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ORIGINAL ARTICLE

Interleukin-17 positive cells accumulate in renal allografts during acute rejection and are independent predictors of worse graft outcome

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Summary

Interleukin-17 (IL-17) plays an important role in the regulation of cellular and humoral immune responses. Recent studies suggest a role for IL-17 in transplantation. Our study investigated whether quantifying IL-17$^+$ cells in renal transplant biopsies during acute rejection could have additional prognostic value for better stratifying patients at risk for nonresponsiveness to anti-rejection therapy and future graft dysfunction. Forty-nine renal biopsies with acute rejection were double immunostained and quantitatively analyzed for IL-17 and CD3 (IL-17$^+$ T-lymphocytes), tryptase (IL-17$^+$ mast cells) or CD15 (IL-17$^+$ neutrophils). Total IL-17$^+$ cell count correlated with total percentage of inflamed biopsy and estimated GFR during rejection. Most IL-17$^+$ cells were mast cells and neutrophils. We could hardly find any IL-17$^+$ T-lymphocytes. IL-17$^+$ mast cells correlated with interstitial fibrosis/tubular atrophy (IF/TA). None of the IL-17$^+$ cell counts had an additional prognostic value for response to anti-rejection treatment. Multivariate analysis correcting for C4d positivity and time from transplantation to biopsy showed that total IL-17$^+$ cell count independently predicts graft dysfunction at the last follow-up, which was validated in an independent cohort of 48 renal biopsies with acute rejection. We conclude that intragraft IL-17$^+$ cell count during acute allograft rejection could have an additional value for predicting late graft dysfunction.

Introduction

With the introduction of potent anti-rejection therapy, there has been a progressive improvement in short-term renal transplant survival. The occurrence of an acute rejection episode, however, still remains a major determinant of late allograft dysfunction. In particular the time point, severity and sensitiveness to anti-rejection therapy determines transplant outcome. Data on intragraft markers that predict responsiveness to conventional steroid-based anti-rejection therapy and late allograft outcome are scarce.

Cellular infiltrates are the hallmark of acute rejection. Immunophenotypic characterization of intragraft infiltrates has generated conflicting results, with various studies reporting the predominance of CD4$^+$ T-lymphocytes, CD8$^+$ T-lymphocytes or macrophages. Acute allograft

rejection was initially thought to be induced by T_h1-mediated cytotoxicity and T_h2 cytokines would blunt the severity of graft rejection by inhibiting the T_h1 response [1]. The identification of additional T-cell subtypes has led to an even more intricate picture of effector mechanisms that mediate rejection. One of these subtypes is the interleukin-17 producing T-helper lymphocyte (T_h17 lymphocyte). Interleukin-17 is member of a cytokine family, which consists of six subtypes (named IL-17A-F) of which IL-17A and IL-17F are the most studied and are considered to be the key subtypes produced by T_h17 cells. However, IL-17 production is not restricted to T_h17 lymphocytes; also $CD8^+$ T-cells, γδ T-cells, neutrophils and macrophages are able to produce IL-17A and IL-17F [2–6]. Mast cells are able to produce IL-17E and IL-17F, but not IL-17A [7,8].

Previous studies have shown that IL-17 cytokines are potent inducers of organ-specific autoimmunity, allergy and defense against microbial infections via mobilization of innate immunity [9,10]. In the kidney, IL-17 stimulates tubular epithelial cells to produce high levels of IL-6, IL-8, monocyte chemotactic protein-1 (MCP-1) and complement component C3. IL-17 is present at cellular and molecular levels during acute rejection of renal allografts [11] and increased levels of IL-17 mRNA were found in lung [12], liver [13] and cardiac transplants [14].

The current study investigated whether intragraft $IL\text{-}17^+$ inflammatory infiltrate during an episode of acute rejection could have additional prognostic value over currently assessed intragraft and clinical surrogate markers of renal allograft outcome.

Patients and methods

Renal allograft recipients

The current study included three cohorts: a test cohort and a validation cohort consisting of 49 and 48 acute renal allograft rejection biopsies respectively and a third control cohort consisting of 10 protocol renal biopsies. Maintenance immunosuppressive regimens included prednisolone, mycophenolic acid and a calcineurin inhibitor. Written informed consent was obtained from all patients and the study was approved by the medical ethics committee of the Academic Medical Center.

