Quantitative polymerase chain reaction profiling of immunomarkers in rejecting kidney allografts for predicting response to steroid treatment

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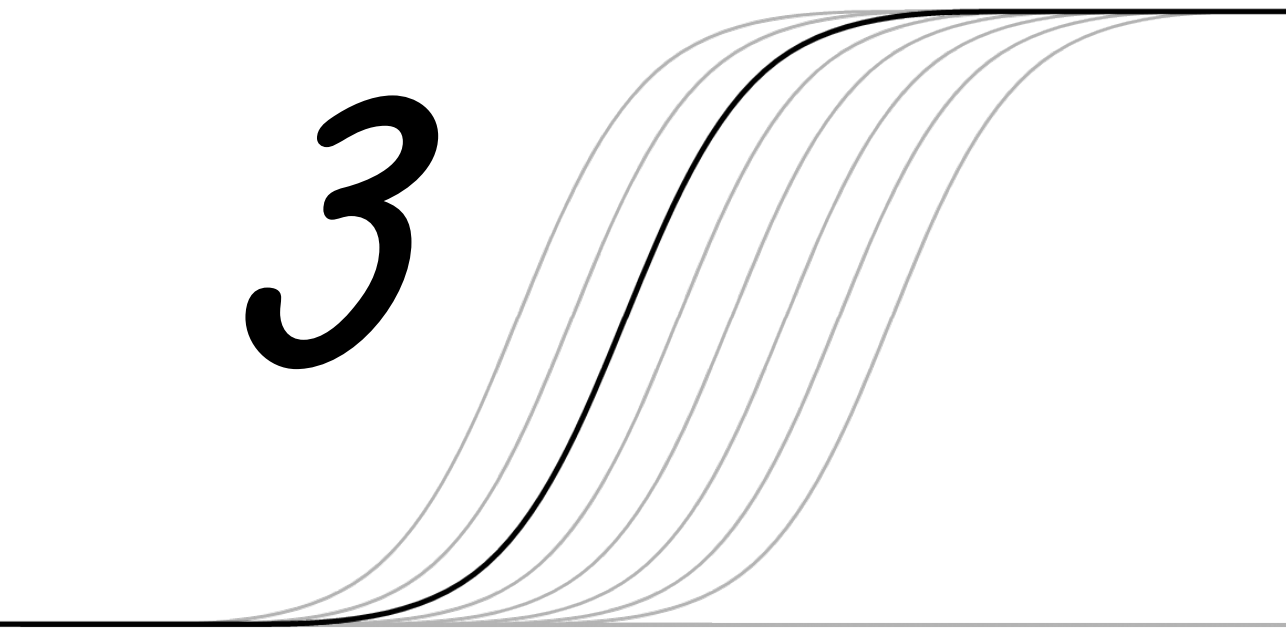

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Abstract

Background. Steroid resistant acute rejection is a risk factor for inferior renal allograft outcome.

Methods. From 873 kidney transplant recipients (1995-2005) 108 patients with a first rejection episode were selected for study using strict inclusion criteria and clinical end point definition. We aimed to predict response to corticosteroid treatment using gene expression of 65 transcripts. These reflect cytokines, chemokines, and surface and activation markers of various cell types including T cells, macrophages, B cells, and granulocytes. Steroid resistance (40% of the patients) was defined as requirement for anti-thymocyte globulin treatment within two weeks after corticosteroid treatment.

Results. None of the clinical and histomorphologic parameters showed a significant association with response to treatment. Univariate logistic regression analysis resulted in eleven mRNA markers, including T cell-related transcripts CD25, LAG-3, Granzyme B, and IL-10, and macrophage-specific transcripts MannoseR and S100A9, which significantly discriminated steroid resistant from steroid responsive rejections ($P<0.05$). In multivariate logistic regression the combination of T cell activation markers CD25:CD3e ratio (OR=8.7, CI: 2.4-31.2) and LAG-3 (OR=3.3, CI: 1.4-7.7) represented the best predictive model for steroid response ($P<0.0001$). Specificity and sensitivity were 78% and 60%, respectively. After internal stratified ten-fold cross-validation the model remained significant. Inclusion of clinical variables into the model with molecular variables did not enhance prediction.

Conclusions. Differences in intragraft expression profiles reflect variability in the response to anti-rejection treatment. In acute rejection, molecular markers, particularly those reflecting T cell activation, offer superior prognostic value compared to conventional parameters.

Introduction

After kidney transplantation, the occurrence of acute rejection is one of the most important risk factors for adverse graft outcome [1-3]. Acute rejection is a complex process in which infiltrating cells of the host immune system cause injury to the allograft. The Banff classification [4-7] represents histomorphologic criteria to determine the severity of the acute rejection based on the site and degree of inflammation in the renal allograft biopsy.

A main parameter determining graft outcome is therapy sensitivity of the acute rejection episode [3;8;9]. In most cases a first rejection episode can be adequately treated with high-dose corticosteroids. However, in approximately 30% of the patients the rejection episode cannot be reversed with corticosteroid therapy alone [2;8;10]. In these cases of steroid resistance the patient requires more rigorous therapy with anti-thymocyte globulin (ATG). Acute rejection episodes leading to incomplete restoration of graft function upon treatment may lead to progression of chronic damage to the graft, and have a detrimental effect on graft outcome [8;9;11].

Once the diagnosis acute rejection has been made, it is difficult to predict the response to anti-rejection treatment using clinical parameters and histopathologic assessment of the biopsy. Availability of molecular markers could help to assess which patients will respond to steroid treatment and which patients are in need of immediate ATG treatment. Indeed, several studies have described cellular and molecular markers in the graft tissue, of which the expression was found to be associated with therapy resistance (reviewed in [11]). Sarwal and colleagues found increased expression of B cell-, cytotoxic T cell-, and natural killer cell signatures in steroid resistant acute rejection in renal allografts of children [12]. In more recent studies investigators have not been able to confirm that the presence of intragraft B cells, on basis of CD20 expression, is associated with therapy response and/or graft function after rejection [13-16]. It has further been shown that high FasL mRNA expression [17] and dense granulysin staining [18] in renal allograft biopsies, as well as low FoxP3 expression in urinary sediments [19], were associates of steroid resistance. The presence of macrophages during acute rejection within the graft was also found to be associated with steroid resistance [20-23]. Together, these results give an indication of the complexity in accurately predicting the response of kidney transplant patients to anti-rejection treatment with steroids.

