Molecular and genetic markers for the prediction of kidney transplant outcome
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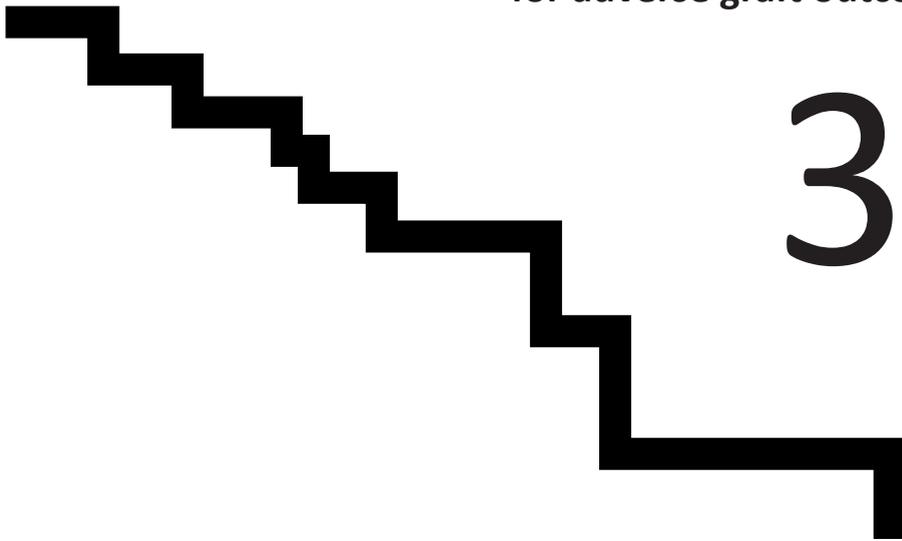
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Elevated intragraft expression of innate immunity and cell death-related markers is a risk factor for adverse graft outcome

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Abstract

Background: Molecules of the innate immune response are increasingly recognized as important mediators in allograft injury during and after kidney transplantation. We therefore aimed to establish the relationship between the expression of these genes at implantation, during an acute rejection (AR) and on graft outcome.

Method: A total of 19 genes, including Toll like receptors (*TLRs*), complement components and regulators, and apoptosis-related genes were analyzed at the mRNA level by qPCR in 123 biopsies with acute rejection and paired pre-transplantation tissue (n=75).

Results: Before transplantation, relative mRNA expression of *BAX:BCL2* ratio (apoptosis marker) and several complement genes was significantly higher in tissue samples from deceased donors compared to living donors. During AR, TLRs and complement genes showed an increased expression compared to pre-transplant conditions, whereas complement regulators were decreased. A relatively high *TLR4* expression level and *BAX:BCL2* ratio during AR in the deceased donor group was associated with adverse graft outcome, independently of clinical risk factors.

Conclusions: Complement- and apoptosis-related gene expression is elevated in deceased donor transplants before transplantation. High *BAX:BCL2* ratio and *TLR4* expression during AR may reflect enhanced intragraft cell death and immunogenic danger signals, and pose a risk factor for adverse graft outcome.

Introduction

The occurrence of an acute kidney allograft rejection, associated with infiltration of recipient immune cells to the kidney, is a risk factor for adverse graft outcome (1). The role of innate immunity including pattern recognition receptors and the complement system in rejection has been appreciated (2, 3). Toll like receptors (TLRs) are a family of transmembrane proteins that are capable of recognizing pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs)(4). TLR stimulation leads to dendritic cell maturation, characterized by upregulation of pro-inflammatory cytokines, chemokines, and co-stimulatory molecules, which initiate an immune response (5). Endogenous ligands including heat-shock proteins (HSP) (6), uric acid (7), high-mobility group box 1 protein (HMGB1) (8, 9), and genomic double-stranded DNA (10) may stimulate TLRs. The interaction between HMGB1 and TLR4 leads to proinflammatory responses in the graft: after kidney transplantation, recipients with a donor graft containing a genotype variant in the coding sequence of TLR4 had lower expression of proinflammatory genes MCP-1 and TNFα and higher expression of anti-inflammatory heme oxygenase 1, and they showed an increased rate of immediate graft function (11). Association of TLR2 and TLR4 expression was found with renal ischemia reperfusion injury (IRI) and early kidney allograft outcomes (12, 13). Other TLRs have not been investigated in the context of delayed graft function (DGF) and acute rejection (AR).

The complement system plays a pivotal role in ischemia reperfusion injury and allograft rejection after transplantation (3). The expression of complement components is significantly increased in deceased donor kidneys after cold ischemia (14, 15). Activation of the complement cascade leads to the release of anaphylatoxins (C3a and C5a) and the formation of the membrane attack complex (MAC) C5b-C9, which mediates the injury following transplantation (16, 17). C2 and C4 are essential components in the classical and lectin pathway, and C3 plays a central role in all pathways of the complement system. Complement regulators act as inhibitors of the complement cascade through various mechanisms (18, 19). For example, the decay acceleration factor (CD55) prevents the formation of C3 convertase. CD46 acts as cofactor for inactivating C3b and C4b by serum factor I. Complement receptor 1 both has decay-accelerating activity and cofactor activity. CD59 prevents the formation of MAC. Deficiency of CD55 and CD59 in experimental settings leads to increased renal ischemia reperfusion injury (20, 21). In C4d-negative biopsy specimens during allograft dysfunction local CD55 expression was related to favorable transplant outcome (22).

The role of apoptosis in IRI after kidney transplantation is increasingly being recognized (23, 24). The anti-apoptotic protein B-cell lymphoma 2 (BCL2) was significantly decreased and pro-apoptotic protein BCL2-associated X protein (BAX) was increased during normothermic ischemia (25). The augmentation of BCL2 protects renal tubular cells from

IRI through reducing renal tubular epithelial cell apoptosis (26). High ratios of *BAX:BCL2* in pre-transplant biopsies are associated with an increased risk of DGF (27).

In the present study, we examined innate-immune-related and apoptosis-related markers in kidney biopsies of 125 patients before transplantation and during an acute rejection episode, and investigated their relation to clinical outcome.

Methods

Patient characteristics
Patients who had received a kidney allograft at the Leiden University Medical Center (LUMC) during 1995-2005 were included. A total of 123 for-cause biopsy samples in case of clinical suspicion of AR were obtained within 6 months after transplantation, and 77 pretransplantation biopsies (75 biopsies paired to the subsequent AR biopsy) were taken at time of transplantation before reperfusion. Patient characteristics are shown in Table 1. Delayed graft function was defined as dialysis-dependency in the first week after transplantation.

Ethics
Written informed consent was obtained from donors for use of part of the human material for scientific purposes. The study were performed in accordance with the Declaration of Helsinki Good Clinical Guidelines and approved by the local medical ethics committee.

Gene selection
The innate immune related genes (*TLR1-TLR10*), potentially acting as initiators of inflammation, were studied. The key complement component (*C2, C3, C4*) and complement regulators (*CR1, CD46, CD55, CD59*), which inhibit complement activation, were included. The apoptosis related genes *BAX* and *BCL2,* which may be associated with IRI and DGF, were also tested.