Immunohistochemical stainings

De Boer *et al.* extensively validated the immunohistochemical stainings for IL-17 and we used the same staining protocols [8,15]. The biopsy material was formalin-fixed and subsequently paraffin-embedded. All available biopsies of the test cohort were double immunostained with IL-17 and CD3, tryptase or CD15 in a sequential manner. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol for 20 min. After heat-induced epitope retrieval (HIER) with Tris-EDTA pH 9.0 for 20 min at 98°C, sections were incubated with goat anti-human IL-17 primary antibody (R&D Systems, Minneapolis, MN, USA), followed by a rabbit anti-goat immunoglobulin (Ig) (RAG, DAKO, Glostrup, Denmark) and an alkaline phophatase (AP)-conjugated anti-rabbit Ig polymer (Immunologic, Duiven, The Netherlands). According to the supplier, the R&D polyclonal antibody raised against IL-17A cross-reacts with IL-17F (IL-17A and IL-17F share 50% sequence homology). AP activity was visualized in blue using Vector Blue (Vector Labs, Burlingame, CA, USA). A second HIER step was applied (10 min, Tris-EDTA pH 9.0, at 98°C) to remove the antibodies from the first staining sequence, but leaving the deposits of blue reaction product unchanged. Then, either one of the following antibodies was applied: monoclonal rabbit anti-human CD3 for T cells (Neomarkers, Fremont, USA), monoclonal mouse anti-human CD15 for neutrophils (Immunologic, Duiven, The Netherlands) and AP-conjugated monoclonal mouse anti-human tryptase for mast cells (Chemicon, Temecula, USA). Except for the directly AP-conjugated anti-tryptase antibody, the second step was either an appropriate anti-rabbit or anti-mouse immunoglobulin AP-conjugated polymer. Secondary AP activity was visualized in red using Vector Red (Vector Labs, Burlingame, CA, USA). Paraffine sections of inflamed tonsillar tissue were used as positive controls and renal biopsy material without incubation with IL-17 antibody was used as negative control.

Histopathological assessment and immunophenotypical quantification

$IL\text{-}17^+$ cells, $CD3^+/IL\text{-}17^+$ cells (T_h17-cells), $tryptase^+/IL\text{-}17^+$ cells (IL-17 producing mast cells) and $CD15^+/IL\text{-}17^+$ cells (IL-17 producing neutrophils) were counted per high power field. Representative photographs are shown in Fig. 1. All biopsies were scored according to the Banff 2009 update [16] by two observers in a simultaneous manner. As described in the Banff 97 update [17], the threshold for a minimal adequate specimen is seven glomeruli and one artery. Only the biopsies that met these criteria were included. The whole cortex was examined except for the immediate subcapsular cortex with a width of 0.5 mm. Data on C4d were already available from previous studies [18,19].

Outcome measures

We used three binary outcome measures. The first outcome measure was response to therapy and was defined

as a decrease in serum creatinine level within 2 weeks after the start of anti-rejection medication to a maximum of 125% of the value before the diagnosed episode of rejection. The same definition of response to therapy has been proposed earlier by Gaber *et al.* [20] and was also used by Haas *et al.* [21] and in three recent publications of our group [18,19,22]. The baseline creatinine value was defined as the lowest creatinine value before the rise in creatinine. The glomerular filtration rate (eGFR) was estimated with use of the CKD-EPI formula [23]. The second and third outcome measures concerned late graft dysfunction: an eGFR of <30 ml/min/1.73 m^2 at the last follow-up and return to dialysis (eGFR of <15 ml/min/1.73 m^2).

Statistical analyses

Cell counts showed a skewed distribution and therefore nonparametric Spearman's rank tests for continuous variables, Kruskal–Wallis rank tests for k-independent variables and Mann–Whitney rank tests for binary variables were used to address associations between cell counts and clinical or histopathological parameters. All significance levels underwent post-hoc Holm-Bonferroni correction. A Mann–Whitney rank test was used to address the relation between IL-17^+ cell count and response to anti-rejection therapy. Parameters involved in transplant dysfunction at the last follow-up were tested with univariate Cox proportional hazard models. Only parameters that significantly associated with transplant dysfunction at the last follow-up were included in the multivariate Cox proportional hazard model ('cph' function in 'rms' package). Parameters that were significant in univariate analyses for <30 ml/min/1.73 m^2 at last follow-up and return to dialysis were included for multivariate analyses. Internal validation was performed on all Cox models via bootstrapping (150 iterations) ('validate.cph' function in 'rms' package). The degree of overfitting is addressed as percentage of slope shrinkage after 150 bootstrap samples were taken. Proportional hazard assumption was validated ('cox.zph' function in 'survival' package). The area under the receiver operating characteristic curve was determined to summarize the discriminative ability of the test ('roc' function in 'pROC' package). The point on the curve with the highest combined sensitivity and specificity (plus their 95% confidence intervals) was used as cut-off value for cross-validation in an independent cohort. Kaplan–Meier estimates with their corresponding 95% confidence intervals were plotted with the 'survplot' function in the 'rms' package. A *P*-value of 0.05 was chosen as statistical threshold. All analyses were performed with SPSS version 18.0 for Macintosh (SPSS Inc. Chicago, IL, USA) and R for Macintosh (http://www.r-project.org).

