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Cytokine Profiles in Children After Pediatric Kidney Transplantation With Acute Cellular Compared to Chronic Antibody-mediated Rejection and Stable Patients: A Pilot Study

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Background . Different patterns of plasma cytokines can be expected in the case of chronic active-antibody-mediated (cAMR) and acute cellular rejection (AR) after kidney transplantation (KTx). **Methods** . IL-2, 4, 6, 10, 17A, tumor necrosis factor alpha, and interferon gamma were measured in 51 pediatric KTx recipients at time of renal biopsy (17 AR, 14 cAMR, 20 normal). Patients were divided into a training (n = 30) and a validation (n = 21) set. **Results.** IL-6 was significantly higher in AR patients and significantly lower in the case of cAMR. In children with s-creatinine increase, IL-6 values were significantly different between AR and cAMR. IL-10 levels showed similar tendencies. For IL-2, 4, 17A, tumor necrosis factor alpha, and interferon gamma, no differences were found. In the independent validation cohort, the receiver operating characteristic area under the curve for IL-6 was 0.79 and 0.70 for AR and cAMR. In children with AR, an IL-6 <1141 fg/ml, and in those with cAMR, an IL-6 >721 fg/ml was associated with a specificity of 86%/76%, a sensitivity of 71%/80%, a positive predictive value of 56%/45%, and a negative predictive value of 92%/94%. **Conclusions.** In this pilot study, the plasma IL-6 level is a promising biomarker to identify pediatric kidney transplant recipients free from AR and cAMR and might help to distinguish between both entities, whereas there is only a nonsignificant trend toward the usability of IL-10. Validation in larger cohorts in combination with other biomarkers are warranted.

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Acute rejection and chronic antibody-mediated rejection (cAMR) are 2 important causes of impaired graft function after kidney transplantation (KTx). Both are identified primarily by indication graft biopsies using the Banff classification,[1,2] and in combination with detection of donor-specific antibodies (DSAs) in plasma in the case of cAMR. The clinical relevance of rejection found on protocol biopsies (subclinical rejections) is still unclear,[3] as the Banff classification was not established for this purpose so that clinical consequences related to such findings remain a matter of debate.[4] Until now, there have been no available biomarkers as a substitute for kidney biopsies that can assess the relevance of subclinical acute rejections.

Cellular and humoral immune responses are important in allograft rejection.[2,5,6] T-cell homeostasis plays a major role in preventing acute rejection after KTx. A balance between T-helper (Th) 1, 2, and 17 cells (Th1, Th2, Th17) is a prerequisite for a stable post-KTx course.[7,8] B-cells primarily produce DSAs that cause chronic humoral rejection.[9] Cytokines mediate B- and T-cell activity. Differentiation of B cells is mediated by interleukin (IL)-7, whereas IL-4, IL-5, IL-6, Il-21, and interferon gamma (IFNγ), produced by Th-cells, activate B-cells.[10,11] The 2 cytokines IL-10 and IL-17 are principally produced by B cells.[8,10,12] IL-10 secreted by B-lymphocytes or plasma cells reduce T-cell activation and increase the number of regulatory T-cells (Treg), curtailing the ongoing immune response.[11] This IL-10 secretion is mainly attributed to regulatory B-cells that are stimulated by a B-cell activation factor.[13] It is associated with tumor necrosis factor alpha (TNFα) production in acute kidney rejection. A high IL-10/

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IFNγ- ratio is associated with normal Th1 cytokines, suppressed Th2 cytokines and poor graft survival.[14] Low levels of the proinflammatory cytokine IL-17 were associated with reduced expression of the Th1 cytokine IFNγ and less graft damage and better survival in a murine model of KTx.[15] In a pretransplant risk model, high soluble IL-17 levels were associated with a higher risk of future rejection; however, no measurements were taken at the time of rejection.[16] In kidney biopsies following acute rejection, IL-17 could be found as a marker of rejection.[17] In the case of inflammation, Treg can be converted into harmful Th17-producing cells. Treatment of inflammation can lead to TNFβ production and thereby a reswitch to Treg that protect the graft from immunological complications.[18] B cells also contribute to enhanced T-cell activation and differentiation, as well as formation of memory T cells by production of the cytokines IL-6 and TNFα.[11] It has been shown in experimental models that the proinflammatory cytokine IL-6 is upregulated in the case of acute rejection.[19] Of additional interest, plasma cells are supported by stromal cells secreting IL-6 in their surviving niches.[11]

