Determinants of acute and chronic renal allograft injury
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Chapter 8

The prognostic significance of glomerular infiltrating leukocytes during acute renal allograft rejection

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Abstract

Transplant glomerulitis, observed in T cell- and antibody-mediated rejection, is histologically characterized by intracapillary mononuclear cell infiltration. However, the prognostic value of counting various glomerular inflammatory cells during rejection has not been elucidated, which is a key step for the introduction of novel biomarkers in the clinics. We immunophenotyped glomerulitis during episodes of acute rejection in order to investigate their predictive value for transplant outcomes. To do so, we included 57 transplant biopsies of 57 renal transplant recipients with biopsy-proven acute rejection with a median follow-up of 4.2 years. We determined average glomerular cell counts for T cells, B cells, Tregs, IL-17+ cells, neutrophils and macrophages. Logistic and Cox regression models were used to investigate the association of glomerular inflammatory cells with response to therapy and graft failure on a population level. We used novel time-dependent ROC curve analyses to investigate the value of glomerular inflammatory cell infiltrates for the prediction of transplant outcomes, applicable to the individual patient. We identified three cell types that were responsible for glomerulitis during rejection: macrophages, T cells and neutrophils. By quantification of glomerular macrophages, an emerging cell type associated with antibody-mediated rejection, we were able to predict the progression towards death-censored graft failure within the first 500 days after the initial episode of rejection. With the use of novel time-dependent ROC analyses, we propose dynamic sensitivities, specificities, and positive and negative predictive values with their corresponding cut-off values for the average amount of glomerular macrophages, depending on what time after rejection death-censored graft failure needs prediction.
Introduction

According to the Banff classification, histologically transplant glomerulitis is characterized by intracapillary glomerular mononuclear cell infiltration in the kidney allograft. In the first Banff classification of renal allograft pathology, tubulitis and intimal arteritis were regarded as the principal lesions indicative of acute rejection. However, this classification did not include glomerulitis as a defining feature (Racusen et al., 1999). Nowadays, glomerulitis is recognized as a form of microcirculatory inflammation in the context of allograft injury related to antibody-mediated rejection (ABMR) (Haas et al., 2014; Racusen et al., 2003), however this feature has also been observed in T cell-mediated rejection (TCMR). The Banff classification has included semi-quantitative lesion grading, which makes it simple and quick to apply (Racusen et al., 1999). However, the reproducibility of lesion scoring might be a limiting factor and true quantification of leukocytes might be more suitable and accurate (Furness et al., 2003; Marcussen et al., 1995). Methodological aspects of histological grading of glomerulitis are the subject of ongoing debate. No clear cut-off value exists to render a diagnosis of glomerulitis in the context of ABMR. Batal et al. addressed this issue in a series of 111 index renal allograft biopsies and compared three different histological methods to grade glomerulitis (defined by the presence of >5 leukocytes/glomerulus) and their correlations with clinical parameters (Batal et al., 2010). They concluded that grading glomerulitis based on the percentage of affected glomeruli (as recommended by the Banff) was superior to grading based on the most inflamed glomerulus, or the presence of capillary loop occlusion by inflammation. As far as glomerulitis is concerned, the Banff definition is based on identification of mononuclear cells in particular and granulocytes are not included, which was the case in the initial study of Batal (Batal et al., 2010). Both cell types might be difficult to discriminate on conventional histological stainings and exclusion of the granulocytes might underestimate the pathogenic role of these cells. A limited number of studies is available concerning the immunohistochemical phenotyping of glomerular leukocytes and their relationship with clinical parameters, response to anti-rejection therapy and renal allograft outcome. Emerging data from several studies show that even though associated with indices of ABMR, transplant glomerulitis associates with death-censored graft loss, independent of C4d or donor-specific antibody (DSA) status (Batal et al., 2010; Einecke et al., 2009; Papadimitriou et al., 2010; Tinckam et al., 2005). Therefore, there is a need to better
define glomerulitis immunophenotypically and to assess the prognostic value of the cell types for the prediction of graft outcomes.

We thoroughly phenotyped glomerular inflammation during episodes of acute rejection. The principle aim of our present study was to investigate the predictive value of the various types of transplant glomerulitis in the context of acute renal allograft rejection for the response to therapy and the development of graft failure, since this is currently lacking. In order to do so, we used novel time-dependent receiver operating characteristics analyses.