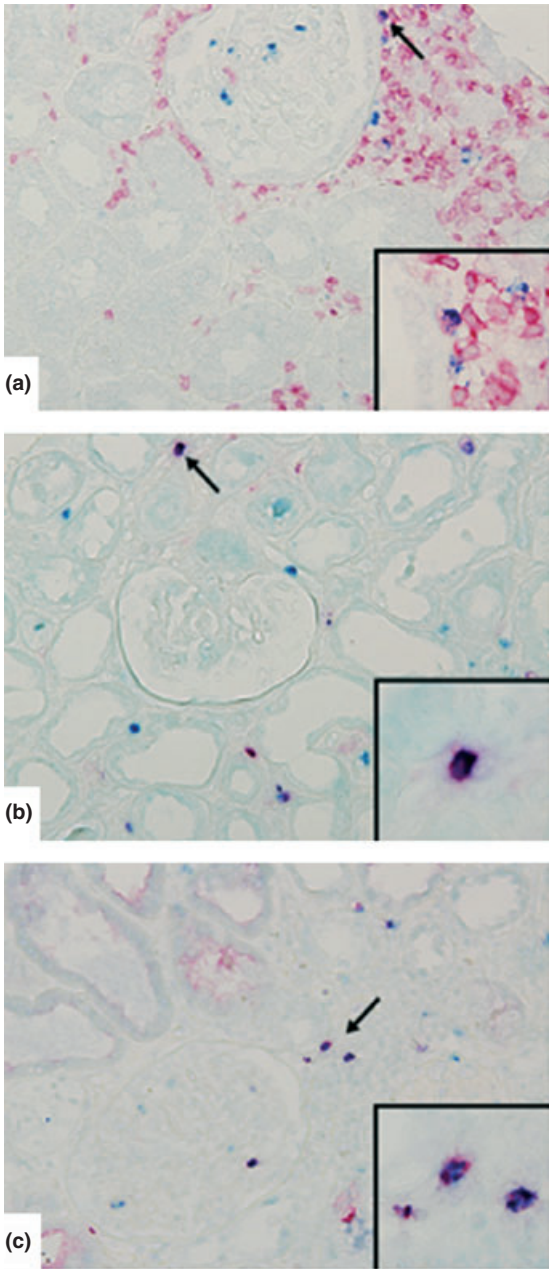

Figure 1 Representative photographs of immunohistochemical double stainings for IL-17 with CD3, tryptase or CD15. (a) Double staining for IL-17A/F in blue and CD3 (T-lymphocytes) in red. The black arrow plus the insert indicate the rarely observed $CD3^+$/IL-17^+ cells (T_h17 lymphocytes). Original magnification ×20, insert ×40. (b) Double staining for IL-17A/F in blue and tryptase (mast cells) in red. The black arrow plus the insert indicate the $tryptase^+$/IL-17^+ cells. Original magnification ×20, insert ×40. (c) Double staining for IL-17A/F in blue and CD15 (neutrophils) in red. The black arrow plus the insert indicate the $CD15^+$/IL-17^+ cells. Original magnification ×20, insert ×40.

Results

Demographic, clinical and histological characteristics of the patients

Table 1 shows the demographic and clinical characteristics of the cohorts. The test and validation cohort differed in some aspects. Pediatric patients were not included in the validation cohort ($P < 0.001$), more HLA mismatches were present in the validation cohort ($P = 0.007$) and more patients in the validation cohort had worse outcomes compared with the test cohort, but this was not statistically significant. Information on the IL-17 low and IL-17 high groups is additionally provided (cut-off at 4 IL-17$^+$ cells/HPF; generation of these groups is described below). Patients in the IL-17 low group showed a tendency to be less frequently presensitized ($P = 0.053$). A prolonged cold ischemia time was significantly more often observed in the IL-17 high group ($P = 0.029$). Ten rejection episodes were treated with plasmapheresis, which were all IL-17 high ($P = 0.001$). The eGFR at time of biopsy was significantly lower in the IL-17 high group (11 ml/min difference compared with the IL-17 low group, $P = 0.007$). C4d positive biopsies were often IL-17 high as well ($P = 0.007$).

Distribution of IL-17$^+$ cell counts over the Banff rejection grades

The distribution of IL-17$^+$ cell counts over the different rejection grades is displayed in Table 2. No significant

Table 1. Characteristics of the included transplant recipients.