Th1 cells mainly produce IFNγ, IL-2, and TNFβ and evoke cell-mediated immunity and phagocyte-dependent inflammation, whereas Th2 cells secrete IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13. Their activation leads to strong antibody responses and eosinophil accumulation but inhibits several functions of the phagocytic cells.[20] The "classical" Th1/Th2 paradigm in allograft response states that Th1 response (IL-2 and IFNγ) is associated with rejection, whereas the Th2 response is linked to the development of tolerance.[21,22] In adults, an increase in the Th1 cytokines IL-6 and IL-10 has been shown in the case of chronic cellular rejection, whereas IL-10 and IFNγ were increased in patients with acute rejection (as defined by Banff 2007 criteria). In those patients with stable graft function, IFNγ and Th2 cytokines were downregulated.[23] In pediatric liver transplantation, an association of increased IL-2 and decreased IFNγ was found in the cases of acute rejection.[24] Recently, it has been shown that in preactivation of endothelial cells with anti-HLA-DR antibody, allogenicity is redirected towards a pro-inflammatory response by decreasing amplification of functional Treg and by further increasing IL-6-dependent Th17 expansion.

According to these findings, it can be hypothesized that acute rejection with acute inflammation might be associated with higher levels of immune activating and proinflammatory cytokines, whereas cytokines that are expressed in protolerogenic states might be primarily found in stable patients. It was the intention of this trial to test this hypothesis in children after KTx.

MATERIALS AND METHODS

Patients

Fifty-one kidney transplant patients under the age of 18 years (21 females, mean age 13.0 ± 3.9 y) were classified into 3 groups according to graft function based on clinical symptoms, Banff classification of graft biopsy, and DSA analysis. Seventeen children were diagnosed with acute celluar rejection (AR), Banff 4, (8 females, mean age 13.0 ± 3.4 y) and 14 children with biopsy-proven chronic humoral rejection, Banff 2 (7 females; mean age 14.7 ± 2.9 y). The control group consisted of 20 children with baseline creatinine and normal protocol biopsy, Banff 1 or 6 (7 females, mean age 11.7 ± 4.5 y). The actual Banff classification based on definitions, Banff Lesion Scores, and Banff Diagnostic Categories[25] during time of biopsy was used in each case. Patients from each group were divided randomly 3:2 into a training (n = 30) and a validation (n = 21) set.

Exclusion criteria were symptoms of infections and severe illnesses as well as mixed Banff classifications (combination of cellular and humoral rejection). Patient characteristics are given in Table 1.

Plasma Samples

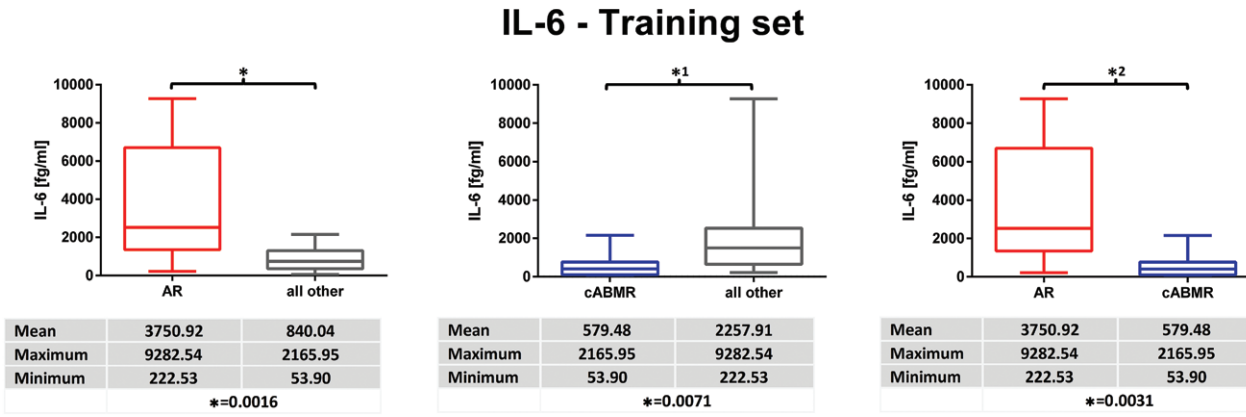
Whole blood samples were collected at a timepoint of renal protocol biopsy 6 months or a later annual control after KTx

TABLE 1.