**Materials & Methods**

**Renal allograft recipients**

In the current study we collected renal transplant patients with a diagnosis of acute rejection (TCMR or mixed TCMR/ABMR rejection) on their index biopsy from the database of the Academic Medical Center. Patients were included when after diagnostic work-up, paraffin embedded biopsy material was still available (excluded patients were in this case considered missing completely at random). An initial group of 28 renal biopsies was analyzed by immunohistochemistry for the presence of T cells, B cells, macrophages, FoxP3+ cells, IL-17+ cells and neutrophils in the glomeruli. From this preliminary assessment, we concluded that B cells, plasma cells, and FoxP3+ cells were hardly present in glomeruli during acute rejection (Figure 1). IL-17 was only positive in neutrophils and we therefore omitted further sub-analysis based on this immunohistochemical staining as well. We completed the group with another 29 renal biopsies with acute rejection and in this cohort, immunostains for T cells, macrophages and neutrophils were performed. We excluded acute rejection biopsies with the coexistence of biopsy-proven polyomavirus nephropathy (N = 3). Complete data analysis was performed on 57 biopsies for which all stainings were available.

**Histopathology**

The biopsy material was formalin-fixed, paraffin-embedded and 4-μm sections were stained for hematoxylin–eosin, periodic acid-schiff and Jones’ silver stain (2-μm sections) as a diagnostic routine. For the additional immunostains, the following
protocol was used: paraffin-embedded tissues (4-μm) were dewaxed. Endogenous peroxidase activity was blocked for 15 min in methanol/H$_2$O$_2$. Antigen retrieval was performed by boiling sections in a buffer containing 10 mM TRIS and 1 mM EDTA (pH 9.0) for 10 min. Sections were labeled with the various primary antibodies followed by labeling with horseradish peroxidase (HRP)-labeled goat-anti-rabbit IgG or goat-anti-mouse IgG (Immunologic, Duiven, The Netherlands) and stained with 3,3′-diaminobenzidine (DAB) (Sigma-Aldrich, Zwijndrecht, The Netherlands). The following primary antibodies were used: CD3 monoclonal antibody (Neomarkers, Suffolk, United Kingdom) for T cells, CD15 monoclonal (Immunologic, Duiven, The Netherlands) for granulocytes, CD68 monoclonal antibody for macrophages (Dako, Heverlee, Belgium), IL-17 polyclonal antibody (R&D Systems, Abingdon, United Kingdom) for all IL-17$^+$ cells, Foxp3 monoclonal antibody (Abcam, Cambridge, United Kingdom) for regulatory T cells, CD20 monoclonal antibody (Dako, Heverlee, Belgium) for B cells and CD138 monoclonal antibody (Immunologic, Duiven, The Netherlands) for plasma cells. Immunohistochemical staining for C4d was also performed on paraffin-embedded tissue using a rabbit polyclonal anti-human C4d antibody (clone Bl-RC4D, Biomedica, Oxford, UK). Renal biopsies with N10% positive peritubular capillaries were considered positive for C4d.

**Histological assessment and immunophenotypical quantification**

All biopsies were reviewed and scored by a renal pathologist according to the latest update of the Banff classification (Haas et al., 2014). Only renal biopsies with at least 7 glomeruli and 1 artery were included for analysis. The total number of immunohistochemically stained cells in the glomeruli were counted and divided by the total number of glomeruli to get an average cell count per glomerulus as a continuous parameter. A total “leukocyte glomerulitis” cell count was produced by adding up the CD68$^+$, CD3$^+$ and CD15$^+$ cell counts.

**Outcome measures**

We used two binary outcome measures. The first primary outcome measure was response to therapy and was defined as a decrease in serum creatinine level within two weeks after the start of anti-rejection therapy 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. (Gaber et al., 1996) and was
also used by Haas et al. (Haas et al., 2002) and in recent publications by our group (Scheepstra et al., 2008; Yapici et al., 2009, 2011). The baseline creatinine value was defined as the lowest creatinine value before the rise in creatinine. The second primary outcome measure was death-censored graft failure, defined as the return to chronic dialysis and/or re-transplantation.