Patient characteristics	Protocol Bx (*N* = 10)	Test cohort (*N* = 49)	Validation cohort (*N* = 48)	*P*‡	IL-17 low* (*N* = 48)	IL-17 high† (*N* = 49)	*P*§
Age recipient at biopsy, median years (range)	53 (25–72)	48 (8–68)	45 (18–69)	0.762	46 (10–65)	45 (8–69)	0.854
Recipient gender, no. of males (%)	5 (50)	21 (43)	30 (63)	0.053	26 (54)	25 (51)	0.756
Pediatric patients, no. (%)	0 (0)	8 (16)	0 (0)	0.003	5 (10)	3 (6)	0.442
Donor age, median years (range)	53 (15–67)	45 (13–65)	51 (7–66)	0.189	51 (15–66)	45 (7–66)	0.276
Living donor, no. (%)	4 (40)	10 (20)	14 (29)	0.318	15 (31)	9 (18)	0.142
Donation after cardiac death	3 (30)	13 (27)	11 (23)	0.680	11 (23)	13 (27)	0.680
Organ perfusion support	3 (30)	26 (53)	23 (48)	0.612	22 (46)	27 (55)	0.361
HLA mismatches, median (range)	3 (0–6)	2 (0–6)	3 (0–6)	0.007	3 (0–6)	3 (0–6)	0.724
0–1 mismatches, no. (%)	2 (20)	16 (33)	6 (13)	0.039††	11 (23)	11 (23)	0.940††
2–4 mismatches, no. (%)	7 (70)	28 (57)	32 (67)		29 (60)	31 (63)	
5–6 mismatches, no. (%)	1 (10)	5 (10)	10 (21)		8 (17)	7 (14)	
Pretransplant sensitized, no. (%)¶	0 (0)	2 (4)	5 (11)**	0.184	6 (13)	1 (2)	0.053
Time of biopsy post-transplantation, median days (range)	214 (185–277)	21 (5–2944)	35 (6–4069)	0.905	33 (6–2509)	20 (5–4069)	0.773
Cold ischemia time, median minutes (range)	925 (155–1680)	1140 (120–2078)	1020 (82–3020)	0.281	930 (120–2078)	1179 (82–3020)	0.029
Delayed graft function, no. (%)	0 (0)	15 (31)	18 (38)	0.474	15 (31)	18 (37)	0.569
Follow-up time from biopsy, median days (range)	1586 (136–3044)	900 (1–2891)	935 (21–3801)	0.419	1254 (28–3639)	556 (1–3801)	0.003
Second transplantation, no. (%)	0 (0)	10 (20)	13 (27)	0.440	11 (23)	12 (24)	0.855
ATG treatment, no. (%)	NA	7 (14)	13 (27)	0.119	8 (17)	12 (24)	0.341
Plasmapheresis, no. (%)	NA	3 (6)	7 (15)	0.171	0 (0)	10 (20)	0.001
eGFR at biopsy, median (range)	48 (28–65)	20 (4–97)	17 (4–54)	0.697	24 (4–97)	13 (4–77)	0.007
eGFR <30 ml/min at last follow-up, no. (%)	0 (0)	25 (51)	32 (67)	0.118	18 (38)	39 (80)	<0.001
Return to dialysis, no. (%)	0 (0)	16 (33)	21 (44)	0.261	9 (19)	28 (57)	0.007
C4d positive acute rejections, no. (%)	NA	8 (16)	8 (17)	0.964	3 (6)	13 (27)	0.007

HLA, human leukocyte antigen; ATG, anti-thymocyte globuline; NA, not available.

*≤4 IL-17$^+$ cells per high power field.

†>4 IL-17$^+$ cells per high power field.

‡Test cohort versus validation cohort.

§IL-17 low versus IL-17 high group (test and validation cohort combined, the control groups was excluded from analysis).

¶Patients were considered sensitized when they had >50% pretransplant panel-reactive antibodies.

**Four patients had missing data concerning pretransplantation panel-reactive antibodies.

††Comparing all HLA mismatch groups with χ^2 test.

Table 2. Distribution of IL-17$^+$ cells over the different Banff rejection classifications.

	TCMR IA		TCMR IB		TCMR IIA		TCMR IIB		TCMR III		*P*
Test cohort	*N* = 29	3 (1–27)	*N* = 5	6 (2–13)	*N* = 8	5 (3–8)	*N* = 7	4 (0–17)	*N* = 0		NS
Validation cohort	*N* = 24	4 (1–18)	*N* = 8	7 (2–26)	*N* = 9	3 (1–28)	*N* = 5	11 (3–22)	*N* = 2	12 (2–22)	NS

TCMR, T cell-mediated rejection; NS, not significant.
All values are expressed as median (range) cell count per high power field.

differences between the groups were observed. Eight biopsies in both the test and the validation cohort were C4d$^+$ (T cell-mediated rejection (TCMR) and antibody-mediated rejection grade II).

Correlations of IL-17$^+$ infiltrates during acute rejection with Banff scores and clinical parameters

Figures 1a–c are representative photographs of immunohistochemical double stainings for IL-17 with CD3, tryptase or CD15 respectively. A median of 4 IL-17$^+$ cells per HPF was observed during rejection (range = 0–27 cells/HPF). The rejection cohorts had significantly higher levels of IL-17$^+$ cells compared with the protocol biopsies (Fig. 2). IL-17$^+$ cell count was excellent in discriminating rejection from nonrejection (AUC = 0.933; 95% CI = 0.881–0.985). The total IL-17$^+$ infiltrate correlated with the Banff 'i' and 'ti' score. An inverse relation was observed with the eGFR at time of biopsy. We further characterized the IL-17$^+$ infiltrate to investigate whether one particular cell type was responsible for the observed associations. CD3$^+$/IL-17$^+$ lymphocytes were rarely observed during rejection (maximum of 1 cell per total biopsy surface). Therefore, statistical analyses were not performed on this cell type. The majority (76%) of mast cells (tryptase$^+$) were also IL-17$^+$ and they represented 25% of total IL-17$^+$ cells. A median of 1 tryptase$^+$/IL-17$^+$ cell per HPF was seen (range = 0–8 cells/HPF) and the cell count was correlated to interstitial fibrosis/tubular atrophy (IF/TA). The majority of IL-17$^+$ cells were neutrophils (68%) and 91% of all neutrophils was IL-17$^+$ (median of 3 cells per HPF, range = 0–22 cells/HPF). IL-17$^+$ neutrophils did not correlate with Banff scores or eGFR at time of biopsy. The correlations between IL-17$^+$ cell counts and the various parameters are listed in Table 3.