RNA extraction and cDNA synthesis
RNA isolation and quality check, and cDNA synthesis were performed as described previously (28).

Real time quantitative PCR analysis
Optimal primers pairs were selected using Primer 3 version 4.0.0. To prevent amplification of genomic DNA, reverse and forward primers were designed to target separate exons, spanning at least one intron with a size of 800 bp or more. All primer sets were tested on control cDNA, and PCR efficiencies were between 90% and 110%. The 15-μL qPCR reaction

contained 3 µL of 25-times-diluted cDNA, 15 pmol forward and reverse primers, 7.5 µL of PCR Mix (Applied Biosystems by Life Technologies, Austin, Texas, USA), and nuclease-free water (29). Relative gene expression levels were normalized to the geometric mean of the reference genes β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Table 1. Demographics of patient cohort.

Variable	Number (%)
Recipient age (>= 50 years)	53 (43.1%)
Recipient gender (Female)	40 (32.5%)
Donor age (>= 50 years)	52 (42.6%)
Donor gender (Female)	74 (60.7%)
Donor type (Living)	24 (19.5%)
Time from transplant to rejection (days, IQR)	14 (9 - 37)
First Transplantation (Yes)	103 (84.4%)
HLA–A/B matching (Yes)	20 (16.4%)
HLA-DR matching (Yes)	43 (35.2%)
Virtual PRA (0-5%)	81 (66.4%)
DGF (Yes)	33 (28.7%)
Steroid responsiveness	68 (56.2%)
Cold ischemia time (<= 18 h)	31 (29.8%)
Banff score	
Glomerulitis (g=0/1/2/3)	74/25/7/3
Interstitial inflammation (i=0/1/2/3)	5/44/36/24
Tubulitis (t=0/1/2/3)	11/39/38/21
Intimal arteritis (v=0/1/2/3)	62/24/7/7
Interstitial fibrosis (ci=0/1/2)	61/41/7
Tubular atrophy (ct=0/1/2)	60/44/5
C4d diffuse positive	14 (11.4%)
Rejection characteristics	
No rejection	7 (5.7%)
Borderline rejection	33 (27.0%)
Interstitial rejection	42 (34.4%)
Vascular rejection	40 (32.8%)
Graft survival (Death censored)	
>1 year	106 (92.2%)
>6 year	101 (87.8%)

HLA, human leukocyte antigen; PRA, panel reactive antibodies; DGF, delayed graft function

Immunohistochemistry

Immunohistochemical studies were performed on an independent set of 34 formalin-fixed and paraffin-embedded (FFPE) kidney biopsy samples: 25 from patients with AR and 9 protocol biopsies from patients with stable graft function. Patients included in this group were transplanted between 2006 and 2015. Monoclonal anti-human antibodies against BAX (ab32503, Abcam, 1:1400 dilution), BCL2 (Sp66, Ventana), TLR4 (ab22048, Abcam, 1:800 dilution), and TLR9 (clone 26C593.2, Novus, 1:800 dilution) were used for immunohistochemistry on sequential 4-μm sections. Staining procedures have been described in a previous publication (30). Semi quantitative scoring of the number of Bcl2-, TLR4-, and TLR9 positive tubular epithelial cells was performed blindly by two observers using a scale from 0 to 5 (0 = 0%, 1 = <10%, 2 = 10-25%, 3 = 26-50%, 4 = 51-75%, 5 = 76-100%).

Statistical analyses

Gene expression differences in paired (PreTx, AR) tissue samples were analyzed using Wilcoxon signed ranks test. Differences in gene expression between deceased and living donors and the occurrence of DGF were assessed by Mann-Whitney U tests (two-sided). Correlations between innate immunity mRNA expression levels and mRNA expression of general inflammation markers were analyzed by Spearman's rank correlation coefficients (two-sided). The Bonferroni method was used to correct for multiple comparisons. Death-censored graft survival curves were created using the Kaplan-Meier method, and differences between curves were calculated using log rank tests. High expression level of inflammatory markers (*CD163, CD68, CD20, CD3e*) was defined as recipients with deceased donor graft with the highest one-third of gene expression. Risk factors affecting graft survival in the deceased donor group were analyzed by multivariate Cox-regression model including the variables showed a borderline significance (P < 0.1) in univariate test. Statistical analyses were performed using SPSS statistics, version 23. Due to the limited number of graft loss events, penalized survival analysis by lasso method, including clinical and molecular risk factors, were performed using the "penalized" R (3.4.0 version) package (31).

Results

Relation of pre-transplant gene expression levels with the type of donor

No significant difference was observed between deceased (n=65) and living (n=11) donors regarding the donor age and donor gender. A shorter cold ischemia time (< 18 hours) was more frequently seen in the living-related donation group. Sixteen genes, including the TLRs and membrane-bound complement regulators *C4* and *BAX*, were not significantly different in their expression between living and deceased-related donors at t0 (Table 2). The expression

of the complement genes *C2* and *C3* was more than 4-fold higher in the cadaveric donors compared to the living-related donors (Table 2). A significantly higher *BAX:BCL2* ratio was observed in biopsies of deceased donor kidneys compared to living-related donor kidneys (Figure 1). Within the deceased donation group, recipients with relatively high expression of *C2, C3* and *BAX:BCL2* did not differ from recipients with relatively low expression in the incidence of DGF, steroid resistant rejection, and graft survival (data not shown).
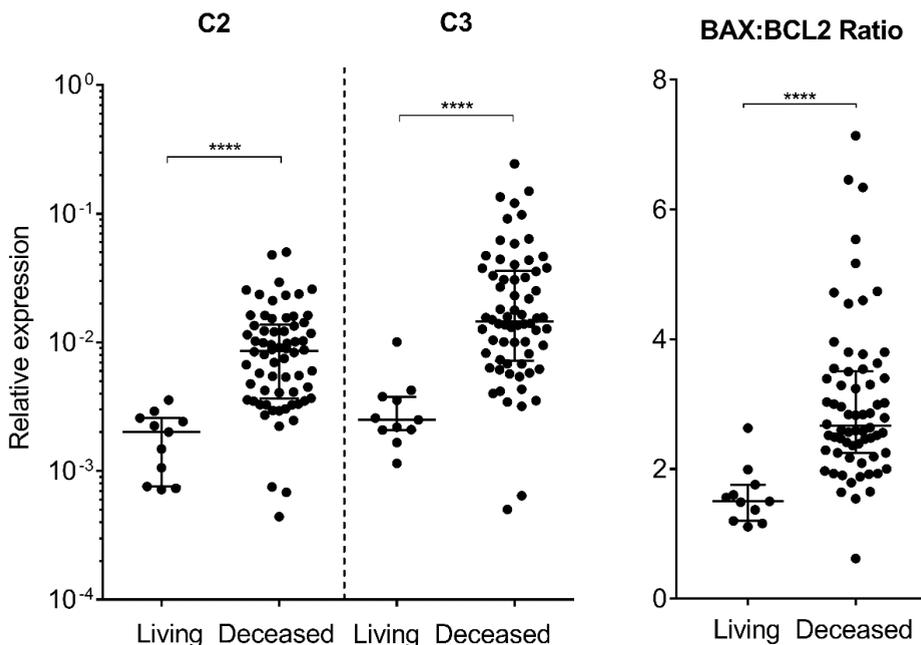
Figure 1. Gene profiling in living and deceased donors at pre-transplant (PreTx). The relative expression of *C2* and *C3* was significantly lower in living donor than that in deceased donor in pre-transplant biopsies. The *BAX:BCL2* ratio was significant lower in living donors in the PreTx biopsies. Flags show median with interquartile range. P values were calculated by Mann-Whitney U tests (two-sided), **** $P<0.0001$. (corrected for Bonferroni)