**Statistical analyses**

Analyses of cell counts showed skewed distributions and therefore non-parametric 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. Univariate logistic regression was used to relate the cell counts (independent variables) with response to anti-rejection therapy (dependent variable). Cox proportional hazards models were used to correlate cell types with death-censored graft failure. To investigate whether cell counts could discriminate patients that did or did not respond to therapy, we used conventional receiver operating characteristics (ROC) analyses. The ability to discriminate between patients that underwent death-censored graft failure or not on follow-up was assessed by cumulative/dynamic ROC analyses as described by Heagerty and colleagues (Heagerty et al., 2000). Cumulative/dynamic ROC analyses take censoring of patient follow-up into account by utilizing the Kaplan–Meier estimator. In this way, time-dependent area under the curves can be calculated, i.e. the discriminative value for graft failure at certain time-points after the assessment of the cell counts during an episode of acute rejection. The AUC at any time point represents the maximum combined sensitivity and specificity, i.e. an AUC of 1.0 indicates perfect discrimination between diseased state and non-diseased state (100% sensitivity and specificity), whereas an AUC of 0.5 indicates no better prediction than chance. Sensitivities, specificities, positive predictive values and negative predictive values were computed by inverse probability of censoring weighting (IPCW) (Blanche et al., 2013). The Youden indices (in percentages) were calculated as (sensitivity + specificity – 100) (Schisterman et al., 2005). In order to provide an estimate of the type II error in case no association between the cell types and clinical parameters
were found, we calculated the hypothetical sample size that was necessary in order to observe a $P \leq 0.05$ for the association between the cell counts and the outcomes (power = 0.80). Sample sizes for survival analyses, as an estimate for type II error, were calculated according the methods described by Hsieh and Lavori (Hsieh and Lavori, 2000). A $P$-value of $b0.05$ was defined as statistical threshold. All analyses were performed with SPSS version 20.0 for Macintosh (SPSS Inc. Chicago, IL, USA) or the R statistical platform for Macintosh version 2.15.2. Modules for the R statistical platform that were used in the current manuscript were: gdata v2.12.0, ggplot2 v0.9.3.1, grid v2.15.2, Hmisc v3.10-1, MASS v7.3-23, plyr v1.8, powerSurvEpi v0.0.6, pROC v1.5.4, timeROC v0.2 and rms v3.6-3.

Results

Baseline characteristics of the patients

Demographic characteristics of the patients are summarized in Table 1. Immunosuppressive medication consisted of induction therapy with the anti-CD25mAb basiliximab (Simulect; Novartis Pharma B.V., Arnhem, The Netherlands), prednisolone, a calcineurin inhibitor: cyclosporine A (Neoral; Novartis Pharma B.V.) or tacrolimus (Prograf; Astellas Pharma, The Netherlands) and mycophenolate mofetil (Cellcept; Roche Nederland B.V.). The median time of indication-biopsy after transplantation was 18 days (interquartile range 9–113 days) and the median follow-up after transplantation was 4.2 years (interquartile range 1.4–6.7 years). Of the 57 patients, 29 (51%) had Banff grade acute T cell-mediated rejection (TCMR) IA, 8 (14%) had grade IB, 11 (19%) had grade IIA, 8 (14%) had grade IIB and 1 (2%) had grade III rejection (Table 1). Nineteen individuals (55%) had a mixed TCMR and antibody-mediated rejection (ABMR) rejection according to the latest Banff classification; of the patients with concomitant ABMR, 5 were considered C4d negative. Fifty-five of the 57 patients were treated with methylprednisolone (MPNS). Of these 55, 5 were subsequently treated with anti-thymocytoglobulin (ATG), 1 was also treated with plasmapheresis and 5 were also treated with the combination of ATG and plasmapheresis. Of the 2 patients that were not treated with MPNS, 1 was only treated with ATG and 1 with ATG and plasmapheresis.
Kinetics of complement-binding HLA antibodies in recipients with acute rejection

Donor-specific antibody (DSA) screening was performed by the complement dependent cytotoxicity (CDC) technique (standard method used in our tissue typing laboratory). Panel-reactive antibody assay (PRA) data were available prior to
transplantation, at time of biopsy and on follow-up. Twenty-four % of our patients had complement-binding HLA antibodies before transplantation that persisted at the time of biopsy and on follow-up. Of the patients who had a C4d-positive biopsy as compared to those who had a C4d-negative renal biopsy, a similar proportion was pre-sensitized (67% versus 57%, P = 0.72), a larger proportion had complement-binding HLA antibodies detectable at time of biopsy (78% versus 39%, P = 0.06) and a similar proportion had complement-binding HLA antibodies detectable on follow-up (78% versus 56%, P = 0.28).