In the current study we investigated a broad panel of immunological markers, measured by quantitative PCR (qPCR), in a large cohort of patients suffering from a first acute rejection episode. We aimed to predict response to anti-rejection treatment on the basis of molecular markers in the allograft biopsy.

Materials and methods

Patients

We reviewed all 873 patients who received a renal allograft in our center between 1995 and 2005. All patients who had suffered from histologically proven acute rejection were included in the study. Further criteria for this study were: 1) only first rejection episode biopsy specimens were included; 2) only patients who received corticosteroids as anti-rejection treatment were included; 3) patients who received ATG as induction therapy were excluded; 4) all studied biopsy cores had been obtained prior to the start of anti-rejection treatment. Frozen material, for RNA extraction and quantitative PCR (qPCR), was available from 108 patients who met the criteria. Nine samples were excluded from analysis: 1 sample due to RNA degradation and 8 samples due to too low RNA yield from the biopsy. In total, 99 patients were included in the statistical analysis (Figure 1).

Seventy patients (70.7%) received no induction therapy and 29 patients (29.3%) received induction therapy with an IL-2 receptor blocker (Daclizumab). Maintenance immunosuppressive medication consisted of a calcineurin inhibitor (Cyclosporine A or Tacrolimus) and prednisone. Fifty-six patients (56.6%) also received an antimetabolite (Mycophenolate Mofetil) as part of the maintenance immunosuppression. Immunosuppression for the treatment of acute rejection consisted of pulse therapy for 3 days with a 1-g bolus of intravenous methylprednisolone daily.

Definition of primary clinical endpoint

The primary clinical endpoint was response to anti-rejection treatment with methylprednisolone. Steroid resistant acute rejection was defined as lack of clinical response (serum creatinine level did not return to within 120% of the pre-rejection baseline value [16;24;25]) to steroid pulse therapy, and a requirement for anti-thymocyte globulin treatment within 14 days after the start of the steroid therapy. In the steroid

resistant group, the time between initiation of steroid treatment and ATG treatment was on average 7.3 ± 3.5 days (in 82% of the patients an interval of ≥ 5 days [25;26]). In seven cases, ATG was given within 5 days after initiation of steroid treatment. Reason was insufficient decline in serum creatinine, stagnation of the decline above the 120% level, or a rising serum creatinine level despite the steroid treatment. A total of 59 patients with steroid responsive acute rejection and 40 patients with steroid resistant acute rejection were evaluated in the study (Figure 1).

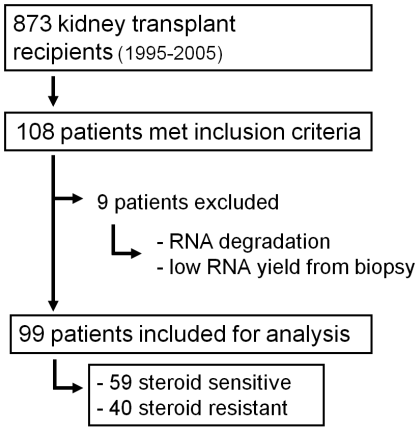

Figure 1. Flow diagram of the sample selection. All patients who received a renal allograft in our center between 1995 and 2005 were reviewed (n=873). All patients who had suffered from histologically proven acute rejection were considered for study. Further selection criteria led to 59 patients with steroid responsive acute rejection and 40 patients with steroid resistant acute rejection for investigation in the study.

Biopsy samples

At least two biopsy cores were collected from each patient. One core was formalin fixed, embedded in paraffin, and used for histochemical stainings. Three independent pathologists (I.B., N.G. and M.G.) blinded to the clinical data of the patients and outcome of the acute rejection revised individual Banff lesions on all biopsies according to Banff 2011 criteria [7]. The pathologists highly agreed on acute and chronic changes and reached a satisfactory inter-observer agreement. The second biopsy core was immediately snap frozen in liquid nitrogen and stored at -80°C. Immunofluorescent staining for C4d was performed on frozen sections from 93 of the 99 (94%) patients with mouse anti-human C4d antibody, as described previously [13;27]. Biopsies with diffuse C4d+ staining were assessed for histomorphologic characteristics indicative of a possible antibody-mediated rejection.

RNA extraction and quality assessment

Eight to ten 10-µm sections were cut with a cryomicrotome from each snap frozen biopsy core. Total RNA from the renal tissue was extracted using the RNeasy Mini Kit (Qiagen, Chatsworth, CA). RNA quality was determined on Nano LabChips with the Aligent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The RNA concentration was 2.75 ± 1.13 µg, mean 28S:18S ratio was 2.1 ± 0.05, and mean RIN was 8.00 ± 0.98.

Real-time quantitative PCR analysis

An average of 0.89 ± 0.19 µg of RNA from each frozen biopsy core was transcribed into cDNA, according to a previously described protocol [28]. To prevent amplification of genomic DNA, forward and reverse primers for each transcript targeted separate exons, spanning at least one intron with a size of 800 bp or more. Primer sequences can be provided on request. All primer sets were tested before use on control cDNA and genomic DNA to ensure optimal performance and no amplification of genomic DNA. Reactions and PCR conditions have been described previously [28]. All PCR efficiencies were between 90% - 110%. Relative gene expression levels were determined on the basis of a standard curve (in duplicate) of five serial dilution points of reference cDNA (qPCR Human Reference Total RNA, Clontech, France). Differences in mRNA expression levels were normalized either to the geometric mean signal of the reference genes GAPDH, 18S rRNA and β-actin (inter-correlation ranged between 0.88 and 0.95), or normalized to the signal of CD3e. For each marker reference-gene-normalized mRNA levels and CD3e-normalized mRNA levels were calculated.

Statistical analysis

Comparison of categorical data between patient groups was evaluated using Pearson chi-square tests. With respect to steroid response of the acute rejection, the predictive value of individual mRNA markers and of clinical and histomorphologic parameters was analyzed using univariate logistic regression analysis. Multivariate logistic regression analysis was used to test if multiple markers combined could provide a model with a higher predictive value. Markers were included stepwise in the model based on the likelihood ratio statistics. The obtained candidate marker set was validated using stratified ten-fold cross-validation. All logistic regression analyses were performed using a classification cutoff of 0.5, and logistic regression data is presented as odds ratios (OR) with 95% confidence interval (CI). Receiver operating

characteristics (ROC) analysis was performed to evaluate the predictive value of multivariate parameters.