No association of gene expression before transplantation with delayed graft function
All recipients with DGF (28.7%) had received a deceased donor renal allograft. Donor age of more than 50 years was a risk factor for DGF. In the pre-implantation tissue of deceased donors, none of the genes investigated were significantly different in expression between patients with DGF and those with no DGF (Table 2).

Table 2. Association between donor type, DGF, and gene expression in the pre-transplant tissues. [a]

	Living (N=11)	Deceased (N=66)	P	DGF (N=22)	No DGF (N=44)	Pf
TLR1	1.0 (0.86-1.95)	1.45 (0.90-2.06)	0.25	1.05 (0.61-1.56)	1.0 (0.65-1.42)	0.72
TLR2	1.0 (0.38-1.65)	1.42 (0.76-2.38)[b]	0.05	1.10 (0.69-1.87)	1.0 (0.56-1.80)[b]	0.73
TLR3	1.0 (0.79-1.55)	1.28 (0.95-1.66)[b]	0.30	0.99 (0.68-1.55)	1.0 (0.75-1.28)[b]	0.9
TLR4	1.0 (0.69-1.19)	0.96 (0.64-1.34)	0.69	1.25 (0.83-1.65)	1.0 (0.70-1.44)	0.17
TLR5	1.0 (0.82-2.29)	1.20 (0.67-1.89)[b]	0.58	1.45 (0.56-2.05)	1.0 (0.70-1.88)[b]	0.43
TLR6	1.0 (0.48-2.17)	1.26 (0.70-2.49)[b]	0.50	1.14 (0.63-2.29)	1.0 (0.54-2.08)[b]	0.69
TLR7	1.0 (0.64-2.03)	1.68 (1.05-3.07)[c]	0.03	0.86 (0.46-2.05)	1.0 (0.67-1.51)[c]	0.73
TLR8	1.0 (0.62-2.47)	1.49 (0.81-2.45)[e]	0.63	1.53 (1.01-2.76)[b]	1.0 (0.59-2.02)[d]	0.1
TLR9	1.0 (0.82-2.70)	1.00 (0.38-3.13)[b]	0.80	1.19 (0.38-3.68)[b]	1.0 (0.54-3.15)	0.9
TLR10	1.0 (0.44-4.29)	2.14 (0.72-6.42)[c]	0.29	0.90 (0.29-2.54)[b]	1.0 (0.31-3.87)[b]	0.74
CD46	1.0 (0.94-1.15)	0.86 (0.67-1.06)	0.02	0.90 (0.72-1.18)	1.0 (0.77-1.21)	0.45
CD55	1.0 (0.92-1.50)	0.90 (0.63-1.32)	0.09	1.06 (0.84-1.55)	1.0 (0.72-1.53)	0.36
CD59	1.0 (0.83-1.16)	0.97 (0.85-1.26)	0.61	0.99 (0.89-1. 25)	1.0 (0.87-1.29)	0.74
C2	1.0 (0.38-1.28)	4.28 (1.81-6.81)	5.20E-6*	1.01 (0.40-1.72)	1.0 (0.48-1.58)	0.95
C3	1.0 (0.83-1.52)	5.81 (2.88-14.43)	5.98E-6*	1.21 (0.74-2.09)	1.0 (0.47-2.97)	0.59
C4	1.0 (0.89-1.65)	2.17 (1.38-3.03)	0.01	0.97 (0.53-1.39)	1.0 (0.70-1.38)	0.53
CR1	1.0 (0.75-2.07)	0.99 (0.67-1.54)[b]	0.60	1.26 (0.89-1.82)	1.0 (0.68-1.81)[b]	0.41
Bcl2	1.0 (0.87-1.49)	0.71 (0.47-1.01)	2.35E-3*	1.11 (0.66-1.66)	1.0 (0.74-1.37)	0.61
BAX	1.0 (0.83-1.24)	1.18 (0.99-1.53)	0.12	1.13 (1.00-1.58)	1.0 (0.85-1.38)	0.13
BAX:BCL2	1.0 (0.80-1.18)	1.78 (1.50-2.34)	8.41E-5*	1.09 (0.89-1.42)	1.0 (0.87-1.30)	0.34

[a] Gene expression data shown as medians with interquartile range

[b, c, d, e] Data missing for one[b], two[c], four[d], five[e] patients.

[f] The expression level of patients with and without DGF was analyzed in the deceased donor group.

* Statistically significant p-values after Bonferroni correction (P<0.0025). P values were calculated by Mann-Whitney U tests (two-sided).

Comparison of pre-transplant and acute rejection tissues

Paired pre-transplant and acute rejection biopsies of 75 patients were available for analysis of gene expression dynamics (Table 3). The expression level of *TLR 6, TLR7, TLR8, TLR9*, and *TLR10* was elevated more than 5.5 fold at the moment of AR, and the expression levels of *TLR1, TLR2, TLR3*, and *C2* were increased 1.2-4.4 fold compared to those before implantation. The expression levels of *TLR4, TLR5, C3* and *CR1* were similar between both biopsies, and levels of *C4*, *BCL2* and the complement regulators (*CD46, CD55,* and *CD59*) were slightly decreased during AR (Figure 2). Patients whose *C3* expression increased between AR and

pre-transplantation did not differ from patients whose *C3* expression decreased in this time interval with respect to incidence of steroid resistant rejection and death censored graft survival (data not show).

Table 3. Pairwise comparison of gene expression between pre-transplant and AR biopsies.