Patient characteristics

	Control group (N = 20)	Acute rejection (N = 17)	Chronic antibody-mediated rejection (N = 14)
Mean Age (y)	11.7 ± 4.5	13.0 ± 3.4	13.9 ± 3.7
Age range	2–18	6–18	5–17
Sex (male/female)	13/7	9/7	7/7
Mean eGFR	65.2 ± 21.8	59.6 ± 27.9	43.5 ± 26.8
Range of eGFR	28.4–95.9	26.0–133.0	16,7–104.0
ø Serum creatinine (μmol/l)	91.2 ± 52.9	101.9 ± 30.8	116.4 ± 43.9
Range serum creatinine (μmol/l)	26.5–195.4	41.5–158.0	51.3–208.6
Mean Time (d) Transplantation – biopsy	1015	418	2379
ø BMI	19.4 ± 3.9	21.7 ± 6.9	20.7 ± 5.5
First transplantation	16	12	13
Second transplantation	3	5	1
Donor LD/DD	4/15	3/14	5/9
Mean level of IFTA on biopsy (ci/ct)	0/1	1/1	1/1
Presence of HLA DSA (timepoint of biopsy)			
Class I only (%)	0	0	1 (7.1)
Class II only (%)	1 (5.2)	5 (31.3)	8 (57.2)
Class I and II (%)	0	0	1 (7.1)
Non-HLA-DSA (ATII Receptor AB) (%)	0	0	4 (28.6)

BMI, body mass index; DD, deceased donor; DSAs, donor-specific antibodies; eGFR, estimated glomerular filtration rate; HLA, human leukocyte antigen; IFTA, interstitial fibrosis and tubular atrophy; LD, living donor.



	AR	all other
Mean	3750.92	840.04
Maximum	9282.54	2165.95
Minimum	222.53	53.90
	*=0.0016	

	cABMR	all other
Mean	579.48	2257.91
Maximum	2165.95	9282.54
Minimum	53.90	222.53
	*=0.0071	

	AR	cABMR
Mean	3750.92	579.48
Maximum	9282.54	2165.95
Minimum	222.53	53.90
	*=0.0031	

FIGURE 1. Plasma concentration of IL-6 of patients with long-term stable graft function (control), patients with acute rejection (acute), and patients with chronic antibody-mediated rejection (cAMR). Results are represented as median, minimum, and maximum concentration. AR, acute cellular rejection; IL, interleukin.

or during episodes of kidney transplant rejection. Samples were drawn by venipuncture in S-Monovette 7.5 mL LH (Sarstedt AG & Co. KG, Nümbrecht, Germany) and centrifuged immediately at 315 g for 10 min at room temperature. Lithium heparin plasma aliquots were stored at −80°C until required for flow cytometric cytokine measurements.

Acute rejection episodes (AREs) were categorized as follows: (1) biopsy-proven acute rejection (BPAR) Banff score ≥IA on indication biopsy; (2) BPAR including borderline findings on indication biopsy, triggering antirejection therapy; (3) overall treated ARE (BPAR plus ARE, where a graft biopsy was either logistically not possible or medically contraindicated, but where antirejection therapy was initiated).

Donor-specific Antibodies

Human leukocyte antigen (HLA) antibodies were measured before engraftment and at least annually posttransplant by the LABScreen single-antigen beads Luminex Kit (One Lambda, Canoga Park, CA) which uses single HLA-coated beads and enables identification of IgG alloantibody specificities against HLA-A, -B, -C, -DRB1/3/4/5, -DQA1, -DQB1, -DPA1, and -DPB1 antigens. Because no clinically validated cutoff for the Luminex assay is recommended by the provider company, a mean fluorescence intensity of ≥1 000 was used to define the cutoff for antibody positivity. For high-resolution typing, CTS-Sequence Kits (Heidelberg, Germany) and Olerup-SSP Kits (Olerup-SSP AB, Stockholm, Sweden) were used.