Glomerular T cells and macrophages both correlate to the existence of endothelialitis but not tubulitis

Figure 1 shows the distribution of glomerular immune cells in our group. In total, 97% of biopsies had any form of glomerulitis. As an internal control, we correlated the Banff g-score with the sum of the T cells, macrophages and neutrophils (total glomerulitis cell count). The total glomerulitis cell count increased with an increasing g-score (P < 0.0001). The amount of macrophages and T cells also correlated to the Banff g-score individually (both P < 0.0001). However, the amount of neutrophils did not correlate to the Banff g-score (P = 0.30). Glomerular macrophages correlated with glomerular T cells (ρ = 0.66, P < 0.0001), whereas neither glomerular T cells (ρ = −0.03, P = 0.80) nor glomerular macrophages correlated to the presence of neutrophilic glomerulitis (ρ = 0.22, P = 0.09). We did not observe a correlation of the cell counts with tubulitis (all P > 0.28), however both T cells and macrophages significantly correlated with the existence of endothelialitis (P = 0.03 and P = 0.008 respectively), a feature that is considered in the context of both T cell- and antibody-mediated rejection according to the latest Banff update (Haas et al., 2014). None of the cell counts associated with indices of chronic allograft injury, i.e. transplant glomerulopathy and vasculopathy, interstitial fibrosis and tubular atrophy (IF/TA) and glomerulosclerosis, which might be explained by the early time-point of biopsy after transplantation.

Immunophenotype of glomerulitis in the context of complement fixation and microvascular inflammation and chronic damage

In our cohort, C4d positivity did not relate to the Banff g-score (P = 0.16) but the total glomerular cell count (sum of macrophages, T cells and neutrophils) was higher in
C4d-positive acute rejections than in C4d-negative ones (median 8.7 versus 4.1 cells/glomerulus, P = 0.04). There was a trend towards higher glomerular cell counts in C4d+ cases for macrophages (median 3.1 versus 1.4, P = 0.07) and T cells (2.6 versus 1.2 cells, P = 0.08). We did not observe a difference in neutrophil cell counts between C4d+ cases and C4d- cases (average 0.9 versus 1.1 cells, P = 0.90).

Table 2 shows the percentages of patients with C4d-positive peritubular capillaries and complement-binding HLA antibodies at time of biopsy per tertile of cell count. We conclude that the total glomerular cell count as represented by the amount of macrophages best fits in the context of antibody-mediated rejection. We divided the glomerular macrophage count into high (>1.89 cells per glomerulus) and low.
Glomerulitis during acute renal allograft rejection

A high glomerular macrophage count associated with microvascular inflammation (Banff g + ptc score; \( P = 0.008 \)), however this was entirely due to the association with the g-score, since the association with the ptc-score was not significant (\( P = 0.40 \)). No association of the glomerular macrophage count with the ti-score (\( P = 0.40 \)) or chronic indices of ABMR were found (transplant glomerulopathy \( P = 0.80 \), IF/TA \( P = 0.20 \)). This indicates the glomerulitis with macrophages might be an early lesion in the context of ABMR.