All statistical tests were two-sided and P values less than 0.05 were considered as significant. Statistical analyses were performed using the SPSS statistical package (SPSS Inc., version 17.0.2, Chicago, IL, US).

Results

Demographics and clinical data

Demographic and clinical characteristics were not significantly different between the steroid responsive (n=59) and steroid resistant (n=40) patients (Table 1). No significant differences were found in rejection severity (P=0.64, Table 2) and C4d positivity (P=0.40, Table 1). In the 13 biopsies showing C4d positivity we investigated histomorphologic characteristics (granulocytic infiltrate, microthrombi, peritubular capillaritis, and necrotizing vascular rejection) indicative of a possible antibody-mediated rejection. None of these biopsies showed such features.

The steroid resistant group had significantly higher serum creatinine levels at 6 months (P=0.008) and 12 months (P=0.008) after the transplantation compared with the steroid responsive group. The graft survival after 12 years was 60.4% for the steroid resistant group and 81.9% for the steroid responsive group.

Table 1. Demographic and clinical data of patients with steroid responsive and steroid resistant acute rejection.

Variable	Steroid responsive (N=59)	Steroid resistant (N=40)	P value
Patient age			0.69
≥ 50 years	26 (44.1%)	16 (40.0%)	
Patient gender			0.89
Female	20 (33.9%)	13 (32.5%)	
Donor age			0.35
≥ 50 years	21 (35.6%)	18 (45.0%)	
Donor gender			0.66
Female	38 (64.4%)	24 (60.0%)	
Year of transplantation			0.15
1995 through 1999	36 (61.0%)	30 (75.0%)	
2000 through 2005	23 (39.0%)	10 (25.0%)	
Donor type			0.30
Living	11 (18.6%)	11 (27.5%)	
Post mortal	48 (81.4%)	29 (72.5%)	
HLA-A matching			0.75
≥ 1 mismatch	38 (64.4%)	27 (67.5%)	
HLA-B matching			0.89
≥ 1 mismatch	45 (76.3%)	31 (77.5%)	
HLA-DR matching			0.36
≥ 1 mismatch	36 (61.0%)	28 (70.0%)	
Virtual PRA			0.74
Immunized (6-100%)	17 (29.3%)	13 (32.5%)	
Cold ischemia time			0.46
> 18 hours	32 (66.7%)	26 (74.3%)	
Induction therapy			0.44
Daclizumab	19 (32.2%)	10 (25.0%)	
None	40 (67.8%)	30 (75.0%)	
Maintenance therapy			0.28
Double therapy	23 (39.0%)	20 (50.0%)	
Triple therapy	36 (61.0%)	20 (50.0%)	
Delayed graft function			0.18
Yes	17 (29.3%)	7 (17.5%)	
Rejection time			0.80
<3 months post Tx	57 (96.6%)	39 (97.5%)	
3-6 months post Tx	2 (3.4%)	1 (2.5%)	
C4d staining			0.40
Positive	6 (11.3%)	7 (17.5%)	
Vascular rejection			0.25
Yes	17 (28.8%)	16 (40.0%)	

PRA, panel reactive antibodies; Tx, transplantation

Table 2. Diagnostic categories according to Banff 2011 criteria of patients with steroid responsive and steroid resistant acute rejection [1].

Diagnostic category	Steroid responsive (N=54)	Steroid resistant (N=36)
Borderline changes	25 (46.3%)	12 (33.3%)
TCMR grade IA	6 (11.1%)	2 (5.6%)
TCMR grade IB	6 (11.1%)	6 (16.7%)
TCMR grade IIA	12 (22.2%)	10 (27.8%)
TCMR grade IIB	2 (3.7%)	3 (8.3%)
TCMR grade III	3 (5.6%)	3 (8.3%)

[1] No significant differences were found between groups (P=0.64). In each group, three biopsies did not contain sufficient cortex to determine a Banff score. Three additional patients had a clinical rejection (with i- and t-scores of 0). TCMR, T-cell mediated rejection

Evaluation of predictive value of clinical and molecular variables with respect to steroid response

Univariate analysis

We questioned whether differences in the expression of immunomarkers between the treatment response groups could predict steroid-refractory acute rejection. No significant differences in the RNA concentration, purity, and quality between the groups were found (data not shown). Expression of 73 markers was quantified by qPCR (see Table S1). Eight markers were excluded from further analysis due to an inferior melt curve in the PCR assay or due to low expression in all biopsies.

We tested whether clinical and histomorphologic parameters influenced the response to steroid treatment. None of these parameters had a significant relationship with steroid response in univariate logistic regression analysis (Table 3). We tested whether mRNA expression of molecular markers can predict response to corticosteroid treatment by using univariate logistic regression analysis. Eleven markers had an odds ratio (OR) below 0.5 or above 2.0 (Table 4).

Table 3. Univariate analysis of predictive value of clinical and histomorphologic parameters for steroid response.

Variable	Steroid responsive (N=59)	Steroid resistant (N=40)	OR	95% CI	P value
Clinical parameters					
Patient age (years)	47.4 (12.81)	45.8 (12.88)	0.99	0.96 – 1.02	0.54
Donor age (years)	44.1 (14.59)	46.7 (13.14)	1.01	0.98 – 1.04	0.38
HLA-AB mismatches	1.71 (1.05)	1.73 (1.04)	1.01	0.69 – 1.49	0.95
HLA-DR mismatches	0.68 (0.60)	0.73 (0.51)	1.16	0.57 – 2.39	0.68
Virtual PRA (%)	11.3 (22.29)	17.1 (29.27)	1.01	0.99 – 1.03	0.27
Cold ischemia time (hours)	20.4 (8.23)	21.9 (11.12)	1.02	0.97 – 1.07	0.47
Rejection time (days)	28.4 (32.24)	21.7 (21.97)	0.99	0.98 – 1.01	0.28
Year of transplantation (2000-2005)	39.0%	25.0%	0.52	0.22 – 1.27	0.15
Delayed graft function (yes)	29.3%	17.5%	0.51	0.19 – 1.38	0.19
Histomorphologic parameters					
Interstitial inflammation	51.8%	51.4%	0.98	0.43 – 2.26	0.97
Tubulitis	44.6%	59.5%	1.82	0.78 – 4.22	0.16
Intimal arteritis	32.1%	48.5%	1.99	0.82 – 4.87	0.13
Interstitial fibrosis	39.3%	37.8%	0.94	0.40 – 2.21	0.89
Tubular atrophy	44.6%	40.5%	0.85	0.36 – 1.96	0.70
C4d positivity	11.3%	17.5%	1.66	0.51 – 5.40	0.40

All clinical parameters were included in the univariate logistic regression analysis as continuous variables, except for year of transplantation (dichotomized for early period [1995-1999] and late period [2000-2005]) and delayed graft function (dichotomized for presence and absence). All histomorphologic parameters were dichotomized with a 1/0 parameter indicating presence or absence of the feature; except for interstitial inflammation and tubulitis, which were dichotomized for mild (0, 1) and severe (2, 3). Values are expressed as mean ± SD, or as percentage (presence or severe). OR, odds ratio; CI, confidence interval; PRA, panel reactive antibodies

Table 4. Univariate and multivariate logistic regression analysis of predictive value of molecular markers for steroid response.