	Pre-transplant (N=75)	Acute rejection (N=75)	P
TLR1	1.0 (0.65 - 1.46)	4.36 (3.24 - 5.34)	5.50E-14*
TLR2	1.0 (0.52 - 1.63)	3.42 (2.63 - 5.21)	4.60E-12*
TLR3	1.0 (0.74 - 1.29)[a]	1.42 (1.20 - 1.86)[a]	5.38E-8*
TLR4	1.0 (0.70 - 1.37)	1.21 (0.95 - 1.56)	0.019
TLR5	1.0 (0.56 - 1.59)	1.40 (1.01 - 1.70)	0.0028
TLR6	1.0 (0.56 - 1.97)	5.59 (4.18 - 8.47)	2.22E-13*
TLR7	1.0 (0.61 - 1.55)[a]	7.40 (4.60 - 9.79)[a]	1.48E-13*
TLR8	1.0 (0.57 - 1.73)[b]	27.04 (18.94 - 34.87)[b]	3.56E-13*
TLR9	1.0 (0.39 - 3.07)[a]	7.66 (4.99 - 13.14)[a]	1.62E-11*
TLR10	1.0 (0.31 - 2.83)[c]	8.96 (4.55 - 14.31)[c]	2.25E-9*
CD46	1.0 (0.78 - 1.22)	0.80 (0.62 - 1.07)	2.19E-3*
CD55	1.0 (0.71 - 1.48)	0.72 (0.58 - 0.84)	4.34E-7*
CD59	1.0 (0.86 - 1.25)	0.84 (0.63 - 1.04)	4.92E-4*
C2	1.0 (0.47 - 1.76)	2.49 (1.71 - 3.56)	4.34E-7*
C3	1.0 (0.43 - 2.35)	2.04 (1.32 - 3.65)	8.41E-3
C4	1.0 (0.65 - 1.44)	0.49 (0.36 - 0.61)	2.19E-10*
CR1	1.0 (0.70 - 1.54)	1.26 (0.87 - 1.97)	0.0087
BCL2	1.0 (0.67 - 1.35)[c]	0.76 (0.60 – 1.06)[c]	3.14E-4*
BAX	1.0 (0.84 - 1.31)[c]	0.98 (0.85 - 1.27)[c]	0.44
BAX:BCL2	1.0 (0.76 - 1.30)[c]	1.24 (0.95 - 1.49)[c]	6.20E-5*

[a, b, c] Data missing for one[a], five[b], or two[c] patients.
* Statistically significant p-values based on Bonferroni correction (P<0.0025), P values were calculated by Wilcoxon signed ranks test.

Gene expression correlated with inflammatory cell markers and Banff score

Since all TLRs showed elevated levels during AR, we investigated whether this upregulation could be ascribed to infiltration of inflammatory cells. Correlations of innate immunity expression levels with expression of key inflammatory markers (*CD163, CD68, CD20, CD3e*) and Banff classification are summarized in Table S2. Except for *TLR2*, *TLR3* and *TLR5*, all TLRs correlated with one or more inflammatory cell marker. *C2* and *C3* were significantly correlated with macrophage makers, whereas *CD46* and *CD59* showed a negative relationship with these molecules. In addition, *CR1* demonstrated relationships with T cell, B cell, macrophage

markers, and interstitial inflammation score. Apoptosis-related genes did not correlate with any of the inflammatory molecules. In summary, the altered gene expression may in part be the result of infiltrating inflammatory cells.
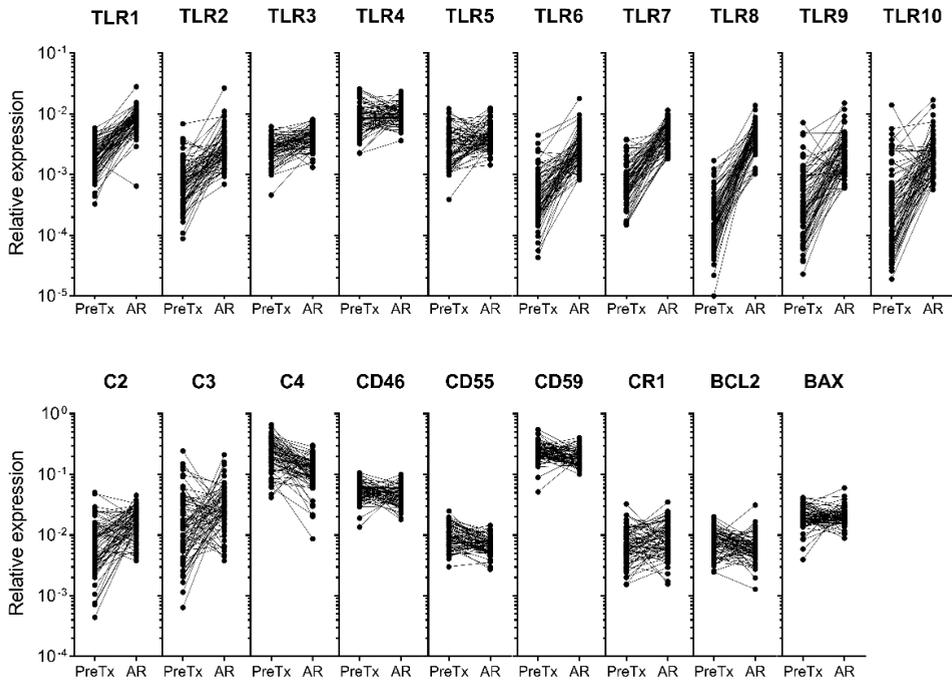

Figure 2. Gene expression dynamics in kidney biopsies. The paired pre-transplant (PreTx) and acute rejection (AR) biopsies of 75 patients were used for comparison. The mRNA levels were quantified by qPCR and normalized to reference genes.

High expression of TLR4 and high BAX:BCL2 ratio during AR predicts inferior graft outcome
The relative expression of TLR4 at the moment of AR in living and deceased patients was comparable. The patients with a deceased donor graft were divided into two groups based on their gene expression levels. One-third of patients who showed the highest *TLR4* expression were defined as high expression group (open circles); and the rest of patients as low expression group (black dots) (Figure 3A). At 12.5 years post transplantation, patients with high *TLR4* expression showed significant inferior graft survival (59.2%) compared to recipients who had relative low *TLR4* expression (79.6%, P= 0.04, Figure 3A). More than 10% of the patients with high *TLR4* expression lost their graft within the first 3 months.

As for the *BAX:BCL2* ratio: patients in the deceased donor group, who had a *BAX:BCL2* ratio that was higher than in the living donation group, were defined as the high ratio group (open circles) (Figure 3B). The group of patients with relatively high *BAX:BCL2* ratio at time of AR had an inferior graft survival (57.9%) compared to patients with a low *BAX:BCL2* ratio (79.8%) and those with a living donor graft (88.3%, P=0.03, Figure 3B). In univariate analysis, Banff classification score did not predict long term graft survival. In multivariate cox regression analysis within deceased donor group (Table 4), only high TLR4 expression (HR=3.46; CI=1.17-10.23; P=0.025) and a high *BAX:BCL2* ratio (HR=4.6; CI=1.44-14.73; P=0.01) were a significant independent risk factor for graft loss. The penalized cox regression model using the lasso showed that high *TLR4* expression, higher donor age (> 50 year) and high *BAX:BCL2* ratio were the most significant (Figure S1). Expression levels in the pre-transplant tissues were not associated with graft survival.