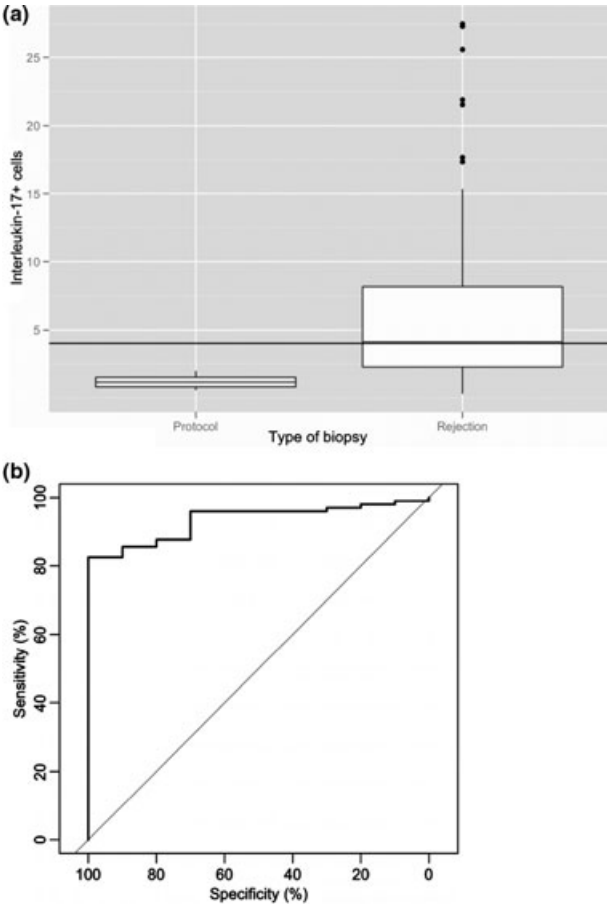

Figure 2 Discrimination between rejection biopsies and protocol biopsies by interleukin-17 cell count. (a) Boxplot of IL-17$^+$ cell counts in rejection biopsies versus protocol biopsies ($P < 0.001$). The horizontal line represents the cut-off value of 4 IL-17$^+$ cells/HPF, which associates with an adverse outcome. None of the protocol biopsies showed IL-17$^+$ cell counts above this threshold. (b) Area under the receiver operating characteristic curve analysis for the diagnosis of acute rejection. AUC = 0.933, 95% confidence interval = 0.8814–0.9846.

Prediction of response to anti-rejection therapy

We assessed whether IL-17$^+$ cell count could have a predictive role for response to anti-rejection therapy. Mann–Whitney testing did not show an association between IL-17$^+$ cells and response to anti-rejection therapy (data not shown). Further sub-analyses were therefore not performed.

Prediction of late graft dysfunction by IL-17$^+$ cells

Univariate Cox proportional hazard models were fitted for late graft dysfunction (Table 4). Time from transplantation to biopsy, total IL-17$^+$ cell count and C4d positivity were significantly associated with both outcome measures of late graft dysfunction. The hazard coefficients for IL-17$^+$ cells, C4d positivity and time from

Table 3. Correlation of IL-17$^+$ cell types with the Banff scores and clinical parameters.

	All IL-17$^+$ cells (N = 49)	Tryptase$^+$/IL-17$^+$ cells (N = 46)	CD15$^+$/IL-17$^+$ cells (N = 29)
Banff t	NS	NS	NS
Banff i	0.48**	NS	NS
Banff g	NS	NS	NS
Banff v	NS	NS	NS
Banff ti†	0.54**	0.40*	NS
IF/TA	NS	0.66**	NS
C4d positive‡	NS	NS	NS
FOXP3$^+$ cell count	NS	NS	NS
eGFR at Bx	–0.47**	NS	NS
Proteinuria at Bx§	NS	NS	NS

IF/TA, interstitial fibrosis/tubular atrophy; C4d, complement component 4d; eGFR, estimated glomerular filtration rate (CKD-EPI formula); FOXP3, forkhead box P3; Bx, biopsy; NS, not significant.
*P < 0.01; **P < 0.001.
†Represented as a continuous variable.
‡Cut-off at 10% positivity (paraffin sections), Mann–Whitney signed rank tests.
§Grams per 24 h.

