in urine. Elevation of chemokine expression was correlated with the immunohistological findings in allograft biopsies.

Our data support the findings of Hu *et al.*[4] and Tatapudi *et al.*[5] that patients with acute rejection episodes have significantly elevated urinary IP-10 mRNA and protein expression compared to patients with stable graft function (Figures 1a and 2a). In addition, the data presented show that IP-10 gene and protein expression in urine of renal transplant recipients is upregulated at earlier time points before invasive needle biopsy is indicated by other clinical parameters like rising serum Cr levels, suggesting IP-10 as a sensitive marker for ongoing rejection (Rx) processes within the graft. The statistical analysis (Mann–Whitney *U*-test) revealed that patients with acute rejection episodes displayed significantly upregulated IP-10 mRNA expression in urinary sediment up to 7 days prior to biopsy compared to patients with stable graft function ($P<0.05$). Since ROC-curve analysis of IP-10 mRNA expression in urine displayed low sensitivities (Figure 1b–d, Table 1), we also calculated the predictive properties for IP-10 protein measurement prior to acute rejection and found higher sensitivities and specificities for a defined cutoff value of approximately 200 pg/ml (Figure 2b–d, Table 1). Interestingly, it would have even been possible to diagnose an ongoing rejection with a specificity of 100% several days before biopsy applying a higher cutoff value as displayed in Figure 2c and d. Mean IP-10 concentrations in urine of patients with developing acute rejection episodes were already higher 6/7 days prior to acute rejection compared to urine samples obtained from control patients but rose strongly over time in contrast to serum Cr levels, because of immune activity within the graft (Figure 3).

The prognostic attributes of IP-10 expression for acute rejection were further analyzed by performing individual kinetic analyses of IP-10 protein levels in urinary samples from patients with biopsy-proven acute rejection. This study was performed to illustrate the feasibility of the assay for individual monitoring in combination with other classical clinical parameters. The data revealed significantly elevated levels of IP-10 gene expression several days before histological diagnosis of acute rejection, sometimes despite normal serum Cr values. Onset of antirejection therapy, either with OKT3 monoclonal antibody or methylprednisolone led to a fast decrease of IP-10 expression (Figure 4a–b). The majority of samples from control patients with stable renal function contained no or low levels of IP-10 protein.

GFR is usually accepted as the best overall index for kidney function since Cr clearance is affected by factors like renal tubular secretion, which results in overestimation of graft function. We calculated the GFR for 3 and 6 months post Tx in 67 patients by the Cockroft–Gault equation[24] and hypothesized that elevated urinary IP-10 expression at early time points might be predictive of an impaired graft function after 3 and 6 months (GFR <45 ml/min per 1.73 m^2). Multivariate analysis of the obtained data derived from urine samples collected during the observation period confirmed only a correlation between elevated IP-10 protein levels in urine and a restricted graft function 6 months post Tx. In contrast, no correlation could be found between a 3 months GFR and IP-10 expression. We examined the mean IP-10 protein expression during the first month post Tx and found that IP-10 levels in urine of patients with a GFR <45 ml/min per 1.73 m^2 were significantly higher than in patients with a 6 month GFR >45 ml/min per 1.73 m^2 (Figure 5a). Moreover, elevated mean IP-10 levels during the first month post Tx were also predictive of impaired graft function even in the absence of acute rejection (Figure 5b).

We suggest that an early induction of IP-10 in the graft because of injury mediated by factors including prolonged cold ischemia or surgical procedure leads to the attraction of IFN-γ-producing CXCR3 + cells. The release of IFN-γ might result in enhanced IP-10 production, which increases the recruitment of activated leukocytes to the graft in a self-sustaining loop. Therefore, we hypothesize that induction of IP-10 mRNA and protein expression in urine could be an early predictor of inflammatory changes within the graft prior to acute rejection diagnosis and that it might be predictive of ongoing (subclinical) immune activation resulting in poor long-term graft function. This is supported by the observation that elevated levels of IP-10 were not related to the occurrence of urinary tract and CMV infections.

Measurement of protein expression provides more accurate evidence than mRNA expression analysis, while urine collection and determination of IP-10 protein expression is simple, fast, and therefore clinically applicable. We suggest that monitoring the IP-10 protein expression in urine of renal transplant recipients may lead to early individualized adjustment of immunosuppressive therapy and therefore reduces the incidence of severe graft damage. In addition, patients with enhanced IP-10 levels are less suitable to drug-weaning procedures, particularly calcineurin inhibitors-free protocols. To further evaluate this marker as a suitable candidate for acute rejection screening approaches, it might be of great interest to study IP-10 expression in other kidney transplantation-associated complications including drug toxicity or delayed graft function. Concerning the latter issue, preliminary results from our laboratory indicate the same predictive properties of urinary IP-10 protein expression for acute rejection independently of delayed graft

function (data not shown). Moreover, simultaneous non-invasive measurement of IP-10 with additional candidate markers at early time points could increase the diagnostic sensitivity and specificity of this approach leading to reduced frequencies of biopsy procedures. We also suggest that the prognostic properties of IP-10 make this molecule and its receptor CXCR3 candidate targets for therapeutic intervention with chemokine antagonists or receptor-blocking agents.