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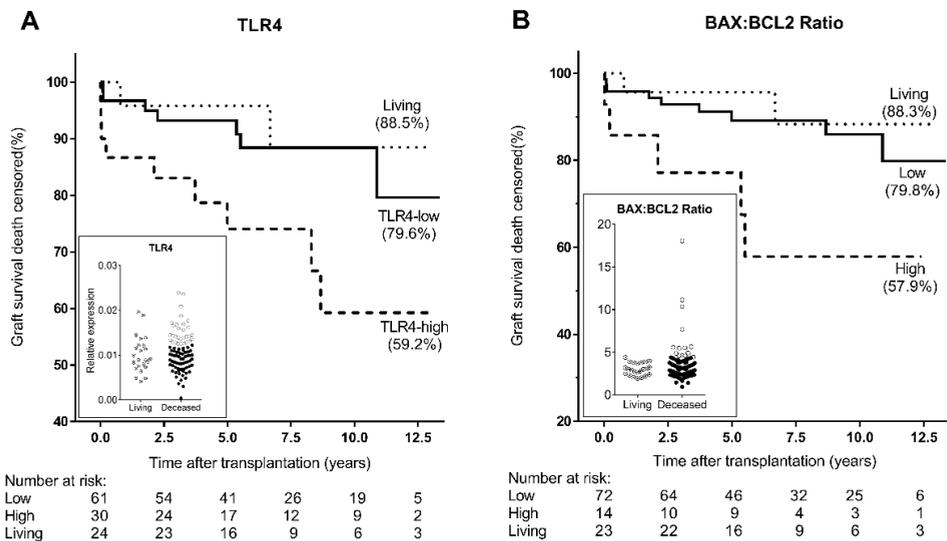
Figure 3. Association between gene expression at moment of acute rejection and kidney graft survival. (A) The *TLR4*-high expression patient group (n=30; dash line) had significantly inferior graft survival compared to the *TLR4*-low expression patient group (n=61; solid line) and living donor group (n=24; dots line). (B) The high *BAX:BCL2* ratio patient group (n=14; dash line) had significantly inferior graft survival rate compared to the low *BAX:BCL2* ratio patient group (n=72; solid line) and the living donor group (n=23; dots line).

Table 4. Cox regression analysis of transplant-related risk factors and post-transplant gene expression levels at time of AR with death censored graft survival.

	Univariate		Multivariate	
	HR (Lower-Upper)	P	HR (Lower-Upper)	P
Recipient age (> 50 year)	1.35 (0.50 - 3.60)	0.55		
Transplantation date (< 1999)	0.93 (0.25 - 3.42)	0.91		
Donor age (> 50 year)	2.10 (0.78 - 5.68)	0.14		
ABDR Mismatching	0.95 (0.27 - 3.34)	0.94		
Cold ischemia time (> 18h)	0.88 (0.25 - 3.14)	0.85		
Delayed Graft function	1.61 (0.60 - 4.33)	0.35		
Vascular rejection	1.28 (0.46 - 3.54)	0.63		
Steroid resistant	1.94 (0.72 - 5.21)	0.19		
Number of transplants (> 1)	2.88 (1.00 - 8.32)	0.05*	–	
CD163 (high expression level)	1.52 (0.55 - 4.20)	0.42		
CD68 (high expression level)	1.78 (0.62 - 5.07)	0.28		
CD20 (high expression level)	0.42 (0.12 - 1.47)	0.17		
CD3e (high expression level)	1.14 (0.41 - 3.16)	0.8		
TLR4 (high expression level)	2.89 (1.08 - 7.78)	0.04*	3.46 (1.17 - 10.23)	0.025*
Ratio *BAX:BCL2* (higher than living)	3.22 (1.09 - 9.51)	0.03*	4.60 (1.44 - 14.73)	0.01*

* Statistically significant difference (P<0.05)

Localization of TLR4, TLR9, and BCL2 expression in renal transplant biopsies

To verify clinically relevant mRNA markers at the protein level and localize their expression in the tissue, immunohistochemical staining for TLR4, BAX and BCL2 were performed on kidney biopsy specimens (Figure 4). In addition, we investigated TLR9 which was increased during AR, and which has been shown to be an inducer of proinflammatory signals (32). Quantification of BAX expression could not be performed since almost no staining was observed in the biopsies (positive area < 10%). TLR4 protein expression was detected in tubular epithelial cells and in inflammatory cells (Figure 4, A and B). Semi-quantitative scoring showed a significantly higher expression during AR than those with stable graft function. Protein expression of TLR9 was predominantly seen in tubular epithelial cells and varied considerably within the AR group (Figure 4, C and D). BCL2 expression was observed in the cytoplasm of tubular epithelial cells and in infiltrating inflammatory cells, and showed a wide range of expression among AR biopsy samples (Figure 4, E and F). The extent of protein expression of BCL2 and TLR9 during AR was increased in comparison to the stable graft group, however this difference was not significant after correction for multiple comparisons (Table 5).
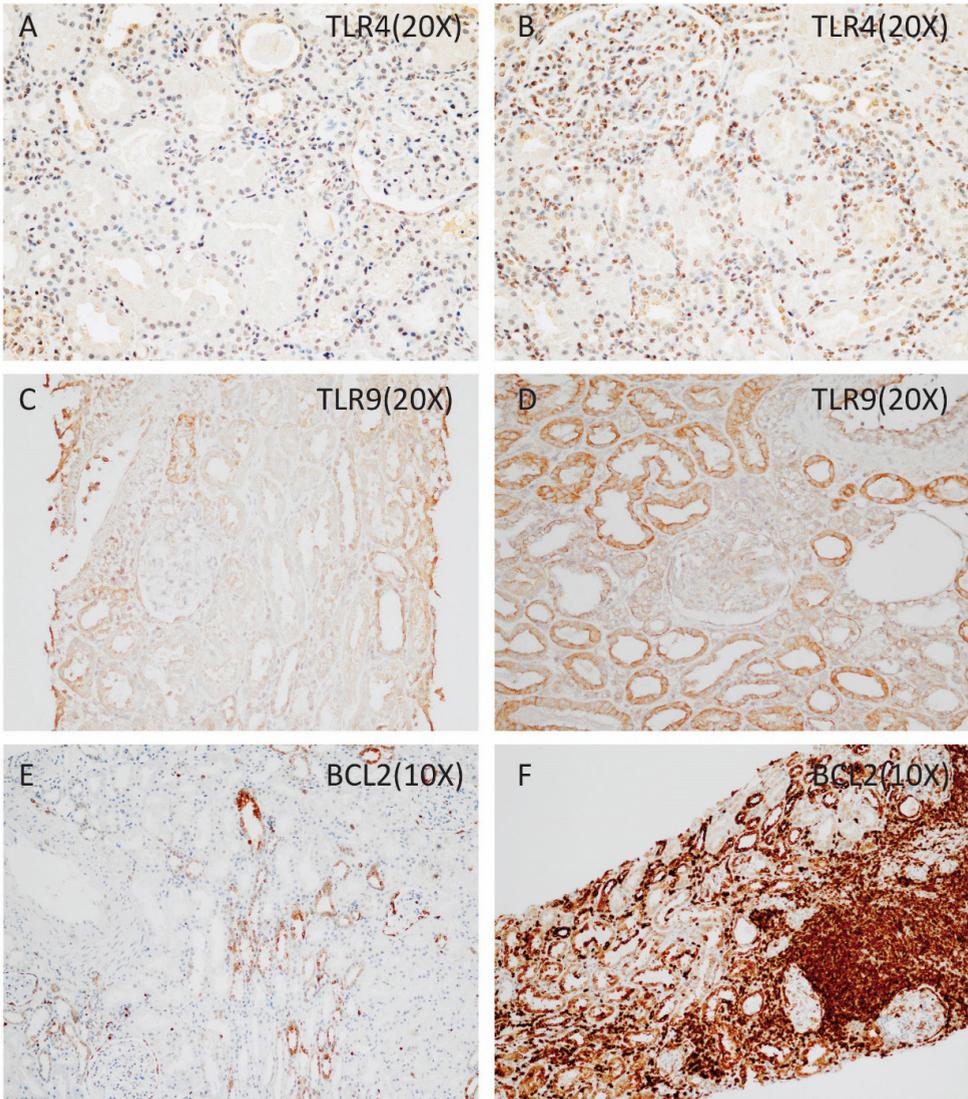
Figure 4. Immunohistochemical staining pattern of TLR4, TLR9, and BCL2 in kidney transplant biopsy specimens. TLR4 protein expression was detected in tubular epithelial cells and in inflammatory cells (A-B). TLR9 was observed in the tubular epithelial cells (C-D). BCL2 was detected in tubular epithelial cells and infiltrated lymphocytes (E-F). Both BCL2 and TLR9 expression varied extensively between acute rejection biopsy specimens. Two representative samples from the acute rejection group are shown.