transplantation to biopsy were all proportional over time (all $P > 0.700$). The multivariate Cox model including all significant parameters showed that total IL-17$^+$ cell count was independently associated with a decreased renal function at last follow-up ($P = 0.0007$) and return to dialysis ($P = 0.0027$) (Table 5). Both multivariate models showed a good overall fit after the bootstrapping procedure (eGFR <30 ml/min/1.73 m^2: P-value χ^2_{Wald} = 0.0012, 24% optimism and return to dialysis: P-value χ^2_{Wald} = 0.0012, 29% optimism). The hazard coefficients of the multivariate Cox model for <30 ml/min/1.73 m^2 and for return to dialysis were proportionally distributed (both $P > 0.600$). The area under the receiver operator characteristic curve (AUROC) of an eGFR <30 ml/min/1.73 m^2 at last follow-up was 0.7517 (95% CI = 0.6105–0.8929) and for return to dialysis 0.7557 (95% CI = 0.5909–0.9205) (Fig. 3a and c). The threshold with the most optimal combined sensitivity and specificity for both late outcome measures was 4 IL-17$^+$ cells per HPF (median of 75–76% sensitivity and 67–75% specificity). We validated the prediction model as obtained from the test cohort in an independent validation cohort. The

Table 4. Univariate Cox proportional hazard regression analyses for late graft dysfunction.

	eGFR <30 ml/min at last follow-up				Return to dialysis			
	HR	95% CI	D_{xy}*	P	HR	95% CI	D_{xy}*	P
Time from Tx to Bx	1.09	1.00–1.18	0.0809	0.0432	1.12	1.04–1.22	0.0068	0.0054
Age recipient at Bx				NS				NS
Gender recipient, male				NS				NS
eGFR at Bx				NS				NS
Proteinuria at Bx				NS				NS
Banff tubulitis (t)				NS				NS
Banff inflammation (i)				NS				NS
Banff endothelialitis (v)				NS				NS
Banff glomerulitis (g)				NS				NS
Banff total inflammation (ti)				NS	4.26	1.24–14.58	–0.5112	0.0211
Banff IF/TA				NS				NS
IL-17$^+$ cells	1.62	1.21–2.17	–0.4079	0.0013	1.65	1.15–2.37	–0.4704	0.0061
C4d$^+$	3.01	1.23–7.35	–0.1756	0.0154	4.43	1.56–12.59	–0.2632	0.0052

eGFR, estimated glomerular filtration rate; HR, hazard ratio; CI, confidence interval; NS, not significant; IF/TA, interstitial fibrosis and tubular atrophy; Tx, transplantation; Bx, index biopsy.
Corrected Somers' D_{xy} after 150 bootstrap samples [2(AUROC – 0.5)].

Table 5. Multivariate Cox proportional hazard regression analysis for late graft dysfunction.

	eGFR <30 ml/min at last follow-up			Return to dialysis		
	HR	95% CI	P	HR	95% CI	P
Time from Tx to Bx	1.05	0.95–1.18	0.3399	1.08	0.97–1.21	0.1673
IL-17$^+$ cells	1.72	1.26–2.35	0.0007	1.83	1.23–2.72	0.0027
C4d$^+$	2.38	0.78–7.30	0.1287	3.04	0.80–11.54	0.1021

eGFR, estimated glomerular filtration rate; HR, hazard ratio; CI, confidence interval; Tx, transplantation; Bx, index biopsy.