**Prediction of response to anti-rejection therapy by glomerulitis**

We assessed if glomerulitis by Banff g-score or by cell count could predict whether recipients responded to their anti-rejection therapy. In our study 29 patients (51%) presented with a clinical response to therapy. The patients who were classified as mixed TCMR/ABMR versus patients with TCMR did not have a worse response.
to treatment (P = 0.42, corrected for plasmapheresis). The g-score (OR = 0.92 per increase in stage 95% confidence interval [CI] = 0.51–1.61, P = 0.80) and the total glomerulitis score (OR = 0.96 per increase in cell/glomerulus 95% CI = 0.88–1.02, P = 0.20) were not associated with a higher risk of resistance to anti-rejection therapy. In line with these findings, glomerular macrophages, T cells and granulocytes were not associated with the risk of therapy-resistant rejection. The cell counts in responders and non-responders with their corresponding ROC AUCs are plotted in Figure 2. C4d was also not related to a worse response to therapy (OR = 1.36, 95% CI = 0.32–6.08, P = 0.68, sensitivity = 18%, 95% CI = 6–37%, specificity = 86%, 95% CI = 68–96%). In order to provide the reader with an idea of the type II error in this cohort, sample size analysis was performed, which indicated that at least 126 patients for neutrophils, 272 patients for T cells and 499 patients for macrophages had to be included to show a theoretical significant difference (power = 80%, α = 5%). It is to be expected that the confidence intervals, but not the ROC AUCs themselves, are influenced by sample size. Based on these results

![Figure 2 | Prediction of clinical response to therapy by glomerular cell counts.](image)

(a–d) Distributions of glomerular cell counts (total leukocyte glomerulitis, macrophages, T cells and neutrophils) between patients that clinically responded or not responded to anti-rejection therapy. (e–h) Corresponding receiver operating characteristics (ROC) curves.
Glomerulitis during acute renal allograft rejection

we did not consider glomerulitis, in each of the above forms tested, as a candidate biomarker for clinical response to therapy.

**Prediction of death-censored graft failure by glomerulitis**

We were wondering whether transplant glomerulitis was able to predict death-censored graft failure, independent of type of rejection diagnosis. Of the 57 patients that underwent acute rejection, 24 lost their graft on follow-up (42%) at a median of 1236 (IQR = 395–2285) days after the diagnosis of rejection. In total there were 219.7 follow-up years, and the incidence rate for death-censored graft failure was 0.11 events/year. As an internal control, patients that did not respond to anti-rejection therapy had indeed worse graft prognosis (HR = 5.82, 95% CI = 2.16–15.67, P < 0.001, overall sensitivity = 79%, 95% CI = 58–93%, overall specificity = 76%, 95% CI = 58–89%). A similar cumulative incidence of graft failure was observed among those patients who classified as mixed TCMR/ABMR versus those who were classified as TCMR (P = 0.32, corrected for plasmapheresis). The Banff g score yielded no increase in risk per increase in category (HR = 1.00, 95% CI = 0.66–1.53, P = 0.99). The more detailed “total leukocyte glomerulitis” cell count (sum of CD3, CD15 and CD68 cell counts) rendered similar results (HR = 0.99, 95% CI = 0.94–1.04, P = 0.68). Glomerular cell counts for T cells, macrophages and neutrophils neither correlated with late renal outcome (plotted per tertile in Figure 3a–d), although the 2nd and 3rd tertile of the glomerular macrophages had a higher HR for death-censored graft failure as compared to the 1st (HR = 3.40, 95% CI = 1.15–10.03, P = 0.03, Figure 3b). We calculated the predictive value for the individual patient by time-dependent ROC analysis according to the methods described by Heagerty and colleagues (Heagerty et al., 2000). The AUC(t) for the “total leukocyte glomerulitis” count was around 0.7 early after rejection and declined to 0.5 around 1500 days after rejection. The 95% confidence interval remained above the AUC(t) = 0.5 cut-off until 500 days after the rejection episode (Figure 3e). The predictive value of the total glomerulitis count could be explained by the predictive value of the CD68+ glomerular macrophage count, which followed the same course (Figure 3f). Table 3 displays the sensitivity, specificity and predictive values of glomerular macrophage cell count cut-off values. We observed a decline in sensitivity at later time-points whereas specificity remained stable. The positive predictive value increased with an increase in cumulative incidence of death-censored graft failure.
Figure 3 | Prediction of death-censored graft failure by glomerular cell counts.
(a–d) Kaplan–Meier curves for the association between glomerulitis (plotted per tertile cell count/glomerulus) and death-censored graft failure. (e–h) The corresponding cumulative/dynamic receiver operating characteristics (ROC) analysis. The solid black line represents the estimated time-dependent area under the curve of the ROC plot for a particular time-point after the episode of acute rejection [the dashed black lines represent the 95% confidence intervals of the AUC(t)]. The solid red line represents an AUC of 0.5, which indicates a predictive value not better than chance. The x-axis represents the time-dependent AUC at the time point as defined by the y-axis. Those events that occur early after the episode of rejection were best predicted (highest AUC).
Table 3 | Prognostic value of glomerular macrophage count for the prediction of death-censored graft failure after an episode of acute rejection.