Variable	Specificity	Sensitivity	Positive PV	Negative PV	OR	95% CI	P value
Univariate analysis							
CD25:CD3e	86.4%	37.5%	65.5%	67.1%	5.76	1.76 – 18.80	0.004
CD25	83.1%	40.0%	61.5%	67.1%	3.70	1.41 – 9.70	0.008
MannoseR	81.4%	30.0%	52.2%	63.2%	4.67	1.42 – 15.36	0.011
S100A9	83.1%	42.5%	63.0%	68.1%	2.33	1.22 – 4.44	0.011
LAG-3:CD3e	81.4%	22.5%	45.0%	60.8%	3.80	1.30 – 11.15	0.015
CXCL13:CD3e	81.4%	37.5%	57.7%	65.8%	2.06	1.10 – 3.83	0.023
LAG-3	84.7%	25.0%	52.6%	62.5%	2.43	1.12 – 5.26	0.025
RORγT:CD3e	84.7%	25.0%	52.6%	62.5%	0.48	0.24 – 0.94	0.032
IL-10	83.1%	25.0%	50.0%	62.0%	3.02	1.07 – 8.63	0.038
STAT6:CD3e	88.1%	20.0%	53.3%	61.9%	0.28	0.09 – 0.93	0.038
Granzyme B	83.1%	17.5%	41.2%	59.8%	2.05	1.01 – 4.17	0.048
Multivariate analysis							
Multivariate model	78.0%	60.0%	64.9%	74.2%			0.00008
CD25:CD3e					8.66	2.40 – 31.21	0.001
LAG-3					3.29	1.41 – 7.69	0.006

All molecular markers with a significant P value in the univariate logistic regression analysis, and the clinical and histomorphological parameters were included in the multivariate logistic regression analysis. Only markers and parameters that reached significance and represent independent variables in the multivariate analysis are shown in the table. PV, predictive value; OR, odds ratio; CI, confidence interval

Multivariate analysis

Multivariate analysis with logistic regression resulted in a predictive model (P=0.00008) that contained the CD25:CD3e ratio (OR=8.7; P=0.001) and LAG-3 (OR=3.3; P=0.006) as independent covariates (Table 4). The specificity and sensitivity of this multivariate predictive model were 78% and 60%, respectively. The receiver operating characteristics (ROC) analysis showed that the predictive value of the multivariate model was significant (P=0.00002). Area under the curve (AUC) was 0.76 with a 95% CI of 0.66 – 0.85 (Figure 2). Inclusion of the clinical and histomorphologic parameters in the multivariate logistic regression model containing the molecular predictors did not enhance predictive power.

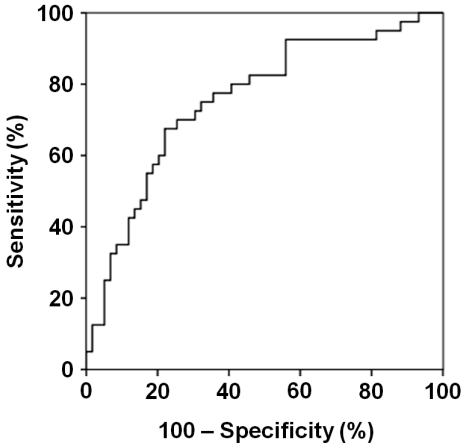

Figure 2. Predictive value of the multivariate logistic regression model for steroid resistant acute rejection. The receiver operating-characteristic (ROC) curve shows the percentage of true-positive results (sensitivity) and false-positive results (100 – specificity) for various cut-off levels of the multivariate logistic regression model, containing the molecular markers CD25:CD3e and LAG-3. The area under the curve of the ROC curve is 0.76 (P<0.001) with a 95% CI of 0.66 – 0.85.

Cross-validation model

For internal validation of the findings, a stratified ten-fold cross-validation was performed. The multivariate logistic regression analysis with cross-validation resulted in the same significant predictive model as before, containing the CD25:CD3e ratio and LAG-3 as independent covariates (mean P-value of 0.0004 ± 0.0004). The specificity and sensitivity of the cross-validated multivariate predictive model were 76% ± 20% and 43% ± 22%, respectively. ROC analysis for the model was significant (P=0.037).

Discussion

Steroid-refractory acute rejection is a risk factor for adverse renal allograft outcome [8;9]. To identify molecular associates of steroid resistant acute rejection, we performed a retrospective cohort study in a large cohort of renal transplant patients with a first rejection episode. None of the clinical and histomorphologic parameters tested showed significant association with treatment response. Eleven transcripts significantly discriminated steroid resistant rejections and steroid

responsive rejections in univariate analysis. In multivariate logistic regression, the combination of T cell activation markers CD25:CD3e ratio and LAG-3 represented the best predictive model for steroid response. The specificity and sensitivity of this multivariate predictive model were 78% and 60%, respectively. After internal cross-validation the predictive model remained significant.