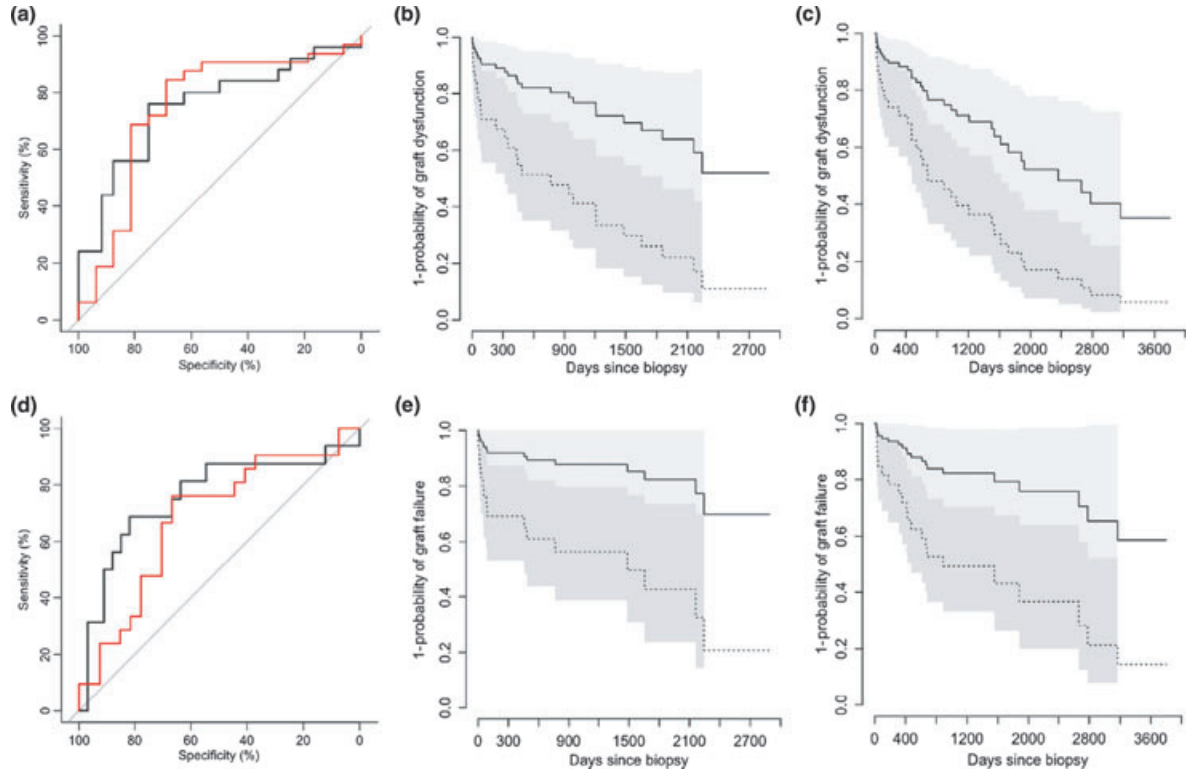

Figure 3 Analyses for the predictive value of IL-17 staining for late graft dysfunction. (a) Area under the receiver operating characteristic curve analysis of total IL-17$^+$ cell count per high power field for the prediction of an eGFR <30 ml/min/1.73 m^2 at the last follow-up. The black line represents the test cohort and the red line represents the validation cohort. (b) Kaplan–Meier curve for an eGFR <30 ml/min/1.73 m^2 at the last follow-up in the test cohort comparing IL-17 high (dotted line) with IL-17 low grafts (full line) with a cut-off at 4 cells/HPF. The grey area depicts the 95% confidence interval of the Kaplan–Meier estimates. (c) Kaplan–Meier curve for an eGFR <30 ml/min/1.73 m^2 at the last follow-up in the validation cohort comparing IL-17 high (dotted line) with IL-17 low grafts (full line) with a cut-off at 4 cells/HPF. The grey area depicts the 95% confidence interval of the Kaplan–Meier estimates. (d) Area under the receiver operating characteristic curve analysis of total IL-17$^+$ cell count per high power field for the prediction of return to dialysis. The black line represents the test cohort and the red line represents the validation cohort. (e) Kaplan–Meier curve for return to dialysis in the test cohort comparing IL-17 high (dotted line) with IL-17 low grafts (full line) with a cut-off at 4 cells/HPF. The grey area depicts the 95% confidence interval of the Kaplan–Meier estimates. (f) Kaplan–Meier curve for return to dialysis in the validation cohort comparing IL-17 high (dotted line) with IL-17 low grafts (full line) with a cut-off at 4 cells/HPF. The grey area depicts the 95% confidence interval of the Kaplan–Meier estimates.

AUROC for prediction of an eGFR <30 ml/min/1.73 m^2 was comparable with the test cohort (0.7480, 95% CI = 0.5827–0.9134) and slightly decreased for return to dialysis (0.6808, 95% CI = 0.5236–0.8379) (Fig. 3a and c). In the validation cohort, 4 IL-17$^+$ cells per HPF corresponded to 69–76% sensitivity and 67–81% specificity, which corresponded to the best combined sensitivity and specificity in this cohort as well. None of the protocol biopsies had IL-17$^+$ cell counts above the cut-off value of four cells per HPF. Figure 3b,c,e and f show the Kaplan–Meier curves of the corresponding outcomes (all log-rank test $P < 0.05$).

Discussion

To the best of our knowledge, this is the first report dealing with intragraft IL-17$^+$ cells and renal allograft outcome. Our study demonstrates that it is possible to stratify patients at risk for an adverse graft outcome based on the amount of intragraft IL-17$^+$ cells. Most IL-17$^+$ leukocytes were either neutrophils or mast cells, whereas IL-17$^+$ lymphocytes were rarely observed.

In the past decades, the incidence of acute renal allograft rejection has decreased, but still 10–20% of patients suffer an episode of acute rejection [24]. Mechanisms underlying this process are complex and incompletely understood. Until recently, allograft rejection was thought to be a consequence of a T_h1 response. The discovery of IL-17 producing T cells (T_h17 cells) has brought new insights into the field of transplantation and has led to a rapid increase of studies on this cell type in recent years. T_h17 cells are a distinct subpopulation of T-helper cells with major functions in the induction of tissue inflammation

and protection against extracellular pathogens [25,26]. T_h17 cells characteristically produce IL-17A (IL-17), IL17-F and IL-22, which leads to the production of pro-inflammatory cytokines, chemokines and metalloproteinases from various tissues and cell types. As a result, neutrophils are recruited to tissues [27].