Table 5. Immunohistochemical scoring of TLR4, TLR9, and BCL2 in stable graft function and acute rejection biopsies.

IHC score	1	2	3	4	5	P
TLR9						0.069
SGF	1	5	1	0	0	
AR	4	4	7	4	4	
TLR4						0.008*
SGF	4	2	1	0	0	
AR	2	7	5	5	3	
BCL2						0.024
SGF	1	3	2	1	0	
AR	0	3	8	8	3	

IHC, Immunohistochemistry; SGF, stable graft function; AR, acute rejection.
* Statistically significant p-values based on Bonferroni correction (P<0.016), P values were calculated by Mann-Whitney Test.

Discussion

In the present study mRNA expression levels of TLRs, key complement components and regulators, and apoptosis-related genes were investigated in biopsies obtained before graft implantation and at time of AR. We found that in deceased donors, *C2* and *C3* expression and *BAX:BCL2* ratio are already elevated before transplantation but were not indicative of DGF. High *TLR4* levels and a high *BAX:BCL2* ratio at the time of an AR were both independent risk factors of graft loss. Results from this exploratory study suggest that innate immune activation occurs both at time of graft implantation and during episodes of acute rejection.

Although the TLR/MyD88 pathway was found to be redundant for host defense against most natural infections (33), depletion of a functional TLR pathway in mice, by knocking out either TLR2, TLR4 or MyD88, protects against IRI and kidney dysfunction, and limits an increase in expression of cytokines, chemokines and in infiltration of inflammatory cells (34, 35). In human kidney transplants, the expression of TLR4 and HMGB1 (an endogenous ligand of TLR4) was significantly elevated in pre-implementation biopsies from deceased donors in contrast to those from living donors (11). However, in our study, we could not confirm these findings (Table 2), and none of the markers we investigated were associated with DGF.

Earlier studies showed that the expression of TLRs is significantly upregulated during allograft rejection mainly because of infiltration of leukocytes (13, 36). However, none of the previous studies have documented gene expression dynamics in a large patient cohort. We

showed that the expression of all TLRs except *TLR4* and *TLR5* was significantly increased in AR biopsies when compared to pre-implementation biopsies (Table 3). Moreover, expression of majority of the TLRs positively correlated with one or more inflammatory cell markers at the moment of AR (Table S1), suggesting that the elevated expression of TLRs is a result of inflammatory cell presence. As for *TLR2* and *TLR3*, which showed only minor increase during an AR but they were not correlated with any inflammatory markers, their expression may be dominant in renal parenchymal tissue. Similar expression patterns of *TLR3* were reported by Dessing et al. (36). *TLR4*, the expression of which correlated with *CD163* and *CD68* but was not increased during AR, may be expressed by both parenchymal and myeloid cells. In addition, patients with relatively high levels of TLR4 during AR exhibited inferior graft survival 12.5 years after transplantation, which may mean that intracellular ligands released after cell damage bind to TLR4 and thereby provide additional inflammatory signals leading to long term graft loss. Expression of TLR4 in the renal allograft biopsy has been described previously (11, 13). TLR4 was expressed in tubular cells and infiltrated lymphocytes, with significantly higher expression during AR compared to stable graft conditions. The possible explanation, that on one hand no increase in *TLR4* mRNA was seen between AR and pre-Tx and on the other hand immunohistochemistry showed significantly higher expression during AR compared to stable graft conditions, may be that the epithelium expresses high levels of mRNA but relatively low level of protein. The endogenous pattern recognition receptor TLR9 is involved in immune complex kidney disease (37). Immunohistochemical staining showed that TLR9 was increased during AR compared to the stable graft group with borderline significance.

The complement system acts as a bridge to the adaptive system and facilitates clearance of immune complexes and cellular debris. It has been shown that the MAC plays a central role in renal IRI and that locally synthesized C3 is important in kidney graft survival (17, 38). In line with a previous study (14), the mRNA levels of *C2* and *C3* in the living donor grafts were significantly lower than those in the deceased donor grafts at time of implantation, which supports the notion that the local C3 expression is induced by donor brain death (15). The observations of a slight increase in C4 expression in deceased donors are in line with those from a previous study (14). However, inconsistent with that study, the expression level of CR1 was comparable between deceased and living donor biopsies in our relatively large cohort. The increased C2 during AR may represent a higher activity of the classical and lectin pathway, whereas the decreased C4 expression may be a result of injury of renal parenchymal cells.

The complement regulators CD46, CD55, and CD59 act as inhibitors of activation of the complement pathway. Hyper-sensitized rats treated with sCR1 displayed significantly prolonged cardiac graft survival (39). Similarly, kidneys of animals treated with CR1 derivatives (APT070) showed less acute tubular injury, and the animals had a significantly higher graft survival rate (40). CD55 had a protective effect on renal function in C4d-negative

grafts and antibody-mediated cardiac allograft rejection (22, 41). We found that expression of *CD46, CD55* and *CD59* was significantly reduced during an AR compared to that in the pre-transplantation tissue. However, none of the complement regulators were predictive for the development of DGF, steroid resistant rejection and graft survival in the present study. Interestingly, Budding et al. showed that serum sCD59 are elevated at the time of bronchiolitis obliterans syndrome (BOS) after lung transplantation, and the patients with higher serum sCD59 titers (>400pg/ml) had a significantly lower chance of BOS free survival. We observed that the expression of complement regulators at time of AR was slightly decreased compared to pre-transplant conditions, and that it negatively correlated with expression of macrophage markers.