<table>
<thead>
<tr>
<th>Predictive value</th>
<th>Cut-off average cell count per glomerulus</th>
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<tr>
<td></td>
<td>1 macrophage</td>
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<tr>
<td>Sensitivity, % (SE)</td>
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</tr>
<tr>
<td>7 days</td>
<td>100 (0)</td>
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<td>30 days</td>
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<td>180 days</td>
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<td>365 days</td>
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<tr>
<td>500 days</td>
<td>83 (11)</td>
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<td>Specificity, % (SE)</td>
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<td>7 days</td>
<td>41 (7)</td>
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<td>30 days</td>
<td>42 (7)</td>
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<td>180 days</td>
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<td>365 days</td>
<td>49 (8)</td>
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<tr>
<td>500 days</td>
<td>46 (8)</td>
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<td>Youden index, %</td>
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<td>41</td>
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<td>30 days</td>
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<td>500 days</td>
<td>29</td>
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<td>PPV, % (SE)</td>
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<td>7 days</td>
<td>17 (6)</td>
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<td>30 days</td>
<td>19 (7)</td>
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<td>500 days</td>
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<td>NPV, % (SE)</td>
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<td>7 days</td>
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<td>365 days</td>
<td>100 (0)</td>
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<tr>
<td>500 days</td>
<td>90 (97)</td>
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</table>

SE = standard error, PPV = positive predictive value, NPV = negative predictive value. The Youden index is calculated as (sensitivity + specificity -100) and is considered the maximum test performance for the cumulative incidence of death-censored graft failure at the mentioned time-point after rejection.
on follow-up. The negative predictive value remained stable high on follow-up. The calculated sample size to show a possible significant difference with a comparable patient population based on these data (graft failure = 42%, power = 80%, $\alpha = 5\%$) was 1172 biopsies for T cells, 1231 for neutrophils and 3972 for macrophages. Again, an increase in sample size influences confidence intervals, but not ROC AUC(t) values. We conclude that the CD68$^+$ macrophage glomerular cell count during an episode of acute rejection is able to predict death-censored graft failure.

**Discussion**

In the current study, which included 57 individuals who underwent a biopsy-proven acute rejection early after transplantation, we show that 1) glomerulitis as defined by the Banff g-score did not associate with indices of ABMR and subsequently did not predict response to therapy and graft failure, 2) macrophages, T cells and neutrophils are the predominant cell types during glomerulitis, 3) glomerular macrophages associate with indices of ABMR, do not predict clinical response to therapy but are predictive of graft failure and 4) neutrophilic glomerulitis represents a different entity that neither correlates to indices of T cell- or antibody-mediated rejection, nor to response to therapy, chronic graft injury or the development of graft failure.

In a series of 240 patients that had to undergo a biopsy for cause (not only rejection), Papadimitriou et al. showed that transplant glomerulitis, consisting predominantly of macrophages and to a lesser extent of T cells was related to the presence of donor-specific antibodies and microvascular injury (Papadimitriou et al., 2010). They suggested that glomerular macrophages are key players in the development of ABMR that have diagnostic and prognostic value. To a certain level, our study is in line with these data, and also with previous work by Magil and colleagues (Magil, 2005; Magil and Tinckam, 2003; Tinckam et al., 2005) and Lefaucheur and colleagues (Lefaucheur et al., 2007) who later observed a similar phenomenon. We also noted an association between an increase in the number of glomerular macrophages and the presence of both circulating complement-binding HLA antibodies and C4d-positive peritubular capillaries. However, we were unable to show a correlation with the existence of transplant glomerulopathy. This may be
caused by the short time interval between biopsy and transplantation in our cohort as compared to the study by Papadimitriou et al. where at inclusion, the patients had a graft survival of at least 1 year (Papadimitriou et al., 2010). We also did not observe a correlation of glomerular cell counts with other indices of chronic damage like IF/TA and transplant glomerulopathy, even though these lesions were represented in the biopsies of our patients (23/57 IF/TA > 0 and 20/57 cv > 0). Unfortunately, for the majority of patients, no Luminex assay was performed for the more sensitive detection of donor-specific antibodies, which can be interpreted as a limitation of our study. We did observe a potential predictive value of glomerular macrophages for the development of graft failure. The difference in clinical significance of the glomerular infiltrates between the several studies might also be explained by the presence of subtypes of macrophages or T cells, which has recently been observed in a rat renal transplant model with preformed DSAs following donor-specific splenocyte transfusion (Huang et al., 2014). Huang et al. demonstrated the influx of M1-type (pro-inflammatory) macrophages and IFN-γ TH1 cells in these pre-sensitized rats, a finding that they confirmed in pre-sensitized renal transplant recipients with ABMR. The clinical significance of these finding remains however to be elucidated, but might be an explanation of the difference in study results between the various studies.