Estimated glomerular filtration rate was calculated using the complete 2009 Schwartz formula.[26]

Cytometric Bead Array Immunoassay

Because of IL-6 and IL-10 levels around the detection limit of the standard set, cytokine plasma levels, including IL-2, IL-4, IL-6, IL-10, IL-17A, TNFα, and IFNγ, were simultaneously quantified with the Human Th1/Th2/Th17 Cytokine Kit and additional Enhanced Sensitivity Flex Set IL6/IL10 (all BD Biosciences Pharmingen, San Diego, CA), according to the instruction manual. Data acquisition and analysis was performed with a FACSVerse flow cytometer, using FACSuite and FCAP Array software (BD Biosciences Pharmingen).

Statistical Analysis

Data were expressed as median and range for each group. The difference between groups was analyzed by the Wilcoxon two-sample test. All analysis, including the receiver operating characteristic (ROC) analysis were performed with GraphPad Prism 6. A $P \leq 0.05$ was considered statistically significant. In the ROC analysis, the patients were grouped as AR versus combined rejection (cAMR + controls) and cAMR versus combined rejection (AR + controls).

This study was approved by the ethics committee of Hannover Medical School (Number 2336-2014) and all families and patients gave informed consent.

RESULTS

Interleukin 6

In patients with AR Banff ≥IA (training set), IL-6 concentration (3 751 SD 3 214 versus 840 SD 645 [fg/ml], P = 0.0016) was significantly higher than in the other patients. In children with cAMR, IL-6 (579 SD 692 versus 2 258 SD 2 559 [fg/ml], P = 0.0071) was significantly lower. Among patients with increase in s-creatinine, IL-6 values (3 751 SD 3 214 versus 579 SD 692 [fg/ml], P = 0.0031) were significantly different between patients with AR and cAMR (Figure 1).

In the training cohort, AR showed an area under the ROC curve (AUC) for IL-6 of 0.84 (95% confidence interval [CI], 0.66-1.03, P = 0.002); for cAMR, the AUC for IL-6 was 0.81 (95% CI, 0.64-1.00, P = 0.16) (Figure 2). AR IL-6 <1631.0 fg/ml and cAMR IL-6 >901.7 fg/ml were associated with a specificity of 80%/88%, a sensitivity of 85%/64%, a positive predictive value of 52%/57%, and a negative predictive value of 96%/91% at 20% prevalence.

In the independent validation cohort, AR showed an AUC for IL-6 of 0.79 (95% CI, 0.55-1.02, P = 0.04); for cAMR, the AUC for IL-6 was 0.70 (95% CI, 0.42-0.98, P = 0.16) (Figure 3). AR IL-6 <1141.0 fg/ml and cAMR IL-6 >721.0 fg/ml were associated with a specificity of 86%/76%, a sensitivity of 71%/80%, a positive predictive value of 56%/45%, and a negative predictive value of 92%/94% at 20% prevalence.

Interleukin 10

Measurements of IL-10 concentration showed the same tendency as for the IL-6 results but no significant differences. Patients with AR Banff ≥IA (training set) showed higher IL-10 concentration (2 686 SD 5 598 versus 519 SD 965 [fg/ml], P = 0.05) than the other patients but not significantly. There