MATERIALS AND METHODS

Patients and sample collection

Adult renal transplant recipients were recruited from the Departments of Surgery and Nephrology, Virchow-Clinic, Universitätsmedizin Charité, Germany between October 2002 and May 2004 and provided informed consent. The study was approved by the local ethical committee. Urinary samples of 100 ml were collected three times/week/patient during hospitalization and twice/month from outpatients. Immunosuppression consisted of methylprednisolone and tacrolimus ± mycophenolate mofetil and most patients additionally received anti-interleukin-2 receptormonoclonal antibody induction. Histology was classified according to the Banff'97 criteria[33] and carried out by an experienced nephropathologist in blinded fashion. Confirmed acute rejection episodes were treated with methylprednisolone bolus or OKT3 monoclonal antibody. To investigate whether elevated IP-10 expression is predictive for acute cellular rejection, 54 patients were selected from our study cohort and analyzed retrospectively. Thirty patients were diagnosed with an acute cellular rejection (Banff I–III) and 24 patients who experienced a needle biopsy after rising serum Cr levels were diagnosed with BL rejection. They were also treated with methylprednisolone. Forty-two renal-transplanted patients with stable graft function served as controls. Patient demographics are summarized in Table 2.

Quantification of urinary mRNA gene expression

For mRNA expression analysis, urine was centrifuged at 10 000 r.p.m. and 4°C for 15 min. Pellets were washed in 1 ml phosphate-buffered saline and centrifuged at 14 000 r.p.m. for 7 min at 4°C. Supernatants were discarded and total RNA was isolated from the pellet using the Rneasy® Mini Kit (Qiagen, Hilden, Germany). Samples were tested for genomic DNA contamination and if tested positive were excluded from the study. cDNA synthesis and real-time reverse transcriptase-polymerase chain reaction of non-amplified mRNA were performed as recently described.[2] Gene expression of Histone mRNA was used for normalization given by the formula $2^{-\Delta C_t}$. All primers and probes were designed using Primer Express software (Applied Biosystems, Darmstadt, Germany).

Quantification of urinary IP-10 protein expression

For quantification of IP-10 protein expression, urine aliquots were stored at −20°C until measurement using an Enzyme-linked immunosorbent assay Kit (HyCult Biotechnology, Beutelsbach, Germany). For the individual kinetic studies, 302 samples from 35 patients with one or more acute rejection episodes were monitored for IP-10 protein during the first 6 months following kidney Tx and were compared to a confidence interval based on 292 control samples derived from 41 patients with stable renal function.

GFR

For calculation of the predictive value of IP-10 protein levels in urine for long-term function of the graft, the 3- and 6-month GFR was estimated by the Cockroft–Gault equation.[24] Samples were divided into two groups of 35/31 patients displaying a 3/6-month GFR of <45 ml/min per 1.73 m^2 and 32/36 patients having a 3/6-month GFR >45 ml/min per 1.73 m^2. Out of the 67 investigated patients, 33 suffered from a biopsy-proven cellular acute rejection. The summary of patients displaying a good or restricted kidney function reflected by a 3- and 6-month GFR $>/<45$ ml/min per 1.73 m^2 are summarized within Table 3.

Statistical analysis

Multivariate statistical analysis was performed using a Nonparametric Analysis of Longitudinal Data (SAS-Macro by Brunner, University Goettingen, Germany). ROC curves and Mann–Whitney *U*-tests were performed applying SPSS 12.0 for Windows. Values of $P<0.05$ were considered statistically significant. The calculation for the overall normalized mRNA ($2^{-\Delta C_t}$) and protein expression of IP-10 was based upon the mean values of analyzed urines/patient with acute rejection Banff I–III or BL rejection and one randomly chosen sample/control patient with stable allograft function (mRNA, $n=26$; protein, $n=41$). For evaluation of the predictive properties of elevated IP-10 levels in urine, ROC-curve analysis of the normalized IP-10 mRNA and protein expression data was performed at three different time points defined as 2/3, 4/5, or 6/7 days before confirmation of acute rejection by pathohistological analysis of biopsies. For IP-10 mRNA expression, 54 samples derived from 32 patients with acute rejection were grouped into the three time points specified above and compared to controls ($n=26$). The same set-up was chosen for IP-10 protein expression in urine analyzing 57 samples from 29 patients with acute rejection episodes and comparing them to controls ($n=41$).

Table 3 | Number of patients analyzed for IP-10 protein expression up to 3 and 6 months post Tx

	aRx (*n*=33)	w/o Rx (*n*=34)
GFR <45 ml/mi/1.73 m²		
3 months (*n*=35)	24	11
6 months (*n*=31)	21	10
GFR >45 ml/min/1.73 m²		
3 months (*n*=32)	9	23
6 months (*n*=36)	12	24

aRx, acute rejection episodes; glomerular filtration rate; IP-10, interferon-inducible protein 10; nRx, stable graft function.

Table 2 | Demographics of kidney transplanted recipients analyzed in the study

	Patients (*n*)	Male/female	Mean age (years)	Living/cadaveric donor	First transplant	Retransplant	aRx Banff'97 (grade)			
							BL	I	II	III
aRx	54	36/18	49 ± 15	7/47	48	6	24	18	11	1
nRx	42	19/23	54 ± 13	9/33	35	7				

aRx, acute rejection episodes; BL, borderline; nRx, stable graft function.

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