Another interesting finding is the presence of neutrophilic glomerulitis in our rejection biopsies. In the study by Papadimitriou et al., these were mostly present during thrombotic microangiopathy and recurrence of glomerulonephritis, two lesions that were not present in our group (Papadimitriou et al., 2010). In the current study, we neither observed a correlation with other forms of glomerulitis, nor with chronic lesions. Lefaucheur et al. demonstrated that in their group of 21 patients with ABMR, 100% of patients had neutrophilic glomerulitis in their first index biopsy (Lefaucheur et al., 2007). In the eight ABMR patients with an eGFR <15 mL/min at the end of follow-up (of which 6 had to be dialyzed), the amount of neutrophils in the glomeruli was higher than in those with an eGFR >15 mL/min at the end of follow-up. With a time-dependent analysis, which is a more detailed analysis in this case, we did not observe a correlation between the presence of neutrophilic glomerulitis and death-censored graft failure on follow-up after rejection. In the report by Lefaucheur et al.,
glomerular neutrophils decreased and glomerular macrophages increased at later biopsies in patients with a bad outcome (Lefaucheur et al., 2007). It might therefore be that the kinetics of the glomerular infiltrating inflammatory cells is the most important indicator of worse outcome comparable, which was also shown for data obtained in the context of a stereotypic injury–repair response as is seen during ischemia–reperfusion injury (Famulski et al., 2012; Halloran et al., 2010). A minority of the glomerular infiltrates during rejection in our cohort consisted of B cells, plasma cells and regulatory T cells. Corroborating on our findings, Jin et al. did not observe regulatory T cells in the context of either C4d positive or C4d negative rejection (Jin et al., 2015). A similar pattern of inflammation was observed by Sun et al. during intima arteritis, where B cells and plasma cells accounted for b 5% of the inflammatory infiltrate (Sun et al., 2011).

Besides the importance of elucidating the pathophysiology of rejection, the discovery and validation of novel biomarkers is of high importance in the field of renal transplantation. Stratification of patients at risk for future graft loss is the first step to tailored therapeutic regimens. Prediction of future events after transplantation goes beyond a correlation between early parameters and the subsequent graft loss and includes analyses of sensitivity/specificity, decision analytic performance and eventually cost-effectiveness (Steyerberg et al., 2012). Using novel time-dependent ROC analyses we observed a role for macrophage glomerulitis in the prediction of death-censored graft failure, at least until 500 days after the episode of acute rejection. It might be that the extent or subtype of macrophage glomerulitis in rejection biopsies that is taken later after transplantation, like in the study of Papadimitriou et al., shows even higher predictive values for transplant outcome (Papadimitriou et al., 2010). The predictive value of these markers should preferably be evaluated in a prospective cohort study blinded for the treating physician and other demographic and clinical data should be taken into account, as they are easier to obtain than an invasive renal biopsy.

In conclusion, we show that macrophages, T cells and neutrophils are the main cell types responsible for transplant glomerulitis in this group of patients with an early episode of T cell- and/or antibody- mediated rejection. Macrophagic (and to a lesser extent T lymphocytic) glomerulitis was associated with indices of ABMR.
including the presence of complement-binding HLA antibodies, C4d-positive peritubular capillaries and endothelialitis, whereas neutrophilic glomerulitis did not. In this study, early glomerulitis with macrophages showed its potential usefulness as a biomarker for the development of death-censored graft failure after an episode of acute rejection, independent of indices of antibody-mediated rejection.