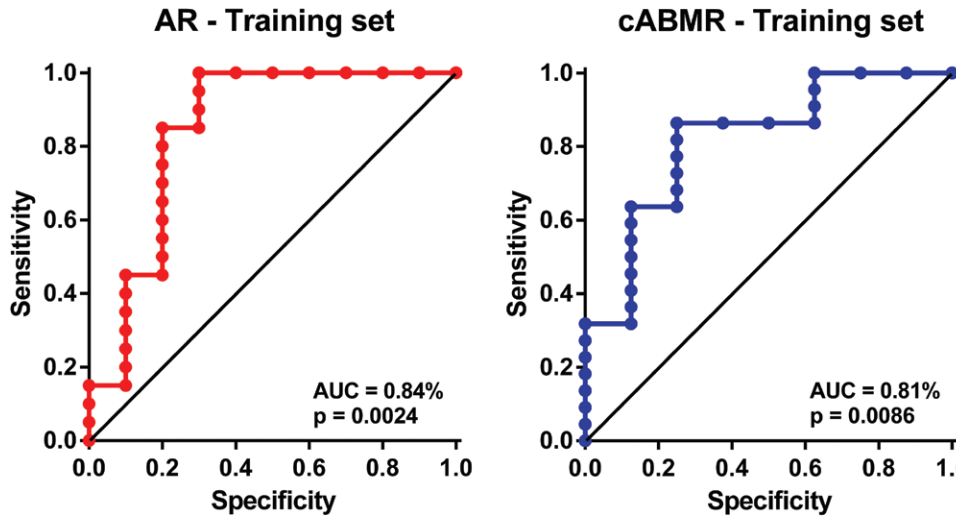


FIGURE 2. In the training cohort, (A) acute celluar rejection (AR) showed an area under the receiver operating characteristic (ROC) curve (AUC) for IL-6 of 0.84%. In cAMR (B), the ROC analysis indicates an AUC for IL-6 of 0.81%. The diagonal lines indicate random guessings associated with an AUC of 50%. cAMR, chronic antibody-mediated rejection; IL, interleukin.

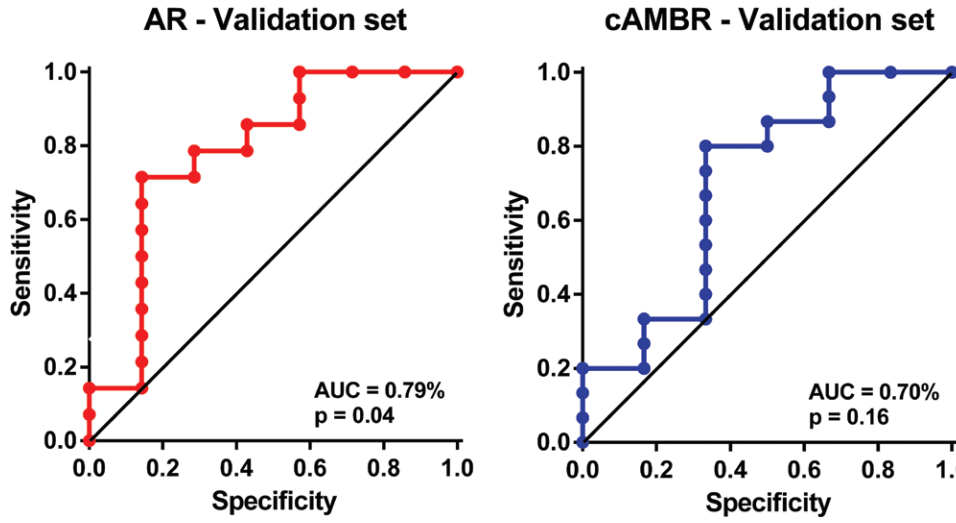
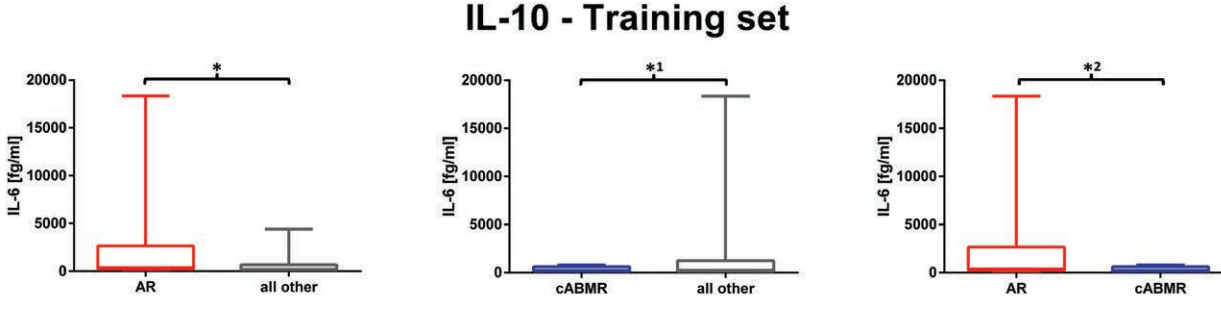


FIGURE 3. In the independent validation cohort, (A) acute celluar rejection (AR) showed an area under the receiver operating characteristic (ROC) curve (AUC) for IL-6 of 0.79%. In cAMR (B), the ROC analysis indicates an AUC for IL-6 of 0.70%. The diagonal lines indicate random guessings associated with an AUC of 50%. cAMR, chronic antibody-mediated rejection; IL, interleukin.