It has been shown that kidney cell apoptosis is involved in IRI and that apoptotic cells are frequently present in AR biopsies (23, 24). In the present study, the mRNA of *BCL2*, an anti-apoptotic molecule, was lower in deceased donor biopsies than in living donor grafts. The higher *BAX:BCL2* ratio suggests that the extent of apoptosis is already increased in deceased donors. The *BAX:BCL2* ratio tended to be higher in the DGF group in the deceased donor cohort, but this was only marginal (Table 2), which is inconsistent with findings from a previous study (27). During an AR, the *BAX:BCL2* ratio was marginally increased and it significantly correlated with expression of macrophage markers. Protein investigations by immunohistochemical staining showed that BAX was rarely detected in the biopsy samples. The BCL2 expression was mainly observed in tubular epithelial cell and inflammatory cells, with a wide range of staining within the AR group. Patients who received a living donor graft had superior graft survival compared to those with a graft from a deceased donor, and thus this group acted as a reference. Moreover, patients with relatively high *BAX:BCL2* ratio during AR in their deceased donor graft demonstrated significantly inferior graft survival rates (57.9%) 12.5 years after transplantation compared to those with a lower ratio or to patients who had received a living donor graft (Figure 3). High *BAX:BCL2* ratio during AR possibly reflects an increased number of apoptotic cells, which leads to attraction of phagocytic cells to the graft (42, 43). The accumulated phagocytes may be triggered by immunogenic danger signals and mediate subsequent chronic allograft loss (44, 45).

In conclusion, complement and apoptosis pathways are elevated before kidney transplantation. Increased expression of the majority of genes partly reflect the infiltration of inflammatory cells during an AR. Relatively high *TLR4* expression and *BAX:BCL2* ratio during AR, possibly reflecting enhanced immunogenic danger signals, were both independent risk factors for adverse outcome after transplantation of a deceased donor kidney. The results of this study suggest that the different impact of AR on outcome between living and deceased donor transplants may partly be ascribed to differences in TLR4 regulation and cell death related mechanisms. They form a basis to further validate and explore the functional relevance of these pathways in relation to transplant outcome.

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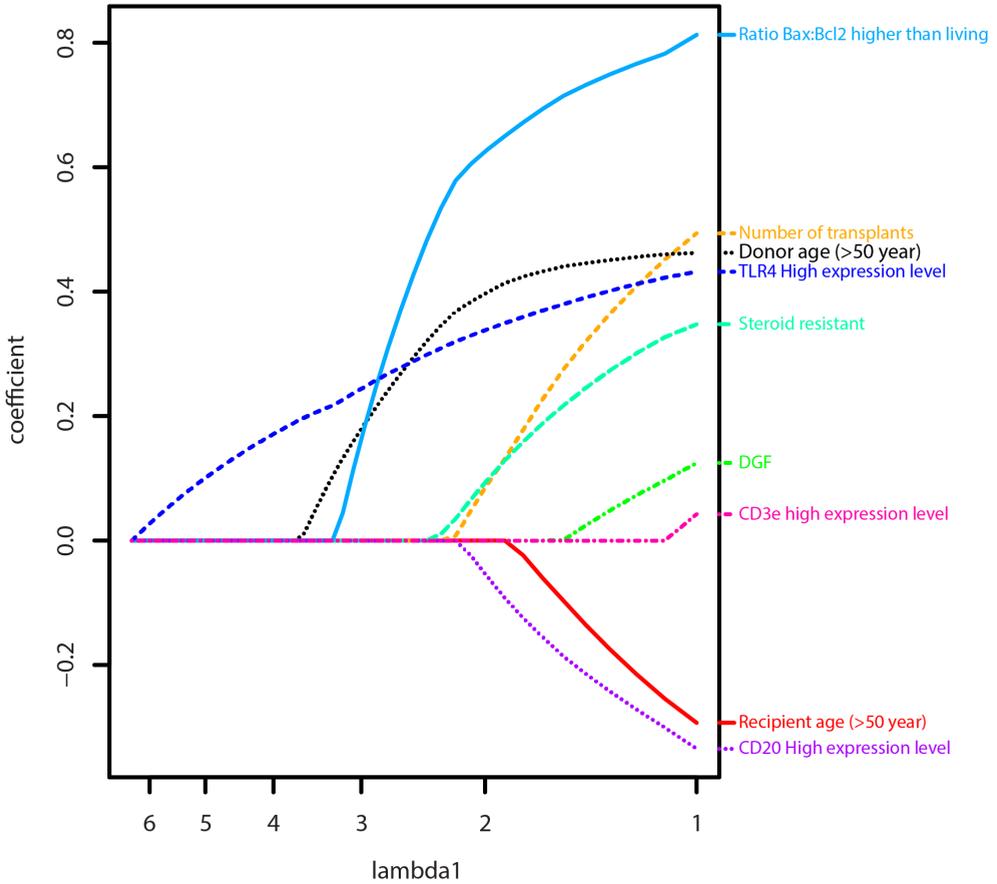
Supplementary Data

Figure S1. Penalized survival analysis of clinical risk factors. The plot shows the effect of lambda on the fitted regression coefficients. High TLR4 expression, donor age >50, and high Bax:Bcl2 ratio were the top three parameters in the penalized lasso model.

Table S1. Primer sequences, amplification efficiency and functional description

Genes	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)	Efficien-cy (%)	Description
TLR1	acaccaagttgtcagcgatg	gggctaattttggatgggca	135	94.5	Detects triacylated lipopeptides
TLR2	gtgataggtgtgaggcaggt	gtggccgccttgattcatag	136	100.8	Detects HSP70, HMGB1, HA
TLR3	gaacctccagcacaatgagc	tgacaagccattatgagacaga	169	99.6	Detects mRNA
TLR4	acctcccttctcaaccaag	ggctctgatatgccccatct	150	106.9	Detects HMGB1, heparan sulfate, S100A9, and LPS
TLR5	tgaagtctttaaacaaccaggga	tccaaacacaggaccggc	142	108.1	Detects flagellin
TLR6	ctctccactctgcttttccca	ctccgaggactggtgcatat	109	107.1	Forms heterodimer with TLR2
TLR7	tgctctcttcaaccagacct	cacgatcacatggttctttgga	196	103.5	Detects RNA, ssRNA
TLR8	taccaccttgaagagagccg	tgctttggttgatgtctgc	112	99.5	Detects ssRNA
TLR9	agatggaggggagaaggtct	caaggtgaagttgagggtgc	112	98.6	Detects DNA
TLR10	tccagagctgccagaagaaa	aaatccagtgtcgttgtggc	102	97.5	Forms heterodimer with TLR2
C46	attcagtgtggagtcgtgct	aattgtgtcgctgccatcg	167	95.8	Cofactor that inactivates C3 convertase
CD55	tcctggcgagaaggactcagtga	agccttgttggcacctcgca	96	98.0	Decay-accelerating activity C3 convertase
CD59	gcgtgtcattaccaaaagct	ccttcttgcagcagtagtacg	127	95.9	Prevents the formation of MAC
C2	tctgaacctctacctgctcc	acatgagactttgggctct	164	92.8	Component in the classical and lectin pathway
C3	agcagtcaaggtctacgcc	ttgtccagccgttcttcca	180	95.3	Central role in complement activation
C4	ctgaacaaccgccagattcg	tggcaggtcgttgttcttcat	149	98.9	Component in the classical and lectin pathway
CR1	tgtttgcgatgaaggttcc	gagttcctgtgtgtctccca	155	101.4	Decay-accelerating activity and cofactor activity
Bcl2	gccctgtgatgactgagta	cttcagagacagccaggaga	135	100.4	Anti-apoptotic protein
BAX	cgcccttttctacttttgcca	ccaatgtccagccccatgatg	92	102.1	Pro-apoptotic protein
B-actin	accacccttctacaatgag	tagcagcctggatagc	161	94.0	Reference gene
GAPDH	accactcctccacctttgac	tccacaccctgttgctgtag	110	98.0	Reference gene
CD3e	cggccatcttagtaaagtaacac	aataccaccatttcttcattacc	131	90	T cell marker
CD163	tggagtgacctgctcagatg	caccgtccttggaatttgat	99	103.8	M2 macrophage maker
CD68	ttccctatggacacctcag	ttgtactccacgccatgta	86	103.9	Pan macrophage marker
CD20	gggggctgtccagattatgaa	ccaggagtgatccggaaata	148	97.2	B cell marker