For the current study, we selected a panel of immune-related genes, which included markers previously reported in association with steroid response [12;17-23;29-33], and which together reflected the full immune repertoire that may be present in the grafts (Table S1). These included cytokines, chemokines, and surface and activation markers of various cell types including cytotoxic T cells, T helper cells, regulatory T cells, classically and alternatively activated macrophages, B cells, plasma cells, natural killer cells, granulocytes, and mast cells. We found that the combination of the CD25:CD3e ratio and LAG-3 represented the best predictive model for steroid response. These markers are mainly expressed by activated T cells and regulatory T cells and represent cell surface proteins involved in signal transduction [34-38]. CD25 is the α-subunit of the IL-2 receptor (IL-2R), which is important in the regulation of the survival and proliferation of T cells [35;36]. Lymphocyte activation gene-3 (LAG-3 or CD223) is an activation-induced marker involved in the negative regulation of homeostasis and T cell function [34;38]. It is for the first time that these markers are linked to steroid resistance in renal transplantation. Interestingly, several studies have demonstrated that T cell characteristics, through disparities in IL-2 responses, play a role in steroid resistance [30;31]. Lee and colleagues identified in patients with steroid refractory ulcerative colitis a characteristic subgroup of peripheral $CD25^{int}$ T helper cells, which continued to proliferate *in vitro* upon activation, despite exposure to glucocorticoids [32]. This finding together with observations from the current study may suggest that steroid resistance resides in specific activated T cell populations and is not a feature of all lymphocytes. Hueso and colleagues have shown that low pretransplant levels of $CD3^{+}CD25^{high}$ lymphocytes were associated with an increased risk for early acute renal allograft rejection [39], which is likely a reflection of low levels of regulatory T cells [40;41]. The CD25:CD3e ratio at the mRNA level, associating with steroid resistant acute rejection, is probably more a reflection of activated T cells, comparable to the $CD25^{int}$ cells described by Lee and colleagues, rather than of regulatory T cells. A potential clinical implication of current findings is the identification of patients who do not respond to steroid treatment and may benefit from immediate ATG treatment after the diagnosis of acute renal allograft rejection.

In other studies, the presence of B cells (CD20) [12;29;33], macrophages (CD68) [20-23], and cytotoxic T cells (Granzyme B, FasL) [17] was associated with steroid resistant acute rejection. With did find by univariate analyses association between expression of Granzyme B and macrophage-specific transcripts (Mannose receptor, S100A9) with steroid resistant rejection. We could not confirm, however, the predictive value of CD20 expression. Complementary B cell specific transcripts, including CD19, CD22 and CD252, gave similar results. Besides the set of immunomarkers, transcripts of glycine amidinotransferase (GATM), phytanoyl-CoA hydroxylase (PHYH), and epidermal growth factor (EGF), of which relatively high levels were related to steroid responsive acute rejection in a previous study [12], were not of prognostic relevance in the current study. One aspect that may have influenced the discrepancy between findings from the current study and some findings from others could be differences in the age of the patients (for example, adults versus adolescents and children), type of immune suppression, the number of HLA mismatches, the sensitization grade of the patients, and the time between transplantation and the rejection episode. Of note, several other investigators also have not been able to confirm the association of CD20 expression with steroid response of the acute rejection [13-16]. Although not significant, in our study a higher percentage of vascular rejection was seen in the steroid resistant group (40%) compared to the steroid sensitive group (29%) (Table 1). Vascular rejection was found to be associated with steroid resistance in previous studies [24;42;43], and its presence may have had an influence on therapy sensitivity in the current study.

We handled strict inclusion criteria, clinical end point definition, and quality controls for RNA processing and PCR assays. We decided to use qPCR rather than microarray analysis to measure intragraft mRNA expression, since the former technique is the best tool to quantify expression. Furthermore, expression of most of the interleukins and chemokines studied cannot be detected by microarray analysis. To have defined clinical criteria for response to anti-rejection therapy, steroid resistance of the acute rejection was adopted when the patient required ATG within two weeks after initiation of the steroid administration.

Some comments need to be made with regard to the current findings. In this retrospective study, none of the C4d-positive biopsies showed histomorphologic characteristics indicative of a possible antibody-mediated rejection. However, we had no information on the presence of donor specific antibodies at the time

of rejection. Therefore, we were unable to firmly conclude on the contribution of antibody-mediated rejection. Furthermore, we observed, as pointed out previously [12], a considerable heterogeneity in transcriptional regulation among the acute rejection biopsies. Presence of multiple mechanisms underlying steroid resistance probably accounts for the restricted predictive power. Firstly, the outcome of acute rejection may be determined by the response of the renal parenchyma to inflammation besides the composition of the infiltrate itself. Secondly, interaction of the glucocorticoid receptor (GR) with its ligand might in part determine therapy response. Polymorphisms in *NR3C1*, the gene encoding for the GR, may affect the ability of this receptor to bind glucocorticoids [44;45]. Further research needs to be performed to test the impact of polymorphisms in the *NR3C1* gene on therapy response during acute rejection.

In conclusion, we evaluated a broad panel of immunological markers by qPCR profiling in a large cohort of renal allografts with acute rejection. Differences in intragraft expression profiles reflect variability in the response to anti-rejection treatment. With respect to therapy response of the rejection episode, molecular markers offer superior prognostic value compared to conventional parameters. The combination of the CD25:CD3e ratio and LAG-3 expression represents the best model for risk assessment of steroid resistance in patients suffering from acute rejection. This is of interest in terms of the pathogenic mechanism of steroid resistance. In the future, the results could be used to identify which patients will not respond to steroid treatment and may benefit from immediate ATG treatment. Our findings indicate that steroid resistance resides in specific T cell populations and is not a feature of all lymphocytes.