Mean	2686.35	519.45
Maximum	18344.71	4411.19
Minimum	107.85	0.00
	*=0.05	

Mean	330.77	1573.02
Maximum	785.76	18344.71
Minimum	103.75	0.00
	*=0.66	

Mean	2686.35	330.77
Maximum	18344.77	785.76
Minimum	107.85	103.75
	*=0.13	

FIGURE 4. Plasma concentration of IL-10 of patients with long-term stable graft function (control), patients with acute rejection (acute), and patients with chronic antibody-mediated rejection (cAMR). Results are represented as median, minimum, and maximum concentration. AR, acute cellular rejection; IL, interleukin.

was a nonsignificant trend for lower levels of IL-10 in children with cAMR than in other patients (331 SD 266 versus 1 573 SD 3 913 [fg/ml], $P = 0.66$). The same pattern could be seen between patients with increased s-creatinine. There was also a nonsignificant trend for lower levels of IL-10 values in patients with cAMR compared to AR (331 SD 266 versus 2 686 SD 5 598 [fg/ml], $P = 0.13$) (Figure 4).

Others

IL-2, IL-4, IL-17, TNFα, and IFNγ measurements were below the detection limit of the Human Th1/Th2/Th17 Cytokine Kit, and therefore, the results cannot be reported.

DISCUSSION

We were able to show that chronic humoral rejection and acute rejection are associated with different cytokine profiles in children. Most particularly, plasma IL-6 and partly IL-10 seem to be possible surrogate markers for rejection status. Because of their high negative predictive value, they might help to identify patients free from rejection.

This is especially interesting as the IL-6 antibody tocilizumab has proven to be an effective treatment for AMR.[27] The IL-6 pathway is active and high IL-6 production is associated with activation of Th17 cells and inhibition of Treg with attendant inflammation.[28] Il-6 drives B-cell activation and differentiation of B-cells to antibody-producing plasma cells. In AR, there is inflammation which leads to the upregulation of IL-6 production, with high levels measured as shown previously.[29] Chung et al[30] have shown that the Th17-L phenotype is increased in patients with chronic graft dysfunction. Serum levels of IL-17, IL-33 and receptor for advanced glycation end-products were increased but, interestingly, not IL-6 levels.[30] In AMR, it can be speculated that no acute systemic inflammation occurs but that both IL-6 and IL-17 primarily bind in the graft and are thereby reduced in the serum.

IL-6 is a pleiotropic cytokine with proinflammatory and anti-inflammatory properties and acts according to 2 different receptor pathways. In classic signaling, target cells are stimulated via IL-6, interacting with the membrane-bound IL-6 receptor (mIL-6R). The resulting IL-6/mIL-6R complex associates with the signaling receptor protein gp130 and activates an intracellular signaling cascade. Only a few cell types express mIL-6R, mainly hepatocytes, neutrophils, monocytes, and some leukocyte subpopulations, as well as some T- and B-cells. IL-6-trans-signaling acts via the soluble IL-6 receptor (sIL-6R). The soluble IL-6/sIL-6R complex can bind to gp130 on cells that lack the membrane-bound IL-6R. Membrane-bound gp130 is omnipresent and thus the spectrum of IL-6 target cells will be enlarged.[31]

In several experiments with mouse models of human disease, it could be shown that IL-6 classic signaling, activating STAT3, represents the anti-inflammatory or regenerative axis of IL-6, whereas IL-6 trans-signaling typifies the proinflammatory part of the IL-6 axis.[32-35] The IL-6/sIL-6R complex seems to promote the shift from acute to chronic inflammation via transition from neutrophil to mononuclear cell infiltrate and activation of the immune system.[36-38] Selective blocking of sIL-6R via sgp130Fc protein inhibits the proinflammatory but not the anti-inflammatory mIl-6R pathway. This could lead to new therapeutic options in chronic rejection after KTx despite tocilizumab therapy. The sgp130Fc was efficient in several preclinical models of inflammation (intestinal inflammation, rheumatoid arthritis, asthma, and inflammation-associated cancer) and initiated phase II clinical trials in patients with active ulcerative colitis.[39]