Until now only a few studies have been performed on IL-17 in acute allograft rejection and a minor part of these studies are performed in renal transplants. The group of van Kooten was the first to report the *de novo* expression of IL-17 by immunofluorescence during acute rejection in human renal biopsies [11]. Loong *et al.* found elevated IL-17 mRNA and protein levels in rat allograft biopsy material with acute rejection as early as day 2 post-transplant. Moreover, by immunohistochemistry, the number of IL-17$^+$ cells was increased in human renal allografts during borderline rejection [28]. In several other organs, IL-17 cell counts were also found to be elevated during acute rejection [12–14,29]. However, in these studies, the clinical relevance of intragraft IL-17 and the cellular sources of IL-17 were not evaluated. Our study is the first to report that intragraft IL-17$^+$ cells during acute rejection prognosticate for late clinical outcome.

An interesting finding in our study was that only a few intragraft T_h17 cells, defined as double positive cells for IL-17 and CD3, could be found during acute rejection. De Boer *et al.* characterized IL-17$^+$ infiltrates in human atherosclerotic plaques and could not find any T_h17 cells. In atherosclerosis, the major sources for IL-17A and IL-17F were neutrophils and mast cells [8]. In a mouse model of Gram-negative bacterial lung infection, the group of Prause found that CD3-negative cells dominate among IL-17$^+$ cells in bronchoalveolar lavage samples [30]. Recently, fate mapping of IL-17 producing cells showed that T_h17 cells can switch towards an interferon-γ producing T_h1 phenotype depending on their local environment [31], which has been shown to be important for chronic transplant rejection [32]. The finding of only a few T_h17 cells in our biopsy material is in line with these studies.

The aim of our study was to show the feasibility of IL-17 immunostaining as a surrogate marker for worse renal allograft outcome. There is a need for biomarkers that predict late graft outcome preferably at a time point where specific therapy still might be beneficial. Immunophenotypical characterization of the intragraft inflammatory infiltrate for the prediction of graft outcome has produced conflicting results, possibly because of broad molecular heterogeneity among individual patients during acute rejection [33]. At present CD20, FOXP3, mast cells, neutrophils, eosinophils and NK cell counts or scores (as reviewed in [34]) could not be validated in such a way that they were clinically applicable. C4d has been proved to be a marker of graft failure [35] and recent studies suggest that the total area of inflamed biopsy tissue also associates with adverse outcome [36,37], independent of interstitial fibrosis. In our multivariate analysis, the total percentage of inflamed tissue and C4d positivity were not independently related to late graft dysfunction when considering IL-17$^+$ cell count.

As total IL-17$^+$ cell count is predictive of late graft dysfunction, which was independent of time of biopsy after transplantation and C4d positivity, we propose that IL-17 staining on biopsy tissue is of value for further stratifying patients at risk for future graft function decline. These findings were validated in an independent cohort of acute renal allograft rejections of equal size, but with some differences in the composition of included patients. The validation cohort included more patients with profound HLA mismatches. However, we did not observe a difference in HLA mismatch composition between the IL-17 low and high group and suggest therefore that the amount of mismatches does not alter the expression of IL-17 in the graft. We observed a longer cold ischemia time (>3 h difference) in the IL-17 high group. In the literature, ischemic renal transplant biopsies with delayed graft function showed higher levels of IL-17 protein compared with pretransplant biopsies [38]. In a recent study by the group of Deteix, renal transplantectomies with high numbers of IL-17$^+$ cells showed a shorter survival time compared with grafts with low numbers of IL-17$^+$ cells. They suggest a role for T_h17 in promoting lymphoid neogenesis, which possibly hastens clinical chronic rejection [39]. The association of IL-17 with shorter transplant survival is in line with our study. However, we only found a few T_h17 cells in our biopsy material without any statistical value and consider neutrophils and mast cells as the major sources of IL-17.

An interesting finding in our study is the relation between the IL-17 high group and C4d positivity. Van Kooten *et al.* showed that IL-17 can stimulate renal proximal tubular epithelial cells to produce increased amounts of complement component C3 [11]. Also fibroblasts produce C3 when stimulated with IL-17 [40] and in inflammatory bowel disease, mRNA levels of IL-17 and C3 showed a strong correlation [41]. Therefore IL-17 expression on leukocytes during acute rejection could possibly be a marker for humoral rejection, especially with the focus on C4d negative antibody-mediated rejection as proposed by Sis and Halloran [42].

In conclusion, we propose that IL-17$^+$ cell counts can be used as an independent surrogate marker for late graft dysfunction during an episode of acute renal allograft rejection. However, prospective standardized cohort studies including not only rejection biopsies but also other

index biopsies will be necessary to validate the predictive value of the intragraft IL-17$^+$ infiltrates.

Authorship

ÜY: performed research, analyzed data and wrote the paper. JK: analyzed data and wrote the paper. FB, JG, KD, RB and MI: provided the biopsy material, collected clinical data and reviewed the manuscript. CL and NC: contributed important reagents. JR: supervised research and reviewed the manuscript. SF: designed and supervised research and reviewed the manuscript.

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