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References

1. Hariharan S, Johnson CP, Bresnahan BA, Taranto SE, McIntosh MJ, Stablein D: Improved graft survival after renal transplantation in the United States, 1988 to 1996. *N Engl J Med* 342:605-612, 2000
2. Pascual M, Theruvath T, Kawai T, Tolkoff-Rubin N, Cosimi AB: Strategies to improve long-term outcomes after renal transplantation. *N Engl J Med* 346:580-590, 2002
3. Wu O, Levy AR, Briggs A, Lewis G, Jardine A: Acute rejection and chronic nephropathy: a systematic review of the literature. *Transplantation* 87:1330-1339, 2009
4. Racusen LC, Solez K, Colvin RB, Bonsib SM, Castro MC, Cavallo T, Croker BP, Demetris AJ, Drachenberg CB, Fogo AB, Furness P, Gaber LW, Gibson IW, Glotz D, Goldberg JC, Grande J, Halloran PF, Hansen HE, Hartley B, Hayry PJ, Hill CM, Hoffman EO, Hunsicker LG, Lindblad AS, Yamaguchi Y, .: The Banff 97 working classification of renal allograft pathology. *Kidney Int* 55:713-723, 1999
5. Solez K, Colvin RB, Racusen LC, Haas M, Sis B, Mengel M, Halloran PF, Baldwin W, Banfi G, Collins AB, Cosio F, David DS, Drachenberg C, Einecke G, Fogo AB, Gibson IW, Glotz D, Iskandar SS, Kraus E, Lerut E, Mannon RB, Mihatsch M, Nankivell BJ, Nickeleit V, Papadimitriou JC, Randhawa P, Regele H, Renaudin K, Roberts I, Seron D, Smith RN, Valente M: Banff 07 classification of renal allograft pathology: updates and future directions. *Am J Transplant* 8:753-760, 2008
6. Sis B, Mengel M, Haas M, Colvin RB, Halloran PF, Racusen LC, Solez K, Baldwin WM, III, Bracamonte ER, Broecker V, Cosio F, Demetris AJ, Drachenberg C, Einecke G, Gloor J, Glotz D, Kraus E, Legendre C, Liapis H, Mannon RB, Nankivell BJ, Nickeleit V, Papadimitriou JC, Randhawa P, Regele H, Renaudin K, Rodriguez ER, Seron D, Seshan S, Suthanthiran M, Wasowska BA, Zachary A, Zeevi A: Banff '09 meeting report: antibody mediated graft deterioration and implementation of Banff working groups. *Am J Transplant* 10:464-471, 2010
7. MengeL M, Sis B, Haas M, Colvin RB, Halloran PF, Racusen LC, Solez K, Cendales L, Demetris AJ, Drachenberg CB, Farver CF, Rodriguez ER, Wallace WD, Glotz D: Banff 2011 Meeting Report: New Concepts in Antibody-Mediated Rejection. *Am J Transplant* 12:563-570, 2012
8. Madden RL, Mulhern JG, Benedetto BJ, O'Shea MH, Germain MJ, Braden GL, O'Shaughnessy J, Lipkowitz GS: Completely reversed acute rejection is not a significant risk factor for the development of chronic rejection in renal allograft recipients. *Transpl Int* 13:344-350, 2000
9. Vereerstraeten P, Abramowicz D, de Pauw L, Kinnaert P: Absence of deleterious effect on long-term kidney graft survival of rejection episodes with complete functional recovery. *Transplantation* 63:1739-1743, 1997
10. Petrie JJ, Rigby RJ, Hawley CM, Suranyi MG, Whitby M, Wall D, Hardie IR: Effect of OKT3 in steroid-resistant renal transplant rejection. *Transplantation* 59:347-352, 1995
11. Eikmans M, Roelen DL, Claas FHJ: Molecular monitoring for rejection and graft outcome in kidney transplantation. *Expert Opin Med Diagn* 2:1365-1379, 2008
12. Sarwal M, Chua MS, Kambham N, Hsieh SC, Satterwhite T, Masek M, Salvatierra O, Jr.: Molecular heterogeneity in acute renal allograft rejection identified by DNA microarray profiling. *N Engl J Med* 349:125-138, 2003
13. Eikmans M, Roos-van Groningen MC, Sijpkens YW, Ehrchen J, Roth J, Baelde HJ, Bajema IM, de Fijter JW, de Heer E, Bruijn JA: Expression of surfactant protein-C, S100A8, S100A9, and B cell markers in renal allografts: investigation of the prognostic value. *J Am Soc Nephrol* 16:3771-3786, 2005
14. Doria C, di Francesco F, Ramirez CB, Frank A, Iaria M, Francos G, Marino IR, Farber JL: The presence of B-cell nodules does not necessarily portend a less favorable outcome to therapy in patients with acute cellular rejection of a renal allograft. *Transplant Proc* 38:3441-3444, 2006
15. Kayler LK, Lakkis FG, Morgan C, Basu A, Blisard D, Tan HP, McCauley J, Wu C, Shapiro R, Randhawa PS: Acute cellular rejection with CD20-positive lymphoid clusters in kidney transplant patients following lymphocyte depletion. *Am J Transplant* 7:949-954, 2007