IL-6 plasma levels of healthy men varied between 0.9 and 30.6 pg/ml (mean 3.1 pg/ml) in line with their circadian rhythms.[40] This data support the finding of low IL-6 plasma in pediatric patients. In healthy individuals, approximately 30% of circulating IL-6 is free-floating in the blood and able to bind to mIL-6R. The larger part (~70%) is bound in IL-6/sIL-6R complexes. A mathematical model demonstrated that a 2-fold increase of sIL-6R results in a 43% decrease of free IL-6 concentration.[41] The finding of low plasma IL-6 in AMR does not implicate the absence of IL-6 production. In fact, circulating IL-6 could be bound to sIL-6R promoting the proinflammatory trans-signal pathway. The circulating IL-6/sIL-6R complex possibly conceals the effective amount of free circulating IL-6. To substantiate our data, future measurements of sIL-6R protein could further clarify the difference between acute and chronic renal rejection. Analyses of IL-8 could verify this additional approach, because IL-8 induces IL-6R shedding from neutrophils.[36,37]

On the one hand, as the measurement of IL-6 (and highly sensitive IL-6) is routine in many laboratories for diagnosis of acute inflammation, this test could easily be introduced in transplanted patients and might therefore be used as a routine surrogate marker for AMR or AR. On the other hand, it has to be taken into account that in the case of elevated values of IL-6 the clinician will have to differentiate between an inflammation because of AR and an impairment of graft function that has been caused by a viral or bacterial infection, eventually in combination with dehydration.

IL-10 is a cytokine with anti-inflammatory and immunomodulation properties. It influences the release of immune mediators, both antigen presentation and the phagocytosis of macrophages. This inhibits the release of proinflammatory mediators such as TNFα, IL-1β, IL-6, and others.[42] IL-10 increase can be induced by elevated IL-6 plasma levels.[43] This confirms our findings of high IL-6 plasma levels in correlation with high IL-10 plasma levels during acute rejection in our study cohort. In fact, IL-10 inhibits the release of proinflammatory mediators from monocytes and macrophages; it acts as a negative feedback loop on IL-6 secretion.[42] In IL-10 deficient mice,[44] as well as in diseases with a relative or absolute IL-10 deficiency (eg, rheumatoid arthritis[45] or after organ transplantation[46]), there is ongoing immune activation. It has been shown that the production of IL-10 is part of an autocrine pathway to reduce uncontrolled activation of IFNγ in Th1 cells. The inability to produce enough IL-10 is associated with an unregulated antidonor response and can be linked to a higher proportion of graft loss over time.[47] It could also be shown that IFNy/IL-10 ratios were higher in patients with AMR as compared with other patients after renal transplantation.[48] These findings correspond to our experience of decreased IL-10 levels in children with AMR.

Our study is limited by the relatively small number of pediatric patients included. As measurements were not performed at regular timepoints before biopsy, no prediction values for the markers for development of cellular or humoral rejection after early detection of changes in IL-6 or IL-10 before renal biopsy could be calculated.

Therefore, future longitudinal studies should evaluate whether IL-6 and IL-10 levels can differentiate earlier between patients with acute rejection and cAMR before renal function

decreases and indication biopsies are perfumed. Consequently, an increase in immunosuppressive therapy based on these biomarkers could be a possible intervention to prevent the full picture of clinical rejection. Second, further studies should determine if IL-6 decrease and IL-10 decrease in patients with cAMR can be detected before cAMR is diagnosed clinically. Renal biopsy could then be performed earlier, and interventions could lead to more timely intensification and change of immunosuppression or IL-6 antibody therapy and thus preserve the graft.

In conclusion, serum IL-6 and possibly IL-10 are promising biomarkers that might help the clinician to identify kidney graft recipients free from acute rejection and cAMR and to distinguish between both entities in the case of creatinine increase. However, this study only represents pilot data; therefore, future longitudinal studies in larger populations must confirm the potential of these 2 cytokines as diagnostic and possibly predictive markers of cellular and humoral rejections of kidney grafts to incorporate these markers in prediction models of rejection.

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