Table S2. Correlations of innate immunity markers with inflammatory markers and Banff lesions

		CD163	CD68	CD20	CD3e	g	i	t	v	ci	ct
TLR1	R	0.46	0.44	0.35	0.35	0.01	0.30	0.25	-0.02	0.13	0.16
	P	7.16E-8*	2.93E-7*	7.20E-5*	7.57E-5*	0.92	1.71E-03	0.01	0.86	0.18	0.09
TLR2	R	0.02	0.09	0.16	0.06	0.04	0.08	0.01	0.01	0.21	0.19
	P	0.84	0.34	0.07	0.53	0.67	0.40	0.01	0.90	0.03	0.05
TLR3	R	-0.19	-0.22	-0.11	0.05	-0.13	-0.21	-0.02	-0.11	-0.08	-0.04
	P	0.04	0.02	0.24	0.61	0.16	0.03	0.87	0.28	0.43	0.65
TLR4	R	0.48	0.52	0.16	0.23	0.10	0.11	0.19	0.12	0.13	0.20
	P	1.73E-8*	8.83E-10*	0.08	0.01	0.29	0.25	0.04	0.22	0.17	0.04
TLR5	R	0.21	0.09	0.24	0.15	-0.08	0.11	0.08	0.02	0.16	0.19
	P	0.02	0.35	0.01	0.1	0.43	0.23	0.39	0.83	0.10	0.05
TLR6	R	0.08	0.1	0.32	0.11	-0.02	0.13	0.09	-0.05	0.24	0.20
	P	0.38	0.25	3.97E-4*	0.22	0.88	0.16	0.38	0.65	0.01	0.04
TLR7	R	0.44	0.52	0.45	0.44	0.03	0.23	0.33	-0.18	0.12	0.07
	P	2.62E-7*	1.12E-9*	1.80E-7*	2.77E-7*	0.77	0.02	4.66E-4	0.08	0.22	0.45
TLR8	R	0.42	0.51	0.17	0.18	0.22	0.27	0.24	0.05	0.17	0.13
	P	1.01E-6*	1.33E-9*	0.06	0.05	0.02	0.00	0.01	0.59	0.08	0.17
TLR9	R	-0.1	-0.05	0.37	0.1	-0.12	0.11	0.10	-0.18	0.25	0.13
	P	0.25	0.58	2.70E-5*	0.26	0.20	0.24	0.31	0.08	0.01	0.18
TLR10	R	-0.1	-0.05	0.5	0.17	-0.11	0.18	0.14	-0.13	0.20	0.09
	P	0.27	0.56	6.87E-9*	0.05	0.27	0.07	0.16	0.21	0.04	0.37
CD46	R	-0.48	-0.47	-0.11	-0.07	-0.11	-0.19	-0.11	-0.23	-0.19	-0.04
	P	1.92E-8*	3.61E-8*	0.25	0.46	0.26	0.04	0.27	0.02	0.05	0.69
CD55	R	0.2	0.1	0.11	0.03	0.03	0.03	1.85E-4	0.07	0.04	0.28
	P	0.03	0.26	0.23	0.73	0.74	0.72	1.00	0.50	0.65	3.07E-3

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Table S2: Continued

CD59	R	-0.37	-0.36	-0.3	-0.18	0.15	-0.17	-0.15	0.02	-0.14	-4.32E-3
	P	3.03E-5*	4.66E-5*	7.912E-04	0.05	0.11	0.07	0.13	0.84	0.15	0.96
C2	R	0.64	0.68	0.21	0.42	0.07	0.32	0.32	0.13	0.29	0.20
	P	3.12E-15*	9.04E-18*	0.02	1.74E-6*	0.50	7.82E-4	7.76E-4	0.19	2.79E-3	0.04
C3	R	0.4	0.4	0.26	0.22	0.09	0.33	0.10	0.08	0.31	0.17
	P	5.41E-6*	6.71E-6*	0.01	0.02	0.34	5.85E-4	0.30	0.45	1.08E-3	0.09
C4	R	-0.23	-0.18	0.05	0.08	-0.14	0.06	0.09	-0.15	-0.07	0.04
	P	0.01	0.05	0.59	0.38	0.14	0.51	0.36	0.14	0.45	0.70
CR1	R	0.7	0.52	0.43	0.33	0.00	0.44	0.27	0.12	0.25	0.26
	P	6.53E-19*	1.16E-9*	8.98E-7*	1.96E-4*	0.96	1.59E-6*	4.18E-3	0.22	0.01	0.01
BCL2	R	-0.28	-0.27	0.22	0.15	-0.26	-0.08	0.06	-0.05	-0.07	-0.08
	P	2.20E-03	2.30E-03	0.02	0.09	0.01	0.42	0.53	0.66	0.47	0.40
BAX	R	0.16	0.04	0.11	0.1	-0.05	0.03	0.03	0.12	0.16	0.13
	P	0.07	0.7	0.24	0.29	0.59	0.79	0.74	0.24	0.10	0.19
BAX:BCL2	R	0.45	0.33	-0.18	-0.18	0.24	0.07	-0.05	0.15	0.24	0.17
	P	2.13E-7*	2.42E-4*	0.06	0.05	0.01	0.47	0.64	0.14	0.01	0.09

g, glomerulitis; i, interstitial inflammation; t, tubulitis; v, intimal arteritis; ci, interstitial fibrosis; ct, tubular atrophy; R, Spearman Correlation Coefficient; P, P value.

* Statistically significant p-values after Bonferroni correction ($P<0.00025$).