16. Scheepstra C, Bemelman FJ, van der Loos C, Rowshani AT, van Donselaar-Van der Pant KA, Idu MM, ten Berge IJ, Florquin S: B cells in cluster or in a scattered pattern do not correlate with clinical outcome of renal allograft rejection. *Transplantation* 86:772-778, 2008
17. Desvaux D, Schwarzinger M, Pastural M, Baron C, Abtahi M, Berrehar F, Lim A, Lang P, Le Gouvello S: Molecular diagnosis of renal-allograft rejection: correlation with histopathologic evaluation and antirejection-therapy resistance. *Transplantation* 78:647-653, 2004
18. Sarwal MM, Jani A, Chang S, Huie P, Wang Z, Salvatierra O, Jr., Clayberger C, Sibley R, Krensky AM, Pavlakis M: Granulysin expression is a marker for acute rejection and steroid resistance in human renal transplantation. *Hum Immunol* 62:21-31, 2001
19. Muthukumar T, Dadhania D, Ding R, Snopkowski C, Naqvi R, Lee JB, Hartono C, Li B, Sharma VK, Seshan SV, Kapur S, Hancock WW, Schwartz JE, Suthanthiran M: Messenger RNA for FOXP3 in the urine of renal-allograft recipients. *N Engl J Med* 353:2342-2351, 2005
20. Vergara E, Gomez-Morales M, Osuna A, O'Valle F, Aguilar D, Masseroli M, Martinez T, Higueras M, Bravo J, Asensio C, Del Moral RG: Immunohistochemical quantification of leukocyte subsets in the long-term prognosis of kidney transplants. *Transplant Proc* 30:2380-2383, 1998
21. Ozdemir BH, Demirhan B, Gungen Y: The presence and prognostic importance of glomerular macrophage infiltration in renal allografts. *Nephron* 90:442-446, 2002
22. Ozdemir BH, Bilezikci B, Haberal AN, Demirhan B, Gungen Y: Histologic evaluation, HLA-DR expression, and macrophage density of renal biopsies in OKT3-treated acute rejection: comparison with steroid response in acute rejection. *Transplant Proc* 32:528-531, 2000
23. Tinckam KJ, Djurdjev O, Magil AB: Glomerular monocytes predict worse outcomes after acute renal allograft rejection independent of C4d status. *Kidney Int* 68:1866-1874, 2005
24. Haas M, Kraus ES, Samaniego-Picota M, Racusen LC, Ni W, Eustace JA: Acute renal allograft rejection with intimal arteritis: histologic predictors of response to therapy and graft survival. *Kidney Int* 61:1516-1526, 2002
25. Bock HA: Steroid-resistant kidney transplant rejection: diagnosis and treatment. *J Am Soc Nephrol* 12 Suppl 17:S48-S52, 2001
26. Shinn C, Malhotra D, Chan L, Cosby RL, Shapiro JI: Time course of response to pulse methylprednisolone therapy in renal transplant recipients with acute allograft rejection. *Am J Kidney Dis* 34:304-307, 1999
27. Botermans JM, de Kort H, Eikmans M, Koop K, Baelde HJ, Mallat MJ, Zuidwijk K, van Kooten C, de Heer E, Goemaere NN, Claas FH, Bruijn JA, de Fijter JW, Bajema IM, van Groningen MC: C4d staining in renal allograft biopsies with early acute rejection and subsequent clinical outcome. *Clin J Am Soc Nephrol* 6:1207-1213, 2011
28. Heidt S, Roelen DL, Eijsink C, Eikmans M, van Kooten C, Claas FH, Mulder A: Calcineurin inhibitors affect B cell antibody responses indirectly by interfering with T cell help. *Clin Exp Immunol* 159:199-207, 2010
29. Hippen BE, DeMattos A, Cook WJ, Kew CE, Gaston RS: Association of CD20+ infiltrates with poorer clinical outcomes in acute cellular rejection of renal allografts. *Am J Transplant* 5:2248-2252, 2005
30. Kam JC, Szefler SJ, Surs W, Sher ER, Leung DY: Combination IL-2 and IL-4 reduces glucocorticoid receptor-binding affinity and T cell response to glucocorticoids. *J Immunol* 151:3460-3466, 1993
31. Leung DY, Martin RJ, Szefler SJ, Sher ER, Ying S, Kay AB, Hamid Q: Dysregulation of interleukin 4, interleukin 5, and interferon gamma gene expression in steroid-resistant asthma. *J Exp Med* 181:33-40, 1995
32. Lee RW, Creed TJ, Schewitz LP, Newcomb PV, Nicholson LB, Dick AD, Dayan CM: CD4+CD25(int) T cells in inflammatory diseases refractory to treatment with glucocorticoids. *J Immunol* 179:7941-7948, 2007
33. Hwang HS, Song JH, Hyoung BJ, Lee SY, Jeon YJ, Kang SH, Chung BH, Choi BS, Choi YJ, Kim JI, Moon IS, Kim YS, Yang CW: Clinical impacts of CD38+ B cells on acute cellular rejection with CD20+ B cells in renal allograft. *Transplantation* 89:1489-1495, 2010

34. Workman CJ, Cauley LS, Kim IJ, Blackman MA, Woodland DL, Vignali DA: Lymphocyte activation gene-3 (CD223) regulates the size of the expanding T cell population following antigen activation in vivo. *J Immunol* 172:5450-5455, 2004
35. Letourneau S, Krieg C, Pantaleo G, Boyman O: IL-2- and CD25-dependent immunoregulatory mechanisms in the homeostasis of T-cell subsets. *J Allergy Clin Immunol* 123:758-762, 2009
36. Driesen J, Popov A, Schultze JL: CD25 as an immune regulatory molecule expressed on myeloid dendritic cells. *Immunobiology* 213:849-858, 2008
37. Triebel F: LAG-3: a regulator of T-cell and DC responses and its use in therapeutic vaccination. *Trends Immunol* 24:619-622, 2003
38. Haudebourg T, Dugast AS, Coulon F, Usal C, Triebel F, Vanhove B: Depletion of LAG-3 positive cells in cardiac allograft reveals their role in rejection and tolerance. *Transplantation* 84:1500-1506, 2007
39. Hueso M, Mestre M, Benavente Y, Bas J, Grinyo JM, Navarro E: Pretransplant low CD3+CD25high cell counts or a low CD3+CD25high/CD3+HLA-DR+ ratio are associated with an increased risk to acute renal allograft rejection. *Transplantation* 92:536-542, 2011
40. Baecher-Allan C, Brown JA, Freeman GJ, Hafler DA: CD4+CD25high regulatory cells in human peripheral blood. *J Immunol* 167:1245-1253, 2001
41. Nadal E, Garin M, Kaeda J, Apperley J, Lechler R, Dazzi F: Increased frequencies of CD4(+) CD25(high) T(regs) correlate with disease relapse after allogeneic stem cell transplantation for chronic myeloid leukemia. *Leukemia* 21:472-479, 2007
42. Regele H, Exner M, Watschinger B, Wenter C, Wahrmann M, Osterreicher C, Saemann MD, Mersich N, Horl WH, Zlabinger GJ, Bohmig GA: Endothelial C4d deposition is associated with inferior kidney allograft outcome independently of cellular rejection. *Nephrol Dial Transplant* 16:2058-2066, 2001
43. Nickeleit V, Zeiler M, Gudat F, Thiel G, Mihatsch MJ: Detection of the complement degradation product C4d in renal allografts: diagnostic and therapeutic implications. *J Am Soc Nephrol* 13:242-251, 2002
44. Bray PJ, Cotton RG: Variations of the human glucocorticoid receptor gene (NR3C1): pathological and in vitro mutations and polymorphisms. *Hum Mutat* 21:557-568, 2003
45. Miura M, Inoue K, Kagaya H, Saito M, Habuchi T, Satoh S: Inter-individual difference determinant of prednisolone pharmacokinetics for Japanese renal transplant recipients in the maintenance stage. *Xenobiotica* 39:939-945, 2009

Supplemental data

Table S1: Molecular markers investigated in the study by qPCR.

Marker:	Full gene name:	Sources / function	Reference
CD markers			
CCR5	Chemokine (C-C motif) receptor 5; CD195	Mo, MΦ, DC, Th1, NK	
CD3e	CD3e molecule, epsilon (CD3-TCR complex)	T	
CD4	CD4 molecule; OKT4	T subset	
CD8	CD8 molecule; OKT8	T subset	
CD11b	CD11 antigen-like family member B; integrin M alpha, Mac-1	Myeloid cells, NK, Mo	
CD19	CD19 molecule; B4	B	
CD20	CD20 molecule; membrane spanning 4 domains (MS4A1)	B	12
CD22	CD22 molecule; B lymphocyte cell adhesion molecule (BL-CAM)	B	
CD25	CD25 molecule; IL-2 receptor alpha chain	Tact, Bact, Treg	
CD28	CD28 molecule	T, plasma cells	
CD56	CD56 molecule; neural cell adhesion molecule (N-CAM)	NK	
CD66b	CD66b molecule	Granulocytes	
CD68*	CD68 molecule	Mo, MΦ	21-23
CD69	CD69 molecule	Tact, B, NK, granulocytes	
CD86	CD86 molecule; B7-2	Mo, DC, Bact, Tact	
CD103	CD103 molecule; integrin αE	Lymph	
CD115	CD115 molecule; c-fms; CSF-1 receptor	Mo differentiation / proliferation	
CD117	CD117 molecule; c-kit	HSC, F, MC	
CD127	CD127 molecule; interleukin 7 receptor (IL-7R)	T, preB, thymocytes	
CD138*	CD138 molecule; Syndecan 1; SDC1	Plasma cells, preB, epithelium	
CD163	CD163 molecule	Mo, MΦ (MΦ2 in vitro)	
CD205	CD205 molecule; DEC-205	DC	
CD252	CD252 molecule; OX40-ligand	B, DC, MC, EC	
CTLA4*	Cytotoxic T-lymphocyte-associated protein 4; CD152	Tact, B	
ICOS	Inducible T-cell co-stimulator; CD278	Tact, Th2	

Table S1: Continued

Marker:	Full gene name:	Sources / function	Reference
LAG-3	Lymphocyte-activation gene 3; CD223	Tact, NK	
MannoseR	Mannose receptor, C type 1 (MRC1); CD206	MΦ (alternative activation)	
PD-1	Programmed cell death 1; CD279	Tact, Bact, thymocytes	
Activation markers			
AIF-1	Allograft inflammatory factor 1	MΦ	
Blimp-1	PR domain containing 1, with ZNF domain; PRDM1	T, B, NK	
EBI3	Epstein-Barr virus induced 3	Treg, regulation of Th1 and Th2	
FasL	Fas ligand; TNF receptor superfamily, member 6; CD178	Tact, N, Mo, NK	[17]
FoxP3	Forkhead box P3	Treg, T	[19]
Granulysin	Granulysin (GNLY); lymphocyte-activation gene (LAG-2)	CTL	[18]
Granzyme B	Granzyme B (Grzb)	CTL, NK	[17]
HLA-DR	Major histocompatibility complex, class II, DR	Tact, Bact, DC, MΦ	[22]
IgG	Immunoglobulin G	B, plasma cells	
IgM	Immunoglobulin M	B, plasma cells	
NOS2A*	Nitric oxide synthase 2A	Produced by MΦ1	
RORγT	RAR-related orphan receptor C; RORC	T, Th17 pathway	
S100A8	S100 calcium binding protein A8; MRP-8	Mo, MΦ	
S100A9	S100 calcium binding protein A9; MRP-14	Mo, MΦ	
Cytokines			
IFN-γ	Interferon gamma	Regulation of immune cells	
IL-1β	Interleukin 1 beta	MΦact	
IL-2*	Interleukin 2; T cell growth factor (TCGF)	T, L proliferation	
IL-4*	Interleukin 4; B-cell stimulatory factor 1 (BSF-1)	T, Th2 pathway	
IL-5*	Interleukin 5; eosinophil differentiation factor (EDF)	T, Th2, E, MC	
IL-6	Interleukin 6; interferon beta 2	T, MΦ, B	
IL-8	Interleukin 8; chemokine (C-X-C motif) ligand 8 (CXCL-8)	Mo, MΦ, EP	
IL-10	Interleukin 10; cytokine synthesis inhibitory factor (CSIF)	Mo, MΦ, T	
IL-15	Interleukin 15	Mo, MΦ	

IL-16	Interleukin 16; lymphocyte chemoattractant factor (LCF)	L (CD8+), E	
IL-18	Interleukin 18; interferon-gamma inducing factor (IGIF)	MΦ	
IL-21	Interleukin 21	Tact, NKT, MC	
TGF-β_1	Transforming growth factor beta 1	Regulation of immune cells	
TNFα	Tumor necrosis factor alpha	Regulation of immune cells	
Chemokines			
CCL-2	Chemokine (C-C motif) ligand 2; MCP-1	MΦ, L, EC, EP	
CCL-3	Chemokine (C-C motif) ligand 3; MIP-1a	MΦ, L, N, E, F, MC	
CCL-4	Chemokine (C-C motif) ligand 4; MIP-1b	MΦ, L, N, F, MC	
CCL-13	Chemokine (C-C motif) ligand 13; MCP-4	Lung, colon, intestine	
CCL-18*	Chemokine (C-C motif) ligand 18; AMAC-1	Lung, lymph, thymus	
CCL-20	Chemokine (C-C motif) ligand 20; MIP-3α	Liver, lung	
CCL-22	Chemokine (C-C motif) ligand 22; ABCD-1	DC, MΦ, T	
CXCL-9	Chemokine (C-X-C motif) ligand 9; MIG	MΦ, N	
CXCL-12	Chemokine (C-X-C motif) ligand 12; SDF-1	Stromal cells	
CXCL-13	Chemokine (C-X-C motif) ligand 13; BCA-1	Liver, spleen, lymph	
Others			
EGF	Epidermal growth factor		[12]
GATA3	GATA binding protein 3	Th2 pathway	
GATM	Glycine amidinotransferase (L-arginine:glycine amidinotransferase)		[12]
PHYH	Phytanoyl-CoA 2-hydroxylase; PAXH		[12]
STAT4	Signal transducer and activator of transcription 4	Th1 pathway	
STAT6	Signal transducer and activator of transcription 6	Th2 pathway	
T-bet	T-cell-specific T-box transcription factor	Th1 pathway	

* Gene expression results excluded from study due to inferior melt curve.

Abbreviations:
B, B cells; Bact, activated B cells; CTL, cytotoxic T lymphocytes; DC, dendritic cells; E, eosinophils; EC, endothelial cells; EP, epithelial cells; F, fibroblasts; HSC, hematopoietic stem cells; iDC, immature dendritic cells; L, lymphocytes; MC, mast cells; Mo, monocytes; MΦ, macrophages; MΦact, activated macrophages; N, neutrophils; NK, natural killer cells; NKT, NKT cells; preB, B cell precursor; T, T cells; Tact, activated T cells; Th1, T helper cells; Th2, T helper 2 cells; Treg, regulatory T cells